

# The Role of Gut Microbiota in Systemic Lupus Erythematosus

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## ABSTRACT

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease with no known cure. Despite years of study, the etiology of SLE is still unclear. Both genetic and environmental factors have been implicated in the disease mechanisms. Gut microbiota as an environmental factor and the immune system interact to maintain tissue homeostasis, but whether this interaction is involved in the pathogenesis of SLE is unclear.

In a classical model of lupus nephritis, MRL/*lpr*, we found decrease of *Lactobacillales* but increase of *Lachnospiraceae* in the gut microbiota. Increasing *Lactobacillales* in the gut by supplying a mixture of 5 *Lactobacillus* strains improved renal function of these mice and prolonged their survival. Further studies revealed that MRL/*lpr* mice possessed a “leaky” gut, which was reversed by increased *Lactobacillus* colonization. Inside the kidney, oral *Lactobacillus* treatment also skewed the Treg-Th17 balance towards a Treg phenotype.

To remove *Lachnospiraceae* that was higher in lupus-prone mice than controls, we administered vancomycin orally to MRL/*lpr* mice after disease onset from 9 to 15 weeks of age. Vancomycin functions by removing Gram-positive bacteria such as *Lachnospiraceae* but sparing *Lactobacillus* spp. The treatment during active lupus reshaped the gut microbiota and significantly ameliorated systemic autoimmunity and kidney histopathology at 15 weeks of age. However, when vancomycin treatment was initiated from a very early age, the beneficial effect was not observed. Strikingly, mice given vancomycin only at the young age exhibited an even

worse disease outcome. Indeed, regulatory B (Breg) cells were found to be reduced after the vancomycin treatment at young age. Importantly, adoptive transfer of Breg cells at 6-7 weeks of age rescued the beneficial effect, which indicates that Breg cells, inducible by vancomycin-sensitive gut microbiota, plays an important role in suppressing lupus disease initiation and progression. Finally, we demonstrated that bacterial DNA from the gut microbiota might be the inducer of Breg cells, as bacterial DNA administration at young age reproduced the beneficial effect seen in the Breg adoptive transfer experiment. Future studies are required to examine the clinical efficacy of targeting gut microbiota as a novel treatment against SLE.

# **The Role of Gut Microbiota in Systemic Lupus Erythematosus**

Qinghui Mu

## **PUBLIC ABSTRACT**

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease with no known cure. SLE affects over 5 million people worldwide, especially women of childbearing age. Lupus nephritis is a manifestation of SLE occurring in the kidney which affects more than 50% of SLE patients and is a major cause of morbidity and mortality in SLE. Current treatments for lupus nephritis are primarily nonselective immunosuppressants, which can cause a higher incidence of severe infections. There is an imperative need for the development of new therapeutic strategies against SLE. Our research team was the first to describe the dynamics of gut microbiota in a mouse model of SLE. My dissertation research studying the role of gut microbiota in the pathogenesis of lupus-like disease in mice showed that there were both pathogenic and beneficial bacteria co-existing in the gut microbiota of lupus-prone mice. My studies revealed not only the effects of different bacteria on lupus pathogenesis, but also the immunological mechanisms by which they exert the effects. The results suggest that modulation of the gut microbiota through diet, probiotics, and/or prebiotics to selectively enhance the abundance and activity of beneficial bacteria may be an attractive strategy for disease prevention and treatment of SLE patients. Nevertheless, studies on human samples and clinical trials are required to confirm the translational application of this strategy.

## DEDICATION

With my deepest love and appreciation, this dissertation is dedicated to my parents-in-law, Zhong Cao and Biqiong Zhang; my parents, Guanglu Mu and Cuixia Zhang; and my lovely wife, Dr. Qian Cao, for their unwavering love, accompany and support.

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## **CHAPTER I**

### **SLE: Another Autoimmune Disorder Influenced by Microbes and Diet?**

**(Literature Review)**

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## **Abstract**

Systemic lupus erythematosus (SLE) is a multi-system autoimmune disease. Despite years of study, the etiology of SLE is still unclear. Both genetic and environmental factors have been implicated in the disease mechanisms. In the past decade, a growing body of evidence has indicated an important role of gut microbes in the development of autoimmune diseases, including type 1 diabetes, rheumatoid arthritis and multiple sclerosis. However, such knowledge on SLE is little, though we have already known that environmental factors can trigger the development of lupus. Several recent studies have suggested that alterations of the gut microbial composition may be correlated with SLE disease manifestations, while the exact roles of either symbiotic or pathogenic microbes in this disease remain to be explored. Elucidation of the roles of gut microbes—as well as the roles of diet that can modulate the composition of gut microbes—in SLE will shed light on how this autoimmune disorder develops, and provide opportunities for improved biomarkers of the disease and the potential to probe new therapies. In this review, we aim to compile the available evidence on the contributions of diet and gut microbes to SLE occurrence and pathogenesis.

## Introduction

The mammalian gut harbors trillions of microorganisms known as the microbiota (1). Increasing evidence in recent years suggest that host microbiota and immune system interact to maintain tissue homeostasis in healthy individuals (2-6). Perturbation of the host microbiota, especially that in the gut, has been shown to be associated with many diseases. Among these are autoimmune disorders that include inflammatory bowel disease (IBD) (7, 8), type 1 diabetes (T1D) (9-12), rheumatoid arthritis (5, 13-15), and multiple sclerosis (16, 17). However, little is known on the role of gut microbiota in systemic lupus erythematosus (SLE) (18).

SLE is an autoimmune disorder characterized by severe and persistent inflammation that leads to tissue damage in multiple organs. According to the Lupus Foundation of America, more than 750,000 Americans currently live with the disease. The prevalence ranges from 20 to 200 cases per 100,000 persons, with higher prevalence for people of African, Hispanic, or Asian ancestry (19, 20). Although the disease affects both males and females, women of childbearing age are diagnosed 9 times more often than men.

Our research team has recently described the dynamics of gut microbiota in a classical SLE mouse model MRL/Mp-Fas<sup>lpr</sup> (MRL/lpr) (21). In young, female lupus mice, we found marked depletion of Lactobacilli, and increase of Clostridial species (*Lachnospiraceae*) together with increased bacterial diversity compared to age-matched healthy controls. Importantly, dietary treatments that improved lupus symptoms in lupus mice also restored gut colonization of *Lactobacillus* and decreased that of *Lachnospiraceae*. In human SLE, a recent cross-sectional study has shown that a lower *Firmicutes* to *Bacteroidetes* ratio was present in women with SLE even after disease remission (22). Similarly, a higher level of *Bacteroidetes* was found in lupus-

prone SNF1 mice with more severe disease (23), though this was not evident in MRL/lpr mice (21). These results suggest a potentially important role of gut microbiota on lupus pathogenesis, in particular a potential role of *Bacteroidetes*, since the relative abundance of these bacteria is increased in human SLE and at least one murine lupus model. In this review, we aim to compile the available evidence that associates gut microbes to SLE.

### **Environmental factors & SLE**

It is well established that genetic factors influence lupus susceptibility. However, the lack of disease concordance between genetically identical twins strongly suggests the role of non-genetic factors, most likely of environmental factors (24). The role of environmental factors in the etiology of SLE is evidenced by the dramatic difference in disease incidence between West Africans and African Americans, both derived from the same ethnic group but exposed to different environments (25). With an obviously higher burden of infections, the frequency of SLE is much lower in West Africa than Africans living in Europe or USA. The mechanism behind this observation is still unclear, but improvement in hygiene and absence of certain microbes may have contributed to the higher incidence and faster progression of lupus disease (26). In addition to microbes, a number of environmental triggering factors have been described to be associated with SLE, including UV light and cigarette smoking, some of which trigger lupus through epigenetic mechanisms (27-30).

### *The hygiene hypothesis*

Increase of SLE occurrence in the developed world has been reported. Data from several regions of USA show that the incidence of SLE increased at least 3 folds within the second half of the 20th century (31, 32). This increase could be related to changes of environmental factors, though better diagnostic methods and increasing awareness of the disease may partially lead to the change in SLE frequency. Similar increase has been observed in a study analyzing the incidence of SLE in Denmark (33). Genome evolution rate seems to be unpersuasive to this increase. In contrast, due to advancements on medicine and vaccination, a number of infectious agents have been gradually eliminated in developed countries, and the sanitation condition has been largely improved. Some have thus proposed that lower exposure to infections leads to the rise of allergies and some autoimmune diseases, such as T1D (34, 35). This is called “The Hygiene Hypothesis.” Considering the rise of SLE frequency in developed countries, it is reasonable to extend the hypothesis to this autoimmune disorder.

Increasing hygiene standards eliminates both pathogenic and non-pathogenic microbes from the environment. Infections from pathogenic microbes, or the lack thereof, are known to be associated with SLE occurrence. Epstein-Barr virus (EBV) and cytomegalovirus (CMV), for example, have been linked to the pathogenesis of SLE by several reports (36-40). Commensal microbes residing inside the host, in return, have been shown to maintain and expand CD8+ memory T cells during CMV infection, supporting the notion that microbiota and CMV cooperatively augment immune activation (41). While EBV and CMV are largely considered triggers of SLE, it is increasingly evident that some infections may be beneficial and the lack of them might actually facilitate SLE. In one surprising report (42), two female SLE patients with severe SLE showed improved disease after experiencing infections for a short period of time.

Before the infections, both patients failed to respond to a long time of immunosuppressive therapy. Neither experienced relapse after the amelioration of SLE symptoms following the infections. One of the patients even had a successful pregnancy, which is known to trigger lupus flares. Unfortunately the study did not identify the causing agent that ameliorated the disease. However, another study has identified hepatitis B virus (HBV) as a protective factor against SLE (43). In their study, 2.5% of SLE patients were found positive for the presence of HBV-core antibody, compared to 10.7% from normal controls, which suggests a potential benefit of HBV infection against the occurrence of SLE. In addition, in a large serologic survey, *Helicobacter pylori* seronegativity was found to be associated with an increased risk and earlier onset of SLE in African Americans, suggesting a protective role of *H. pylori* in SLE patients (46, 47). These studies suggest that, in developed countries where HBV and *H. pylori* infections are decreasing (48-50), the risk for developing SLE could become higher. T cell exhaustion during chronic infection may explain the ability of these pathogens to down-regulate inflammation and ameliorate SLE (44, 45).

In lupus-prone mouse models, beneficial roles of some pathogenic microbes have also been suggested. Chen et al. reported that with the infection of *Toxoplasma gondii*, New Zealand Black (NZB) × New Zealand White (NZW) F1 (NZB/W F1) mice had significantly decreased mortality, ameliorated proteinuria level and reduced anti-DNA IgG in serum. IFN $\gamma$  and IL-10 expression was reduced in the spleen in the presence of *T. gondii*, suggesting the suppression of T helper 1 (Th1) and Th2 responses, respectively, both demonstrated to be pathogenic in murine lupus (42, 51). In addition, when examining NZB/W F1 mice treated with live *Plasmodium chabaudi*, another prevalent parasite, several independent groups have found that the malaria-causing microbe can prevent clinical symptoms of murine lupus and protect the animals against



lupus nephritis (52-54). This is perhaps due to the changed cytokine profile and redox status in both liver and kidney of the mice. Moreover, virus infection has also been found to improve murine lupus symptoms in addition to parasites. For instance, the infection of lactate dehydrogenase elevating virus (LDV) has been shown to significantly suppress the production of anti-nuclear antibody (ANA) and the development of glomerulonephritis in NZB/W F1 mice (26, 55-58). The beneficial effect is hypothesized to be associated with superoxide anion production from macrophages and modulation of prostaglandin E. While LDV and *P. chabaudi* do not infect humans, results from these mouse studies suggest that some infections might be associated with decreased severity of SLE.

### ***Antibiotics and SLE***

Antibiotics, which can remove gut bacteria, are known to trigger lupus flares. These include sulfa drugs such as trimethoprim-sulfamethoxazole (Septra), tetracycline-related antibiotics such as minocycline, and penicillin-related antibiotics such as amoxicillin. Increased sun sensitivity with antibiotics may be one mechanism behind the observations. However, antibiotics also cause diarrhea and remove beneficial microbes from the intestinal tract. Could it be the removal of “good” bacteria a mechanism by which antibiotics induce flares in SLE patients? In addition, bacterial metabolites produced by gut microbes can modulate immune function. Recently, several groups have found that metabolites produced by gut bacteria, especially butyrate produced by Clostridia, can promote the differentiation of regulatory T cells (Tregs) in the colon, spleen and lymph nodes to suppress inflammation (59-62). Thus, removal of certain gut commensals with antibiotics could potentially lead to decreases of bacterial metabolites, such as

homoserine lactone, N-acetylmuramic acid and N-acetylglucosamine (63)—which could be immunosuppressive—thereby facilitating lupus progression. Incidentally, African Americans have used antibiotics much more frequently than people in West African countries (64, 65) and this may have impacted the differences in lupus prevalence and severity between the two populations.

### ***Dietary components and SLE***

Diet, one of the main environmental factors with known effects on gut microbiota, has been studied extensively in both SLE patients and lupus-prone mice. Vitamin D (VD), vitamin A (VA) and omega-3 polyunsaturated fatty acids (PUFAs), for instance, have been found to modulate lupus onset or flares. Current knowledge suggests that dietary components can influence SLE through changing the composition and function of gut microbiota, modulating immunological pathways, and/or exerting epigenetic changes (18, 30, 66, 67). Here we summarize the recent updates on the roles of VD, VA and PUFAs on lupus.

VD deficiency is increasingly common, resulting in increased risks for multiple disorders (68, 69). Although VD can be synthesized by the body in sunlight, adequate VD in diet is recommended. VD plays an important role in the homeostasis of the immune system, through a nuclear receptor existing in all immune cells, VD receptor (VDR). Polymorphisms of VDR have been recently reported to be associated with SLE susceptibility (70). In SLE patients, lower VD levels are associated with higher SLE activity. Handono and colleagues found that 1,25(OH)<sub>2</sub>D<sub>3</sub> can inhibit neutrophil extracellular trap (NET) formation in cultured cells from SLE patients with hypovitamin D (71). Inhibition of NETs prevents endothelial damage that promotes the progression of lupus disease (72), suggesting a possible benefit of supplying VD in

SLE patients with suboptimal VD levels. Recently, it has been reported that VD supplementation increases the number of Treg cells and induces the shift towards Th2 response in pre-menopausal female SLE patients, although a direct efficacy towards disease activity was not observed (73, 74). Likewise, no correlation was found between SLE-associated cytokine profiles and VD levels (75). However, in juvenile-onset SLE, which is more aggressive than adult SLE, dietary intake of VD has been reported to preclude disease progression in several recent studies (75-78). It is worth noting that the doses of VD utilized in these studies were different—one was rather intensive (50,000 international units or IU/week) and the other was more standard (2,000 IU daily)—but the outcomes were similar with improvement of SLE Disease Activity Index. Further studies are required to verify these findings in juvenile-onset SLE, and to explore the mechanisms of why a lack of response to VD was seen in adult SLE.

VA has long been recognized as an immune regulator. VA exerts its effects mainly via *all-trans*-retinoic acid (tRA), an active metabolite of VA. For SLE, the role of VA has been revealed through oral administration of tRA to either SLE patients or lupus-prone mice. In SLE patients, some benefit of tRA to ameliorate lupus nephritis and proteinuria has been reported (79, 80). For murine lupus, several mouse models, including NZB/W F1 and MRL/lpr, showed reduced proteinuria and renal damage when supplemented with tRA (81-85). In our study (85), although tRA treatment improved lupus-like kidney disease in MRL/lpr mice, there were serious side effects: worsened inflammation in the skin, brain and lung, as well as increased levels of circulating autoantibodies. Our findings suggest the need to monitor diverse organs in SLE patients if tRA were used as a treatment, avoiding any potential damage to organs other than the kidneys.

PUFAs, with the main representative being omega-3 fatty acid, have been studied as complementary or alternative treatments for SLE for many years. Omega-3 PUFAs cannot be synthesized by the human body or other mammals. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are two well-recognized members of omega-3 PUFAs that are found in deep sea cold water fish. Fish oil is thereby utilized in some animal studies and clinical trials to test the efficacies of omega-3 PUFAs. In 1980s, DHA and EPA were both demonstrated to ameliorate renal disease, reduce anti-dsDNA autoantibody levels, and prolong lifespan of NZB/W F1 mice (86-88). It was found that fish oil prevents murine lupus by reducing levels of various pro-inflammatory cytokines, including IL-1 $\beta$ , IL-6, TNF $\alpha$  and TGF $\beta$ , and increasing the expression of antioxidant enzymes (89-95). In addition, Fernandes and colleagues found that DHA-enriched fish oil, compared to EPA-enriched fish oil, was better at attenuating renal disease and increasing the survival of NZB/W F1 mice (90). This suggests that the relative abundance of EPA and DHA in fish oil might impact the outcomes of experiments designed to examine the effects of fish oil on SLE. Moreover, a recent study reported that omega-6 PUFAs did not have the same beneficial effect on lupus nephritis as omega-3 PUFAs (96). The disease-ameliorating effect of omega-3 PUFA against murine lupus was further confirmed in several lupus-prone mouse models other than NZB/W F1 (86, 97, 98). Starting late 1980s, more than 10 interventional studies with omega-3 PUFAs as treatments have been done in patients with SLE. Some studies showed promising results, especially for SLE patients with cardiovascular disease, which has emerged as an important cause of death in patients with SLE (99, 100).

While VD, VA and PUFAs are known to change the composition of gut microbiota (21, 101, 102), how different dietary components modulate the microbiota of SLE patients and subsequent disease is unclear. One recent study has described diet-mediated increases of specific

microbial genera that are known to be lower in SLE (103). Further studies are necessary to determine whether the modulation of diet—likely to be less expensive and safer than immunosuppressive drugs—can be effective at establishing a healthy balance between the host and symbiotic microbiota in the gut of SLE patients. If so, diet modulation might become a cost-effective approach for the management of SLE.

### **Bacterial antigens & SLE**

Bacteria constitute a large part of the symbiotic microbiota living in our body. Diverse components of Gram-positive and Gram-negative bacteria have been reported to contribute to the initiation and maintenance of lupus disease through stimulating TLRs, especially TLR2 and TLR4. TLRs are pattern recognition receptors that can recognize invading microorganisms bearing pathogen-associated molecular patterns (104). Details of TLR signaling pathways and their effects on autoimmune diseases, including SLE, have been reviewed elsewhere (105). In the current review, we will focus on the roles of bacterial antigens in lupus and their possible link to the sex bias observed in SLE. We hypothesize that commensal bacteria naturally present in our microbiota might provide autoantigens that mediate the development of SLE.

#### ***Lipopolysaccharide (LPS)***

LPS is a Gram-negative cell wall component that can be recognized by TLR4. In SLE patients, soluble CD14 (sCD14), which is released by monocytes in response to LPS, is increased in the blood (106). The level of sCD14 is highly correlated with disease activity parameters, suggesting the involvement of LPS in lupus development. In addition, repeated injections of LPS into lupus-

prone mice resulted in increased autoantibody production and development of glomerulonephritis (107-111). Activation of TLR4 also promotes lupus disease activity in transgenic mice (107, 112, 113). Lupus spontaneously develops in mice with overexpression of a molecular chaperone of TLR4 that increases its responsiveness; but when commensal bacterial flora was deleted through treatment with antibiotics, the enhanced lupus phenotype was largely ameliorated (107). This suggests that TLR4 hyperresponsiveness to gut flora (which contains LPS) plays an essential role in lupus development. Moreover, Ni and colleagues found increased levels of serum autoantibodies and more severe lung injury when challenging apolipoprotein E-deficient (ApoE<sup>-/-</sup>) mice with LPS (114). Furthermore, immunization of nonautoimmune mice (C57BL/6 or BALB/c) with phospholipid-binding proteins induced lupus-like disease, and this was facilitated by the presence of LPS (115-117). Taken together, these data suggest that enhanced TLR4 signaling by LPS stimulation is sufficient to induce SLE. LPS might do so by inducing neutrophil activation and migration (118-120), key processes that promote the development of SLE (72). Inhibition of TLR4, on the other hand, reduces autoantibody production and decreases glomerular IgG deposits in the kidney for some lupus-prone murine models (121, 122). However, in TLR4-knockout MRL/lpr mice, disease activity was not modified (123). This may be due to the different genetic backgrounds of the mice strains. Further testing of disease outcome through TLR4 knockout should be done in additional strains of lupus-prone mice to determine the role of TLR4 deficiency in lupus.

In addition to the effect of LPS on neutrophil activation (118-120), several recent studies have explored the mechanisms by which LPS induces lupus. Qin et al. reported that the interaction of TLR4 and LPS strongly induced CD40 expression in macrophages and microglia (124). It was also found that LPS had the ability to increase CD40 mRNA expression in various

tissues, including liver and kidney, in NZB/W F1 mice (125). CD40 silencing reduced the glomerular deposits of IgG and C3 in these mice, revealing a possible role of LPS-TLR4-CD40 signaling in the pathogenesis of lupus. Another possible role for LPS-TLR4 in lupus is to induce autoantibody production or isotype switching towards more pathogenic immunoglobulins, like IgG (126). Both MyD88- and TRIF-mediated signaling pathways are believed to contribute to increased autoantibody levels, though TRIF may play a more important role in driving autoantigen-specific IgG response (126). Moreover, it has been found that IL-18 is induced by LPS stimulation and this cytokine may cooperate with LPS-TLR4 in breaking the tolerance in mice with lupus nephritis (127).

SLE is a female-biased disorder. Accumulating evidences have linked TLR4 function to estrogen and estrogen receptor  $\alpha$  ( $ER\alpha$ ). Studies by Gilkeson's group have found that female SLE patients possess more active monocytes with enhanced TLR4 responsiveness than male SLE patients (128). In lupus-prone mice,  $ER\alpha$  deficiency ameliorated renal damage and prolonged survival compared to  $ER\alpha$ -sufficient controls (129). Importantly, knocking out  $ER\alpha$  in both lupus-prone and control mice resulted in impaired TLR4 activation in immune cells, indicating that estrogen and ER signaling can influence TLR4 responsiveness (130, 131). These results suggest possible contribution of TLR4 activation to sex bias in SLE.

### ***Other bacterial antigens***

Lipoteichoic acid (LTA), a major component of Gram-positive bacterial wall, is also reported to be involved in lupus pathogenesis. LTA is a ligand for TLR2, whose expression is increased in T cells, B cells and monocytes from SLE patients (132). Increased TLR2 leads to enhanced IL-17A

and IL-17F production and is associated with inflammatory response of CD4<sup>+</sup> T cells. In mice, TLR2 activation is known to trigger lupus nephritis (133). In both B6/lpr mice and pristinely induced lupus mice, TLR2 knockout resulted in decreased autoantibody levels and ameliorated lupus-like symptoms (121, 134, 135). However, like the deficiency of TLR4, in MRL/lpr mice, TLR2 deficiency did not affect lupus pathogenesis (123, 136), possibly due to mouse strain differences.

Another bacterial antigen and component of bacterial biofilms, amyloid fiber (curli), has been reported to induce autoantibody production (137). Amyloid fibers can tightly bind to extracellular DNA that exists in many bacterial biofilms. Amyloid-DNA composites have been found to be strong stimulators of both innate and adaptive responses, with the ability to promote IL-6 and TNF $\alpha$  production and type I interferon response in NZB/W F1 mice (138). Importantly, injection of curli-DNA composites greatly increased the autoantibody level in lupus-prone mice, and even stimulated autoantibody production in wild-type mice. Using an amyloid-induced lupus model, Cao and colleagues have recently uncovered important roles of natural killer cells and IFN $\gamma$  in SLE pathogenesis downstream of type I interferon response (139).

### **The “SLE Microbiota”**

The significance of symbiotic microbiota in the development of T1D has been shown in non-obese diabetic mice, which spontaneously develop T1D with a bias towards females (11, 12). The function of microbiota in T1D is found to be highly associated with sex hormones. Fecal



transplant of male gut microbiota to female mice ameliorated the disease and increased testosterone. For SLE, although the initial comparison between lupus-prone mice in germ-free vs. conventional housing conditions showed no difference in disease severity (140), emerging evidences in both SLE patients and lupus-prone mice point to a potential link between lupus and microbiota (**Fig. 1**).

Intestinal dysbiosis has been reported in SLE patients. Compared to age- and sex-matched healthy controls, the fecal *Firmicutes/Bacteroidetes* ratio was found to be significantly lower in SLE patients even during remission (22). The same research group also described alterations in the composition and metabolic functions of gut microbiota in SLE (63). In mice, a recent study has shown that ANA production, a hallmark feature of autoimmune diseases that include SLE, is affected by neonatal colonization of gut microbiota (141). Using mice deficient of lymphotoxin- $\beta$  receptor (LT $\beta$ R)—the signaling of which controls development of secondary lymphoid organs—the authors found that LT $\beta$ R-expressing ROR $\gamma$ T<sup>+</sup> innate lymphoid cells, located in the intestinal lamina propria, were important for the maintenance of immunological tolerance. Importantly, it was found that antibiotics-mediated removal of segmented filamentous bacteria (SFB) inhibited the development of ANA (141). However, in another recent study, SFB were found to be unassociated with the outcome of lupus in (SWR $\times$ NZB)-F1 (SNF1) mice (23). When given acidic pH water, SNF1 mice showed slower development of nephritis and a lower level of circulating ANA, and the improved outcome was associated with changes of gut microbiota unrelated with SFB (23). In their study, the relative abundance of *Lactobacillus* and the ratio of *Firmicutes/Bacteroidetes* were higher in mice with lower lupus severity (23). These changes were consistent, respectively, with our results in MRL/lpr mice (21) and the findings of microbiota composition in human SLE patients (22). The same authors have also reported the

role of gut immune cells in female-biased development of lupus in SNF1 mice (142). Compared to male counterparts, the gut mucosa of female SNF1 mice has a higher frequency of gut-imprinted  $\alpha 4\beta 7$  T cells, higher expression of type I interferons, and a larger number of cells secreting IL-17, IL-22 and IL-9 (142). Altogether the intestinal microenvironment, including microbiota, immune cells and cytokines, could contribute to the development of lupus.

Our research group has recently found that, in female lupus-prone mice, there are significant reduction of *Lactobacillaceae* and increase of *Lachnospiraceae* both prior to disease onset and in the late stage of disease with severe lupus symptoms (21). We also found that lupus-like symptoms, including nephritis, were improved with oral treatment of tRA. Importantly, the improvement was highly associated with the ability of tRA to restore *Lactobacilli* (21). Our work shows the potential benefits of modulating gut microbiota, especially by increasing the level of *Lactobacilli*, in the treatment of lupus. *Lactobacilli* can be introduced as probiotics, which are known to be beneficial to the host when administered in adequate amounts. Proposed health benefits provided by the consumption of *Lactobacilli* include prevention of constipation, hepatic disease, infections, allergies, and as recently suggested, inhibition of autoimmune diseases such as IBD and T1D (143-149). Some *Lactobacillus* strains have been demonstrated to exert specific effects that include modulation of host microbiota, inhibiting the formation of NETs, improving antioxidant status, or increasing the expression of genes encoding junction and adhesion proteins (150-152). This suggests an attractive prospective of utilizing certain strains of *Lactobacillus* in disease management for SLE.

To directly examine the potential effects of sex and gut microbiota on SLE, one approach would be to correct the imbalanced microbial composition associated with SLE with fecal transplantation—from healthy individuals to patients, or from males to females—and see if

the correction ameliorates disease symptoms. This is yet to be reported for either lupus-prone mouse models or SLE patients, and remains an area that researchers actively explore.

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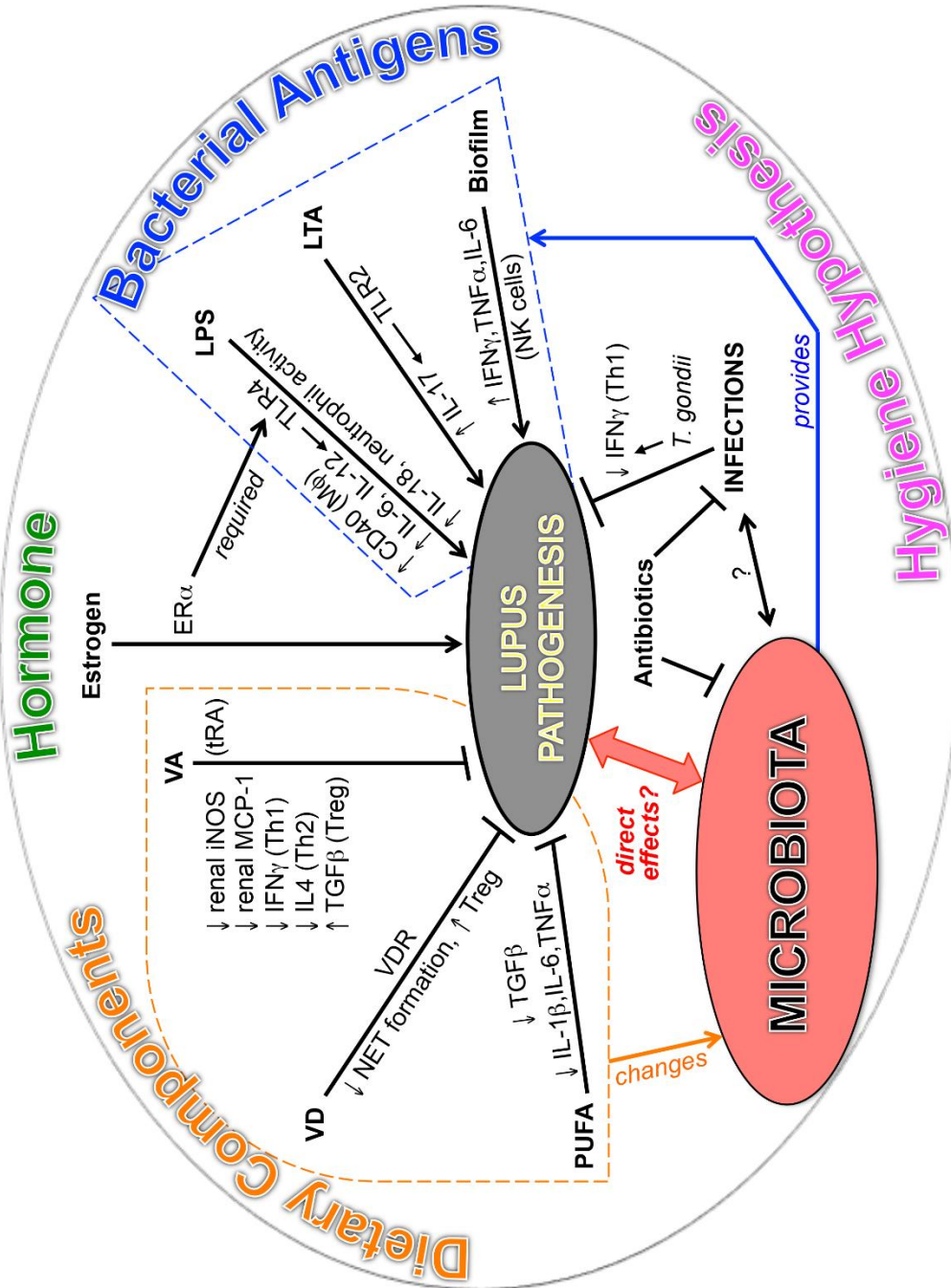
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# FIGURES

Figure 1. Emerging evidences point to a potential link between SLE and microbiota.



## CHAPTER II

### **Leaky Gut as a Danger Signal for Autoimmune Diseases**

**(Literature Review)**

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## **Abstract**

The intestinal epithelial lining, together with factors secreted from it, forms a barrier that separates the host from the environment. In pathologic conditions, the permeability of the epithelial lining may be compromised allowing the passage of toxins, antigens, and bacteria in the lumen to enter the blood stream creating a “leaky gut.” In individuals with a genetic predisposition, a leaky gut may allow environmental factors to enter the body and trigger the initiation and development of autoimmune disease. Growing evidence shows that the gut microbiota is important in supporting the epithelial barrier and therefore plays a key role in the regulation of environmental factors that enter the body. Several recent reports have shown that probiotics can reverse the leaky gut by enhancing the production of tight junction proteins; however, additional and longer-term studies are still required. Conversely, pathogenic bacteria that can facilitate a leaky gut and induce autoimmune symptoms can be ameliorated with the use of antibiotic treatment. Therefore, it is hypothesized that modulating the gut microbiota can serve as a potential method for regulating intestinal permeability and may help to alter the course of autoimmune diseases in susceptible individuals.

## Introduction

For digestion and absorption purposes, mammals have developed a very complicated and highly specialized gastrointestinal system maintained by the mucosal barrier (1). However, apart from absorbable nutrients, the intestinal mucosa also faces tremendous exterior antigens, including food antigens, commensal bacteria, pathogens, and toxins. Thus, a specialized barrier function is required to block the entry of diverse exterior antigens while absorbing nutrients. Impressively, in the intestine, the front line of this barrier is maintained by only a single layer of specialized epithelial cells that are linked together by tight junction proteins. Many other factors aid in support of this barrier including mucins, antimicrobial molecules, immunoglobulins, and cytokines. If any abnormalities occur among these factors, the intestinal permeability may increase, which is termed a “leaky gut.” A leaky gut allows the entry of exterior antigens from the gut lumen into the host which may promote both local and systemic immune responses. Multiple diseases may arise or be exacerbated due to a leaky gut, including autoimmune diseases such as inflammatory bowel disease, celiac disease, autoimmune hepatitis, type 1 diabetes (T1D), multiple sclerosis, and systemic lupus erythematosus (SLE) (2-6). Numerous factors can affect gut permeability, such as various diet-derived compounds, alcohol consumption, and gut microbiota dysbiosis. While this review is focused on chronic inflammation and gut barrier functions in mammals, it is worth noting that leaky gut is a phenomenon that is widespread in both mammalian and non-mammalian animals (7). Thus, studies in systems outside of mammals, such as zebrafish (7, 8), can be also helpful in our understanding of the relationship between inflammation and the intestinal barrier.

The gut microbiota has drawn intense attention in the past decade (9). Although scientists have studied gut microbiota for many years, recent advancements in molecular biology including next generation sequencing technology has enabled researchers to gain new insight in this research field. While we are still far away from clearly understanding the exact roles and effecting modes of gut microbiota, growing evidence suggests that gut microbiota is important in modulating gut permeability and intestinal barrier functions. In this review, we summarize recent advances in the understanding of the leaky gut, bacterial translocation, and gut microbiota dysbiosis, with a particular focus on their association with extraintestinal autoimmune diseases, such as T1D and SLE.

### **The Intestinal Barrier**

A large variety of exogenous substances colonize the gut lumen, such as microorganisms, toxins and antigens. Without an intact and properly functioning intestinal barrier, these substances can penetrate the tissues beneath the intestinal epithelial lining, diffuse into blood and lymphatic circulations, and disrupt tissue homeostasis. However, there is an efficient multi-faceted intestinal barrier system with physical, biochemical and immunological components that prevents the entry of most pathogens (Fig. 1). These components coordinate with each other to prevent uncontrolled translocation of luminal contents into the body. Below is a brief synopsis of the main components comprising the intestinal barrier.

#### ***Physical barrier***

In humans, the intestinal epithelium covers as large as 400 m<sup>2</sup> of surface area (1). Though only a single layer of cells, the intestinal epithelial cells (IECs) are the mainstay of the intestinal barrier and serve as a physical barrier (Fig. 1). There are at least seven types of functional IECs – enterocytes, goblet cells, paneth cells, microfold cells (M cells), enteroendocrine cells, cup cells and tuft cells, although the functions of the last two cell populations are not well understood (10). Among all these cell types, enterocytes represent the absolute majority, accounting for at least 90% of crypt cells or villus cells. Enterocytes are absorptive cells and vital for nutrient uptake. However, growing evidence indicates that the functions of enterocytes are not limited to nutrient absorption. For example, enterocytes can control the abundance of Gram-positive bacteria by expressing RegIII $\gamma$ , one type of antimicrobial proteins (AMPs) (11-13). All epithelial cell types originate from Lgr5<sup>+</sup> intestinal epithelial stem cells, which reside within the crypts (14). The turnover rate of IECs is high and the cells are renewed every 3-5 days in the mammalian intestine (10, 15), with the exception being the paneth cells, which have a life span of about 2 months.

The intestinal epithelial cell lining is continuous, and the contact between IECs is sealed by tight junctions (TJs) (16). The paracellular pathway, in contrast to transcellular pathway, allows the transport of substances across the gut epithelium through the spaces between IECs. A large variety of molecules, mainly proteins, control the plasticity of TJs. More than 40 TJ proteins have been recognized, including occludin, claudins, junctional adhesion molecule A and tricellulin (17). Under various pathological conditions, paracellular permeability may be increased, resulting in the entry of unwelcome, potentially harmful molecules.

On top of the gut epithelium there are two layers of mucus, the inner and outer layers, that cover the whole intestinal epithelial lining and provide physical protection to separate

luminal microorganisms from the epithelium. Organized by its major component, a highly glycosylated gel-forming mucin MUC2, the mucus contains diverse molecules including IgA as well as enzymes and proteins, such as lactoferrin (18). Goblet cells are the central cell type for the formation of mucus. They not only produce MUC2 mucin, but also secrete other mucus components such as ZG16, AGR2, FCGBP, CLCA1 and TFF3 (19, 20). Colitis would spontaneously develop in Muc2-deficient mice, indicating a critical role for MUC2 in mucosal protection (21). In addition to gel-forming mucins, there is another type of mucin that is in close proximity to epithelial cells, called transmembrane mucins. Enterocytes are the main producers of transmembrane mucins (20).

The gut commensal bacteria have been described as one component of the intestinal physical barrier primarily due to its two major functions (22). The first is to promote resistance to the colonization of harmful or pathogenic bacteria species by competing for nutrients, occupying attachment sites, and releasing antimicrobial substances (23, 24). Additionally, the gut microbiota regulates the digestion and absorption of nutrients to supply energy to epithelial cells, which are a major component of the physical barrier (25). A good example of the direct energy supply is the production of short-chain fatty acids by the gut microbiota, which are used by colonocytes for their development and metabolism (26). Taken together, IECs, the mucus layers, and gut microbial residents serve as the physical barrier to limit the entry of unfriendly luminal contents into host tissues.

### ***Biochemical barrier***

Biochemical molecules with antimicrobial properties exist in the mucus as well as far into the lumen, and include bile acids and AMPs (27, 28) (Fig. 1). These diverse molecules form a



complicated network to reduce the load of colonized bacteria and decrease the chance of contact between luminal antigens and host cells. They are a good supplement to the physical barrier and an essential component of the intestinal barrier function.

The proximal small intestine harbors very few microorganisms (29). But as the distance from the stomach increases, the pH rises and the number of colonized bacteria escalates (30). Facing a large number of microorganisms, which likely outnumber the number of host cells, multiple AMPs are generated to fight against invaders. These AMPs are divided into several types, including  $\alpha$ - and  $\beta$ -defensins, C-type lectin, cathelicidin, lysozyme, and intestinal alkaline phosphatase (IAP) (27). Their detailed antimicrobial mechanisms are discussed elsewhere (31). As a major, but not exclusive, producer of AMPs, paneth cells support and mediate the biochemical barrier function.

### ***Immunological barrier***

Below the intestinal epithelium there are organized lymphoid follicles, including the Peyer's patches and isolated lymphoid follicles. Inside the follicles, a variety of immune cells, including B cells, T cells, dendritic cells (DCs) and neutrophils, orchestrate the immune response by presenting antigens, secreting cytokines, and producing antigen-binding antibodies (Fig. 1). In the intestinal epithelium where lymphoid follicles are found, M cells are present that transcytose antigens across the intestinal epithelium to the Peyer's patches underneath (14). In addition, goblet cells present acquired luminal antigens to CD103<sup>+</sup> DCs in lamina propria in small intestine by forming goblet cell-associated antigen passages (GAPs) (32, 33). Interestingly, spontaneous antigen presentation was also observed in the colon, but only when the mice were

raised germ-free (GF), or housed conventionally but with oral antibiotic treatment (34). This suggests that the antigen uptake process and formation of GAPs are regulated by the colonic microbiota (35). In addition, goblet cells and GAPs are capable of sensing invasive pathogens and inhibiting the translocation of pathogenic bacteria into the host immune system (36). Furthermore, intestinal mononuclear phagocytes can sense and sample luminal contents (37, 38). CX3CR1-expressing cells are responsible for this process, and antigen sampling is dependent on structures called trans-epithelial dendrites (TEDs) (39, 40). The formation of TEDs is regulated by CX3CR1<sup>+</sup> macrophages and the expression of CX3CL1 by certain IECs (41, 42).

Another component of the immunological barrier is secretory IgA (SIgA). As the most abundant immunoglobulin in the body, IgA resides primarily on intestinal mucosal surfaces. While some people with selective IgA deficiency appear to be healthy, SIgA is important as it presumably interacts with commensal bacteria to provide protection against pathogens. A unique feature about SIgA is that it is structurally resilient in protease-rich environments allowing it to remain functionally active compared to other antibody isotypes on mucosal surfaces (43). In adult humans, about 50 mg/kg of SIgA is produced daily by plasma cells residing in the intestinal lamina propria. Finally, SIgA can be transcytosed through the epithelium and secreted into the gut lumen.

Though not mentioned here, self-modulating factors, such as nerves and diverse cytokines, are also important for maintaining the normal functions of the intestinal barrier.

## Gut Microbiota and the Intestinal Barrier

Microbiota can be sensed by the host through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs). In the gut, the bacteria-host communications are largely dependent on the recognition of microbe-associated molecular patterns (MAMPs) by PRRs expressed on immune and non-immune cells. Certain microbiota, bacterial products and metabolites affect the intestinal barrier function and are responsible for the subsequent breakdown of tissue homeostasis. When there is a leaky gut, commensal bacteria in gut lumen, together with their products, are able to escape the lumen of the gut, which may induce inflammation and cause systemic tissue damages if translocated into peripheral circulation (Fig. 1). This process of translocation is called microbial translocation (44).

Evidence from GF animals suggests that the development and function of the intestinal barrier are dependent on microbiota. In GF animals, due to the lack of bacterial stimulations, the thickness of the mucus layers is extremely reduced (45-48). The important role of gut microbiota in modulating mucin production from goblet cells is further evidenced in animals with lower loads of bacteria (49, 50). The thinner mucus layers would allow for bacteria penetration, which may initiate inflammation and inflammatory diseases such as colitis (46, 51). Commensal bacteria, or bacterial products such as lipopolysaccharide (LPS) and peptidoglycan, can restore the mucus layers (46, 47). A balance exists between commensal bacteria and the mucus layers, and together they contribute to the maintenance of gut homeostasis (48). Within the mucus layers, there are diverse secreted AMPs that can clear pathogens and control the colonization of commensal bacteria. Reciprocally, the production of some AMPs are regulated by microbiota and/or their products. For instance, RegIII $\gamma$  is the AMP necessary for physically separating

commensal bacteria from intestinal epithelium (11). RegIII $\gamma$  has been shown to be suppressed in alcoholic patients and mice receiving ethanol treatment (52, 53). Prebiotics administration, or increasing probiotic *Lactobacilli* and *Bifidobacteria*, has been shown to restore the properties of RegIII $\gamma$  and control bacterial overgrowth (53). Ang4, a member of Angiogenin family, is another example where gut commensals are known to modulate AMP production. In one study, Gordon and coworkers found that the production and secretion of Ang4 from mouse Paneth cells were induced by a predominant gut microflora, *Bacteroides thetaiotaomicron* (54). Therefore, the antibacterial activity of Ang4 against microbes in gut lumen is, in turn, dependent on the existence of certain commensal species.

In addition, an interaction exists between gut microbes and AMPs, such as IAP. Predominately produced by IECs, IAP is active either anchored on the epithelium membrane or secreted into gut lumen (55, 56). In IAP-deficient mice, it was noted that there were fewer microbes and an altered bacteria composition compared to control wild type animals. In particular, the researchers noted a decrease in *Lactobacillaceae* (57, 58). Upregulated IAP activity can selectively increase LPS-suppressing bacteria (e.g., *Bifidobacterium*) while reducing LPS-producing bacteria (e.g., *E. coli*) (59). Having the capacity to inactivate LPS *in vivo*, IAP is vital in preventing the translocation of LPS, the pro-inflammatory stimulus originated from bacteria (60, 61). Of note, the expression of IAP relies on the presence of microbiota. In GF zebrafish, the colonization of commensals, or even supplying LPS alone, could sufficiently induce IAP expression (62). It is worth mentioning that IAP can also regulate TJ proteins to enhance barrier function through increasing ZO-1, ZO-2 and Occludin expression (63). Several others have also reported on the various types of AMPs and their function in the microbiota (64, 65).

IECs compose the single layer of intestinal epithelium, and the generation of new IECs from local intestinal stem cells is vital in maintaining the barrier function due to the high frequency of apoptosis and shedding of IECs (66). As much as 10% of all the gene transcriptions, especially genes related to immunity, cell proliferation and metabolism, in IECs are regulated by gut microbiota (67). In GF and antibiotic-treated mice, epithelial proliferation rate is reduced, suggesting the role of microbiota on epithelium cell renewal (68, 69). LPS from *E. coli* can induce cell shedding in a dose-dependent manner (70, 71). Colonization of *Bifidobacterium breve*, or more precisely its surface component, exopolysaccharide, can positively modulate LPS-induced epithelium cell shedding through epithelial MyD88 signaling (70). The renewal of IECs relies on the activity of intestinal stem cells which are located at the base of crypts and express TLR4, the LPS receptor. TLR4 activation has been demonstrated to inhibit proliferation and promote the apoptosis of Lgr5<sup>+</sup> intestinal stem cells. In mice bearing selective TLR4 deletion in intestinal stem cells, LPS is no longer able to inhibit the renewal of IECs (72). This process was found to be mediated by the p53-upregulated modulator of apoptosis (PUMA) as TLR4 activation in mice lacking PUMA was unaltered. Apart from LPS, bacterial metabolites, particularly butyrate, have also been identified as inhibitors of intestinal stem cell proliferation (73). The intestinal crypt architecture protects the intestinal stem cells from the negative effect of butyrate. As gate-keepers for the paracellular pathway, TJ complexes are also major targets of microbiota regulation (74). This is particularly true for certain probiotic species including, but not limited to, *Lactobacillus rhamnosus* (75-78), *Streptococcus thermophiles* (79), *Lactobacillus reuteri* (80), and *Bifidobacterium infantis* (81).

## Mechanisms of Leaky Gut

A large variety of gut barrier disruptors and/or gut microbiota disturbers may potentially result in microbial translocation and subsequent inflammation locally and systemically. These include diet, infections, alcohol consumption, and burn injury.

### *Diet-induced gut leakiness*

Nutrients and food ingredients have been reported to contribute to the maintenance or alterations of gut microbiota and the intestinal barrier function (82). A recent review by De Santis et. Al. detailed many dietary factors that may modulate the intestinal barrier. (83). Here, we review some recent publications and emphasize the effects of diet-induced alterations of gut microbiota on compromising the gut barrier function. Vitamin D (VD) has been recognized as an intestinal permeability protector by inducing the expression of TJ proteins ZO-1 and claudin-1. In VD receptor (VDR)-knockout mice, more severe experimental colitis has been observed, suggesting the protective effect of VD on the mucosal barrier (84). However, another group have recently found that VDR-deficiency lowers, whereas VD treatment upregulates, the expression of claudin-2, a pore-forming TJ protein, which renders the intestinal epithelium leaky (85). Further analysis confirmed that VDR enhanced claudin-2 promoter activity. The exact role of VD and VDR on modulating intestinal permeability is therefore unclear and should be investigated carefully in association with gut microbiota. In a recent study by Desai et al, a low-fiber diet consumption was found to trigger the expansion of mucus-degrading bacteria, including *Akkermansia muciniphila* and *Bacteroides caccae* (45). As a result, the thickness of mucus is significantly decreased in mice fed with fiber-deficient diets, although the transcription of *Muc2*

gene was surprisingly heightened, possibly as a compensatory response. The thinner mucus and compromised intestinal barrier function lead to a higher susceptibility to certain colitis-causing pathogens (45). Moreover, a diet high in saturated fat has been shown to greatly decrease *Lactobacillus* and increase *Oscillibacter*, and these changes were correlated with significantly increased permeability in the proximal colon (86). Furthermore, studies revealed that the abundance of the *Oscillospira* genus was negatively correlated with the mRNA expression of barrier-forming TJ protein ZO-1.

### ***Stress-induced gut leakiness***

Under certain circumstances, stress-induced alterations of gut microbiota and the impaired intestinal barrier would allow the occurrence of microbial translocation. Burn injury and alcohol consumption are examples of such stress. Burn injury results in increased intestinal permeability, which is mediated by increased activity of Myosin light chain (MLC) kinase (87, 88). It is known that MLC phosphorylation or kinase activation can trigger epithelial TJ opening (89-91). In burn injury, TJ proteins, including ZO-1, occludin and claudin-1, are redistributed, which can be reversed by adding an MLC phosphorylation inhibitor (87). In addition, both humans and mice experiencing burn injury undergo similar alterations of gut microbiota, in particular, with increases of the abundance of bacteria from the *Enterobacteriaceae* family (88). Importantly, microbial translocation of these Gram-negative aerobic bacteria has been observed. Another research group, using a different burn injury mouse model reported increased colonic permeability together with reduced aerobic and anaerobic bacterial populations in the gut microbiota, particularly those producing butyrate (92). As a consequence, the butyrate level in

the stool was significantly decreased in mice with burn injury. Interestingly, when the experimental mice received fecal microbiota transplant, their altered bacterial counts and impaired mucosal barrier function were reversed, suggesting direct involvement of microbiota in causing gut leakiness after burn injury.

Chronic alcohol consumption is responsible for intestinal barrier dysfunction, alterations on both the quality and quantity of gut microbiota, LPS translocation and alcoholic liver disease (ALD). In both human and mouse, it has been well established that alcohol can disrupt intestinal barrier function, which is closely related to increased tumor necrosis factor (TNF) production from intestinal monocytes/macrophages and enterocytes bearing TNF-receptor 1, followed by downstream activation of MLC kinase (93). Notably, when mice given chronic alcohol also received oral antibiotic treatment, to remove the microbiota, the level of TNF production and intestinal permeability decreased to levels comparable to those in control mice (93). This indicates that the alcohol-induced, TNF-mediated gut leakiness is greatly dependent on gut microbiota. Indeed, though the mechanism is unknown, alcohol administration alters microbiota qualitatively and quantitatively in both human and mouse (94). Bacterial overgrowth has been observed with alcohol consumption, whereas antibiotics can decrease the bacterial load and attenuate ALD (53, 93, 95-97). Interestingly, probiotic *Lactobacillus* is significantly suppressed during alcohol consumption (53, 97). Directly supplying *Lactobacillus* strains or indirect stimulation of *Lactobacilli* with prebiotics or diets can decrease bacterial overgrowth, restore mucosal integrity of the intestine, and suppress microbial translocation (53, 94, 98, 99). Microbial translocation, especially the translocation of LPS, is involved in ALD development and progression as evidenced by the lack of ALD in mice deficient of TLR4 (100, 101). It is worth noting that some bacteria species can produce alcohol, including *E. coli* and *Weissella*



*confusa*, and this may be the mechanism by which they compromise the intestinal barrier function (102, 103).

Infections can play a role in regulating the mucosal barrier. A good example is *Helicobacter pylori*, a Gram-negative bacterium infecting the human stomach (104). *H. pylori* is known to directly increase epithelial permeability by redistributing TJ protein ZO-1 (105, 106). In addition, bacteriophages, which are usually not considered pathogenic to mammals, can have an impact on the leaky gut. When rats were given a bacteriophage cocktail containing phages against *Salmonella enterica*, disruption of the intestinal barrier integrity was observed (107). The authors speculated that the gut microbiota might have been affected by bacteriophages, but sequencing data was not supplied to support their claims.

Taken together, perturbation of gut microbiota, which may be the consequence of diverse interventions, can lead to increased intestinal permeability and translocation of bacterial components and products. Such microbial translocation can subsequently trigger an abnormal immune response, causing inflammation and/or tissue damage in extraintestinal organs.

### **Leaky Gut and Autoimmune Disorders**

Several disease states have been associated with gut microbiota dysbiosis, intestinal barrier dysfunction, and microbial translocation. These include Alzheimer's disease, ALD, cancer, and multiple autoimmune disorders. Autoimmune disorders are characterized by the generation of autoantibodies against self-antigens that attack the body's own tissues, resulting in damage.

Genetic and environmental triggers have been long known as the major contributors to the development of autoimmunity. Increasing evidence in recent years suggests that microbial translocation and intestinal barrier dysfunction, which may be affected by gut microbiota, are another important causative element for autoimmune disorders (2-6). T1D and SLE are examples discussed below that reveal advancements in the understanding of the mechanisms behind the interaction between the leaky gut and autoimmune disorders.

### ***T1D***

T1D is an organ-specific autoimmune disorder characterized by an autoimmune response against the host's own pancreatic  $\beta$  cells, leading to insufficient insulin production from the pancreas (108). Some argue that the leaky gut is only an outcome of disease progression rather than an initiator or exacerbator of disease (109), but this should not be the case for T1D. This is supported by the following evidences. Firstly, studies utilizing human subjects affected by T1D or T1D-prone animal models have indicated that impaired intestinal barrier function occurs before disease onset (110-112). Secondly, the pathogenic role that increased intestinal permeability plays in T1D is zonulin-dependent, and the production of zonulin relies on bacterial colonization (113). Reversion of intestinal barrier dysbiosis by adding a zonulin inhibitor ameliorated T1D manifestations in disease-prone rats (114). Thirdly, a recent study has provided evidence that microbial translocation contributes to T1D development (115). In streptozotocin-induced T1D, mice treated with streptozotocin harbor a distinct microbiota compared to vehicle-treated controls. Importantly, gut bacteria were shown to be able to translocate into pancreatic lymph nodes (PLNs) and contribute to T1D development (115). When mice were treated with

oral antibiotics, PLNs appeared to be sterile and the disease was attenuated. Further analysis revealed that the translocated bacteria in PLNs triggered NOD2 activation and exacerbated T1D. Altogether, these results suggest an essential role for the leaky gut in driving the progression of T1D.

### ***SLE***

SLE, or lupus, is an autoimmune disorder characterized by severe and persistent inflammation that leads to tissue damage in multiple organs (116). Although SLE affects both men and women, women of childbearing age are diagnosed about 9 times more often than men. LPS, a cell wall component of Gram-negative bacteria, can promote SLE development and disease progression upon penetration of the intestinal epithelium and translocation into tissues (117). In SLE patients, the higher level of soluble CD14 suggests an increase in LPS, as soluble CD14 is released from monocytes when the cells are exposed to LPS (118). Activation of TLR4 exacerbates lupus development (119-121). Mice spontaneously develop lupus when TLR4 responsiveness is increased, whereas the exacerbated disease phenotype can be significantly ameliorated when the commensal gut flora is removed by antibiotic treatment (121). This clearly indicates that TLR4 hyper-responsiveness to gut flora (which contains LPS) contributes to the pathogenesis of SLE. Moreover, the development of lupus in wild type mice (C57BL/6 or BALB/c) immunized with phospholipid-binding proteins can be facilitated by the administration of LPS (122-124). Conversely, inhibition of TLR4 results in reduced autoantibody production and lowered renal glomerular IgG deposits in lupus-prone mice (125, 126). Taken together, these data suggest LPS stimulation and TLR4 activation as disease-initiating factors for SLE.

Lipoteichoic acid (LTA), a component of the Gram-positive bacterial cell wall, can also promote lupus disease. The expression of TLR2, the receptor of LTA, has been reported to be increased in SLE patients (127). In lupus-prone mice, TLR2 activation triggers lupus nephritis whereas TLR2 knockout attenuates lupus-like symptoms (125, 128-130). Recently, another bacterial antigen that may mimic self-antigens has been recognized to induce autoantibody production (131).

Several downstream proteins in the TLR signaling cascade are highly relevant to the pathogenesis of SLE and are potential therapeutic targets, including MyD88, IRAKs, and IFN $\alpha$  (132). Deficiency of MyD88, in particular, has been shown to ameliorate lupus disease in MRL/lpr mice (133, 134), suggesting a potential role for TLRs to communicate with harmful bacteria in the gut microbiota. Conversely, there is a paucity of data pertaining to members of the NLR family. The most extensively characterized NLRs are associated with inflammasome formation (135, 136). Loss of NLRP3 and AIM2 inflammasome function was found to significantly contribute to lupus pathogenesis (137). Interestingly, both of these inflammasomes were found compromised in NZB mice, a lupus-prone model. Consistent with this finding, loss of ASC (apoptosis-associated speck-like protein containing CARD), a common adaptor protein required for inflammasome formation in B6-*Fas<sup>lpr</sup>* mice led to exacerbation of lupus-like disease (138). These results suggest a potential role for NLRs to recognize protective bacteria in the gut microbiota. Therefore, it appears that TLRs and NLRs make distinct contributions to lupus pathogenesis by sensing harmful and protective bacteria, respectively. Both types of bacteria can come from gut microbiota through microbial translocation, especially in the presence of a leaky gut.

## Reversing the Leaky Gut as a Potential Therapy

Considering the contributions of leaky gut and bacterial translocation to inflammation and multiple diseases, reversing gut leakiness appears to be an attractive therapeutic strategy. Prebiotics and probiotics, for example, can be used to reduce intestinal permeability (139). Diverse probiotic species have been uncovered that possess the properties to protect the intestinal barrier through targeting different components of the mucosal barrier system. The human commensal *Bacteroides fragilis* may serve as such a probiotic (140). In a mouse model, autism spectrum disorder (ASD) has been shown to be accompanied by intestinal barrier dysfunction, gut microbiota dysbiosis and leakiness of 4-ethylphenylsulfate (4EPS), which originates from the commensal bacteria. When 4EPS was given to wild type mice, it directly caused behavioral abnormalities similar to ASD mice. Treatment with *B. fragilis* reduced the translocation of disease-causative 4EPS, and significantly ameliorated the behavior defects. The therapeutic benefit of *B. fragilis* is believed to be due to its ability to alter microbial composition and enhance intestinal barrier function (140). *B. fragilis* is also known for its capability to induce the development of Foxp3<sup>+</sup> regulatory T (Treg) cells, a process regulated by another product of *B. fragilis*, polysaccharide A (PSA) (141, 142). *B. fragilis* and PSA are beneficial against inflammatory diseases, such as colitis and experimental autoimmune encephalomyelitis (141, 143). The application of *B. fragilis* to prevent the leaky gut and reverse autoimmunity warrants further investigation. In a practical point of view, probiotic candidates with different targets on reversing the leaky gut may synergistically act to attenuate disease as thus may serve as a probiotic cocktail. As probiotics are generally considered safe, it is anticipated that they will become cost-effective treatment options for people with autoimmune diseases in the foreseeable future. This is a very young but exciting field in which much still remains to be learned.

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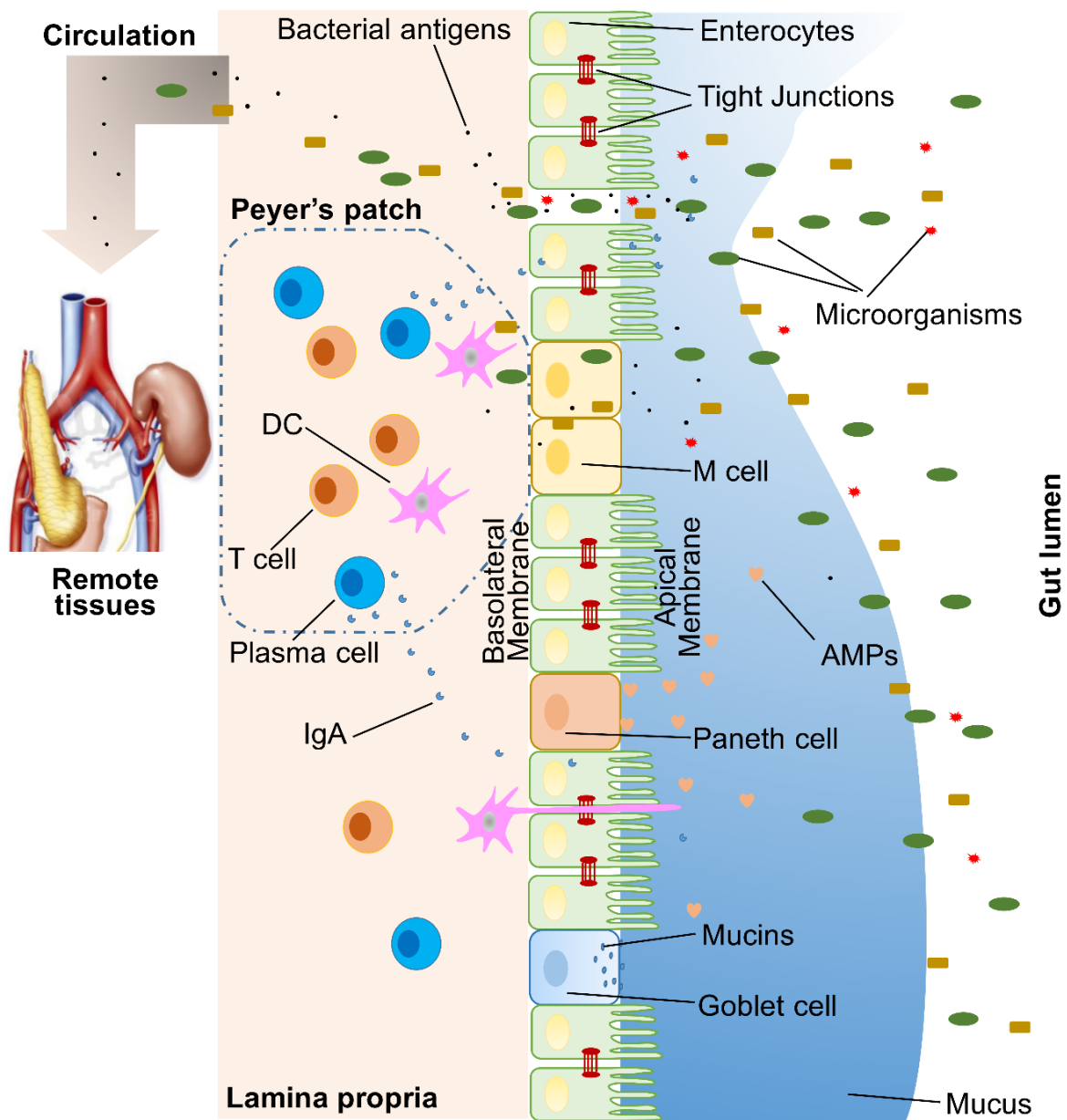


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## FIGURES

**Figure 1.** Illustration of host intestinal barriers, including physical barrier (epithelium, TJs, mucus, commensal bacteria), biomedical barrier (AMPs) and immunological barrier (lymphocytes and IgA). Also shown is the microbial translocation to remote tissues (for example kidney and pancreas) in the presence of a leaky gut.



## CHAPTER III

### Control of Lupus Nephritis by Changes of Gut Microbiota

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## ABSTRACT

**Background:** Systemic lupus erythematosus, characterized by persistent inflammation, is a complex autoimmune disorder with no known cure. Immunosuppressants used in treatment put patients at a higher risk of infections. New knowledge of disease modulators, such as symbiotic bacteria, can enable fine-tuning of parts of the immune system, rather than suppressing it altogether.

**Results:** Dysbiosis of gut microbiota promotes autoimmune disorders that damage extraintestinal organs. Here we report a role of gut microbiota in the pathogenesis of renal dysfunction in lupus. Using a classical model of lupus nephritis, MRL/*lpr*, we found a marked depletion of *Lactobacillales* in the gut microbiota. Increasing *Lactobacillales* in the gut improved renal function of these mice and prolonged their survival. We used a mixture of 5 *Lactobacillus* strains (*oris*, *rhamnosus*, *reuteri*, *johnsonii* and *gasseri*), but *L. reuteri* and an uncultured *Lactobacillus* sp. accounted for most of the observed effects. Further studies revealed that MRL/*lpr* mice possessed a “leaky” gut, which was reversed by increased *Lactobacillus* colonization. *Lactobacillus* treatment contributed to an anti-inflammatory environment by decreasing IL-6 and increasing IL-10 production in the gut. In the circulation, *Lactobacillus* treatment increased IL-10 and decreased IgG2a that is considered to be a major immune deposit in the kidney of MRL/*lpr* mice. Inside the kidney, *Lactobacillus* treatment also skewed the Treg-Th17 balance towards a Treg phenotype. These beneficial effects were present in female and castrated male mice, but not in intact males, suggesting that the gut microbiota controls lupus nephritis in a sex hormone-dependent manner.

**Conclusions:** This work demonstrates essential mechanisms on how changes of the gut microbiota regulate lupus-associated immune responses in mice. Future studies are warranted to determine if these results can be replicated in human subjects.

## BACKGROUND

Perturbation of gut microbiota is known to promote autoimmune disorders that include inflammatory bowel disease, type 1 diabetes, rheumatoid arthritis, and multiple sclerosis. However, little is known on the role of gut microbiota in systemic lupus erythematosus (SLE). SLE is a very complex autoimmune disorder with no known cure. It is characterized by severe and persistent inflammation that damages multiple organs, including skin, kidney, lung, joint, heart, and brain [1]. The prevalence ranges from 20 to 200 cases per 100,000 persons, with higher prevalence for people of African, Hispanic, or Asian ancestry. Although the disease affects both males and females, women of childbearing age are diagnosed 9 times more often than men. African-American women suffer from more severe symptoms and a higher mortality rate. More than half of SLE patients suffer from kidney inflammation, or lupus nephritis (LN), which is the leading cause of mortality by SLE. Current treatments for LN are primarily nonselective immunosuppressants [2]. While immunosuppression can effectively treat symptoms, unwanted side effects are a major cause of concern. Patients taking long-term immunosuppressants are prone to higher incidence of and more severe infections [3]. Therefore, there is an imperative need for new treatment strategies against LN. To accomplish this task, a better understanding of disease pathogenesis is required.

Current knowledge on the relationship between gut microbiota and SLE is limited [4]. In human SLE, a recent cross-sectional study showed dysregulated fecal microbiota of SLE individuals with a lower *Firmicutes* to *Bacteroidetes* ratio [5] that is consistent with gut dysbiosis observed in other autoimmune conditions [6, 7]. In mice, it has been reported that the lupus-prone MRL/Mp-*Fas*<sup>lpr</sup> (*lpr*) mouse model exhibits similar disease manifestations under specific pathogen-free and germ-free conditions [8]. This suggests that complete removal of microbiota

does not affect disease progression in these mice. The same phenomenon was observed in the pristane-induced lupus model [9]. However, completely depleting the microbiota might have neutralized the respective effects of “good” and “bad” microbes. Studies on germ-free New Zealand Black mice showed mixed results, with less renal disease but more anti-nuclear antibodies [10-12]. Our research team has recently described the dynamics of fecal/colonic microbiota in *lpr* mice that suggests a critical role of gut microbiota on lupus pathogenesis [13]. However, whether the change of gut microbiota is a driving force in SLE, or merely a result of disease status, remains unclear.

Here we show that intestinal permeability is increased in female *lpr* mice preceding the onset of kidney disease (i.e., a “leaky” gut), and that increasing gut colonization of *Lactobacillales* restores the mucosal barrier function and reduced kidney pathology. Such change in gut microbiota promotes an anti-inflammatory environment in the gut, suppressing expression of IL-6 in the mesenteric lymph node (MLN) while increasing the levels of IL-10 in circulation and periphery. In addition, the production and renal deposition of pathogenic IgG2a is repressed with increased *Lactobacillales*, suggesting a potential mechanism for the reduced kidney pathology. Moreover, we show that *Lactobacillus* spp. rebalances T cell subsets in the kidney, increasing regulatory T (Treg) cells and suppressing pathogenic T helper (Th)17 cells. This suggests another potential mechanism by which gut microbiota can modulate renal function. Interestingly, the effects of *Lactobacillus* spp. are only present in female and castrated male *lpr* mice, but not in intact males, indicating a role for sex hormones in the regulatory function of gut microbiota on lupus disease. Taken together, our results suggest that the presence of *Lactobacillus* spp. in the gut can attenuate kidney inflammation in lupus-prone mice in a sex hormone-dependent manner.



## RESULTS

### ***Lactobacillus* spp. attenuate LN**

When comparing the bacterial composition in the gut microbiota of lupus-prone *lpr* mice vs. MRL control mice, we found that female *lpr* mice had a significantly lower abundance of *Lactobacillales* in the gut microbiota than MRL controls at 5 weeks of age and prior to the onset of lupus-like disease (Figure S1A). However, it was unclear whether the change was a cause or result of disease initiation. Therefore, we performed reciprocal cecal microbiota transplantation experiments from MRL to *lpr* mice (Figure S1B) and vice versa. While the disease in MRL mice did not change after the transfer of cecal content from *lpr* mice (data not shown), MRL-to-*lpr* cecal transplantation led to significantly reduced production of autoantibodies against double-stranded (ds)DNA from the lower gastrointestinal tract (Figure S1C). Since the gut microbiota of young MRL mice contained a higher abundance of *Lactobacillales* than *lpr* mice, we sought to determine if the decrease in disease could be due to the elevated *Lactobacillales* in *lpr* mice that were transferred from MRL mice upon cecal transplantation. Indeed, *lpr* mice receiving MRL cecal content had more abundant *Lactobacillales* in the gut microbiota than untreated controls (Figure S1D), suggesting a positive correlation between a higher abundance of gut-colonized *Lactobacillales* and improved lupus symptoms.

The bacterial Order *Lactobacillales* includes *Lactobacillus* spp. that are known as beneficial bacteria. We thus examined the effect of these beneficial bacteria on *lpr* mice by directly inoculating freshly cultured *Lactobacillus* isolates (Figure S1E). We used a mixture of 5 *Lactobacillus* strains—*L. oris*, *L. rhamnosus*, *L. reuteri*, *L. johnsonii* and *L. gasseri*. Different *Lactobacillus* strains have been reported to exert different immunological functions [14, 15].

Among the 5 strains, all except *L. oris* are known to colonize the gut. To improve engraftment of *Lactobacillus* spp., we pre-treated the mice with ampicillin, neomycin, vancomycin and metronidazole for 2 days, followed by 2 days of resting to allow for excretion of the antibiotics prior to *Lactobacillus* treatment. The brief antibiotic treatment at the time of weaning did not change the disease severity (Figures S1F and S1G). We found that weekly gavages of *Lactobacillus* spp. significantly increased the relative abundance of *Lactobacillales* in the gut microbiota at weeks 5 and 7 (Figure 1A and Table S1), significantly reduced the level of autoantibodies in the circulation (Figure 1B), and significantly decreased proteinuria (Figure 1C) and renal pathology scores (Figure 1D). Spleen and MLN weights were not changed (Figure S1H). Importantly, *Lactobacillus* treatment significantly increased the survival of female *lpr* mice (Figure 1E). It is noteworthy *Lactobacillus* treatment was given starting from 3 weeks of age and before disease establishment. When given after the onset of lupus disease, *Lactobacillus* treatment had a trend to reduce lupus disease, but the difference was not statistically significant (data not shown). These results suggest that the introduction of more “good” bacteria in the gut microbiota—in this case, *Lactobacillus* spp.—may be able to prevent disease progression in lupus-prone mice. This supports the notion that gut microbiota can directly control LN. How the increase of Lactobacilli in the gut affects disease pathogenesis in the kidney, which is extraintestinal, was unknown. Therefore, we next sought to identify potential “messengers” that transduced the disease-modulating signal from the gut to the kidney.

### **A “leaky” gut in lupus-prone mice**

While 5 *Lactobacillus* strains were inoculated, we found by using 16S ribosomal RNA gene sequencing that, unexpectedly, two bacterial species accounted for >99% of the Order *Lactobacillales* regardless of treatment status. The species were *L. reuteri* and an uncultured *Lactobacillus* sp. (Figure 2A). The same phenomenon was observed for MRL mice (data not shown). This suggests that *L. reuteri* and the uncultured *Lactobacillus* sp. accounted for most of the observed effects. As *L. reuteri* is known to enhance epithelial barrier function of the gut [16, 17], we measured the level of endotoxin in the blood, and found it to be significantly higher in *lpr* mice compared to the age-matched MRL controls (Figure 2B). Interestingly, increasing colonization of *Lactobacillales* in the gut significantly decreased endotoxemia in *lpr* mice (Figure 2C). These results suggest that the gut of *lpr* mice may be “leaky” and allow bacterial components (e.g., lipopolysaccharide, or LPS/endotoxin) to enter the blood stream. *L. reuteri* and the uncultured *Lactobacillus* sp., on the other hand, may be able to correct the leakiness. To test if the gut barrier was leaky in *lpr* mice, we gavaged them with FITC-dextran and found significantly more FITC-dextran in the blood compared to MRL mice. When we treated the *lpr* mice with *Lactobacillus* spp., the levels of FITC-dextran in the circulation significantly decreased (Figure 2D).

Two mucus layers cover the epithelial cells in the lower gastrointestinal tract [18]. Underneath the mucus layers, permeability of the intestinal epithelium is controlled by functions of tight junction proteins [19]. To determine whether *lpr* mice had alterations in epithelial cell junctions, we isolated intestinal epithelial cells and measured the level of tight junction protein transcripts. We found that treatment with *Lactobacillus* spp. significantly increased the expression of barrier-forming junction transcripts (*ZO1*, *occludin* and *Cldn1*) without affecting the level of pore-forming junction transcript *Cldn2* (Figure 2E), suggesting enhanced barrier

function of the intestinal epithelium with a higher abundance of *Lactobacillales* in the gut microbiota. Immunohistochemical analysis confirmed that the level of ZO-1 was increased by *Lactobacillus* treatment in both ileum and colon (Figure 2F). We also found that epithelial expression of IL-18, a cytokine important for tissue repair [20] and limiting colonic Th17 differentiation [21], was significantly enhanced with *Lactobacillus* treatment (Figure 2E). Interestingly, IL-18 can also be detrimental and promote inflammation in *lpr* mice [22]. We found that unlike epithelial expression, the level of IL-18 produced by MLN was significantly decreased by *Lactobacillus* treatment (data not shown). It is likely that *Lactobacillus* spp. can attenuate lupus disease through modulating the production of IL-18 from epithelial vs. immune cells.

In addition to strengthening intestinal mucosal barrier function, *L. reuteri* and the uncultured *Lactobacillus* sp. may also enhance LPS clearance by increasing the expression of intestinal alkaline phosphatase (IAP). IAP is a brush border enzyme expressed on the microvillus membranes of enterocytes [23] that can dephosphorylate LPS, leading to a 100-fold reduction in LPS toxicity [24]. In our studies, epithelial expression of IAP (*Alppl2* and *Alpi*) was significantly upregulated after *Lactobacillus* treatment in *lpr* mice compared to the controls (Figure 2G). The upregulation of IAP was confirmed with immunohistochemical analysis (Figure 2H). Interestingly, IAP has been reported to support growth of Gram-positive bacteria [25], which may explain the increase of *Bifidobacteria* in *Lactobacillus*-treated mice (Figure 1A). *Bifidobacteria* can also promote gut epithelial integrity by strengthening tight junctions [26]. Together, these results suggest that gut microbiota can restore intestinal mucosal barrier function that is compromised in lupus-prone *lpr* mice.

## Control of gut inflammation in lupus

With an enhanced gut mucosal barrier, fewer bacteria are able to translocate across the intestinal epithelium leading to reduced activation and migration of CX3CR1<sup>+</sup> and/or CD103<sup>+</sup> antigen-presenting cells (APC) to the draining lymph nodes of the lower intestinal tract [27-29]. The decrease in APC migration may decrease the activation of CD4<sup>+</sup> T cells. Indeed, we found significantly decreased levels of *Cx3cr1* and *Itgae* (a subunit of CD103) specifically in the MLN with *Lactobacillus* treatment (Figures S2A and S2B) suggesting that *L. reuteri* and the uncultured *Lactobacillus* sp. may reduce the migration of APC to the MLN. We next determined whether the activation of T cells was affected by the decrease of APC in the MLN. Upon activation, MLN T cells upregulate integrin  $\alpha 4\beta 7$  and chemokine receptor CCR9 for homing to the gut mucosa [30]. We found that *Lactobacillus* treatment significantly reduced the expression of both *Itga4* and *Ccr9* in the MLN (Figures S2B and S2C), suggesting decreased activation of T cells. Consistent with this observation, migration of T cells to the intestinal lamina propria was reduced after mice were treated with the *Lactobacillus* spp. (Figure S2D).

Among many pro-inflammatory cytokines produced by activated APC and T cells, IL-6 is known to promote antibody production from B cells [31] and suppress Treg cells [32], which are important for lupus progression in *lpr* mice [33-35]. We measured the transcript level of *Il-6* in the MLN vs. spleen, and found that it was significantly reduced by *Lactobacillus* treatment specifically in the MLN (Figure 3A). CD4<sup>+</sup>CD8<sup>-</sup> T cells appeared to be a source of IL-6 in the MLN of *lpr* mice (Figure 3B). As decreased IL-6 would theoretically allow for differentiation of Treg cells [32], we next evaluated the levels of TGF $\beta$  and IL-10. Both cytokines were significantly increased at the transcriptional level in the MLN, but not spleen, with *Lactobacillus*

treatment (Figure 3C), suggesting gut-specific immunosuppression. Serum TGF $\beta$  level was also significantly enhanced with the treatment (Figure 3D), while the level of IL-6 in the circulation did not change (data not shown). Importantly, the induction of IL-10 with more *Lactobacillales* in the gut microbiota was not only in the MLN, but also systemic (Figure 3E), suggesting that *L. reuteri* and the uncultured *Lactobacillus* sp. may exert a global anti-inflammatory function in *lpr* mice through inducing IL-10 in the gut. Indeed, we also observed a significant elevation of IL-10 transcript levels in the kidney of *lpr* mice with *Lactobacillus* treatment compared to untreated controls (Figure 3F). Further analysis of MLN cells revealed that most IL-10-producing cells in the gut were CD4<sup>+</sup>Foxp3<sup>-</sup> type 1 regulatory T (Tr1) cells (Figure 3G). This observation is consistent with published results on IL-10-producing Tr1 cells in *lpr* mice [36]. Together, these results suggest that gut microbiota can promote an anti-inflammatory environment in the gut of lupus-prone mice, leading to induction of IL-10 that enters the circulation to provide systemic immunosuppression.

### **Control of renal inflammation in lupus**

IL-10 can inhibit kidney disease in *lpr* mice through preventing IFN $\gamma$ -mediated production of IgG2a, a major immune deposit in the kidney of these mice [37]. We found that *Lactobacillus* treatment significantly reduced the level of IgG2a in the blood (Figure 4A) and its deposition in the kidney (Figure 4B). This suggests that IgG2a may act as another “messenger” (in addition to IL-10) to transduce the disease-modulating signal from the gut to the kidney. The levels of IgG1 and total IgG did not change with the treatment (data not shown). Interestingly, the level of IgA was reduced by *Lactobacillus* treatment in the circulation (Figure 4C), suggesting a potential

effect of *L. reuteri* and the uncultured *Lactobacillus* sp. on class-switched antibodies. Indeed, the expression level of *Aicda*, whose gene product mediates class switch recombination [38], was significantly lower in the MLN of *lpr* mice treated with *Lactobacillus* spp. (Figure 4D). The change of IgA did not appear to be related to attenuation of LN, as it was not detectable in the kidney.

Different immune cell populations, including T, B, neutrophils, dendritic cells and macrophages, have been demonstrated to infiltrate in the kidney with LN. To determine how *Lactobacillus* treatment affects immune cell migration to renal tissue, we evaluated various immune cell populations and found marked influx of CD3<sup>+</sup> T cells, particularly CD8<sup>+</sup> T cells, into the kidney of *Lactobacillus*-treated *lpr* mice (Figure 4E). As CD8<sup>+</sup> T cells are generally considered protective in lupus [39-41], it would suggest that renal infiltration of these cells exerts a suppressive effect on the development of LN. In addition, the number of Foxp3<sup>+</sup> Treg cells significantly increased (Figure 4F), while that of pathogenic Th17 cells significantly decreased (Figure 4G), with *Lactobacillus* treatment. Together, these results suggest that gut microbiota may attenuate LN by limiting renal deposition of IgG2a and skewing the Treg-Th17 balance in the kidney towards Treg.

### **Sex hormones and gut microbiota cooperatively regulate LN**

SLE is a female-biased disease with women getting disease nearly 9:1 over men. The results shown so far were obtained from female mice. However, in *lpr* mice both sexes get LN similarly. To investigate whether sex hormones and gut microbiota cooperatively regulate LN in *lpr* mice, we treated male mice with the same *Lactobacillus* strains after mock or castration surgery

(Figure S3A). Bacterial profiling showed that *Lactobacillus* treatment increased gut colonization of *Lactobacillales* in both mock and castrated mice (Figure S3B and Table S2). Strikingly, *Lactobacillus* treatment significantly decreased proteinuria (Figure 5A) and renal pathology (Figure 5B) only in the castrated mice but not the intact animals, suggesting a possible role of androgenic hormones in suppressing the effects of *Lactobacillus* spp. The level of anti-dsDNA IgG was not changed with *Lactobacillus* treatment (Figure S3C). However, the total weight of lymph nodes (including mesenteric, renal, inguinal, lumbar, superficial, axillary/brachial, mediastinal lymph nodes) increased after mice were castrated, an effect reversed by *Lactobacillus* treatment (Figure S3D). In addition, increasing gut colonization of *Lactobacillales* significantly decreased serum levels of IgG2a and IgA in castrated male mice, but not in the mice receiving mock surgery (Figure 5C). The decrease of IgA appears to have originated from the colon (Figure S3E), where the majority of *Lactobacillus* spp. (in terms of total number) resided [42]. Importantly, we found that unlike mice receiving in mock surgery, *Lactobacillus* treatment significantly increased the transcript levels of TGF $\beta$  and IL-10 in the MLN in castrated male *lpr* mice (Figure 5D). *Lactobacillus* treatment also significantly increased circulating IL-10 in castrated animals only (Figure 5E). Together, these results suggest that *Lactobacillus* treatment was not effective in intact male *lpr* mice, while the response of castrated males to *Lactobacillus* treatment parallels that of female *lpr* mice.

As testis is the only source of testosterone in mice, castration surgery completely removed the male hormone regardless of *Lactobacillus* treatment (Figure 5F). We then measured two hormones regulated by testosterone, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). Both are known to be repressed by testosterone [43-45]. As anticipated, castration surgery increased the levels of LH and FSH when the mice were not treated with



Lactobacilli (Figures 5F and S3F). However, *Lactobacillus* treatment significantly decreased serum level of LH, bringing it back to the level where testosterone was still present. We took the ratio of LH to testosterone, and found it to be negatively correlated with serum IL-10 level (Figure 5G). Whether LH directly affects IL-10, or vice versa, requires further investigation. Together, these results suggest that gut microbiota control LN in *lpr* mice in a sex hormone-dependent manner. To determine the effect of Lactobacilli on sex differences, in future studies, we will transfer the cecal contents of young females to male mice to determine whether the interaction between sex hormones and *Lactobacillus* treatment is required for the observed changes in autoimmune response and/or disease phenotype.

## DISCUSSION

The goal of this study was to understand the role of gut microbiota in the pathogenesis of SLE-associated kidney inflammation. In the *lpr* model of LN, we found marked depletion of *Lactobacillales* in the gut microbiota compared to MRL controls. Increasing *Lactobacillales* in the gut microbiota improved the renal function of *lpr* mice. Since *Lactobacillus* spp. are known to enhance mucosal barrier function, the level of circulating endotoxin was measured. Endotoxin can accelerate nephritis in lupus-prone mice [46-48], and significantly higher endotoxemia was observed in *lpr* mice preceding the onset of kidney disease. This suggests a “leaky gut” in pre-disease *lpr* mice. *Lactobacillus* treatment significantly decreased intestinal permeability in these mice and likely prevented detrimental bacteria and their antigens from penetrating the intestinal epithelium. *Lactobacillus* treatment also decreased CX3CR1 and CD103 expression in the MLN.

CX3CR1- and CD103-expressing cells are primarily APC [29, 49, 50] that can capture bacteria from the gut lumen and transport them to the MLN, where they present antigens and activate CD4<sup>+</sup> T cells to produce IL-6 that suppresses Treg, which is vital to lupus pathogenesis in *lpr* mice. By preventing barrier compromise and decreasing microbial translocation, increased gut colonization of *Lactobacillus* spp. may reduce activation and migration of APC to the MLN, hence suppressing IL-6 production and allowing for Foxp3<sup>-</sup> Tr1 cells to produce IL-10, which subsequently represses the synthesis and renal deposition of IgG2a. Inside the kidney, the Treg-Th17 balance was skewed towards Treg with *Lactobacillus* treatment. These effects of Lactobacilli, illustrated in Figure S4, were absent in male mice unless castrated, suggesting that gut microbiota attenuates LN in a sex hormone-dependent manner. It is noteworthy that in these experiments, *Lactobacillus* treatment was given before disease establishment. It appears that *L. reuteri* and the uncultured *Lactobacillus* sp. have a preventative instead of curative effect on the development of LN.

Compromised intestinal barrier function has been reported in autoimmune conditions such as the inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC). It has been shown by using cecal biopsies that intestinal permeability is significantly increased in both CD and UC patients with irritable bowel syndrome-like symptoms than those with quiescent IBD without the symptoms [51]. This increase was accompanied by downregulation of the tight junction protein ZO-1. Endotoxemia in SLE patients that suggests disrupted gut mucosal barrier function in human SLE has also been reported [52]. In our studies, we show that the intestinal epithelium is compromised in lupus-prone *lpr* mice, and that *Lactobacillus* treatment can restore mucosal barrier function by increasing the expression of ZO-1. The effect of Lactobacilli on gut barrier function may also be attributed to the increase of

Muc2, a mucin protein secreted by goblet cells that functions primarily to protect the intestinal epithelium [53].

The imbalance between anti-inflammatory Treg and inflammatory Th17 cells is widely recognized as being causative in the onset of both murine lupus and human SLE [54]. It is well established that environmental factors can promote plasticity between Treg and Th17 cells including the presence of inflammatory cytokines [55]. This cellular flexibility is due to effects of these inflammatory cytokines on the expression and function of the lineage-defining transcription factors Foxp3 and ROR $\gamma$ t, which promote Treg and Th17 cell fates, respectively [56, 57]. Intriguingly, changes in the composition of gut microbiota, particularly those of *Clostridia* and segmented filamentous bacteria (SFB) in mice and *Bacteroides fragilis* in humans, have been shown to alter the balance between Treg and Th17 cells [12]. We show here that increasing *Lactobacillales* in the gut microbiota can promote renal Treg cells and suppress disease-causing Th17 cells to attenuate kidney inflammation in lupus-prone mice.

## CONCLUSIONS

Environmental triggers initiate SLE in susceptible individuals. Since the gastrointestinal system serves as a first line of defense against various pathogens, delineating the type of flora and understanding the role the microbiota plays in determining disease susceptibility in SLE patients is paramount. We show in lupus-prone mice that *Lactobacillus* spp. in the gut microbiota exert anti-inflammatory effects by repairing the damaged gut barrier, suppressing pro-inflammatory

factors in the lymphatic circulation, and improving the ratio of regulatory versus pathogenic T cells, thereby attenuate kidney inflammation. While the relative abundance of *Lactobacillales* appears to be normal in SLE patients in remission (without active disease) [5], this does not preclude the possibility that beneficial bacteria capable of strengthening the gut barrier are lacking in SLE patients with active disease, especially those with LN. SLE is a very diverse disease; therefore, it is important to separately analyze the gut microbiota of SLE patients with different clinical manifestations. If the results of our mouse studies that *L. reuteri* and the uncultured *Lactobacillus* sp. have a preventative effect on the development of LN can be replicated in humans, this may be a new avenue to identify at risk individuals and provide protection in SLE-prone populations.

## METHODS

**Mice.** MRL/Mp (MRL), MRL/Mp-*Fas*<sup>lpr</sup> (MRL/*lpr* or *lpr*, stock number 000485) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained in a specific-pathogen-free facility according to the requirements of Institutional Animal Care and Use Committee at Virginia Polytechnic Institute and State university. Reciprocal cecal microbiota transplantation experiments were performed by diluting, under anaerobic conditions, contents of a cecum collected from one 3-week-old MRL or MRL/*lpr* donor mouse in 5mL PBS. The cecal material was then suspended by vortexing, and the suspension was introduced by oral gavage into recipient mice at 0.2mL/mouse when the mice were 3-weeks old and weaned. Another donor mouse was sacrificed on the next day and the same procedure was repeated once.

All *Lactobacillus* strains, including *L. oris* (F0423), *L. rhamnosus* (LMS201), *L. reuteri* (CF48-3A), *L. johnsonii* (135-1-CHN) and *L. gasseri* (JV-V03) were obtained from BEI Resources. All 5 strains were freshly cultured every week, mixed and inoculated to MRL/lpr mice from 3 weeks old of age until dissection. For experiment involving male castration, the testes and epididymis were removed through a scrotal incision under isoflurane inhalant anesthesia. Skin was closed using wound clips. Mock orchidectomy was performed on an equal number of mice to serve as surgical controls. The mock group of mice were prepared and anesthetized, and a scrotal incision was made, however the incision was closed with a wound clip, without gonad removal. All mice were administered ketoprofen, diluted to 0.5mg/ml in sterile PBS, subcutaneously at 3.5mg/kg as an analgesic post-operatively.

**Microbiota sampling and analysis.** Fecal microbiota samples were obtained by taking individual mice out of their cage and collecting a fecal pellet. To avoid cross-contamination, each microbiota sample was collected by using a new pair of sterile tweezers. Samples were stored at -80°C till being processed at the same time. Sample homogenization, cell lysis and DNA extraction were performed as previously described [13]. PCR were performed and purified amplicons were sequenced bidirectionally on an Illumina MiSeq at Argonne National Laboratory.

**Renal function.** Urine was collected biweekly and all samples were stored at -20°C till being analyzed at the same time with a Pierce Coomassie Protein Assay Kit (Thermo Scientific). When mice were euthanized at 14 weeks of age, kidneys were fixed in formalin for 24h, paraffin embedded, sectioned, and stained with Periodic acid–Schiff (PAS) at the Histopathology Laboratory at Virginia Maryland Regional College of Veterinary Medicine. Slides were read with an Olympus BX43 microscope. All slides were scored in a blinded fashion by a certified

veterinary pathologist. Glomerular lesions were graded on a scale of 0 to 3 for each of the following five categories: increased cellularity, increased mesangial matrix, necrosis, the percentage of sclerotic glomeruli, and the presence of crescents. Tubulointerstitial lesions were graded on a scale of 0 to 3 for each of the following four categories: presence of peritubular mononuclear infiltrates, tubular damage, interstitial fibrosis, and vasculitis.

***Endotoxin quantification and ELISA.*** Separated serum after blood clotting was saved at -20°C until use. Serum endotoxin level was measured by using a Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific). Anti-dsDNA IgG was measured according to a previously described method [58]. Serum IgG, IgA, IgG2a and IL-10 concentrations were determined with mouse IgG, IgA, IgG2a (Bethyl Laboratories) and IL-10 (Biolegend) ELISA kits, respectively, according to the manufacturers' instructions.

***Immunohistochemistry.*** Kidneys and 0.5-cm length ileal and colonic sections were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek) and rapidly frozen in a freezing bath of dry ice and 2-methylbutane. Frozen OCT samples were cryosectioned and unstained slides were stored at -80°C. Frozen slides were warmed to room temperature and let dry for 30 min, followed by fixation in -20°C cold acetone at room temperature for 10 min. After washing in PBS, slides were blocked with PBS containing 1% BSA for 20 min at room temperature. Slides were then incubated with fluorochrome-conjugated antibody mixture at room temperature in a dark humid box. Slides were mounted with Prolong Gold containing DAPI (Life Technologies). The following antibodies were used in immunohistochemical analysis: anti-mouse IgG2a-FITC (eBioscience), rabbit anti-mouse ZO1 and FITC-conjugated goat anti-rabbit IgG secondary antibody (Thermo Scientific), rabbit anti-mouse IAP primary antibody (GeneTex). Slides were

read and pictured with EVOS FL microscope (Advanced Microscopy Group) and a 20× objective.

***Intestinal permeability.*** *In vivo* intestinal permeability assay was performed by using FITC-conjugated dextran (Sigma-Aldrich). Briefly, mice were deprived of water overnight and then orally gavaged with FITC-dextran dissolved in PBS at 40mg/100g body weight (around 300µL/mouse). Mice were anesthetized after 4h and blood was collected and saved in dark until serum separation. Serum was then diluted 1:1 with PBS and added to a 96-well microplate in duplicate, followed by determination of FITC concentration with Glomax (Promega) at an excitation of 485nm and an emission wavelength of 528nm using serially diluted FITC-dextran as the standard.

***Organ cultures.*** Ileum and colon of 1-cm length were collected and opened longitudinally. Intestinal sections were thoroughly washed by PBS and cultured in 48-well plate with 500µL C10 media at 37°C. Supernatant was collected after 24h and analyzed by using ELISA.

***RT-quantitative PCR.*** Spleen, MLN and isolated intestinal epithelial cells (IECs; see below for isolation procedure) were homogenized with Bullet Blender homogenizer (Next Advance) and total RNA was extracted with RNeasy Plus Mini Kit (Qiagen) according to the manufacturers' instructions. Genomic DNA was removed by digestion with RNase-free DNase I (Qiagen). Reverse transcription was performed by using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) and ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Relative quantities were calculated using *L32* (MLN and Spleen) and *Villin* (IECs) as the housekeeping gene. Primer

sequences for mouse *L32*, *Villin*, *ZO1*, *Occludin*, *Cldn1*, *Cldn2*, *IL18*, *IL6*, *Tgfb1*, *IL10*, *Acida*, *CX3CR1*, *CCR9*, *Itgae*, *Itgb7* and *Itga4* are available upon request.

**Cell isolation and flow cytometry.** Spleen, MLN and Peyer's patches were collected and mashed in 70- $\mu$ m cell strainers with C10 media. For splenocytes, red blood cells were lysed with RBC lysis buffer (eBioscience). To isolate lamina propria lymphocytes, intestine was opened longitudinally and cut into pieces. The pieces were incubated twice in EDTA-DTT solution and intensively vortexed to remove epithelial cell layer (saved as IECs enriched fractions). After the second EDTA incubation, the pieces were cut and placed in digestion solution containing 1mg/mL collagenase D (Roche), 0.1mg/mL DNase I (Sigma), and 10 $\mu$ g/mL Dispase (Fisher). After digestion, the solution was passed through a 100 $\mu$ m cell strainer. The same process was repeated for three times and supernatants of three digestions were combined and added onto 40:80 Percoll gradient to separate lymphocytes [59]. For surface marker staining, cells were blocked by anti-mouse CD16/32 (eBioscience), stained with fluorochrome-conjugated antibodies, and analyzed with Attune NxT flow cytometer (Thermo Scientific). For intracellular staining, Foxp3 Fixation/Permeabilization kit (eBioscience) was used. Anti-mouse antibodies used in this study include: CD3-APC-eFluor 780, IL-6-FITC, CD8-PE-Cy7, Tbet-PerCP-Cy5.5, CD4-PerCP-Cy5.5, ROR $\gamma$ T-PE (eBioscience); CD45-FITC, Foxp3-Alexa Fluor 647, IL-10-BV421, IL-17A-APC (Biolegend); CD19-PerCP-Cy5.5, CD4-PE-Cy7, CD8a-V450 (BD Biosciences). Flow cytometry data were analyzed with FlowJo.

**Hormone measurements.** Serum samples were saved at -80°C until analysis. Testosterone, luteinizing hormone and follicle-stimulating hormone were measured at University of Virginia Center for Research in Reproduction, which is supported by the Eunice Kennedy Shriver NICHD/NIH (NCTRI) Grant P50-HD28934.



**Statistical analysis.** For the comparison of two groups, unpaired student's *t*-test was used unless specified. For the comparison of more than two groups, one-way ANOVA and Tukey's post-test were used. Results were considered statistically significant when  $P < 0.05$  (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$ ). All analyses were performed with Prism GraphPad.

## ABBREVIATIONS

CD	Crohn's disease
dsDNA	double-stranded DNA
IAP	intestinal alkaline phosphatase
IBD	inflammatory bowel disease
IEC	intestinal epithelial cells
LN	lupus nephritis
<i>lpr</i>	MRL/ <i>lpr</i>
LPS	lipopolysaccharide
MLN	mesenteric lymph node
PAS	Periodic acid-Schiff
SFB	segmented filamentous bacteria
SLE	systemic lupus erythematosus
Th17	T-helper 17 cells
Tr1	type 1 regulatory T cells
Treg	regulatory T cells
UC	ulcerative colitis

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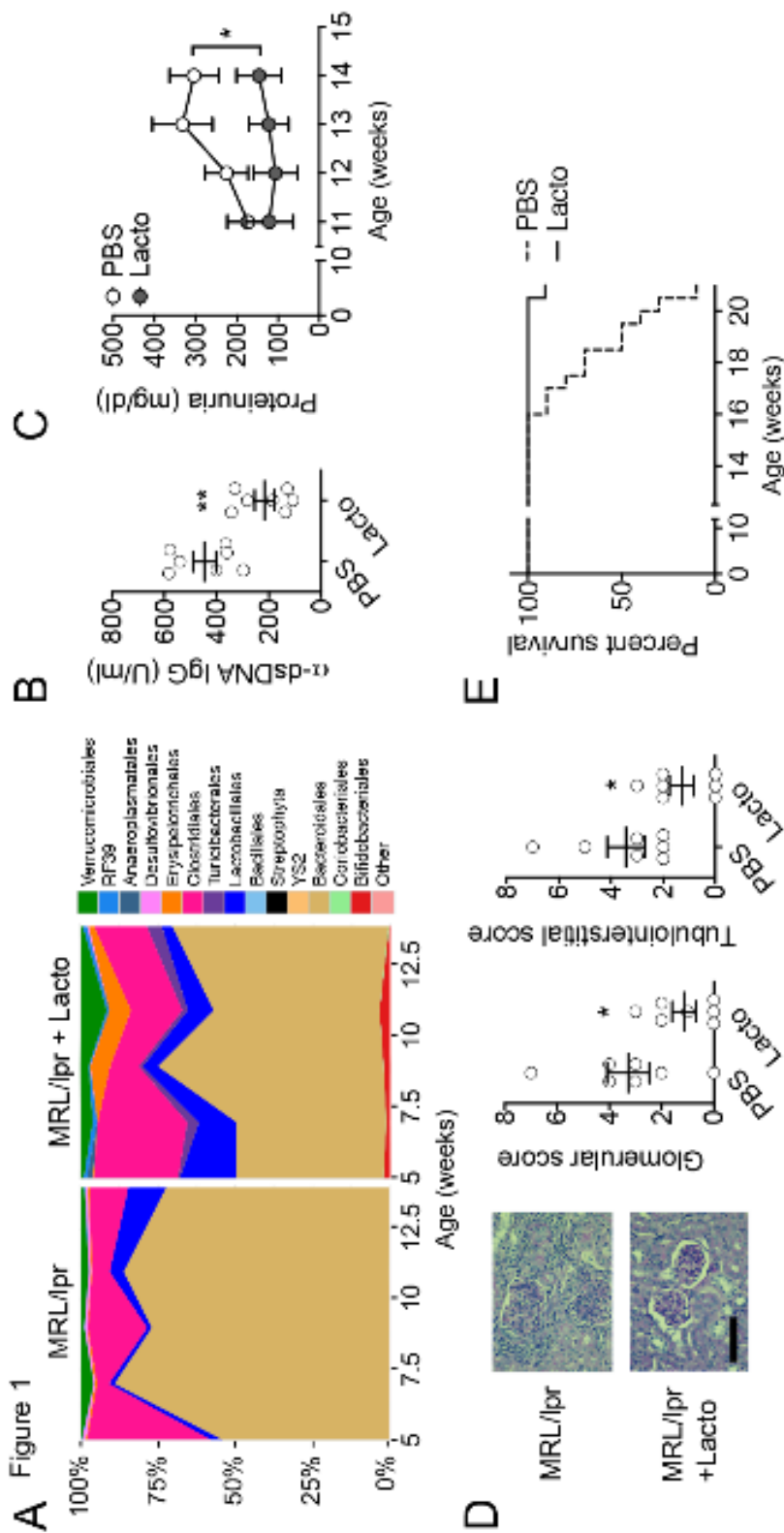
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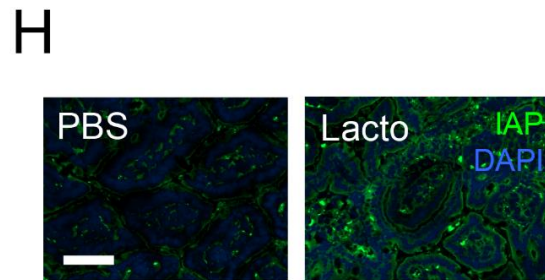
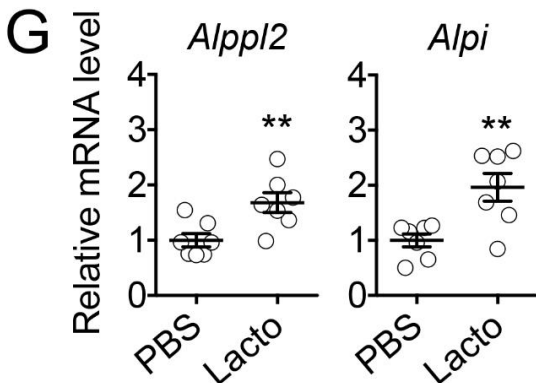
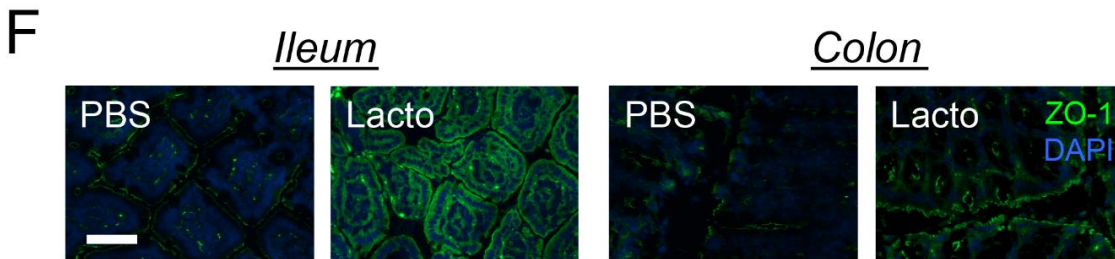
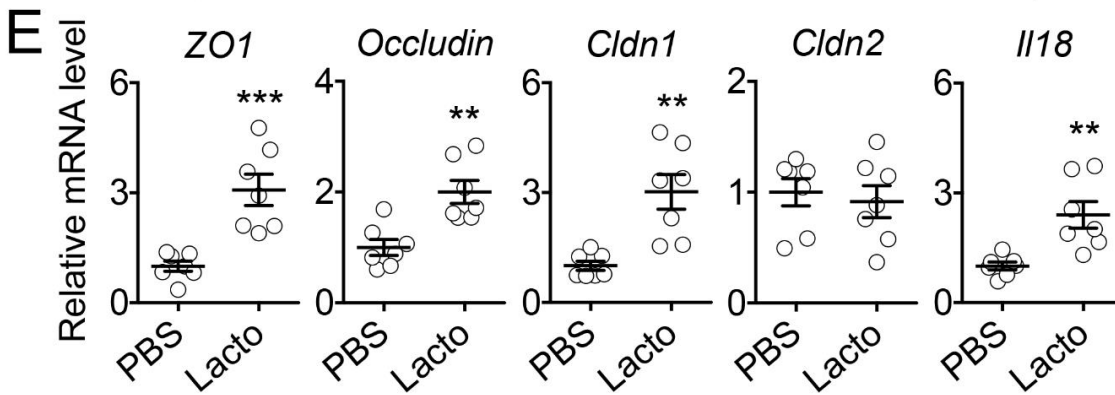
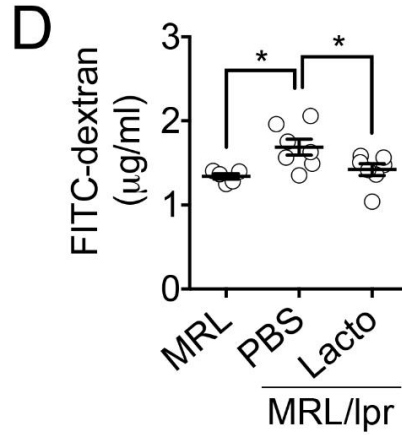
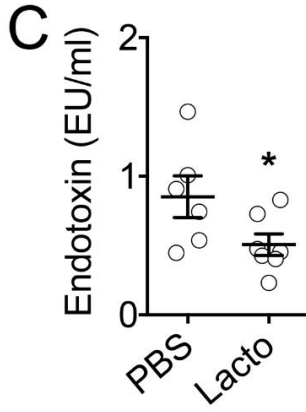
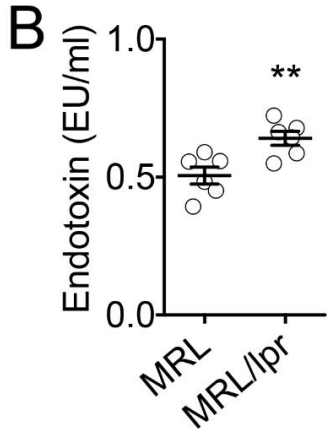
FIGURES



**Figure 1. *Lactobacillus* spp. protect female *lpr* mice from LN.** (A) Time-dependent changes of fecal microbiota upon PBS or *Lactobacillus* (Lacto) treatment (n=4 mice per group). (B) Level of anti-dsDNA IgG in the blood of 10-week-old mice (n=7 mice per group; \*\* $P<0.01$ ). (C) Level of proteinuria over time (n=7 mice per group; paired *t*-test; \* $P<0.05$ ). (D) Renal histopathology at 14 weeks of age (n=7 mice per group; Chi-square test; \* $P<0.05$ ). Left: PAS-stained kidney sections; bar equals 100  $\mu$ m. (E) Survival rate (n=10 mice per group; Chi-square test; \*\*\* $P<0.0001$ )

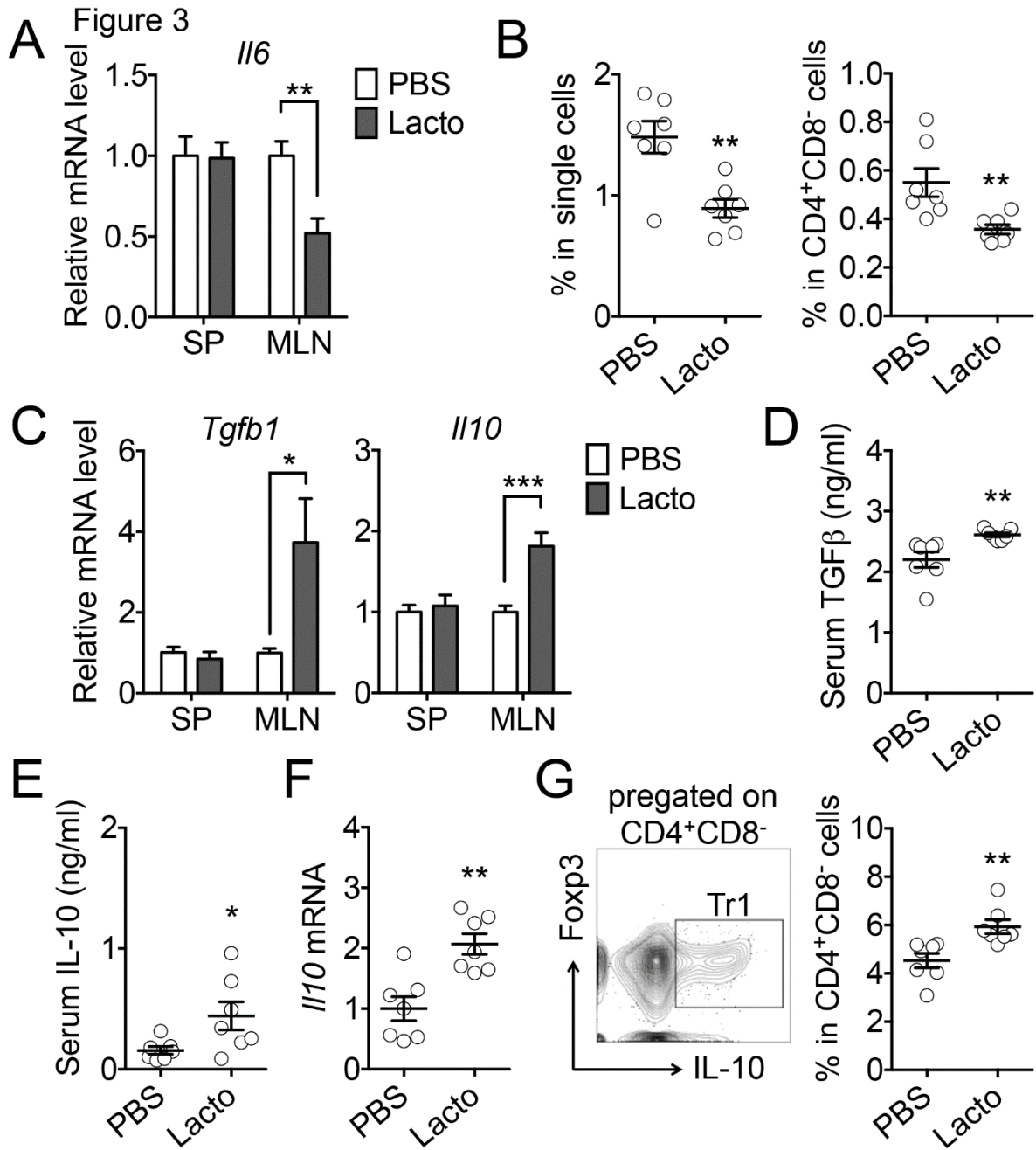
**A** Figure 2

	MRL/lpr	MRL/lpr + Lacto
<i>Lactobacillus reuteri</i> (%)	4.61 ± 0.69	12.85 ± 0.97
Uncultured <i>Lactobacillus</i> (%)	95.2 ± 0.58	86.64 ± 1.12
Total % in Lactobacillales	99.8	99.5

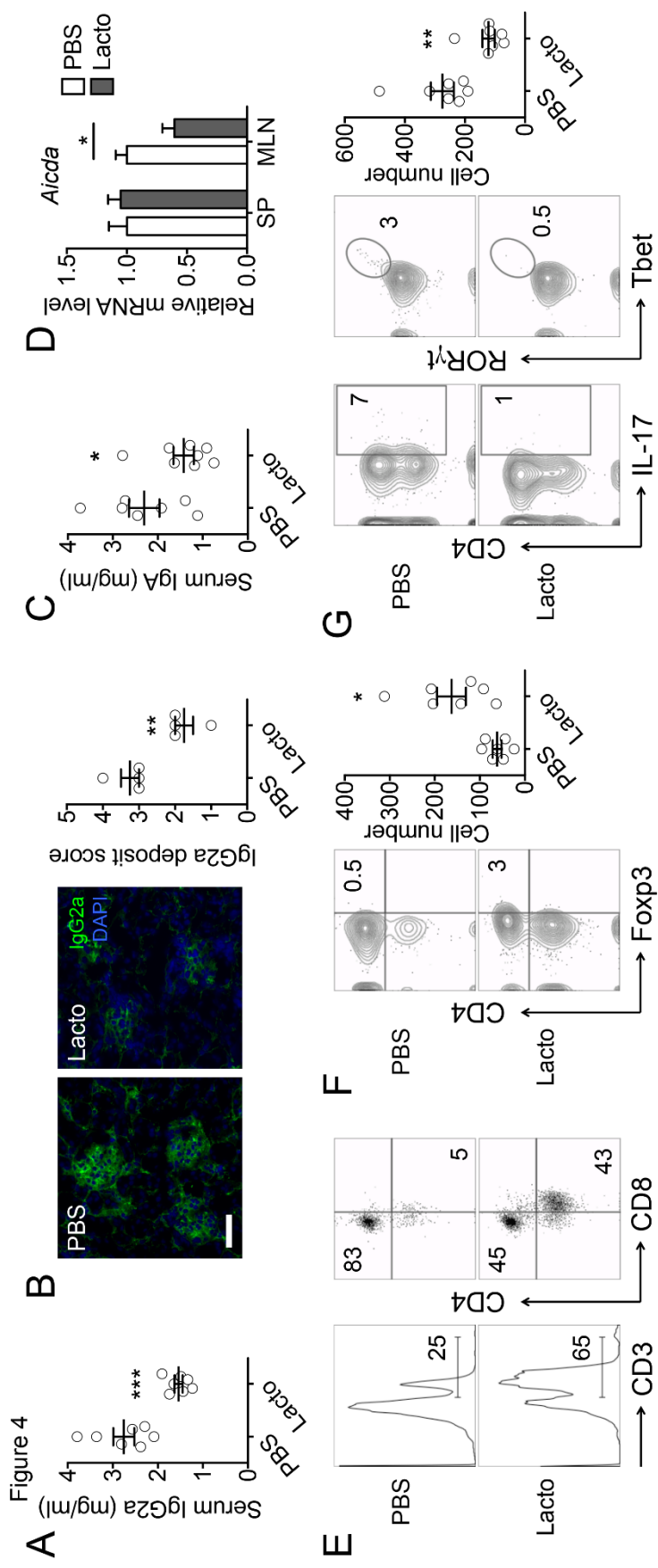




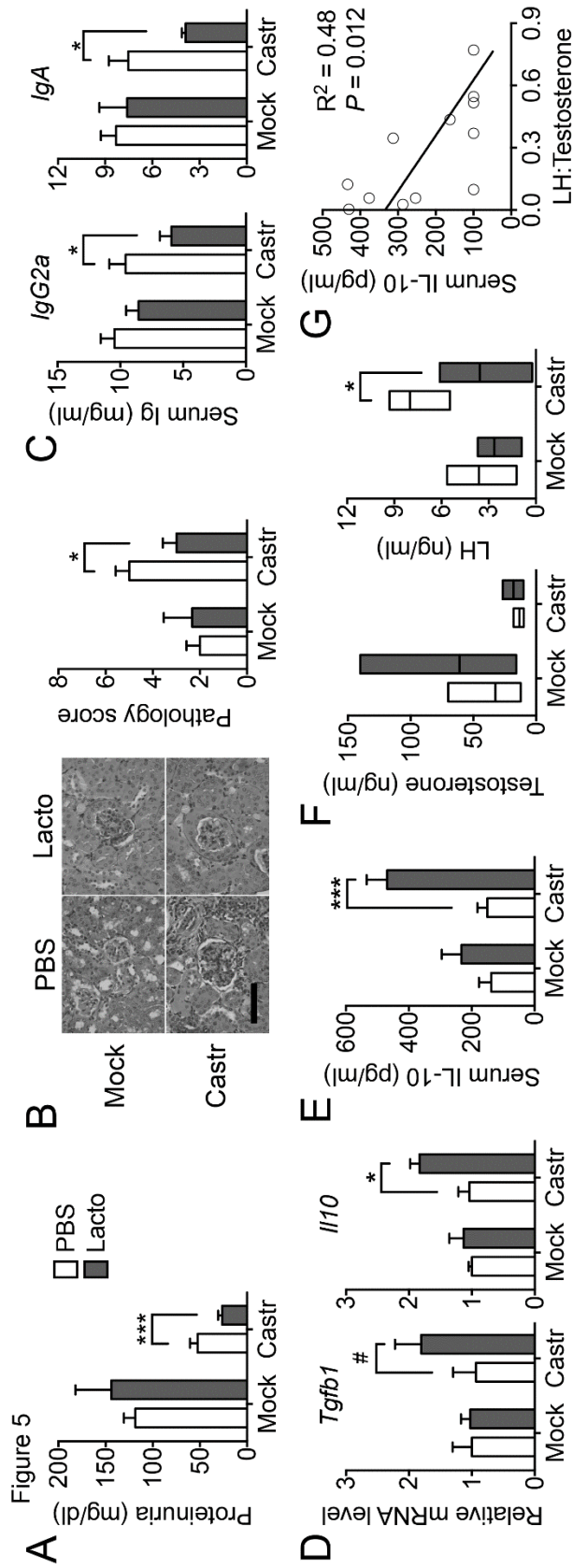
**Figure 2. *Lactobacillus* spp. restore gut mucosal barrier function in female *lpr* mice.** (A) Percentage of *Lactobacillus* strains in the Order *Lactobacillales* (n=4 per group). (B) Level of endotoxin in the blood of 6-week-old *lpr* mice (n=6 mice per group; \*\* $P<0.01$ ). (C) Level of endotoxin in the blood of 10-week-old *lpr* mice with or without *Lactobacillus* treatment (n=6 or 7 mice per group; \* $P<0.05$ ). (D) Level of FITC-dextran diffused to the blood (n=5 or 7 mice per group; \* $P<0.05$ ). (E) Transcript levels of tight junction proteins and IL-18 in intestinal epithelial cells of 14-week-old *lpr* mice (n=7 mice per group; \*\* $P<0.01$ , \*\*\* $P<0.005$ ). (F) Immunohistochemical stains of ZO-1 (green) in the ileum or colon. Nuclear stain (DAPI) is shown in blue. Bar equals 75  $\mu\text{m}$ . (G) Transcript levels of IAP genes in the epithelium (n=7 mice per group; \*\* $P<0.01$ ). (H) Immunohistochemical stains of IAP (green) in the ileum. Bar equals 75  $\mu\text{m}$ .



**Figure 3. Control of intestinal inflammation by gut microbiota in female *lpr* mice.** (A) Transcript level of IL-6 in the spleen (SP) and MLN (n=7 mice per group; \*\* $P<0.01$ ). (B) Percentage of IL-6-expressing cells in the MLN (n=7 mice per group; \*\* $P<0.01$ ). (C) Transcript levels of TGF $\beta$  and IL-10 (n=7 mice per group; \* $P<0.05$ , \*\*\* $P<0.005$ ). (D) Serum level of TGF $\beta$  (n=7 mice per group; \*\* $P<0.01$ ). (E) Serum level of IL-10 (n=7 mice per group; \* $P<0.05$ ). (F) Transcript level of IL-10 in the kidney (n=7 mice per group; \*\* $P<0.01$ ). (G) FACS analysis of IL-10-expressing Tr1 cells in the MLN. Percentages of Tr1 cells in CD4<sup>+</sup>CD8<sup>-</sup> cells are shown (n=7 mice per group; \*\* $P<0.01$ ).



**Figure 4. Control of renal inflammation by gut microbiota in female *lpr* mice.** (A) Serum level of IgG2a (n=7 mice per group; \*\*\* $P<0.005$ ). (B) Immunohistochemical stains of IgG2a (green) in the kidney. Bar equals 75  $\mu$ m. Pathological scores are shown on the right (n=4 mice per group; \*\* $P<0.01$ ). (C) Serum level of IgA (n=7 or 8 mice per group; \* $P<0.05$ ). (D) Transcript level of *Aicda* (n=7 mice per group; \* $P<0.05$ ). (E) Percentages of T cells and subpopulations in the kidney. (F) Percentage of CD4<sup>+</sup>Foxp3<sup>+</sup> Treg cells in the kidney. Absolute Treg cell numbers are shown on the right (n=7 mice per group; \* $P<0.05$ ). (G) FACS analysis of IL-17-producing CD4<sup>+</sup> cells and percentage of RORγt<sup>+</sup>Tbet<sup>+</sup> pathogenic Th17 cells in the kidney. Absolute pathogenic Th17 cell numbers are shown on the right (n=7 mice per group; \*\* $P<0.01$ ).



**Figure 5. Sex hormones and gut microbiota cooperatively regulate LN.** (A) Proteinuria after surgery (Mock vs. Castr/castration)

and treatment (PBS vs. Lacto) of male *lpr* mice (n=5 mice per group; Mann-Whitney test; \*\*\* $P < 0.005$ ).

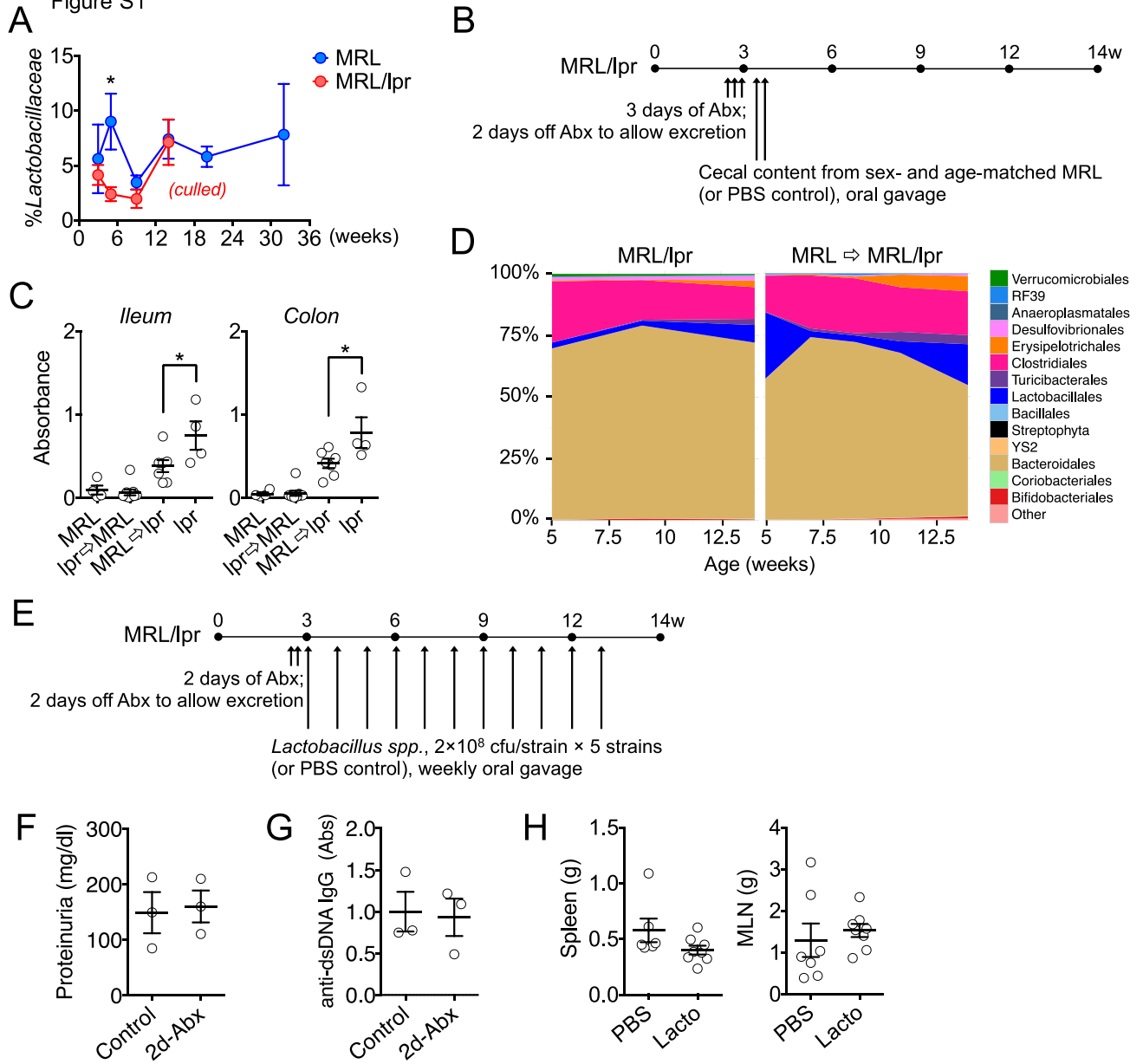
(B) Renal histopathology (n=5 mice per group; Mann-Whitney test; \* $P < 0.05$ ). Left: PAS-stained kidney sections; bar equals 100  $\mu$ m.

(C) Serum levels of IgG2a and IgA (n=5 mice per group; \* $P < 0.05$ ). (D) Transcript levels of TGF $\beta$  and IL-10 in the MLN (n=5 mice per group; # $P < 0.1$ ,

\* $P < 0.05$ ). (E) Serum level of IL-10 (n=5 mice per group; \*\*\* $P < 0.005$ ). (F) Levels of testosterone and luteinizing hormone (LH) in

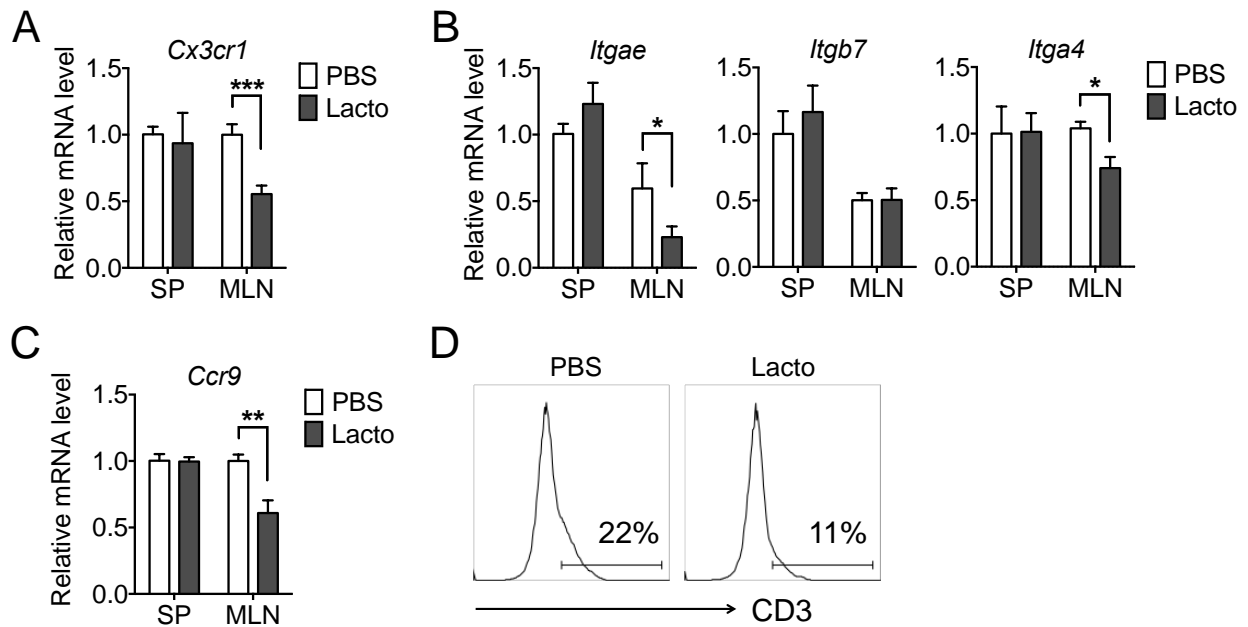
the blood (n=3 mice per group; \* $P < 0.05$ ). (G) Negative correlation between serum IL-10 and the ratio of LH to testosterone.

Figure S1



**Figure S1.** (A) Relative abundance of *Lactobacillaceae* in fecal microbiota (n=4 per group; \* $P$ <0.05 at 5 weeks of age). (B) Study design of cecal transplantation from MRL to *lpr* mice. (C) Level of anti-dsDNA IgG produced by 1-cm long ileal or colonic organ culture after 24 h incubation (n>3 per group; \* $P$ <0.05). (D) Time-dependent changes of fecal microbiota upon cecal transplantation. Abundant bacterial OTU (>0.1%) were summarized (n=4 per group). (E) Study design of *Lactobacillus* treatment of *lpr* mice. (F-G) Female MRL/*lpr* mice were treated with PBS control or mixed antibiotics (Abx) for 2 days at 3 weeks of age and sacrificed at 14 weeks of age (n=3 per group). The levels of proteinuria (F) and anti-dsDNA antibodies (G) at 14 weeks of age are shown. The differences were not significant. (H) Weight of spleen and MLN of *lpr* mice upon *Lactobacillus* treatment.

Figure S2

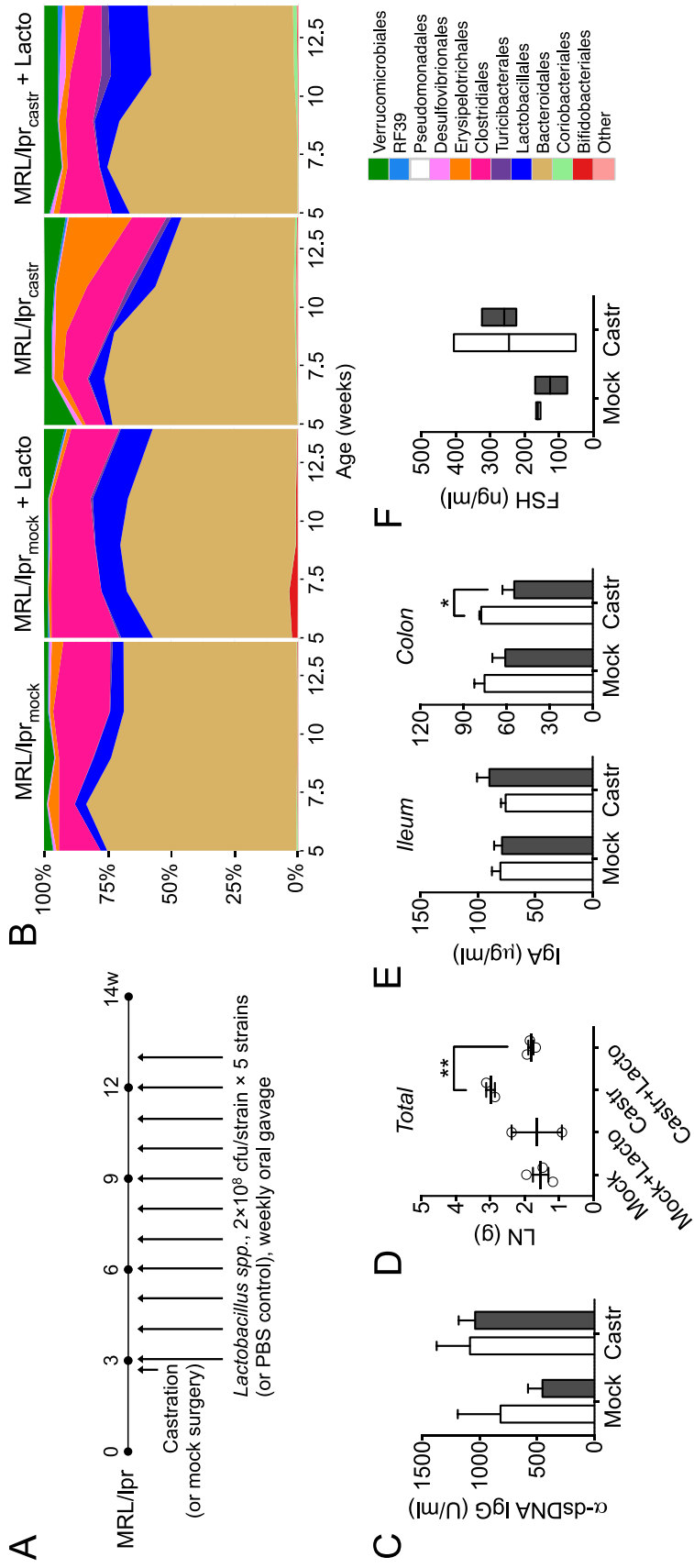


**Figure S2.** (A) Transcript level of CX3CR1 in lymphoid tissues of *lpr* mice treated with PBS or *Lactobacilli*. (B) Transcript levels of CD103 (*Itgae* and *Itgb7*) and  $\alpha4\beta7$  (*Itga4* and *Itgb7*). (C) Transcript level of CCR9. (D) Percentage of CD3<sup>+</sup> T cells in the intestinal lamina propria.

\* $P<0.05$ , \*\* $P<0.01$ , \*\*\* $P<0.001$ .



Figure S3



**Figure S3.** (A) Study design of surgery and treatment in male *lpr* mice. (B) Time-dependent changes of fecal microbiota. Castr, castration. (C) Level of anti-dsDNA IgG in the blood (n=5 per group). (D) Total weight of lymph nodes (LN) from multiple sites (\*\* $P < 0.01$ ). (E) Level of IgA produced by 1-cm sections of ileal or colonic organ culture after 24 h incubation (\* $P < 0.05$ ). (F) Level of FSH in the blood.

Figure S4

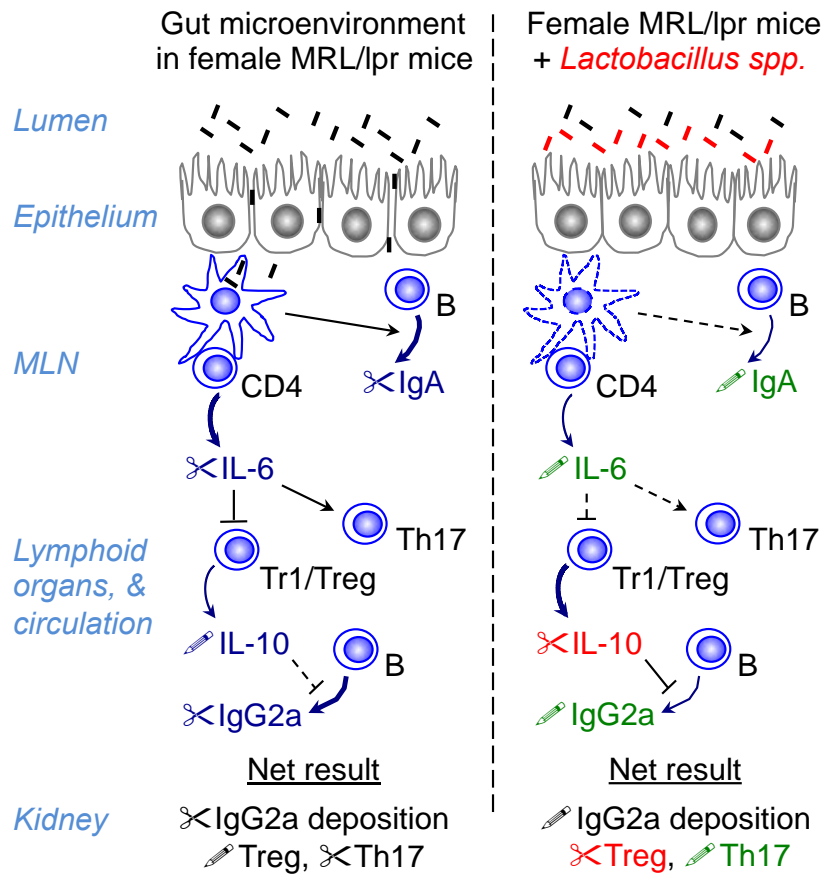


Figure S4. Working model (see text for details).

## CHAPTER IV

### **Antibiotics Ameliorate Lupus-like Symptoms in Mice**

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## ABSTRACT

Gut microbiota and the immune system interact to maintain tissue homeostasis, but whether this interaction is involved in the pathogenesis of systemic lupus erythematosus (SLE) is unclear. Here we report that oral antibiotics given during active disease removed harmful bacteria from the gut microbiota and attenuated SLE-like disease in lupus-prone mice. Using MRL/lpr mice, we showed that antibiotics given after disease onset ameliorated systemic autoimmunity and kidney histopathology. They decreased IL-17-producing cells and increased the level of circulating IL-10. In addition, antibiotics removed *Lachnospiraceae* and increased the relative abundance of *Lactobacillus* spp., two groups of bacteria previously shown to be associated with deteriorated or improved symptoms in MRL/lpr mice, respectively. Moreover, we showed that the attenuated disease phenotype could be recapitulated with a single antibiotic vancomycin, which reshaped the gut microbiota and changed microbial functional pathways in a time-dependent manner. Furthermore, vancomycin treatment increased the barrier function of the intestinal epithelium, thus preventing the translocation of lipopolysaccharide, a cell wall component of Gram-negative *Proteobacteria* and known inducer of lupus in mice, into the circulation. These results suggest that mixed antibiotics or a single antibiotic vancomycin ameliorate SLE-like disease in MRL/lpr mice by changing the composition of gut microbiota.

## INTRODUCTION

Little is known on the role of gut microbiota in systemic lupus erythematosus (SLE) <sup>1,2</sup>. Our research team has described the dynamics of gut microbiota in a classical SLE mouse model MRL/Mp-*Fas*<sup>lpr</sup> (MRL/lpr) <sup>3</sup>. In female lupus mice we found marked depletion of Lactobacilli, and increase of Clostridial species (*Lachnospiraceae*) together with increased bacterial diversity compared to age-matched healthy controls. Importantly, treatments that improved lupus symptoms in lupus mice also restored gut colonization of *Lactobacillus* spp. and decreased that of *Lachnospiraceae*. This suggests that attenuation of lupus disease may be achieved by changes of gut microbiota, but experimental evidence is lacking. Notably, MRL/lpr mice raised under germ-free (GF) conditions exhibit similar disease course and severity as mice housed under conventional conditions <sup>4</sup>, indicating that complete removal of gut microbiota started early in life—including both pathogenic and beneficial microbes—does not attenuate lupus. However, it remains unknown whether and how the removal of gut microbiota after lupus onset would affect the disease. The removal of commensal bacteria post disease onset can be achieved with appropriate antibiotics <sup>5</sup>. This would be more clinically relevant than GF experiments as treatments are usually given after the appearance of clinical signs.

Antibiotics are known to improve symptoms in rheumatoid diseases. Dr. Thomas M. Brown (1906-1989), a renowned rheumatologist, had used antibiotics to successfully treat many patients with rheumatoid arthritis (RA). While no studies have been reported yet that associate antibiotic use to SLE (except rare cases of sun sensitivity with Septra or vancomycin), several clinical trials have suggested that the use of antibiotics is associated with a clinically significant improvement in disease activity in RA without notable side effects <sup>6</sup>. In animal models, the removal of commensal bacteria by either GF housing or antibiotic treatment reduces the severity

of RA-like disease <sup>7,8</sup>, although one study has shown exacerbation of collagen-induced arthritis with partial depletion of gut flora <sup>9</sup>. In lupus-prone mice, acidified water that reduced bacterial diversity in the gut delayed the onset of nephritis and decreased the level of circulating anti-nuclear antibodies <sup>10</sup>. Besides rheumatic diseases, antibiotics have been recently shown to affect the severity of another autoimmune disease type 1 diabetes <sup>11-16</sup>. Most of these studies suggest that antibiotics exacerbate type 1 diabetes.

Here, we showed that antibiotic treatment initiated post disease onset ameliorated lupus-like symptoms likely by decreasing IL-17-producing cells in the spleen and kidney and increasing circulating IL-10. Importantly, antibiotics given at active-disease stage significantly altered the composition of gut microbiota, and most notably, increased the relative abundance of *Lactobacillus* spp. while decreasing that of *Lachnospiraceae*. Moreover, we showed that vancomycin, a single antibiotic known to remove Clostridia (to which *Lachnospiraceae* belongs to) and enrich Lactobacilli in both human and mouse <sup>17-20</sup>, was able to recapitulate the attenuated disease phenotype seen with the mixed antibiotic treatment. Furthermore, mathematical and functional analyses of the microbiome revealed vancomycin-induced changes that were related to Gram-negative bacteria and lipopolysaccharide (LPS). Vancomycin decreased intestinal permeability and reduced the concentration of LPS, a known inducer of murine lupus <sup>21-25</sup>, in the circulation, thus attenuating lupus. Together, these results suggest that antibiotics may be beneficial as a treatment for lupus.

## RESULTS

### *Antibiotics given post disease onset attenuated lupus*

The onset of autoimmune responses in female MRL/lpr mice is as early as 6 weeks of age<sup>26</sup>. To determine the effects of antibiotics on active disease in lupus-prone MRL/lpr mice, we treated female mice with a combination of antibiotics (ampicillin, neomycin, metronidazole and vancomycin<sup>5</sup>) started at 9 weeks of age and post disease onset. The treatment led to enlarged ceca as expected for antibiotic treatments, whereas the overall body weight did not change (data not shown). Spleen and mesenteric lymph node (MLN) weights were significantly decreased with antibiotic treatment compared to controls (Fig. 1A). The serum level of IgG autoantibodies against double-stranded (ds) DNA was also significantly reduced by the mixed antibiotic treatment (Fig. 1B). As kidney inflammation (or lupus nephritis) affects up to 60% of lupus patients<sup>27,28</sup>, we determined the renal function by measuring proteinuria and kidney histopathology. Both glomerular and tubulointerstitial scores were significantly decreased with antibiotic treatment compared to controls (Fig. 1C). The antibiotics also significantly reduced proteinuria (Fig. 1D), suggesting improvement of renal function. Together, these results indicate amelioration of lupus-like disease in female MRL/lpr mice with post-disease-onset antibiotic treatment.

We next sought to understand the underlying mechanism of how antibiotic treatment ameliorated lupus in female MRL/lpr mice by examining immune cell differentiation and inflammatory mediator production (Fig. 2). IL-6, known to promote lupus disease in both human and mouse due to its ability to increase T-helper (Th)17 differentiation and IL-17 production<sup>29,30</sup>, was significantly decreased in the serum with antibiotic treatment (Fig. 2A). We then



quantified IL-17-producing cells in the spleen and kidney by using flow cytometry. As anticipated, antibiotic treatment significantly reduced the percentages of IL-17<sup>+</sup> cells (Fig. 2B), CD3<sup>+</sup>CD4<sup>+</sup>RORγ<sup>+</sup> Th17 cells and Lin<sup>-</sup>CD4<sup>+</sup>RORγ<sup>+</sup> group 3 innate lymphoid cells (ILC3s) (Fig. 2C). Both Th17 cells and ILC3s are known producers of IL-17 in the spleen<sup>31</sup>, and their decrease suggests systemic downregulation of IL-17 with antibiotic treatment. In the kidney, the double negative (DN) T cells and Th17 cells are major sources of IL-17<sup>32</sup> and they both were significantly reduced with post-disease-onset antibiotic treatment (Fig. 2D and Fig. 2E, respectively). These results suggest that antibiotics may attenuate lupus-like disease in female MRL/lpr mice by suppressing the production of IL-17 from Th17 cells and ILC3s in the spleen and from DN-T and Th17 cells in the kidney. Interestingly, we also found a significant increase in serum IL-10 with antibiotic treatment (Fig. 2F) that correlated with an increase in the number of IL-10-producing cells in the MLN (Fig. S1A). IL-10 is known as a protective cytokine in MRL/lpr mice<sup>33</sup>.

### ***Antibiotics given post disease onset reshaped gut microbiota***

It has been recently reported that Th17 cells can migrate from the gut to the kidney to facilitate the development of lupus nephritis<sup>34</sup>, whereas the generation of Th17 cells in the gut is dependent on the gut microbiota<sup>35</sup>. We thus characterized the gut microbiota in antibiotics-treated mice. While antibiotic treatment initiated post disease onset did not decrease the bacterial diversity (Fisher index, Fig. S1B), it did reduce the bacterial load of fecal microbiota by 2 magnitudes (Fig. S1C). Based on 16S rRNA sequencing analysis, the overall structure of the remaining gut bacteria was distinct from untreated animals (Fig. 3A;  $p < 0.01$ , PERMANOVA;

red vs. blue symbols). In addition, the overall structure of the gut microbiota was different between the time points before (3 and 8 weeks of age) and after (11 and 15 weeks of age) antibiotic treatment initiation at 9 weeks of age (also in Fig. 3A;  $p < 0.01$ , PERMANOVA; comparison within the red symbols). Moreover, the antibiotic-treated group before given the treatment (3 and 8 weeks of age) shared similar gut microbiota composition with the Control group, but the antibiotic treatment initiated at 9 weeks of age appears to have altered the bacterial composition at 11 and 15 weeks of age (Fig. 3B). Further analysis on specific bacteria groups revealed that antibiotic treatment significantly increased the abundance of *Lactobacillus* spp. and significantly decreased the abundance of *Lachnospiraceae* (Fig. 3C), two groups of bacteria previously shown to be associated with improved or deteriorated symptoms in MRL/lpr mice, respectively<sup>3</sup>. *L. agilis*, *L. brevis*, *L. mucosae* and *L. reuteri*, in particular, were below detection limit before antibiotic treatment, whereas their abundance increased to about 5% after antibiotics-mediated enrichment. In addition to these changes, treatment with antibiotics removed significant amounts of *Bacteroidales* and *Clostridiales* while increasing the relative abundance of *Bacillales* (Fig. 3D). These results suggest that antibiotics given post disease onset reshaped the gut microbiota, removing potentially harmful bacteria (e.g., *Lachnospiraceae*) and enriching those that are associated with better disease outcomes (e.g., Lactobacilli).

### ***Vancomycin recapitulated the disease-attenuating effects of mixed antibiotics***

A cocktail of 4 different antibiotics is impractical to implement as a treatment for lupus and more likely to induce resistance. Vancomycin alone, however, can remove Gram-positive bacteria such as Clostridial species (*Lachnospiraceae*) but spares Lactobacilli<sup>17-20</sup>, making it a favorable

choice as a potential intervention against lupus progression in MRL/lpr mice. Importantly, vancomycin is not absorbed in the intestine<sup>36,37</sup> and its effects are limited to targeting commensal bacteria in the gut lumen. We thus examined whether oral treatment of vancomycin initiated at 9 weeks of age could attenuate lupus. As anticipated, the relative abundance of *Lactobacillus* spp. was significantly elevated with vancomycin treatment (Fig. 4A). While the overall body weight did not change (Fig. S2A), vancomycin treatment significantly decreased the weight of spleen, MLN and major lymph nodes (Fig. 4B). Furthermore, vancomycin significantly reduced the level of circulating anti-dsDNA IgG (Fig. 4C), proteinuria (Fig. 4D), and renal histopathological scores (Fig. 4E). In contrast, neomycin, an antibiotic with a broad spectrum of activity against both Gram-positive and Gram-negative bacteria<sup>38</sup>, did not affect the severity of lupus disease when given starting from 9 weeks of age. This indicates that the decrease in bacterial load, achieved by both vancomycin and neomycin treatments, was not the reason for disease attenuation. Together, these results suggest that vancomycin given post disease onset recapitulated the attenuated disease phenotype seen with mixed antibiotic treatment.

### ***Vancomycin reshaped the gut microbiota and differentially affected KEGG pathways***

We collected weekly fecal samples from vancomycin-treated mice and determined longitudinal changes of gut microbiota composition by using 16S rRNA sequencing. The diversity of gut microbiota was largely reduced upon vancomycin administration (Fisher index, Fig. S2B). Similar to the mixed antibiotic treatment, vancomycin removed *Clostridiales* right after treatment initiation and *Bacteroidales* at most of the observed time points (Fig. 5A). Many other

groups of bacteria were also removed by vancomycin, including *Desulfovibrionales* and *Turicibacterales*, whereas *Enterobacteriales* were enriched. *Anaeroplasmatales*, on the other hand, was increased by vancomycin treatment at the later time points. These results suggest that vancomycin given during active disease reshaped the gut microbiota in MRL/lpr mice.

We next established mathematical networks to model the gut microbiota changes at the phylum level (Fig. 5B). While some relationships remain the same with and without vancomycin treatment, such as the positive influence of *Bacteroidetes* on *Verrucomicrobia*, overall, treatment with vancomycin significantly affected the interaction among different bacterial groups. *Firmicutes*, for example, were shown to positively influence *Tenericutes* in untreated mice vs. *Verrucomicrobia* in the vancomycin-treated group. Both *Lactobacillaceae* ("good" bacteria) and *Lachnospiraceae* (possibly "harmful" bacteria in this model) belong to the phylum *Firmicutes*. Another example is *Proteobacteria*, which are commonly used to represent Gram-negative bacteria. In untreated mice, *Proteobacteria* were involved in a complex interaction network with *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia*, suggesting that Gram-negative bacteria may contribute to lupus pathogenesis in MRL/lpr mice. However, these interactions were absent in vancomycin-treated mice, suggesting that Gram-negative bacteria no longer play an important role to promote lupus in the presence of vancomycin.

To get insights into functional categories and pathways affected by antibiotics treatment, we performed PICRUSt analyses, and analyzed KEGG level 3 pathways with DESeq2. The analysis showed that the presentation of functional pathways was relatively stable over time for the control group, whereas treatment with vancomycin produced the most significant changes of the functional pathways at 9 weeks of age and 4 days after the initiation of antibiotic treatment (Fig. 6A). Some of the changes sustained beyond 9 weeks of age in the vancomycin group, while

others returned to the baseline levels. Among the pathways with significant changes (Table S1), many exhibited similar trends as in our previous publication<sup>3</sup>. These include the vancomycin-mediated upregulation of *Peptidoglycan biosynthesis* and *Transcriptional factors* pathways that were associated with improved lupus-like symptoms in MRL/lpr mice, as well as vancomycin-mediated downregulation of *Glyoxylate and dicarboxylate metabolism*, *Histidine metabolism*, and *Phenylalanine, tyrosine and tryptophan biosynthesis* pathways that were associated with deteriorated lupus-like symptoms in MRL/lpr mice.

In addition to analysis of the time course, we also determined differential presentation of functional pathways between control and vancomycin groups when data from 9-15 weeks were averaged (Fig. 6B). This analysis showed 75 functional pathways that were significantly altered, including the *Phenylalanine, tyrosine and tryptophan biosynthesis* pathway that was associated with more severe lupus disease<sup>3</sup> and significantly downregulated by vancomycin treatment. Another important pathway, *Lipopolysaccharide biosynthesis*, was also significantly downregulated by vancomycin regardless of sampling time. Detailed analysis of PICRUSt data on the ortholog level (Fig. S3 and Table S2) revealed vancomycin-mediated downregulation of 12 functional genes within the *Lipopolysaccharide biosynthesis* pathway. When the relative levels of these genes were plotted over time, they exhibited the same pattern of a sharp decrease at 9 weeks of age, followed by gradual recovery from 10-11 weeks of age (Fig. 6C). Importantly, a majority of these genes were *Lpx* genes involved in lipid A biosynthesis<sup>39</sup>. Lipid A is the endotoxic component of LPS. These analyses suggest that vancomycin may attenuate lupus-like disease in MRL/lpr mice by downregulating the relative abundance of Gram-negative bacteria and the LPS endotoxin.

### ***Vancomycin decreased intestinal permeability and the plasma level of LPS***

LPS accelerates lupus progression in several lupus-prone mouse models<sup>21-25</sup>. A leaky gut may allow for the translocation of Gram-negative bacteria across the intestinal epithelium, leading to an increase of LPS—a cell wall component of Gram-negative bacteria—in the circulation. We thus determined the intestinal permeability of vancomycin-treated mice by measuring the diffusion of orally gavaged FITC-conjugated dextran. The result showed that vancomycin treatment significantly decreased intestinal permeability (Fig. 7A). In addition, vancomycin significantly increased the epithelial expression of barrier-forming tight junction transcripts *Occludin*, *ZO-1* (Fig. 7B), *Cldn1* and *Cldn3* (Fig. S4A), whereas the transcript level of pore-forming tight junction protein *Cldn2* did not change with vancomycin treatment (data now shown). Further studies that directly measured LPS in the circulation indicated that vancomycin indeed significantly decreased the serum level of LPS (Fig. 7C). Together, these results suggest that vancomycin may attenuate lupus-like disease in MRL/lpr mice by reducing the “leakiness” of the gut epithelium and preventing the translocation of LPS and/or LPS-containing bacteria from the gut lumen to the circulation.

## DISCUSSION

In previous studies we have found that the gut microbiota of lupus-prone MRL/lpr mice contain “good” and “bad” commensal bacteria that may attenuate or facilitate disease progression, respectively<sup>3</sup>. Complete removal of gut microbiota, achieved by GF housing, does not affect the disease outcome as both “good” and “bad” bacteria are removed leading to neutralization of their respective effects. Consistent with this, our preliminary observations suggest that antibiotic treatment initiated from 3 weeks of age (the time of weaning) till the endpoint does not influence disease activity in MRL/lpr mice (unpublished results). Antibiotic treatment started post disease onset, on the other hand, appear to target “bad” bacteria (*Lachnospiraceae*) for removal while enriching “good” bacteria (*Lactobacillus* spp.), thereby attenuating lupus. Our observations are highly relevant to human SLE, as a similar and significant increase of *Lachnospiraceae* was also found when comparing the feces of SLE patients to those of healthy individuals, whereas Lactobacilli were not detectable in either group of people (unpublished results). This suggests that an appropriately selected antibiotic may attenuate disease flares in SLE patients by targeting *Lachnospiraceae* for removal.

Our results have shown that a single antibiotic vancomycin can recapitulate the beneficial effect of mixed antibiotics (ampicillin, vancomycin, neomycin and metronidazole) against lupus progression in MRL/lpr mice. Interestingly, while both treatments reduced the bacterial load, vancomycin was able to decrease the bacterial diversity but the mixed antibiotics were not. Increased gut bacterial diversity is associated with more severe lupus in mice<sup>3</sup> whereas decreasing bacterial diversity with acidified water attenuated lupus<sup>10</sup>. Mixed antibiotics have been shown to decrease gut bacterial diversity in other mouse models<sup>40,41</sup>, but the microbiota community structure can be resilient to antibiotic treatment for days or even weeks<sup>42</sup>. It is

currently unclear why the mixed antibiotics did not reduce the bacterial diversity in MRL/lpr mice when given from 9-15 weeks of age, but the antibiotics were initiated after the microbiota had been established, and that a lower dose of each antibiotic (1 g/l) than vancomycin alone (2 g/l) was used. In future investigations, we will perform dose response experiments to elucidate the relationships between the dose of antibiotics, gut bacterial diversity and the severity of lupus disease.

Vancomycin is known to remove Gram-positive bacteria but spares Lactobacilli. In the current study, when the lupus-prone MRL/lpr mice were treated with vancomycin, their gut microbiota also showed a marked increase in the relative abundance of Lactobacilli. With the reshaped gut microbiota, the intestinal permeability also decreased. This may be due to the upregulation of tight junction proteins in the intestinal epithelium, which are responsible for blocking the paracellular passages between the intestinal epithelial cells<sup>43</sup>. These results are consistent with a recent report showing that vancomycin, rather than amoxicillin and metronidazole, results in less permeable intestines<sup>44</sup>. The increased abundance of Lactobacilli might be the mechanism by which vancomycin enhanced the intestinal barrier function. Numerous *Lactobacillus* strains, such as *L. rhamnosus* and *L. reuteri*, have been reported to improve the gut barrier function<sup>45,46</sup>.

Vancomycin treatment significantly reduced the level of LPS/endotoxin in the circulation. This observation correlates well with our mathematical modeling result that *Proteobacteria* (representing Gram-negative bacteria), while influencing the relative abundance of *Bacteroidetes*, *Actinobacteria* and *Verrucomicrobia* in control mice, were no longer able to affect these bacteria in vancomycin-treated mice. In addition, the decrease in circulating LPS is also consistent with the result of our PICRUSt analysis where the *Lipopolysaccharide*



*biosynthesis* pathway was significantly downregulated by vancomycin treatment. Interestingly, however, vancomycin did not significantly reduce the relative abundance of LPS-containing Gram-negative bacteria (*Proteobacteria*). Within the phylum *Proteobacteria*, the order *Enterobacteriales* was significantly upregulated, whereas the order *Desulfovibrionales* was significantly downregulated, by vancomycin treatment. This led to an averaged effect of no change in the relative abundance of *Proteobacteria* between control and vancomycin-treated groups. Importantly, bacteria in the order *Desulfovibrionales* are resistant to vancomycin<sup>47</sup>, suggesting that the downregulation of *Desulfovibrionales* may be due to their interaction or symbiotic relationship with vancomycin-sensitive Gram-positive bacteria. Moreover, it is speculated that the decrease in *Desulfovibrionales* might be due to reduced proliferation of the bacteria rather than cell death—as they are resistant to vancomycin-mediated killing—so that free LPS is not released to the circulation. The reduction of circulating LPS with vancomycin treatment, on the other hand, could be due to increased intestinal barrier function and reduced translocation of Gram-negative bacteria, such as those in the order *Enterobacteriales*, from the gut lumen to the circulation.

Increased intestinal permeability, or a leaky gut, has arisen in recent years as one contributing factor for autoimmune disease<sup>48,49</sup>. Studies of type 1 diabetes have provided strong evidences to support this notion<sup>50,51</sup>. In SLE, however, the investigation on the interaction between the leaky gut and disease development is still in its infancy. We have previously shown that MRL/lpr mice, compared to age-matched healthy controls, exhibit a higher serum level of LPS before disease onset (unpublished data). Here, the beneficial effects of vancomycin treatment are accompanied by a reversal of the leaky gut and likely less translocation of LPS across the intestinal epithelium. Although the effects were not as significant, the mixed antibiotic

treatment also reversed the leaky gut by decreasing the intestinal permeability (Fig. S4B) and increasing the expression of barrier-forming tight junction transcripts (Fig. S4C). Together, these results suggest that a leaky gut may drive the initiation and/or progression of lupus disease at least in mice.

In the past decades, SLE occurrence has increased several folds in the developed world<sup>2</sup>. The Western diet—high in fat but low in fiber—could have contributed to this by affecting the gut microbiota<sup>52-54</sup>. Notably, the gut leakiness and microbial translocation are also involved in this process<sup>54</sup>. Future investigations are necessary to study the interactions among the leaky gut, microbial translocation and correspondingly the aggravated chronic diseases, such as SLE. At the same time, factors that can reverse gut leakiness, including probiotics and polyunsaturated fatty acids<sup>55,56</sup>, should be considered as part of the disease management strategies.

The cause of lupus is unclear and there is no known cure. Current treatments for SLE are primarily nonselective immunosuppressants. They can effectively treat symptoms, but the side effects are a major cause of concern. Patients taking long-term immunosuppressants are prone to higher incidence of and more severe infections. There is an imperative need for new treatment strategies against SLE, for which a better understanding of disease pathogenesis is required. The results of this study showed that antibiotic treatment given after disease onset in MRL/lpr mice ameliorated lupus-like symptoms, reducing the size of lymphoid organs, decreasing the level of circulating autoantibodies, and attenuating lupus nephritis. The decrease in disease activity was accompanied by decreases in various IL-17-producing cells and an increase of circulating IL-10. In addition, antibiotic treatment reshaped the composition of the gut microbiota, increasing the relative abundance of *Lactobacillus* spp. (“good” bacteria) while decreasing that of *Lachnospiraceae* (“bad” bacteria). Importantly, the therapeutic benefit of the mixed antibiotic

treatment could be recapitulated by a single antibiotic vancomycin, which also favored *Lactobacillus* spp. Detailed analyses of the microbiota through mathematical and functional approaches indicate that *Proteobacteria*, or Gram-negative bacteria, and/or their structural component LPS may contribute to lupus progression in MRL/lpr mice. Further studies revealed that vancomycin reduced intestinal permeability and decreased the translocation of LPS and/or LPS-containing gut microbiota through the intestinal epithelium, thus preventing LPS from accelerating lupus disease. Taken together, these results suggest that antibiotics, especially vancomycin, may be beneficial as a treatment for lupus through reshaping the gut microbiota.

## METHODS

### *Mice and antibiotic treatment*

MRL/lpr mice (stock #000485) were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in a specific pathogen-free facility. All mice used were female as lupus has a strong female bias. Antibiotic mixture (1 g/l ampicillin, 1 g/l neomycin, 1 g/l metronidazole and 0.5 g/l vancomycin) was given in the drinking water starting from 9 weeks of age till euthanasia at 16 weeks of age. For single antibiotic treatment, 2 g/l vancomycin or 2 g/l neomycin was given in the drinking water from 9 weeks of age till euthanasia at 15 weeks of age. The drinking water with antibiotics was refreshed every 5 days. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Virginia Tech College of Veterinary Medicine (Animal Welfare Assurance Number: A3208-01). For anesthesia and euthanasia, isoflurane and CO<sub>2</sub> were used, respectively, according to the IACUC protocol. All experiments were performed in accordance with relevant guidelines and regulations.

### *Microbiota 16S and PICRUSt inferred metagenomics analyses*

Fecal microbiota samples were obtained by taking an individual mouse out of the cage and collecting a fecal pellet. The 9-week fecal pellet was taken 4 days after the initiation of antibiotic treatment. To avoid cross-contamination, each microbiota sample was collected by using a new pair of sterile tweezers. All samples were stored at -80°C till being processed at the same time. Sample homogenization, cell lysis and DNA extraction were performed as previously described<sup>3,57</sup>. The V4 region of purified 16S rRNA gene amplicons were sequenced

bi-directionally (paired-end 150 bp) on an Illumina MiSeq. Microbiome data analysis was performed as previously described<sup>3,57</sup>. Briefly, OTUs were picked by usearch, taxonomy was assigned against Greengenes reference database. Microbial diversity measures including Fisher and observed species were calculated by using QIIME. The datasets generated and analyzed during the current study are available in the NCBI number SRP102626.

Bacterial metagenomes were predicted using PICRUSt<sup>58</sup> by comparing 16S to database gg13.5 then to Integrated Microbial Genomes (IMG). The OTUs were mapped to gg13.5 database at 97% similarity by QIIME's "pick\_closed\_otus" script. The OTUs abundance was normalized using 16S rRNA gene copy numbers from known bacterial genomes. The normalized OTUs were used for metagenomes prediction in PICRUSt. The predicted Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs<sup>59</sup> was summarized to level-3 functional categories and compared among groups by using the Statistical Analysis of Metagenomic Profile package<sup>60</sup>. Differentially represented gene families were identified by two-sided Welch's *t*-test with Storey's false-discovery-rate correction. DESeq2 analysis<sup>61</sup> in R environment was performed with read count data for statistical analysis [R 2016, version 3.3.2 <https://www.r-project.org/>]. We compared between treatments and control for each week (vancomycin vs. control at 8 week, 9 week, etc.). We also compared consecutive weeks for each genotype respectively (e.g. 8 week vs. 9 week for vancomycin), and a time course analysis which tests interaction between time and treatment. Functional categories were considered significantly differentially abundant between conditions if their adjusted *P* value was  $\leq 0.001$  and their absolute value of  $\log_2$  fold change was  $\geq 2$ . Categories with low average count were filtered before the analysis. Read counts for each KEGG category were normalized by DESeq2 and averaged between replicates. The average read counts were centered and scaled before used for clustering analysis to produce a heatmap.

### *Mathematical modeling*

A standard way in mathematically describing the temporal dynamics of interacting species is the generalized Lotka-Volterra or predator-prey system, which reads as follow

$$\mathbf{y}'(t) = \text{diag}(\mathbf{y}(t))(\mathbf{r} + \mathbf{A}\mathbf{y}(t)), \quad (1)$$

where  $\text{diag}(\mathbf{y}(t))$  is the diagonal matrix with the state variable  $\mathbf{y}(t) = [y_1(t), \dots, y_n(t)]^T$  (describing the temporal abundances of the different organisms),  $\mathbf{r}$  is the vector of intrinsic growth rate parameters, and  $\mathbf{A}$  is the interaction matrix that characterizes the influences within the network of each species, e.g., the entry  $a_{ij}$  of the matrix  $\mathbf{A}$  describes the influence of species  $j$  on the growth of species  $i$  [M. Chung, J. Krueger, and Mihai Pop. Robust Parameter Estimation for Biological Systems: A Study on the Dynamics of Microbial Communities. In revision at *Mathematical Bioscience*, 2017. [\[arXiv Preprint\]](#)].

Hence, the network of the species is identified by this interaction matrix  $\mathbf{A}$ .

To determine the interaction  $\mathbf{A}$  we assumed that the dynamics were at an asymptotically stable equilibrium  $\bar{\mathbf{y}}$ , where  $\bar{\mathbf{y}}$  was determined by the data (Fig. 5A). Mathematically such equilibria are given by solution of the nonlinear equation

$$\text{diag}(\bar{\mathbf{y}})(\mathbf{r} + \mathbf{A}\bar{\mathbf{y}}) = \mathbf{0} \quad (2)$$

$$\text{subject to the constraint } \max_j \Re(\lambda_j) < 0,$$

where  $\Re$  is the real part of  $\lambda_j$  and  $\lambda_j$  is the  $j^{\text{th}}$  eigenvalue of the matrix  $\text{diag}(\bar{\mathbf{y}}) + \text{diag}(\bar{\mathbf{y}})\mathbf{A}$ . Hence, we need to find  $\mathbf{A}$  such that the above equations are fulfilled. Note that this is a nonconvex problem and multiple solution may exist. More precise, multiple network configuration may describe the same asymptotically stable equilibrium  $\bar{\mathbf{y}}$ . We used a repeated

Monte Carlo sampled direct search method [Wright, Stephen and Nocedal, Jorge, Numerical Optimization, Springer, 2006] to identify solution **A** of above problem, see Fig. 5B.

### ***Renal function***

Urine samples were collected biweekly and all samples were stored at -20°C till being processed at the same time. We used Pierce Coomassie Protein Assay Kit (Thermo Scientific) to test the total protein level in the mouse urine. When mice were euthanized at 14 weeks of age, kidneys were fixed in formalin for 24 h, paraffin embedded, sectioned, and stained with Periodic acid–Schiff (PAS) at the Histopathology Laboratory at Virginia Maryland Regional College of Veterinary Medicine. Slides were read with an Olympus BX43 microscope. All slides were scored in a blinded fashion by a certified veterinary pathologist (Cecere). Glomerular lesions were graded on a scale of 0 to 3 for each of the following five categories: increased cellularity, increased mesangial matrix, necrosis, the percentage of sclerotic glomeruli, and the presence of crescents. Similarly, tubulointerstitial lesions were graded on a scale of 0 to 3 for interstitial mononuclear infiltration, tubular damage, interstitial fibrosis, and vasculitis.

### ***Endotoxin quantification and enzyme-linked immunosorbent assays (ELISA)***

Separated serum after blood clotting was saved at -20°C until use. We used Pierce LAL Chromogenic Endotoxin Quantitation Kit (Thermo Scientific) to measure serum endotoxin level by following the kit's instructions. For detection of anti-double-stranded DNA (dsDNA) IgG, we used previously described methods<sup>26</sup>. Serum IgG, IL-6 and IL-10 concentrations were determined with mouse IgG (Bethyl Laboratories), IL-6 (Biolegend) and IL-10 (Biolegend) ELISA kits according to the manufacturer's instructions.

### ***Intestinal permeability***

*In vivo* intestinal permeability assay to assess barrier function was performed using Fluorescein isothiocyanate conjugated dextran (FITC-dextran, Sigma-Aldrich) method. Briefly, mice were water starved overnight then orally gavaged with FITC-dextran (40 mg/100 g body weight). After 4 h, mice were anesthetized and blood was collected and saved in the dark. Serum was then prepared from the blood, diluted 1:1 with PBS, and measured in a 96-well microplate in duplicates for the concentration of FITC in the serum by Glomax (Promega) with an excitation wavelength of 485 nm and an emission wavelength of 528 nm, using serially diluted FITC-dextran as the standards.

### ***Cell isolation and flow cytometry***

Spleen, MLN and kidney were collected and mashed in 70- $\mu$ m cell strainers with C10 media (RPMI 1640, 10% fetal bovine serum, 1 mM sodium pyruvate, 1% 100 MEM non-essential amino acids, 10 mM HEPES, 55  $\mu$ M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, all from Life Technologies, Grand Island, NY). For splenocytes, red blood cells were lysed with RBC lysis buffer (eBioscience, San Diego, CA). To isolate intestinal epithelial cells (IECs), intestine was opened longitudinally and cut into pieces. The pieces were incubated twice in EDTA-DTT solution and intensively vortexed to harvest IEC-enriched fractions. For surface marker staining, cells were blocked with anti-mouse CD16/32 (eBioscience), stained with fluorochrome-conjugated antibodies, and analyzed with Attune NxT flow cytometer (Thermo Scientific). For intracellular staining, Foxp3 Fixation/Permeabilization kit (eBioscience) was used. Anti-mouse antibodies used in this study include: CD3-APC-eFluor



780, CD8-PE-Cy7, CD4-PerCP-Cy5.5, ROR $\gamma$ T-PE, CD3e-biotin (eBioscience); CD45-APC-Cy7, IL-10-BV421, IL-17A-APC, CD49b-biotin, CD19-biotin (Biolegend, San Diego, CA); Biotin-FITC (MACS). Flow cytometry data were analyzed with FlowJo.

### ***Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)***

Isolated IECs were homogenized with Bullet Blender homogenizer (Next Advance, Averill Park, NY) and total RNA was extracted with RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturers' instructions. Genomic DNA was removed by digestion with RNase-free DNase I (Qiagen). Reverse transcription was performed by using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) and ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Grand Island, NY). Relative quantities were calculated using *Villin* as the housekeeping gene. Primer sequences for mouse *Villin*, *ZO-1*, *Occludin*, *Cldn1* and *Cldn3* are available upon request.

### ***Statistical analysis***

Statistical analyses were performed by using R version 3.0.2 (sequencing data) or Prism GraphPad (non-sequencing data). Principal coordinate analysis was tested for significance by a permutational multivariate method PERMANOVA<sup>62</sup>. Analysis of non-sequencing data was performed with Student *t*-test or one-way ANOVA with Tukey's post-test. The results were considered statistically significant when  $p < 0.05$ .

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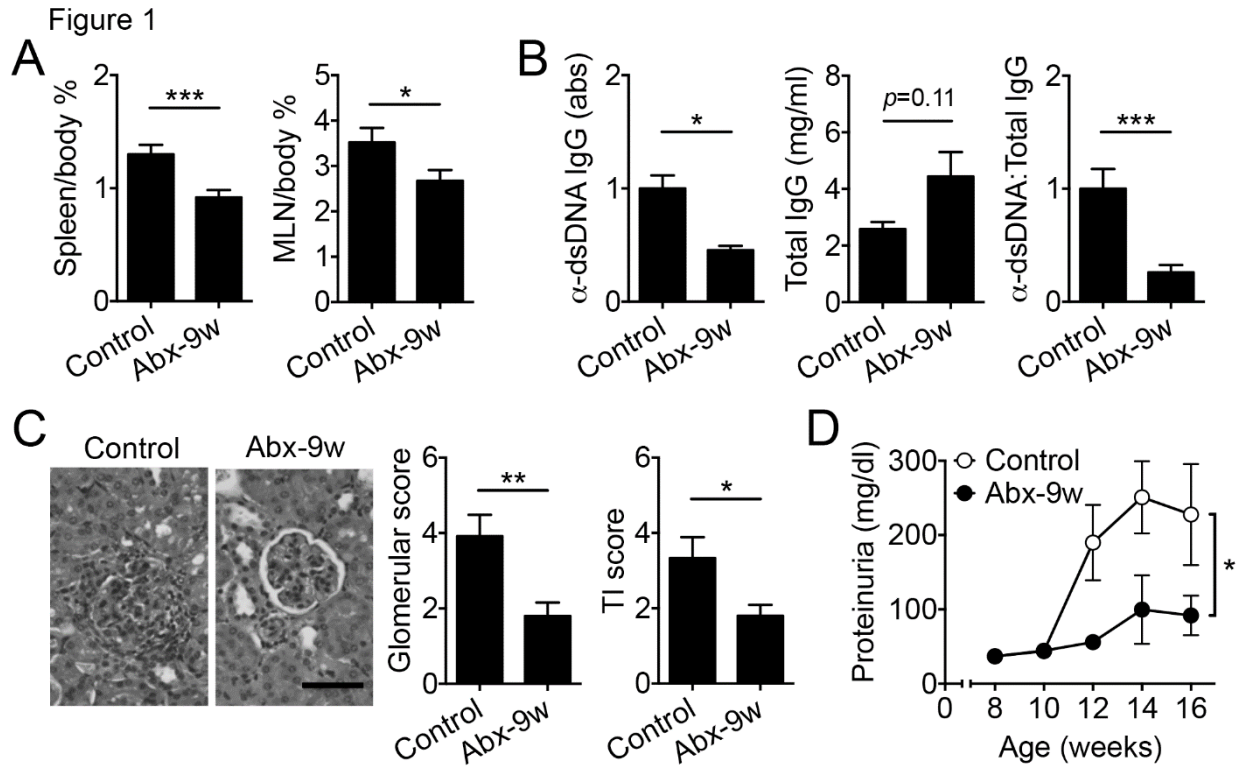
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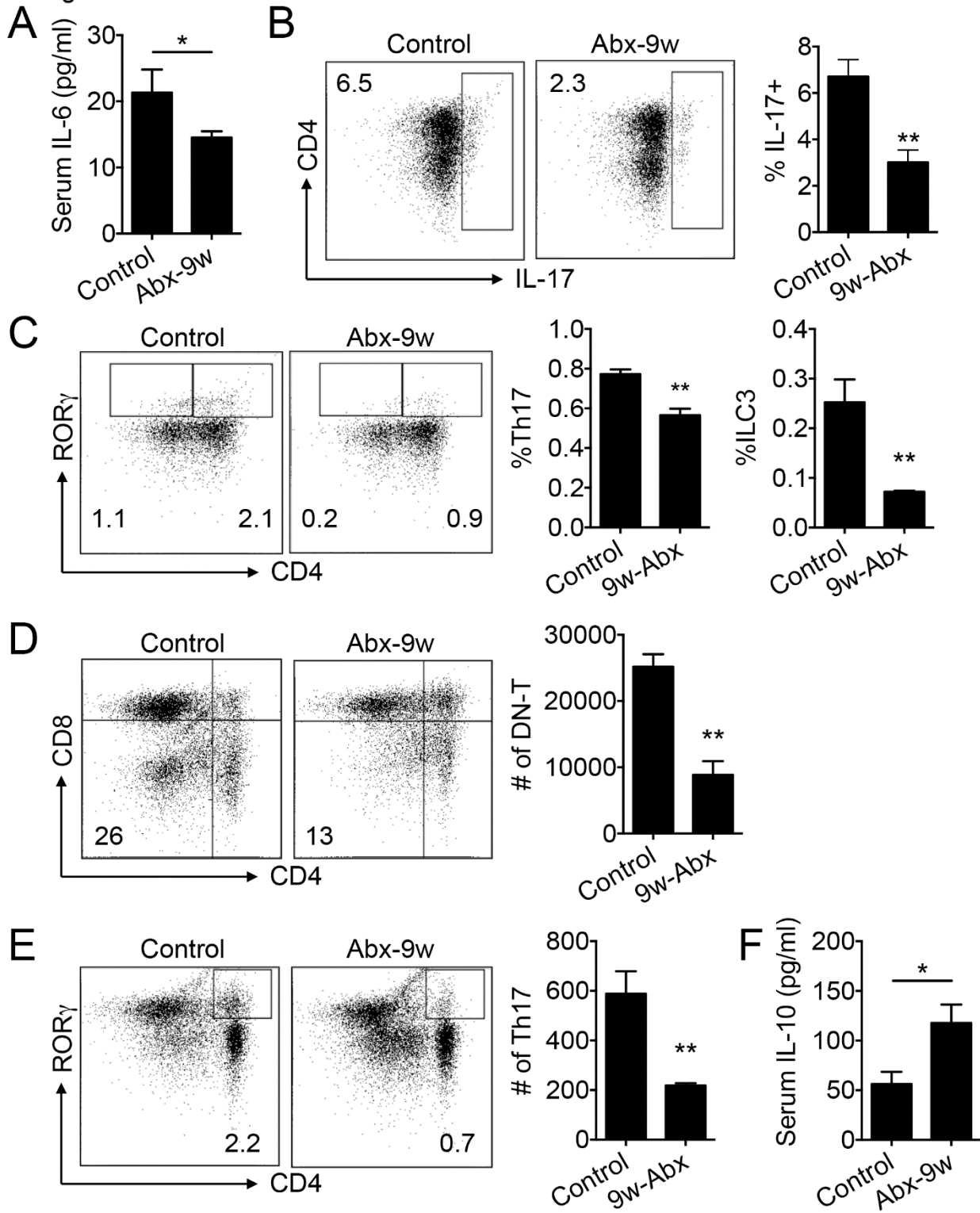
## FIGURES



**Figure 1. Antibiotic treatment started after lupus onset led to disease attenuation. (A)** Spleen and MLN tissue weight to body weight ratio (%) at 16 weeks of age ( $n \geq 8$  per group). Control: no antibiotics. Abx-9w: oral antibiotics were given starting from 9 weeks of age and post disease onset. **(B)** Level of anti-dsDNA IgG in the mouse serum, total IgG, and the ratio of anti-dsDNA IgG to total IgG at 16 weeks of age ( $n \geq 8$  per group). **(C)** Renal histopathology at 16 weeks of age ( $n \geq 8$  per group). Left: representative PAS-stained kidney sections; bar equals 200  $\mu$ m. Middle: glomerular score. Right: tubulointerstitial (TI) score. **(D)** Level of proteinuria over time ( $n \geq 8$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

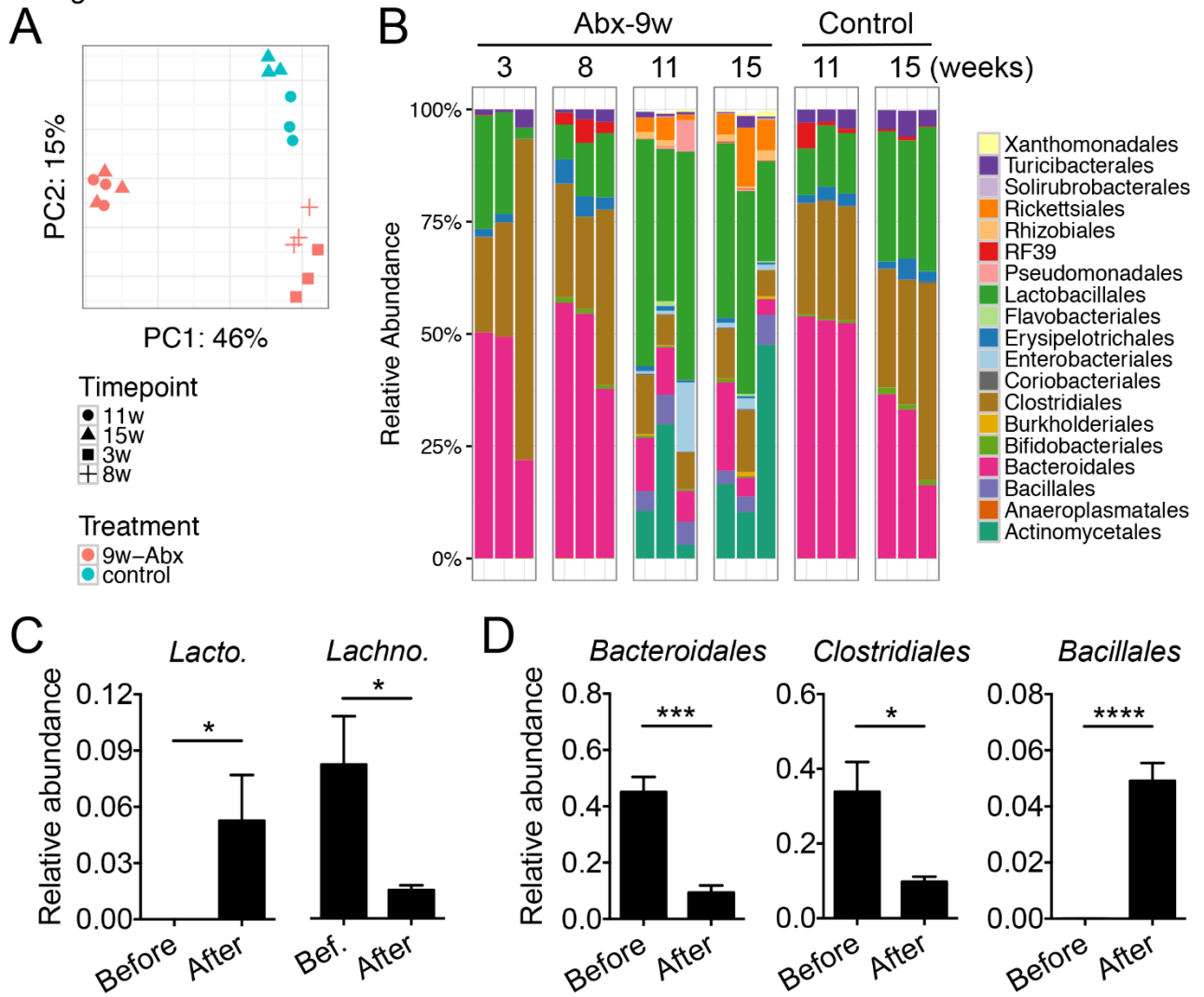


Figure 2



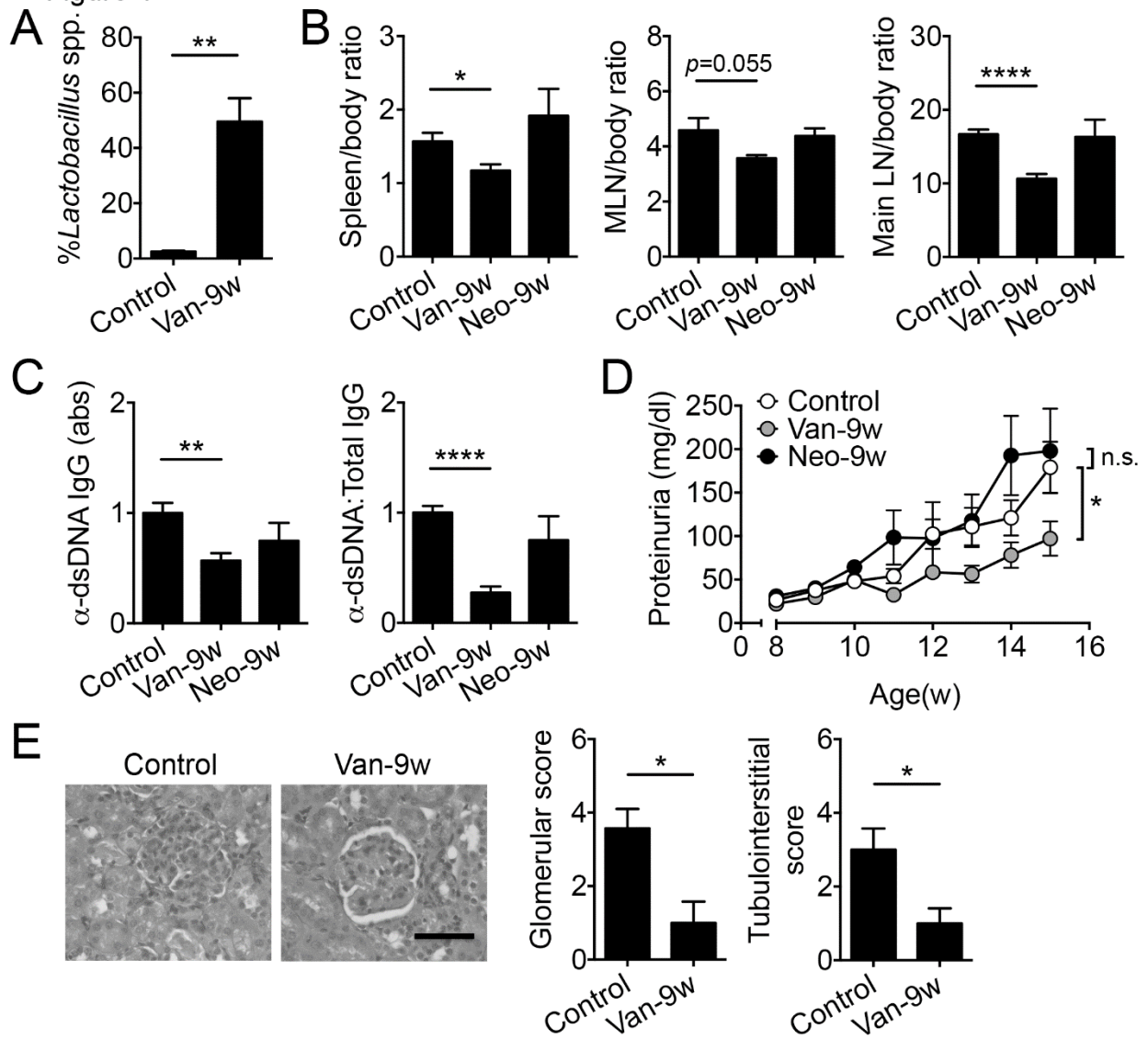
**Figure 2. Antibiotic treatment decreased IL-17-producing cells in the spleen and kidney and increased IL-10 in the circulation.** (A) Serum level of IL-6 at 16 weeks of age (n≥7 per group). (B) Intracellular staining of IL-17 and the percentage of IL-17-producing cells in the spleen at 16 weeks of age (n=4 per group). (C) FACS analysis of CD4<sup>+</sup>RORγ<sup>+</sup> Th17 cells and CD4<sup>-</sup>RORγ<sup>+</sup> ILC3 cells in the spleen at 16 weeks of age (n=4 per group). (D-E) FACS analysis of DN-T cells (D) and Th17 cells (E) in the kidney at 16 weeks of age (n=4 per group). (F) Serum level of IL-10 in the mouse serum at 16 weeks of age (n≥7 per group). \* $p < 0.05$ , \*\* $p < 0.01$ .

Figure 3



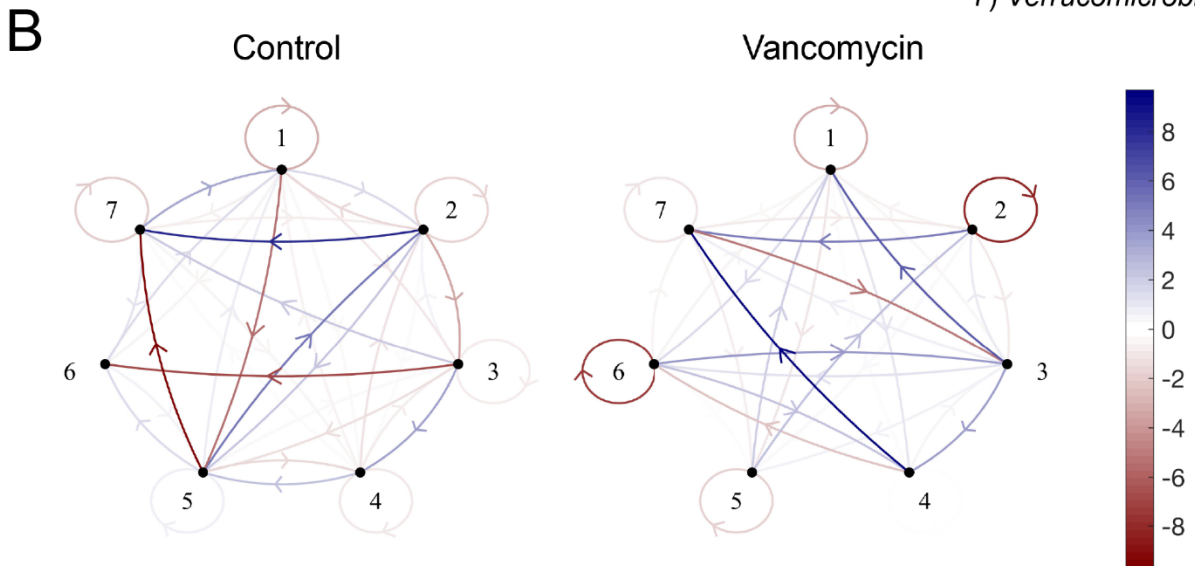
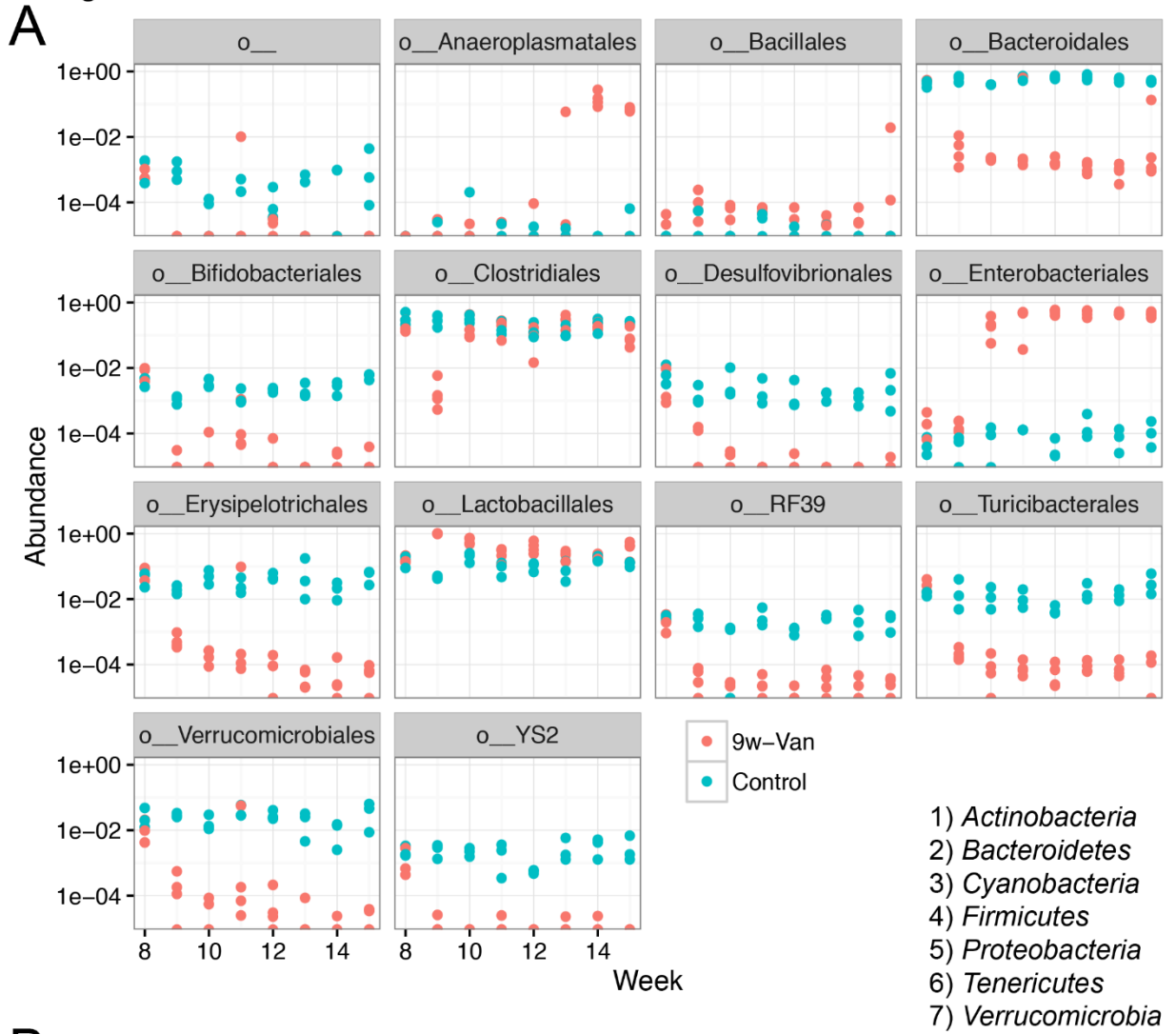
**Figure 3. Composition of gut microbiota changed with antibiotic treatment.** (A) Principal component analysis of fecal microbiota. w: weeks of age.  $p < 0.01$ , PERMANOVA. (B) Time-dependent changes of fecal microbiota. Bacterial taxa at the order level are shown. (C) Relative abundance of detectable *Lactobacillus* spp. (*Lacto.*, left panel) and that of *Lachnospiraceae* (*Lachno.*, right panel) (n=6 per group). The detectable *Lactobacillus* spp. were *L. agilis*, *L. brevis*, *L. mucosae* and *L. reuteri*, and the sum of their relative abundance is shown. Before: 3 and 8 weeks of age. After: 11 and 15 weeks of age. (D) Relative abundance of *Bacteroidales*, *Clostridiales*, and *Bacillales* before and after antibiotic treatment initiated at 9 weeks of age (n=6 per group). \* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ .

Figure 4



**Figure 4. Vancomycin but not neomycin treatment started post disease onset ameliorated lupus-like disease.** (A) Relative abundance of *Lactobacillus* spp. in the fecal microbiota at 15 weeks of age (n=4 per group). Control: no antibiotics. Van-9w: vancomycin was given starting from 9 weeks of age. (B) Tissue to body weight ratio (%) for the spleen, MLN and major lymph nodes (main LN) including mesenteric, renal, inguinal, lumbar, superficial, axillary/brachial, mediastinal lymph nodes at 15 weeks of age. Neo-9w: neomycin was given starting from 9 weeks of age. (C) Level of anti-dsDNA IgG in the mouse serum and its ratio to total IgG at 15 weeks of age. (D) Level of proteinuria over time. (E) Renal histopathology at 15 weeks of age. Left: representative PAS-stained kidney sections; bar equals 200  $\mu$ m. Middle: glomerular score. Right: tubulointerstitial score. In B-E, n=12 in Control and Van-9w groups, n=4 in the Neo-9w group. \* $p$ <0.05, \*\* $p$ <0.01, \*\*\*\* $p$ <0.0001, n.s.: not statistically significant.

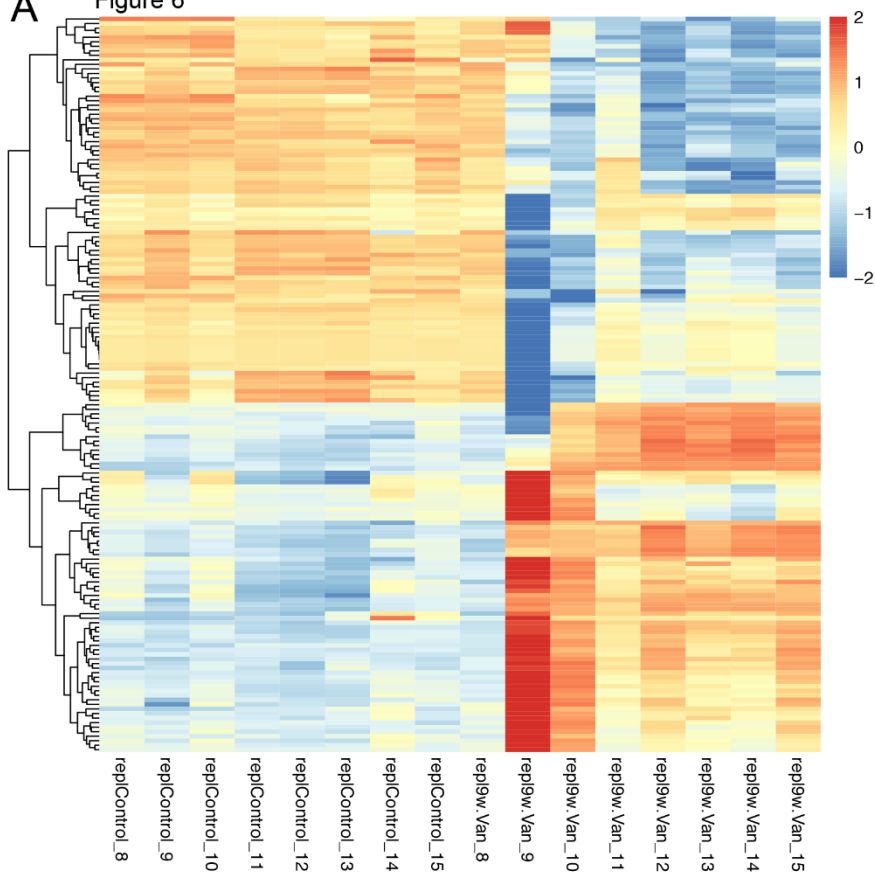
Figure 5



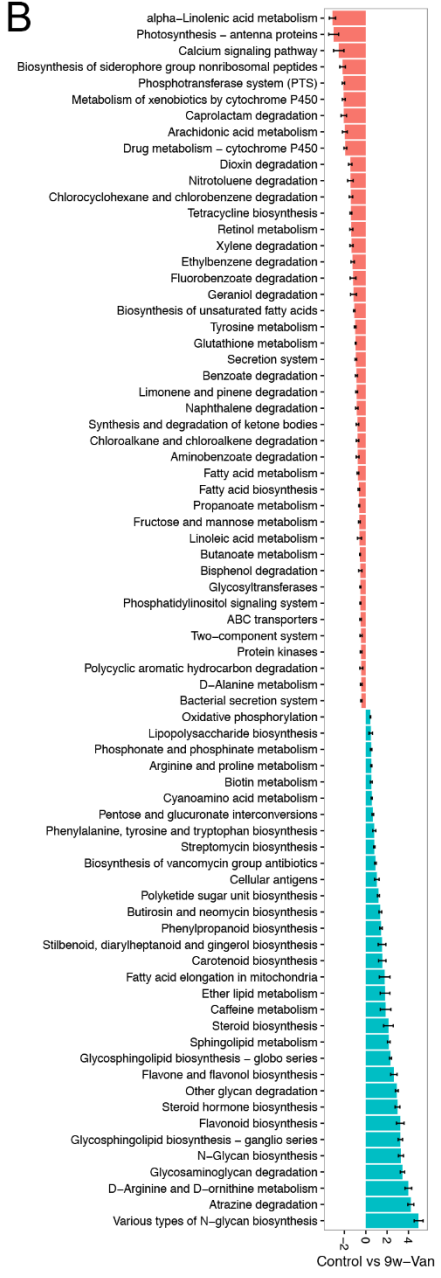
**Figure 5. Vancomycin treatment reshaped the gut microbiota.** (A) Time-dependent changes of the relative abundance of gut bacteria at the order level. The first label “o\_\_” means an order with uncultured bacteria. (B) Mathematical networks generated based on the longitudinal changes of gut microbiota at the phylum level. 1) *Actinobacteria*, 2) *Bacteroidetes*, 3) *Cyanobacteria*, 4) *Firmicutes*, 5) *Proteobacteria*, 6) *Tenericutes*, and 7) *Verrucomicrobia*. Blue, positive influence. Red, negative influence. The thicker the line, the stronger the relationship.



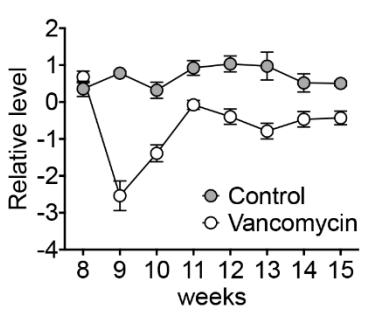
**A** Figure 6



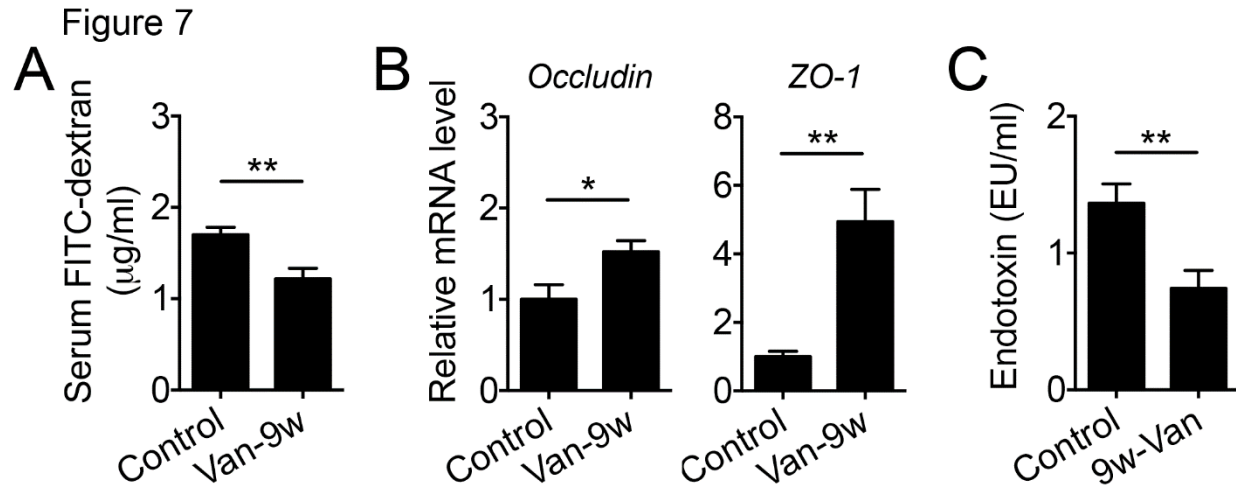
**B**



**C**

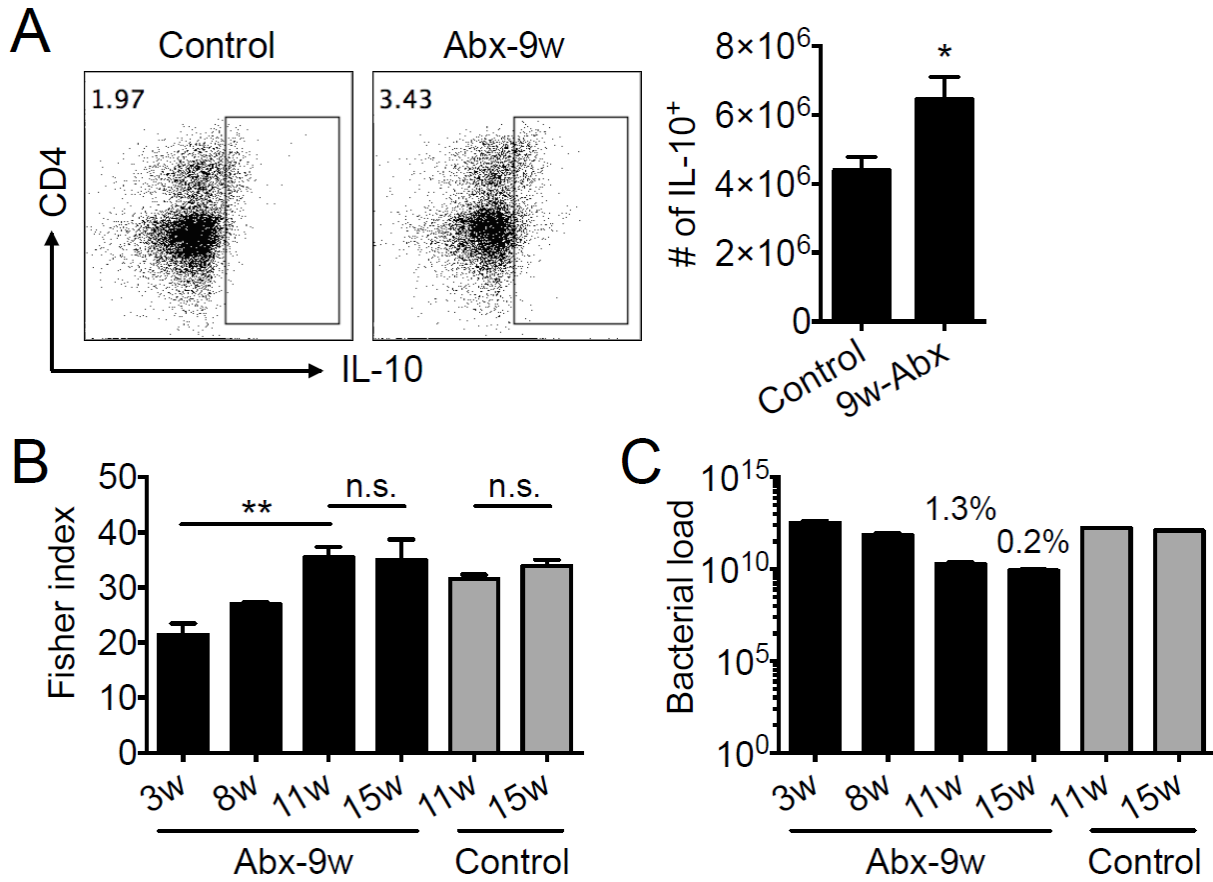


**Figure 6. Vancomycin treatment differentially affected KEGG pathways in a time-dependent manner.** (A) Changes of level 3 functional pathways over time. The result of PICRUSt analysis was plotted with DESeq2. Raw data can be found in Table S1. Note that the 9-week microbiota samples were collected 4 days after the initiation of vancomycin treatment for the Van group. (B) Changes of level 3 functional pathways when data from 9-15 weeks were averaged within each treatment group. 9w-Van, vancomycin was given starting from 9 weeks of age. (C) Changes of the average level of 12 LPS-related functional genes over time. PICRUSt analysis was performed at the ortholog level. Raw data with the names of the LPS-related functional genes can be found in Table S2.



**Figure 7. Vancomycin treatment decreases intestinal permeability and the serum level of LPS.** (A) Diffusion of FITC-conjugated dextran into the circulation after oral gavage as a direct measurement of intestinal permeability ( $n \geq 8$  per group). Mice were at 15 weeks of age when the assay was performed. (B) Transcript levels of barrier-forming tight junction proteins in the intestinal epithelium in 15-week-old MRL/lpr mice ( $n = 4$  per group). (C) Serum level of LPS in 15-week-old MRL/lpr mice ( $n \geq 12$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ .

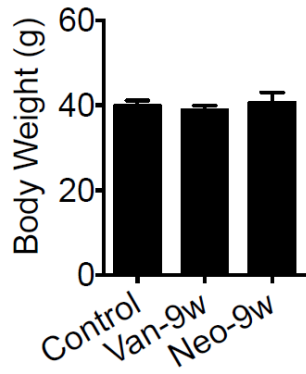
Supplemental Figure 1



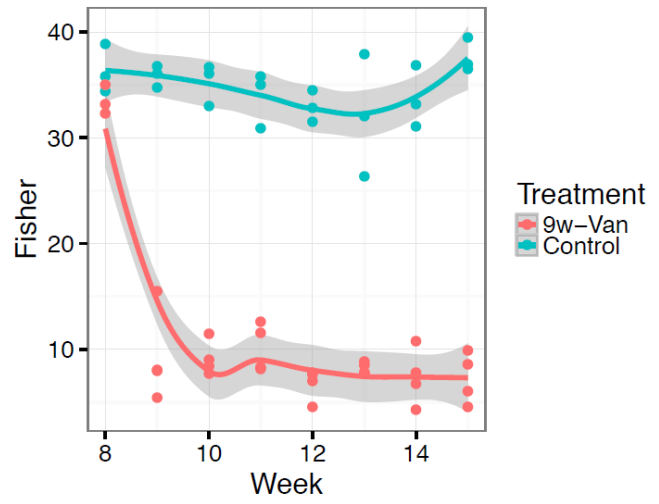
**Supplemental Figure 1.** (A) Percentage and number of IL-10-producing cells in the MLN. (B) Bacterial diversity upon antibiotic treatment. Fisher index is shown. (C) Bacterial load upon antibiotic treatment. The numbers shown are percentage of bacteria left in the gut microbiota. \* $p < 0.05$ , \*\* $p < 0.01$ . n.s., not significant.

Supplemental Figure 2

**A**

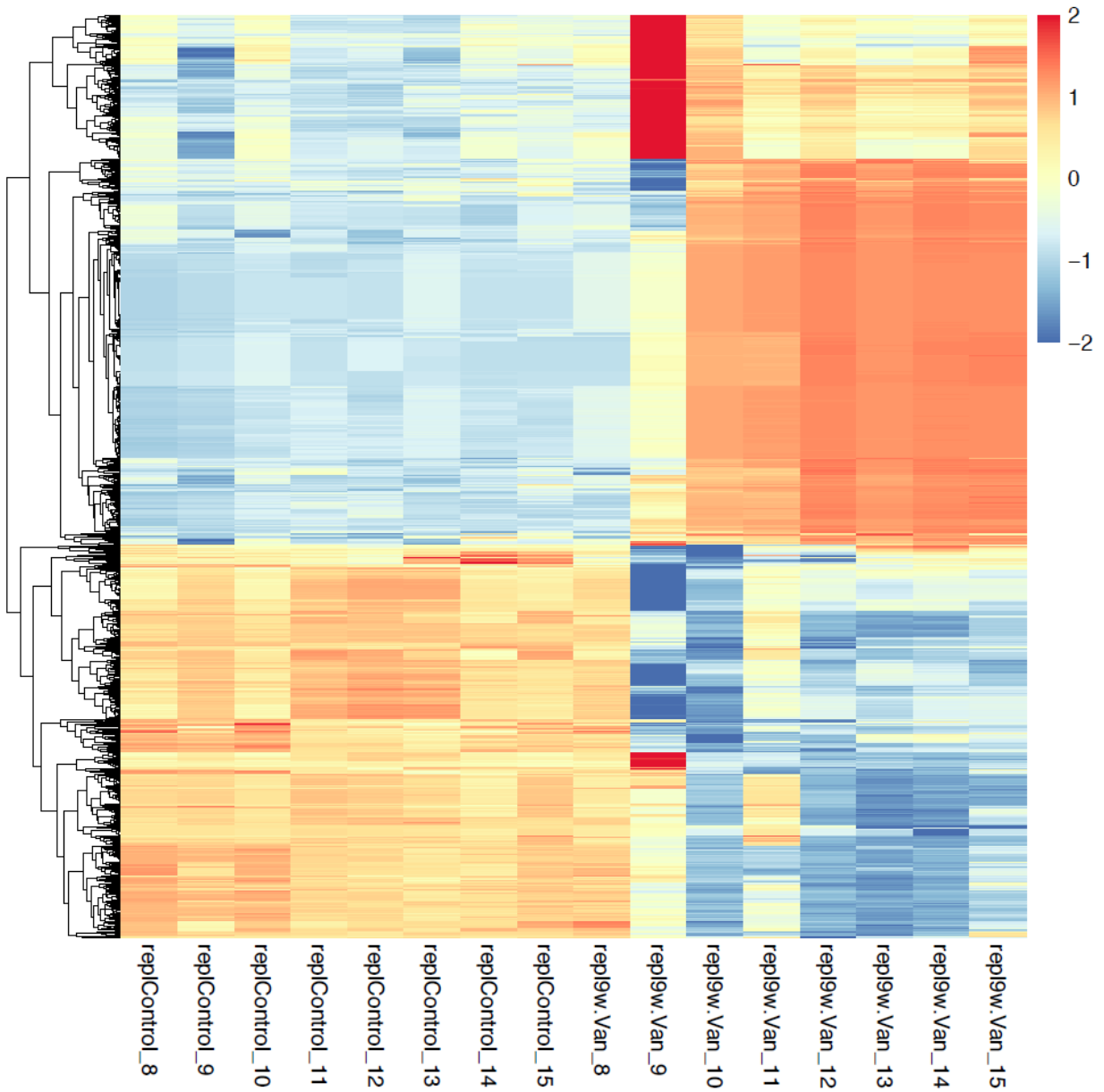


**B**



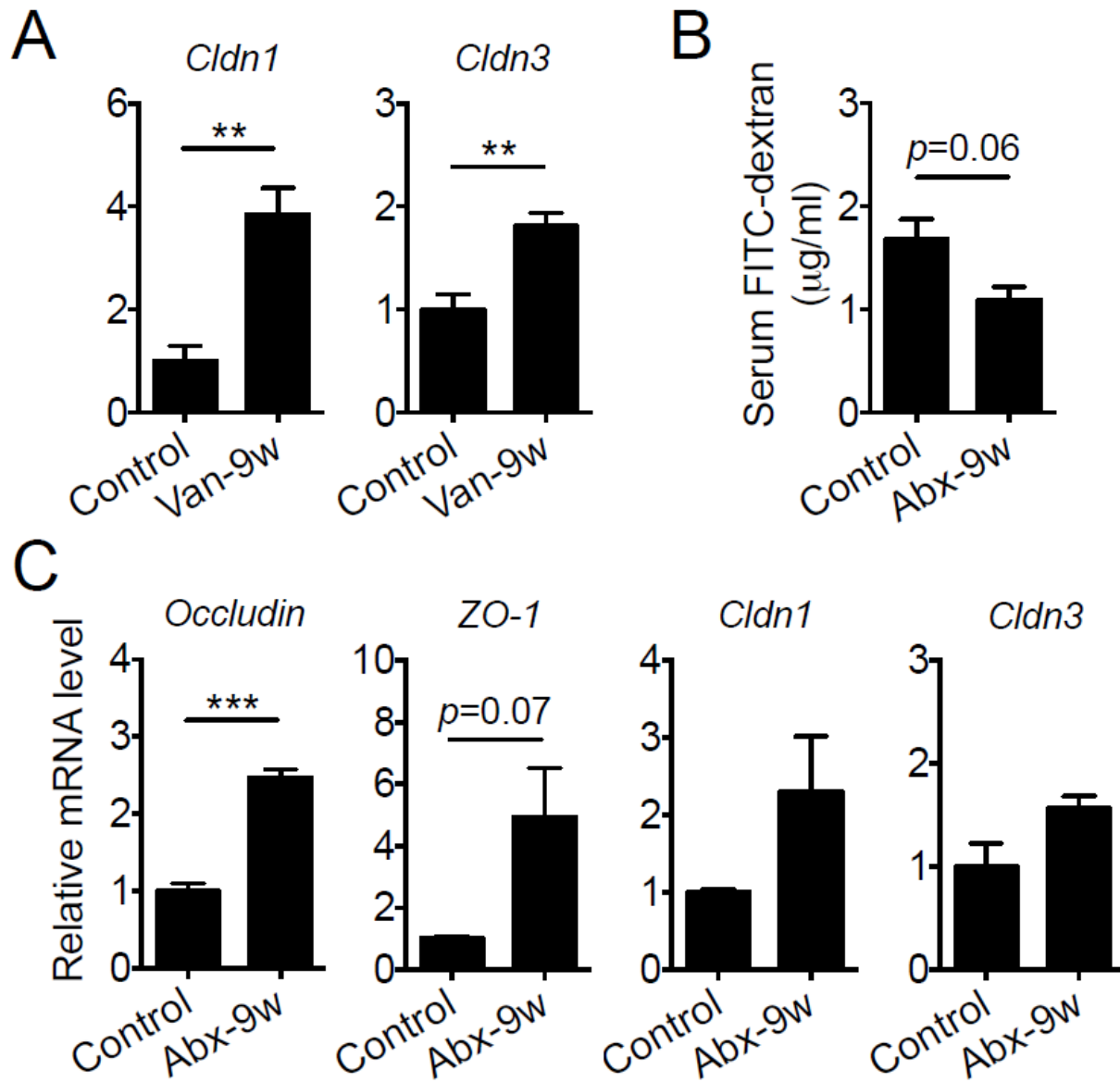
**Supplemental Figure 2.** (A) Body weight of vancomycin- or neomycin- treated mice. (B) Bacterial diversity upon vancomycin treatment. Fisher index is shown.

Supplemental Figure 3



**Supplemental Figure 3.** KEGG analysis (PICRUSt) at the ortholog level.

Supplemental Figure 4



**Supplemental Figure 4.** (A) Relative transcript levels of *Cldn1* and *Cldn3* in IECs upon vancomycin treatment. (B) Diffusion of FITC- dextran to the blood upon mixed antibiotic treatment. (C) Relative transcript levels of *Occludin*, *ZO-1*, *Cldn1* and *Cldn2* in IECs upon mixed antibiotic treatment. \*\*p<0.01, \*\*\*p<0.001.

## CHAPTER V

### **Induction of Regulatory B Cells by Bacterial DNA in the Gut Microbiota at Early Age is Beneficial in Lupus-prone Mice**

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## ABSTRACT

Systemic lupus erythematosus, or SLE, is a complex autoimmune disorder where the hallmark of disease is severe and persistent inflammation that can damage many peripheral organs. There is no known cure for this disease. We previously reported that oral vancomycin treatment of female lupus-prone MRL/lpr mice during active disease (from 9 to 15 weeks of age) could significantly ameliorate disease symptoms. The disease onset in MRL/lpr mice is around 8 weeks of age. However, when vancomycin treatment was initiated from an earlier age (from 3 to 15 weeks), the beneficial effect was not observed. Strikingly, mice given vancomycin during the pre-disease stage (from 3 to 8 weeks) exhibited exacerbated lupus disease. As vancomycin works by removing parts of the gut microbiota, we hypothesized that the regulatory immunity induced by gut microbiota at early age is essential in hampering lupus disease development in MRL/lpr mice. To test the hypothesis, we analyzed diverse regulatory cell types from the mice receiving vancomycin from 3 to 8 weeks of age. Regulatory B (Breg) cells, in particular, were found to be reduced in both the percentage and absolute number in multiple lymphoid organs. Importantly, adoptive transfer of Breg cells at 6-7 weeks of age to long-term vancomycin-treated mice (from 3 to 15 weeks of age) improved lupus-like symptoms. This clearly indicates that Breg cells, inducible by vancomycin-sensitive gut microbiota, plays an important role in suppressing lupus disease initiation and progression. We next sought to determine Breg inducers in the gut microbiota. As the serum level of bacterial DNA was found to be significantly lower in mice treated with vancomycin, we hypothesized that bacterial DNA, rich in unmethylated CpG motif, could induce Breg cells and ameliorate lupus nephritis. Indeed, early administration of bacterial DNA reproduced the beneficial effect seen in the Breg adoptive transfer experiment. Together, these results suggest an important protective mechanism against lupus initiation that involves

bacterial DNA in the gut microbiota and the induction of Breg cells. The administration of bacterial DNA may be a new and attractive therapeutic strategy for SLE, especially juvenile lupus.

## INTRODUCTION

Recent evidences suggest that there is an association between the composition of gut microbiota and the pathogenesis of systemic lupus erythematosus (SLE), a systemic autoimmune disease affecting over 5 million people worldwide (data from Lupus Foundation of America) [1-3]. Our research team has reported intestinal dysbiosis in SLE patients and the dynamics of the gut microbiota in different lupus-prone mouse models [4, 5]. In female MRL/Mp-*Fas*<sup>lpr</sup> (MRL/lpr) mice, there was significant depletion of Lactobacilli but increase of *Lachnospiraceae*.

Importantly, when these mice were orally given a mixture of 5 *Lactobacillus* strains, the lupus disease symptoms were largely attenuated [6]. Several reports from other groups also suggest that remodeling the gut microbiota could change lupus disease progression in lupus-prone mice [7-9]. Notably, germ-free MRL/lpr female mice exhibited very similar lupus disease course and clinical parameters compared to mice housed under conventional conditions [10]. This indicates that complete removal of gut microbiota throughout the lifespan does not attenuate or exacerbate lupus. However, we recently found that the removal of gut microbiota, achieved by mixed antibiotics (ampicillin, neomycin, metronidazole and vancomycin) or vancomycin alone, after lupus onset ameliorated lupus nephritis in female MRL/lpr mice [11]. Thus, we hypothesize that the effects of gut microbiota on lupus disease is time-dependent.

In the past decade, tremendous advances have been achieved in understanding the development and function of regulatory B (Breg) cells [12]. Capable of producing anti-inflammatory cytokines, Breg cells have been recognized as a critical regulator in both normal and aberrant immune responses, especially in autoimmune disorders [13]. Numerical impairment of Breg cells has been observed in SLE patients, particularly those with active lupus nephritis [14]. The protective role of Breg against lupus disease has been illustrated by multiple mouse

studies [13, 15, 16]. The initial finding that B cell-deficient lupus-prone mice exhibited exacerbated disease outcome brought the suppressive functions of Breg cells to light [16]. Further studies revealed that the exacerbated disease phenotype was seen when B cells were depleted early in life [17]. In contrast, B cell depletion during late stage of disease was beneficial, consistent with the functions of B cells to produce pathogenic autoantibodies and present autoantigens to T cells in lupus [18, 19]. This suggests that Breg-mediated protection from lupus may be restricted to the pre-disease stage. However, direct experimental evidence is lacking to support the hypothesis that the effect of Breg cells on lupus is time-dependent.

The association between gut microbiota and Breg development remains to be elusive, although one study has linked microbiota driven IL-1 $\beta$  and IL-6 to Breg induction in an induced arthritis mouse model [20]. We hypothesize that Breg cells can be induced by bacterial DNA in the gut microbiota. Compared to normal mice, B cells isolated from lupus-prone mice produce much more IL-10 in response to the stimulation of CpG oligonucleotides, but not to B-cell receptor or CD40 ligation [21]. Moreover, B cells express toll-like receptor 9 (TLR9), the receptor of CpG-DNA; and TLR9-deficient lupus mice exhibit exacerbated disease suggesting a protective role for TLR9 ligation in lupus [22]. Together, these data support our hypothesis that bacterial DNA from gut microbiota, rich in unmethylated CpG motifs [23], may promote the protective effects of Breg cells against lupus by inducing their IL-10 production.

In the present study, we show that unlike the treatment during active disease (from 9 to 15 weeks of age) that significantly ameliorated disease symptoms in MRL/lpr mice, oral vancomycin is not beneficial when initiated from an earlier age (from 3 to 15 weeks). Strikingly, mice given vancomycin during the pre-disease stage (from 3 to 8 weeks) exhibit an even worse disease phenotype. The exacerbated disease is associated with a marked decrease of Breg cells in

multiple organs. Importantly, adoptive transfer of IL-10 producing Breg cells at 6-7 weeks of age, but not 11-12 weeks of age, to vancomycin-treated mice improves lupus-like disease. As the level of bacterial DNA was significantly reduced in the gut and serum of mice treated with vancomycin at the young age, we hypothesized that bacterial DNA, rich in unmethylated CpG motif, could induce Breg cells and ameliorate lupus disease. Indeed, early administration of bacterial DNA reproduces the beneficial effect seen in the Breg adoptive transfer experiment. Together, these results suggest an important protective mechanism against lupus initiation that involves bacterial DNA in the gut microbiota and the induction of Breg cells.

## RESULTS

*Vancomycin given at pre-disease stage exacerbated lupus nephritis.* We previously reported that when MRL/lpr females were orally treated with vancomycin starting at 9 weeks of age and post disease onset, the disease severity at 15 weeks of age was largely reduced [11]. Vancomycin treatment during the same time frame [9 to 15 weeks, or Van(9-15)] in male MRL/lpr mice led to a similar beneficial effect, including significantly reduced spleen and mesenteric lymph node (MLN) weights, a lower serum autoantibody level, and less proteinuria (Fig. S1A-C). This suggests that the beneficial effect of vancomycin on diseased MRL/lpr mice is not sex-dependent. However, vancomycin is not currently used in the clinic as a treatment for SLE. Indeed, when we extended the time course of vancomycin treatment to 3 to 15 weeks [Van(3-15)], the beneficial effects disappeared in both female (Fig. 1A-E) and male MRL/lpr mice (Fig. S1A-C). As the 3-15 week time frame covers both pre-disease and active-disease stages, we asked whether vancomycin treatment during pre-disease stage [3 to 8 weeks, or Van(3-8)] could promote lupus disease development. Strikingly, splenomegaly in female MRL/lpr mice was aggravated with Van(3-8) treatment (Fig. 1A). In addition, the serum level of IgG autoantibodies against double-stranded (ds)DNA and the ratio of anti-dsDNA to total IgG were significantly elevated (Fig. 1B). As kidney inflammation (or lupus nephritis) affects more than half of SLE patients [24], we determined the renal function by measuring proteinuria and renal lymph node (RLN) weight, and performing kidney histopathological analysis in a blinded fashion. Both the proteinuria level and RLN weight were significantly increased in the Van(3-8) group compared to controls (Fig. 1C-D). Early vancomycin treatment also resulted in significantly higher pathological scores in glomerular and tubulointerstitial (TI) examinations (Fig. 1E). Taken

together, these results indicate that vancomycin treatment during pre-disease stage could exacerbate lupus-like disease in female MRL/lpr mice.

***Early vancomycin treatment promoted pro-inflammatory cytokines and reduced Breg cells.*** To begin to decipher the mechanism by which early vancomycin treatment worsened lupus disease, we examined immune cell populations and production of inflammatory mediators. As we had previously shown that decreased IL-6 and IL-17 levels contributed to the attenuated disease phenotype observed upon Van(9-15) treatment [11], we focused our attention on IL-6 and IL-17. Here, we found that the long-term Van(3-15) treatment led to a similar level of IL-6 in the serum but the value was significantly upregulated in mice with Van(3-8) treatment (Fig. 2A). We next quantified splenic double-negative (DN) T cells and T-helper (Th17) cells (Fig. S2A), two major cellular resources of IL-17 in both human and mouse lupus [25]. As anticipated, the percentage and absolute number of DN T cells were significantly increased in the Van(3-8) group (Fig. 2B), together with a trend of reduced proportion of CD8<sup>+</sup> T cells, though insignificant, which are thought to be protective in lupus nephritis (Fig. S2B) [26, 27]. Moreover, significant immune imbalance towards Th17 cells rather than regulatory T (Treg) cells was noted (Fig. 2B). These data suggest a higher production of IL-17 in the spleen of Van(3-8) group mice. As IL-17 was too low to be detected in the circulation (data not shown), we measured IL-17 mRNA expression in the spleen that may reflect the systemic level of IL-17. Consistent with the flow cytometry data, the transcript level of IL-17 was significantly increased in both spleen and kidney (Fig. 2C). Furthermore, we examined the level of IFN $\gamma$ , a cytokine known to promote lupus disease in both human and mice [28]. An elevated level of IFN $\gamma$  was found in the serum of early vancomycin treated mice (Fig. 2D). In the spleen of female MRL/lpr mice, almost all the IFN $\gamma$  producing

cells were T cells (data not shown). Further analysis showed that mice treated with Van(3-8) had significantly more IFN $\gamma$  producing CD4<sup>+</sup> T cells (or Th1 cells) in the spleen (Fig. 2D and S2C).

To delineate the mechanism that led to systemic inflammation at the endpoint (15 weeks of age), we analyzed the phenotypes of immune cells in 8-week-old mice right after Van(3-8) treatment. Interestingly, we found significantly decreased Breg but not Treg cells when comparing the effect of vancomycin to the control (Fig. 2E and S2E). Breg cells produce large amounts of IL-10 and IL-35, two anti-inflammatory cytokines known as key mediators of the regulatory function of Breg cells in diverse immune disorders including lupus [12]. In our study, early administration of vancomycin [Van(3-8)] dampened the serum levels of both IL-10 and IL-35 (Fig. 2E), suggesting a functional loss of Breg cells. In addition, the bias towards DN T cell differentiation was already observed at this age in vancomycin-treated mice (Fig. S2E), suggesting that the enhanced IL-17 production was initiated early. In contrast, the production of IFN $\gamma$  was not affected at 8 weeks of age (data not shown). Notably, the percentage of Breg cells in 15-week-old mice did not differ between control and vancomycin and regardless of the time of vancomycin treatment (Fig. S2D). It is likely due to the enrichment of Breg inducers during the active-disease stage of lupus, for example self DNA complexes and multiple elevated pro-inflammatory cytokines [20, 29]. Taken together, these results suggest that removal of gut microbiota by vancomycin treatment during the pre-disease stage of lupus reduced Breg cells, resulting in a pro-inflammatory response that may contribute to the exacerbation of lupus-like disease in female MRL/lpr mice.

***Adoptive transfer of Breg cells at early age attenuated lupus in vancomycin-treated mice.***

Considering the potential role of Breg cells in regulating the autoimmunity in lupus-prone mice, we adoptively transferred Breg cells into vancomycin-treated mice. In addition to the control and



vancomycin treatment [Van(3-15)] groups, we have 3 vancomycin-treated groups with cell transfer: 1) sorted IL-10<sup>+</sup> Breg cells, or B10 cells (Fig. S3A), injected at pre-disease stage (6 and 7 weeks old); 2) other cells with Breg cells depleted, injected at 6 and 7 weeks of age; 3) Breg cells injected at active-disease stage (11 and 12 weeks old). Compared to control mice and mice with only vancomycin treatment, early transfer of Breg cells significantly reduced spleen and MLN weights (Fig. 3A), decreased IgG autoantibody level (Fig. 3B), and ameliorated lupus nephritis: a reduced proteinuria level, smaller RLN and a lower pathological score of the kidney glomeruli (Fig. 3C-D). Neither the other-cell control at the pre-disease stage nor Breg transfer at the active-disease stage exerted any beneficial effects (Fig. S3B-C). The lack of beneficial response to late Breg injection suggests that the immunosuppressive function of Breg cells may be more pronounced at young age and during the pre-disease stage of lupus development.

We next examined immune cell populations and cytokine production with and without adoptive transfer. IL-17 production was blocked with Breg transfer at the pre-disease stage, reflected by significantly decreased DN T cell proportions and the balance of Treg/Th17 towards Treg cells (Fig. 3E). Additionally, the percentage of IFN $\gamma$  producing CD4<sup>+</sup> T cells was significantly lowered in mice transferred with Breg cells at early age (Fig. 3E). The level of IFN $\gamma$  in the circulation was not significantly affected, although there was a trend for decline (Fig. S3D). In summary, restoring Breg cells in the vancomycin-treated mice with hampered Breg functions significantly reduced IL-17 and IFN $\gamma$  producing cells and attenuated systemic and nephritic symptoms of lupus. These results clearly indicate that the gut microbiota removed by vancomycin may be responsible for inducing Breg development at the pre-disease stage, whereas Breg cells would in turn dampen the initiation of lupus disease.

***Restoration of bacterial DNA attenuated lupus disease in vancomycin-treated mice.***

Vancomycin targets Gram-positive bacteria such as Clostridia that are producers of short-chain fatty acids (SCFAs) [30, 31]. Therefore, we tested the hypothesis that treatment of vancomycin during the pre-disease stage removed SCFAs that are inducers of Breg cells. We first quantified the levels of fecal SCFAs with gas chromatography. In female MRL/lpr mice, the level of total SCFAs increased from 3 to 5 weeks of age and plateaued after 5 weeks of age (Fig. S4A).

Vancomycin treatment significantly lowered the level of fecal SCFAs, consistent with vancomycin-mediated removal of Clostridia (Fig. S4B). Further analysis showed that 3 most abundant SCFAs—acetate, propionate and butyrate—were all significantly reduced in the feces upon vancomycin treatment (Fig. S4C). In contrast, the fecal heptanoate level significantly increased, although its role in immune regulation and autoimmunity is unknown. In light of the effect of SCFAs in inducing Treg cells [32], it would be of interest to examine whether restoration of SCFAs could educate Breg cells and attenuate lupus. We thus supplied acetate, propionate and butyrate in the drinking water for mice receiving vancomycin treatment. No difference was observed for Breg cells between mice with or without SCFA treatment (Fig. S4D). Consequently, SCFAs supplement did not attenuate either systemic autoimmunity (Fig. S4E) or lupus nephritis (Fig. S4F). These results suggest that the reduction of SCFAs upon vancomycin administration was not the reason for Breg dysfunction and disease exacerbation.

The removal of Clostridia by vancomycin also led to a significant decrease in the total bacterial load in the gut (Fig. 4A) as well as a significant reduction of bacterial DNA in the circulation (Fig. 4B). We thus asked the question whether bacterial DNA could be Breg inducers that could protect vancomycin-treated mice from the exacerbated disease phenotype.

Vancomycin-treated female MRL/lpr mice were orally gavaged with endotoxin-free *E. coli* DNA

once a week from 4 to 7 weeks of age. The administration of bacterial DNA led to significant decreases of spleen and MLN weights (Fig. 4C), and a significantly lower level of anti-dsDNA autoantibodies in the serum (Fig. 4D). We also monitored the proteinuria level weekly after disease onset at 8 weeks of age and witnessed significantly lowered values in mice treated with bacterial DNA (Fig. 4E). In addition, the kidney glomerular pathological score was significantly decreased (Fig. 4F), suggesting improved renal function with bacterial DNA administration. These results suggest that bacterial DNA is protective against lupus, and that the removal of bacterial DNA by vancomycin treatment during the pre-disease stage may be the cause of lupus exacerbation.

We next investigated the effects of bacterial DNA on inflammatory mediators in the circulation and their cellular sources in the spleen. The serum IL-6 concentration was dramatically decreased by bacterial DNA treatment (Fig. 5A). Correspondingly, splenic Th17 and DN T cells, the major IL-17 producers, were significantly reduced, together with a significant increase of the proportion of CD8<sup>+</sup> T cells (Fig. 5B). In both spleen and kidney, the expression of IL-17 mRNA was significantly decreased with bacterial DNA administration (Fig. 5C). In addition, IFN $\gamma$  production from splenic CD4<sup>+</sup> T cells was inhibited, resulting in a significant decrease of serum IFN $\gamma$  (Fig. 5D). Moreover, the expression of CD44 in CD8<sup>+</sup> T cells was significantly elevated in the spleen of bacterial DNA-treated mice (Fig. 5E), resulting in a significantly higher percentage of CD44<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 5F). Furthermore, treatment with bacterial DNA significantly decreased the transcript levels of type 1 and type 2 hyaluronan synthases (Has) in the kidney, enzymes that mediate the secretion of hyaluronate (HA) (Fig. 5G) [33]. HA is known as a positive contributor to lupus nephritis by inducing the production of several inflammatory cytokines including IL-1 $\beta$ , TNF $\alpha$  and IL-6 [34]. We noted that the

expression of TNF $\alpha$  mRNA in the kidney was significantly inhibited (Fig. S5). This may be another mechanism by which the administration of bacterial DNA attenuated lupus nephritis in vancomycin-treated female MRL/lpr mice.

## DISCUSSION

An important role of gut microbiota in the pathogenesis of SLE has been suggested [1]. We found different gut microbiota structures in SLE patients with active disease as well as lupus-prone mouse models compared to their respective controls [4, 5]. MRL/lpr mice, in particular, showed a significantly reduced abundance of Lactobacilli [4]. More importantly, restoration of Lactobacilli in the gut largely ameliorated lupus-like disease, together with reversal of a leaky gut and lower IL-17 production [6]. Moreover, removal of gut microbiota by antibiotics treatment after disease onset resulted in alleviated clinical parameters [11]. However, complete removal of gut microbiota throughout life, achieved with germ-free housing, did not affect the disease outcome in MRL/lpr mice [10]. Consistently, we observed no change in disease manifestations in MRL/lpr mice receiving mixed antibiotics from the weaning age till the endpoint (data not shown). The contradictory results between long-term vs. active-disease stage antibiotic treatment suggest that the gut microbiota as a whole, or some specific bacterial species within the gut microbiota, may play an important role during the pre-disease stage to educate an immune response against lupus. Indeed, antibiotic treatment during the pre-disease stage promoted lupus progression (Fig. 1). The exacerbated disease was associated with increased production of IL-6, IL-17 and IFN $\gamma$  (Fig. 2A-D), which are known contributing factors of lupus pathogenesis [28, 35, 36]. While the percentage of Breg cells at 15 weeks of age did not differ with or without antibiotic treatment at the pre-disease stage (Fig. S2D), the development of Breg cells was significantly inhibited at an earlier time point and right after the cessation of antibiotic treatment (8 weeks of age; Fig. 2E). Remarkably, adoptive transfer of Breg cells into long-term vancomycin-treated mice during the pre-disease stage led to attenuated disease (Fig. 3). This suggests that restoration of Breg cells during the pre-disease stage could overcome the adverse

effects of early gut microbiota depletion. Although diverse gut bacterial species and their metabolites were changed due to vancomycin treatment, the loss of bacterial DNA appears to have inhibited Breg development. Restoration of bacterial DNA regenerated the alleviated outcomes seen with the adoptive transfer experiment (Fig. 4).

B cells are known as a positive contributor to autoimmunity due to their capabilities to produce autoantibodies and stimulate autoreactive T cells [37]. However, many studies have confirmed the regulatory function of a subpopulation of B cells, namely Breg cells [12]. Through producing the anti-inflammatory cytokine IL-10, Breg cells play a critical role in the regulation of both normal and aberrant immune responses [13]. Unlike Treg cells that express *Foxp3*, Breg cells do not have a common transcription factor and any B cells, including mature B cells, immature B cells and plasmablasts, may differentiate into IL-10 producing Breg cells in response to the right stimuli [12]. The gut microbiota has been demonstrated as an important environmental stimulus as wild-type mice treated with mixed antibiotics have fewer Breg cells [20], a phenomenon observed in our lupus-prone model as well (Fig. 2E). TLR9 activation promotes IL-10 production from B cells in different lupus models [21, 38]. Interestingly, unlike TLR7, TLR9 deficiency in lupus mice led to more severe disease development [22, 39, 40]. TLR9 recognizes CpG DNA; and bacterial DNA, in contrast to mammalian DNA, is rich in CpG islands. In our study, we found that vancomycin treatment at early age significantly removed bacterial DNA from the gut and circulation (Fig. 4A-B). As a result, the development of Breg cells at 8 weeks of age was dramatically inhibited (Fig. 2E), leading to more severe clinical outcomes at the late-disease stage (Fig. 1). Importantly, restoration of bacterial DNA in the antibiotic-treated mice downregulated IL-17 and IFN $\gamma$  related immune responses (Fig. 5B-D) and significantly attenuated lupus nephritis (Fig. 4). These results are consistent with those of an

earlier study in NZB/W F1 mice, where immunization with bacterial DNA was protective against lupus nephritis [41]. However, no mechanism was shown in the earlier study, and the administration route of bacterial DNA was different. We administered bacterial DNA orally to mimic the contribution of gut microbiota and present here a Breg-mediated mechanism by which bacterial DNA protects against lupus nephritis.

There is a numerical impairment of Breg cells in SLE patients, particularly those with active nephritis [14]. Defective IL-10 production and reduced immunosuppressive ability were observed in Breg cells isolated from the peripheral blood of SLE patients [42]. This indicates impairment of the regulatory function of Breg cells in human SLE, highlighting them as a potential therapeutic target. Breg cells also play an important role in lupus-prone mice. NZB/W F1 mice deficient of CD19, compared to unaltered NZB/W F1 mice, showed an earlier onset of lupus nephritis and exhibited a reduced survival rate, though the emergence of autoantibodies was also delayed [16]. In another study, the depletion of all mature B cells, including Breg cells, accelerated disease onset [17]. However, the exacerbation of lupus was only seen in NZB/W F1 mice with B-cell depletion initiated very early in life – from 4 weeks of age. In contrast, when B-cell depletion started at 12- to 28 weeks of age, the disease was significantly inhibited. These results not only suggest an important role of Breg cells in regulating disease development in NZB/W F1 mice, but also provide evidence that the suppressive effect of Breg cells in lupus is time-dependent: they are only effective during disease initiation. Although using a different lupus-prone mouse model, we observed a very similar phenomenon on the development and effect of Breg cells. Early removal of the environmental inducers of Breg cells significantly reduced the percentage of Breg cells in the spleen (Fig. 2E), resulting in earlier disease initiation (data not shown) and exacerbation of lupus (Fig. 1). On the contrary, the same antibiotic dosage

given at a later time (9 to 15 weeks of age) attenuated lupus-like disease in the same mouse strain [11]. Another supporting evidence is that adoptive transfer of Breg cells ameliorated disease parameters in vancomycin-treated mice when injected early rather than late (Fig. 3 and S3). These results clearly indicate that the immunosuppressive function of Breg cells is only effective during lupus initiation. In addition, we found that the percentages of Breg cells were similar at the late-disease stage (15 week of age) regardless of treatment (Fig. S2D). This is because self DNA complexes are highly accumulated during active disease, which, together with elevated pro-inflammatory cytokines, dominate the induction of Breg cells [29]. Thus, the effect of bacterial DNA at the active-disease stage is insignificant, which can well explain why antibiotic treatment during the active-disease stage is beneficial [11] even with the removal of bacterial DNA.

In MRL/lpr mice, the results are somewhat controversial regarding the role of Breg cells. One report described Breg cells as protective, as the transfer of *in vitro* anti-CD40-generated B cells greatly improved lupus nephritis through an IL-10-dependent mechanism [15]. However, in another study, B-cell-specific IL-10 deletion did not affect lupus progression, implying that endogenous IL-10 producing Breg cells are ineffective in suppressing autoimmunity in MRL/lpr mice [43]. We think that other anti-inflammatory mediators originated from Breg cells compensated the loss of IL-10 in regulating autoimmunity. Indeed, IL-10 independent immune suppression by Breg cells also occur [12]. For example, a unique subset of IL-35 producing B cells overlaps with the IL-10<sup>+</sup> Breg subset. The promotion of IL-35<sup>+</sup> Breg cells *in vivo* conferred protection against autoimmune disease [44]. In this study, the reductions of IL-10<sup>+</sup> Breg cells and serum IL-10 were accompanied by reduced serum IL-35 (Fig. 2E), suggesting that IL-35 may be responsible for the regulatory function of Breg cells on lupus in



MRL/lpr mice. In addition, Breg cells are able to suppress autoimmune inflammation through elevated expression of PD-L1 [45]. One study, though not in an autoimmune disease model, confirmed PD-L1 expression as another mechanism by which Breg cells suppress effector T cells [46].

While we have proposed that the induction of Breg cells by bacterial DNA is responsible for the attenuation of lupus nephritis in vancomycin-treated mice, other events evoked by bacterial DNA treatment may also be involved in the regulation of autoimmunity. In this study, we showed that oral bacterial DNA treatment upregulated the expression of CD44 in CD8<sup>+</sup> T cells but not in CD4<sup>+</sup> or DN T cells (Fig. 5E-F). CD44 is important for T cell migration into inflammatory sites such as nephritic kidney [47]. Due to their potential protective effect against lupus [48], more CD8<sup>+</sup> T cells infiltrating the kidney may be beneficial. HA is known as the principle extracellular CD44 ligand [33]. CD44-HA interaction mediates the recruitment of diverse immune cells, in particular T cells, and contributes to disease activity in lupus [49-51]. In patients and mice bearing active lupus nephritis, the secretion of HA is enhanced in kidney, which correlates with lymphocyte infiltration and kidney damage [52, 53]. Importantly, the inhibition of HA in lupus-prone mice improved disease parameters, at least partially by reducing pro-inflammatory cytokine expression in the kidney [34]. This suggests a contributing role of HA in the pathogenesis of lupus nephritis. Up to date, 3 mammalian HA synthases have been found (Has1, Has2 and Has3). Interestingly, in mice treated with bacterial DNA, the intrarenal synthesis of Has1 and Has2 were significantly decreased (Fig. 5G). Notably, anti-dsDNA antibodies have been recognized as an inducer of HA production in human kidney cells, and this induction is dependent on the expression of Has2 [52]. Therefore, the significantly lower anti-dsDNA antibody level in the circulation with bacterial DNA treatment (Fig. 4C) may explain the

reduction of HA synthesis. Subsequently, decreased HA in the kidney could lead to fewer CD4<sup>+</sup> and DN T cell infiltration, reflected by the decrease in IL-17 mRNA expression (Fig. 5C). The loss of HA might also impair pro-inflammatory cytokine induction in the kidney (Fig. S5). As a result, the kidney damage during lupus progression was attenuated (Fig. 4E-F).

In conclusion, our results suggest an important protective mechanism against lupus that involves bacterial DNA in the gut microbiota and the induction of Breg cells. Further studies are required to examine the potential of using bacterial DNA as a preventive method against lupus.

## METHODS

**Mice and treatments.** MRL/Mp-*Fas*<sup>lpr</sup> (MRL/lpr, stock number 000485) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred and maintained in a specific pathogen-free facility according to the requirements of the Institutional Animal Care and Use Committee (IACUC) at Virginia Tech (Animal Welfare Assurance Number: A3208-01). CO<sub>2</sub> was used for euthanasia according to the IACUC protocol. All experiments were performed in accordance with relevant guidelines and regulations. Vancomycin (2 g/l) was given in the drinking water during the indicated periods of time. The drinking water containing vancomycin was replenished once a week. Endotoxin-free *E. coli* bacterial DNA was purchased from InvivoGen (San Diego, CA). Eighty micrograms of bacterial DNA was orally gavaged to vancomycin-treated mice once a week for 4 consecutive weeks at 4, 5, 6 and 7 weeks of age.

**Breg cell isolation and adoptive transfer.** IL-10 producing B cells were isolated from the spleen and MLNs of sex, age-matched female MRL/lpr donor mice by using mouse regulatory B cell isolation kit purchased from Miltenyi Biotec (Gladbach, Germany). The recipient mouse was injected with 1 million Breg cells each time through tail vein i.v. injection. Each recipient mouse received two injections either at 6 and 7 weeks of age (during pre-disease stage) or at 11 and 12 weeks of age (during active-disease stage). Mice in control cell group were injected with cells depleted of Breg cells at 6 and 7 weeks of age.

**Renal function.** Urine was collected by squeezing out weekly starting from disease onset at 8 weeks of age, and all samples were stored at -20 °C till analyzed at the same time with a Pierce Coomassie Protein Assay Kit (Thermo Scientific). When mice were euthanized at 15 weeks of age, the kidney was fixed in formalin for 24 h, paraffin embedded, sectioned, and stained with

periodic acid-Schiff at the Histopathology Laboratory at Virginia-Maryland College of Veterinary Medicine. Slides were read with an Olympus BX43 microscope. All the slides were scored in a blinded fashion by a certified veterinary pathologist. Glomerular lesions were graded on a scale of 0 to 3 for each of the following 5 categories: increased cellularity, increased mesangial matrix, necrosis, the percentage of sclerotic glomeruli, and the presence of crescents. TI lesions were graded on a scale of 0 to 3 for each of the following four categories: presence of peritubular mononuclear infiltrates, tubular damage, interstitial fibrosis, and vasculitis.

***Cell isolation and flow cytometry.*** The spleen was collected and mashed in 70- $\mu$ m cell strainers with complete media (RPMI 1640, 10% fetal bovine serum, 1 mM sodium pyruvate, 1% 100 $\times$  MEM non-essential amino acids, 10 mM HEPES, 55  $\mu$ M 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin-streptomycin, all from Life Technologies, Grand Island, NY).. Red blood cells were lysed with RBC lysis buffer (eBioscience). For surface staining, cells were blocked with anti-mouse CD16/32 (eBioscience), stained with fluorochrome-conjugated antibodies, and analyzed with BD FACSAria II flow cytometer (BD Biosciences, San Jose, CA). For intracellular staining, Foxp3 Fixation/Permeabilization kit (eBioscience) was used. Zombie Aqua fixable viability kit (Biolegend) was used to exclude dead cells. Anti-mouse antibodies used in this study include the following: CD3-APC, CD4-PE-Cy7, CD8-PerCP/Cy5.5, IL-10-PerCP/Cy5.5, IFN $\gamma$ -APC/Cy7, IL-17A-FITC, CD44-FITC, CD62L-APC/Cy7, Foxp3-PE, B220-PE, CD19-APC (Biolegend); ROR $\gamma$ t-PE (eBioscience). Flow cytometry data were analyzed with FlowJo (BD).

***RT-qPCR.*** The spleen and kidney were homogenized with Bullet Blender homogenizer (Next Advance), and total RNA was extracted with RNeasy Plus Universal Kit (Qiagen) according to

the manufacturers' instructions. Genomic DNA was removed by digestion with RNase free DNase I (Qiagen). Reverse transcription was performed by using iScript cDNA Synthesis Kit (Bio-Rad). Quantitative PCR was performed with iTaq Universal SYBR Green Supermix (Bio-Rad) and ABI 7500 Fast Real-Time PCR System (Applied Biosystems). Relative quantities were calculated using *L32* as the housekeeping gene. Primer sequences for mouse *L32*, *IL-17A*, *Has1*, *Has2*, *Has3*, *IL-1 $\beta$* , *TNF $\alpha$*  and *IL-6* are available upon request.

**ELISA.** Anti-dsDNA IgG was measured according to a previously described method [54]. Total IgG and cytokine concentrations were determined with mouse IgG (Bethyl Laboratories), IL-10, IL-6, IFN $\gamma$  (Biolegend) and IL-35 (LifeSpan BioSciences) ELISA kits, respectively, according to the manufacturers' instructions.

**Microbiota sampling and analysis.** Fecal microbiota samples were obtained by taking a mouse out of the cage and collecting a fecal pellet. To avoid cross-contamination, each microbiota sample was collected by using a new pair of sterile tweezers. Samples were stored at  $-80^{\circ}\text{C}$  till being processed at the same time. Sample homogenization, cell lysis, and DNA extraction were performed as previously described [13]. Quantitative PCR were performed to measure the total bacteria load and the relative abundance of several bacteria species. The primer sequences for these species are available upon request. For gas chromatography measurement of SCFAs, fecal samples were acidified using phosphoric acid immediately before analysis. The injector settings were: temperature- $200^{\circ}\text{C}$ ; carrier-hydrogen; injection mode-split (ratio 2:1). The temperature program was: initial temperature  $80^{\circ}\text{C}$  held for 3 minutes, then increase temperature at a rate of  $6^{\circ}\text{C}$  per minute to  $140^{\circ}\text{C}$  and hold for 1 minute. The Flame Ionization Detector Settings were: Temperature- $250^{\circ}\text{C}$ ; Hydrogen Flow-35 ml/minute; Air Flow-350 ml/minute; Makeup Flow (Nitrogen)-15 ml/minute; Total Makeup (makeup + column flow)-30 ml/minute.

**Statistical analysis.** Analysis of non-sequencing data was performed with nonparametric Mann-Whitney test for the comparison of 2 groups, and nonparametric Kruskal-Wallis test for the comparison of 3 groups. The results were considered statistically significant when  $p < 0.05$ . All analyses were performed with Prism GraphPad (San Diego, CA).

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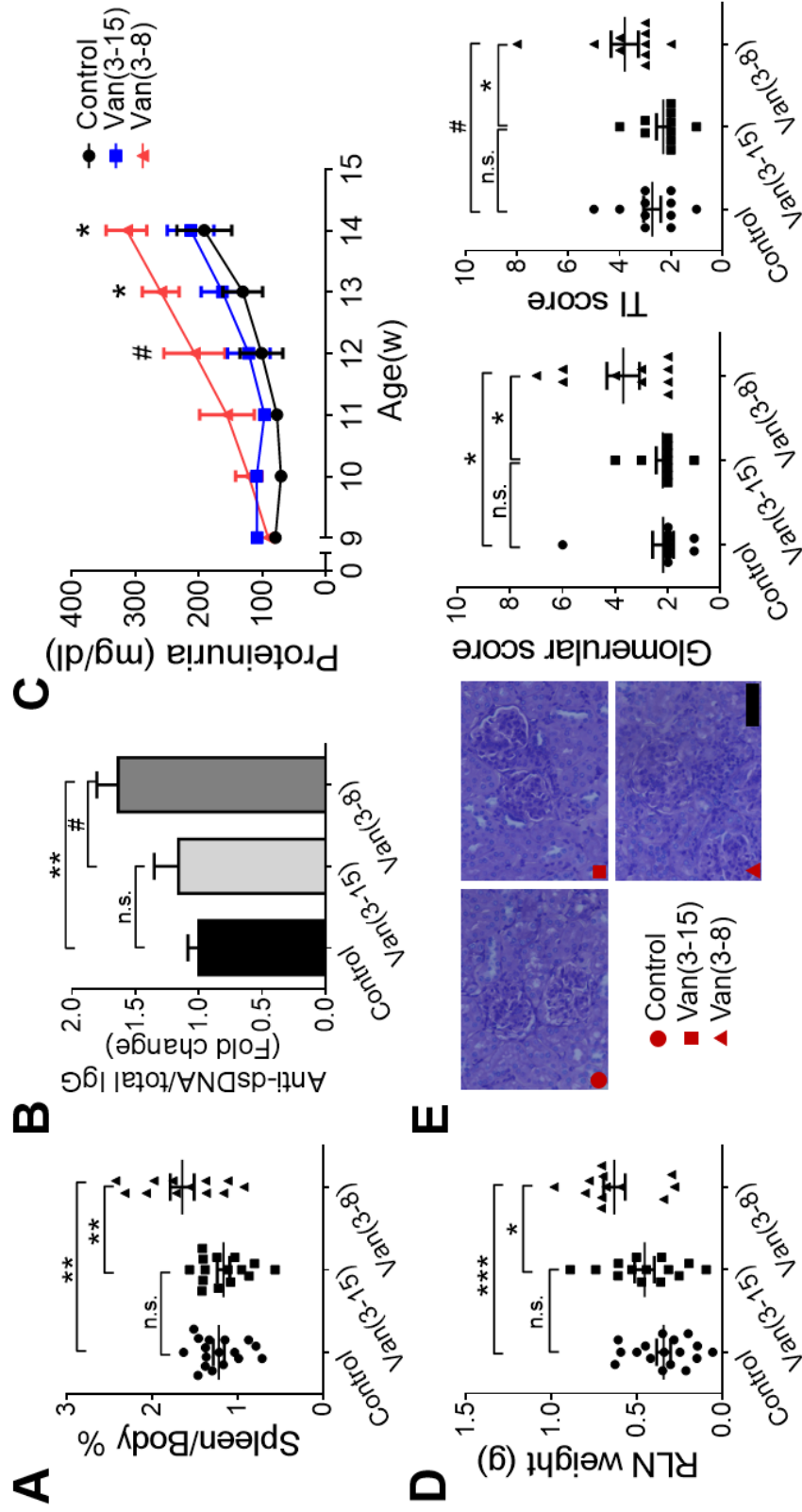


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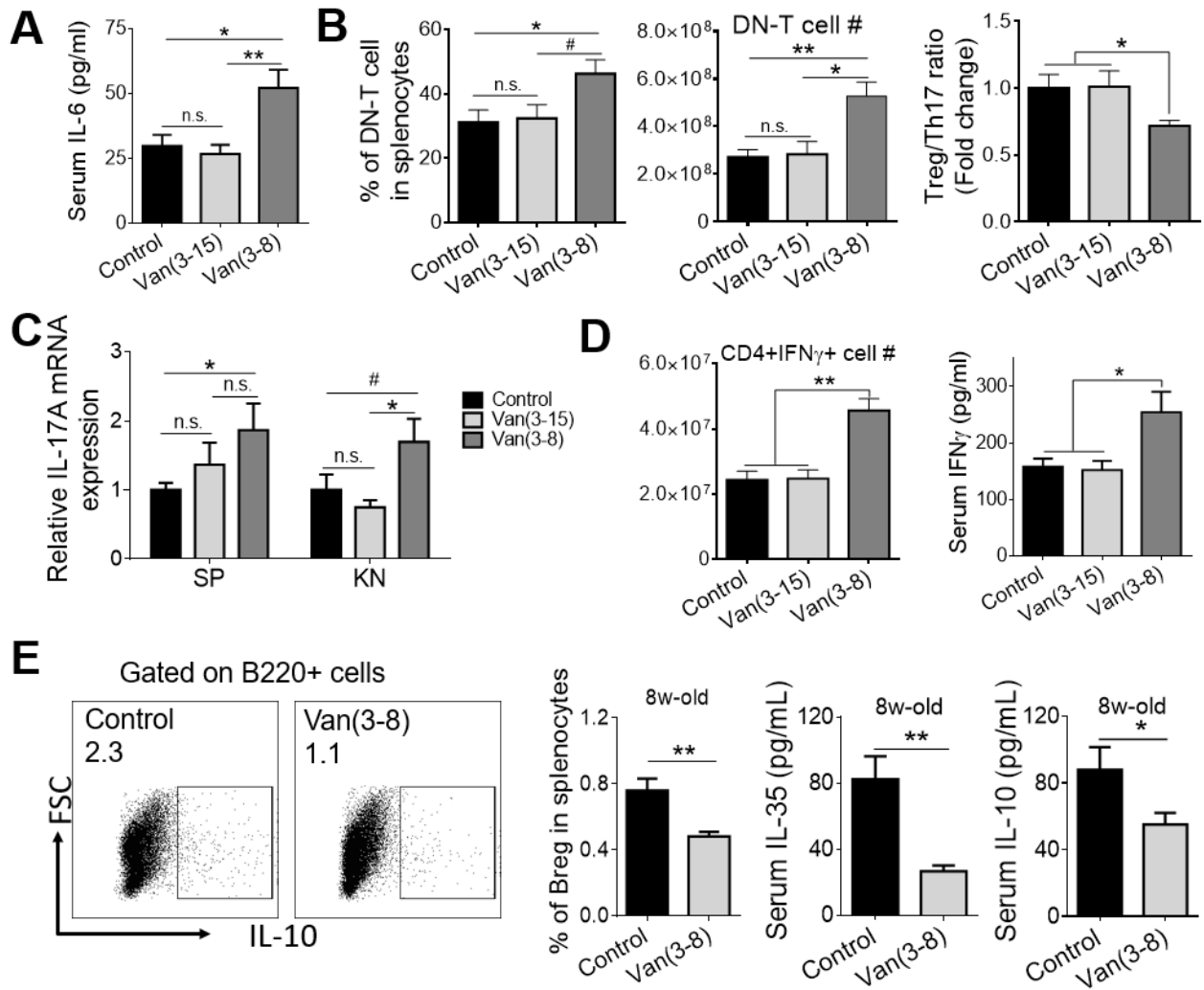
FIGURES

Fig 1.



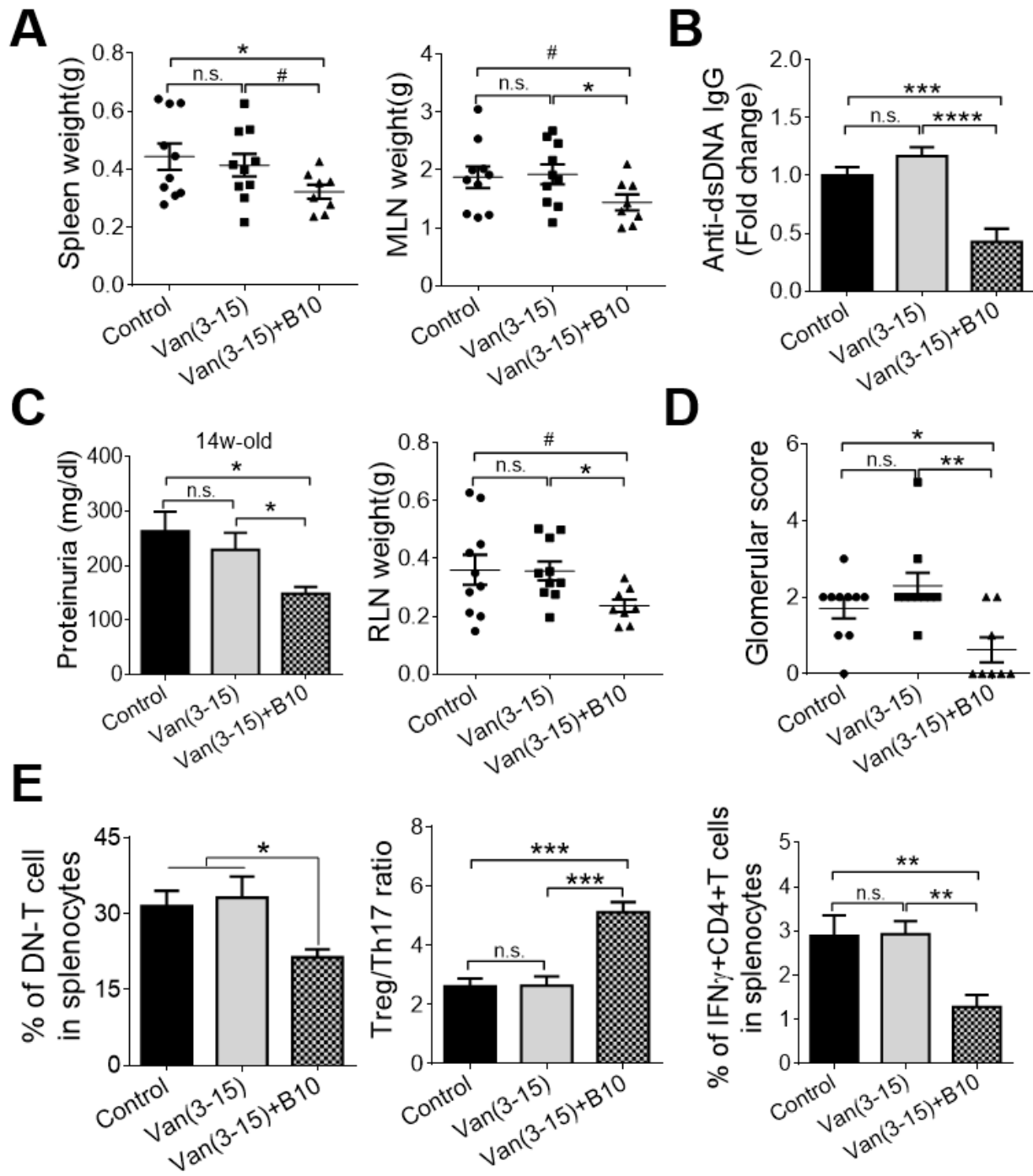
**Fig 1. Vancomycin given at pre-disease stage exacerbated lupus nephritis.** Group: Control: without any antibiotics. Van(3-15): mice received vancomycin treatment from 3 weeks to 15 weeks old. Van(3-8): mice received vancomycin treatment from 3 weeks to 8 weeks old. **(A)** Spleen weight to body weight ratio (%) at 15 weeks of age. **(B)** The ratio of anti-double stranded (ds)DNA IgG to total IgG in mouse serum at 15 weeks of age ( $n \geq 10$  per group). **(C)** Level of proteinuria over time ( $n \geq 10$  per group). **(D)** Renal lymph node (RLN) weight at 15 weeks of age. **(E)** Renal histopathology at 15 weeks of age. Left: representative PAS-stained kidney sections; bar equals 400  $\mu\text{m}$ . Middle: glomerular score. Right: tubulointerstitial (TI) score. # $p < 0.1$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Fig 2.



**Fig 2. Early vancomycin treatment promoted inflammatory immune response and reduced Breg cells.** (A) Serum level of IL-6 at 15 weeks of age ( $n \geq 7$  per group). (B) At 15 weeks of age: Left: the percentage of double negative (DN) T cells in splenocytes. Middle: the absolute number of DN-T cells in spleen. Right: the Treg cell to Th17 cell ratio in spleen. ( $n \geq 5$  per group). (C) Transcript level of IL-17A gene in the spleen and kidney at 15 weeks of age ( $n \geq 7$  per group). (D) The absolute number of IFN $\gamma$  producing CD4<sup>+</sup> T cells in spleen (left) and the IFN $\gamma$  level in mouse serum (right) at 15 weeks of age ( $n \geq 5$  per group). (E) FACS analysis of IL-10<sup>+</sup> Breg cells in spleen at 8 weeks of age. The percentage of Breg and serum IL-35 and IL-10 levels are shown ( $n=10$  per group). # $p < 0.1$ , \* $p < 0.05$ , \*\* $p < 0.01$ .

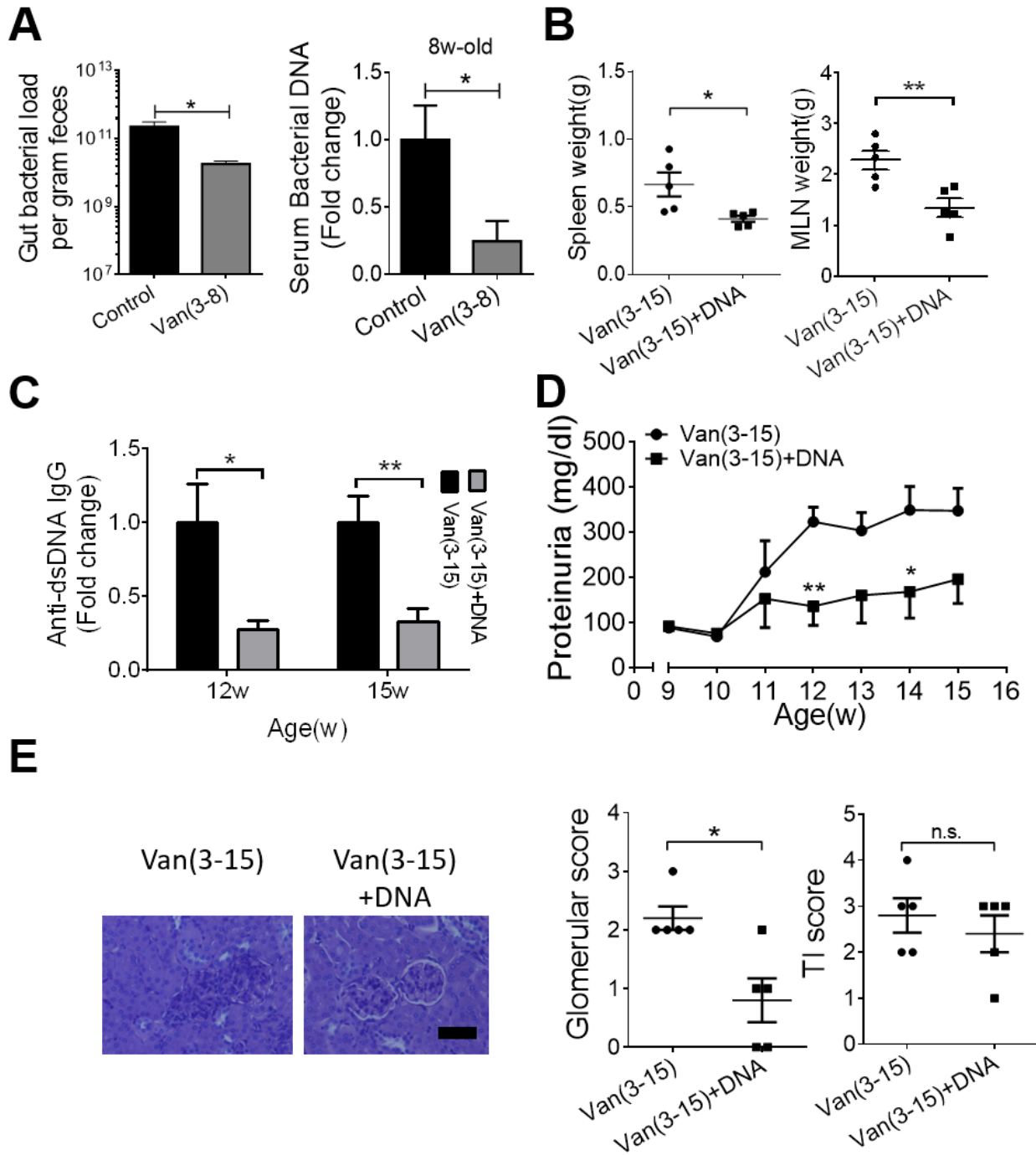
Fig 3.



**Fig 3. Adoptive transfer of Breg cells at early age attenuated lupus in vancomycin-treated mice.** Van(3-15)+B10: van(3-15) mice received IL-10 producing Breg cells adoptive transfer at 6 and 7 weeks of age. **(A)** Spleen weight (left) and MLN weight (right) to body weight ratio (%) at 15 weeks of age. **(B)** Anti-dsDNA IgG level in mouse serum at 15 weeks of age (n≥8 per group). **(C)** Left: proteinuria level at 14 weeks of age (n≥8 per group). Right: RLN weight at 15 weeks of age. **(D)** Kidney glomerular score at 15 weeks of age. **(E)** The percentage of DN T cells (left), Treg to Th17 cells ratio (middle) and the percentage of IFN $\gamma$  producing CD4<sup>+</sup> T cells (right) in the mouse spleen at 15 weeks of age. #p<0.1, \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.



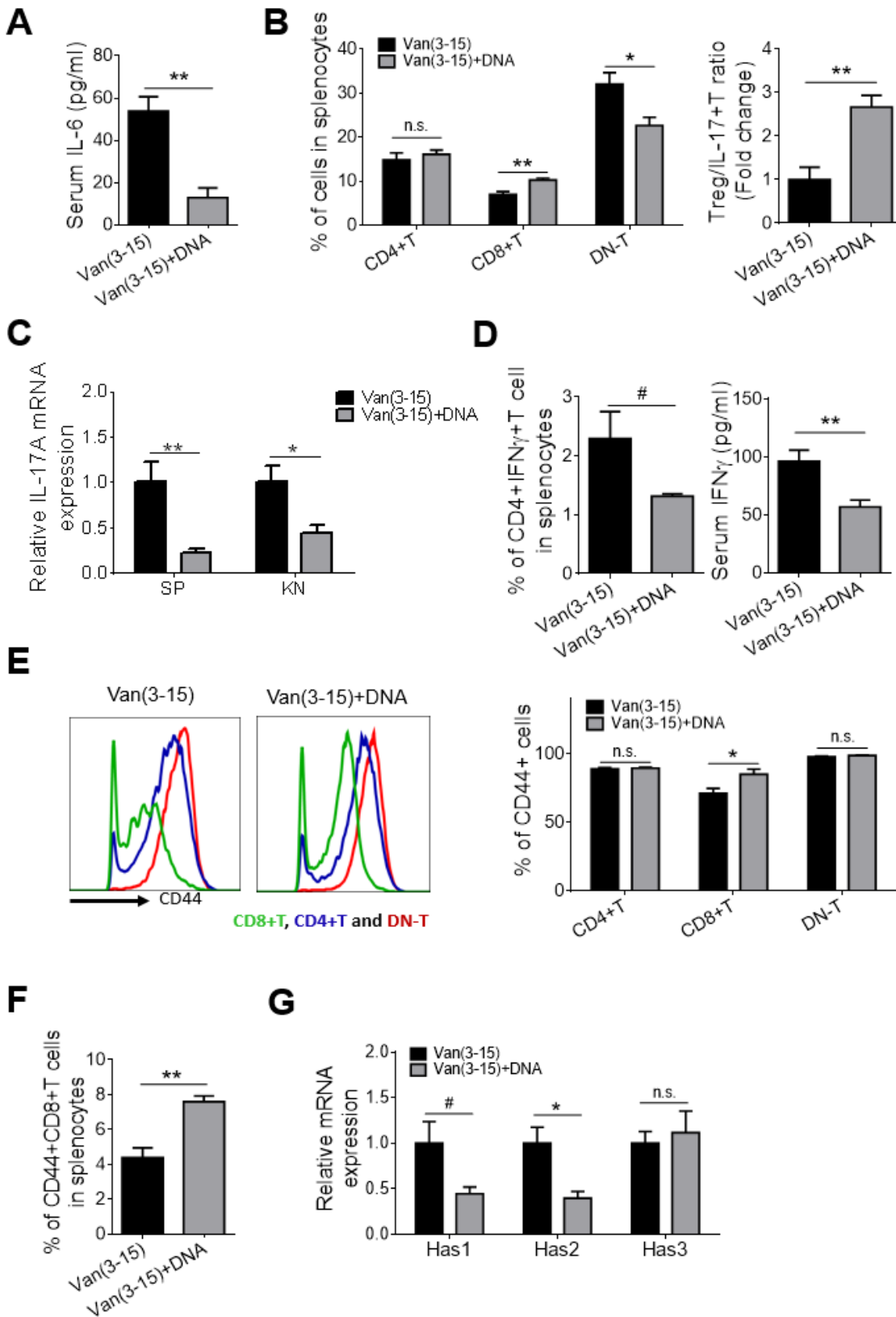
Fig 4.



**Fig 4. Restoration of bacterial DNA attenuated lupus disease in vancomycin-treated mice.**

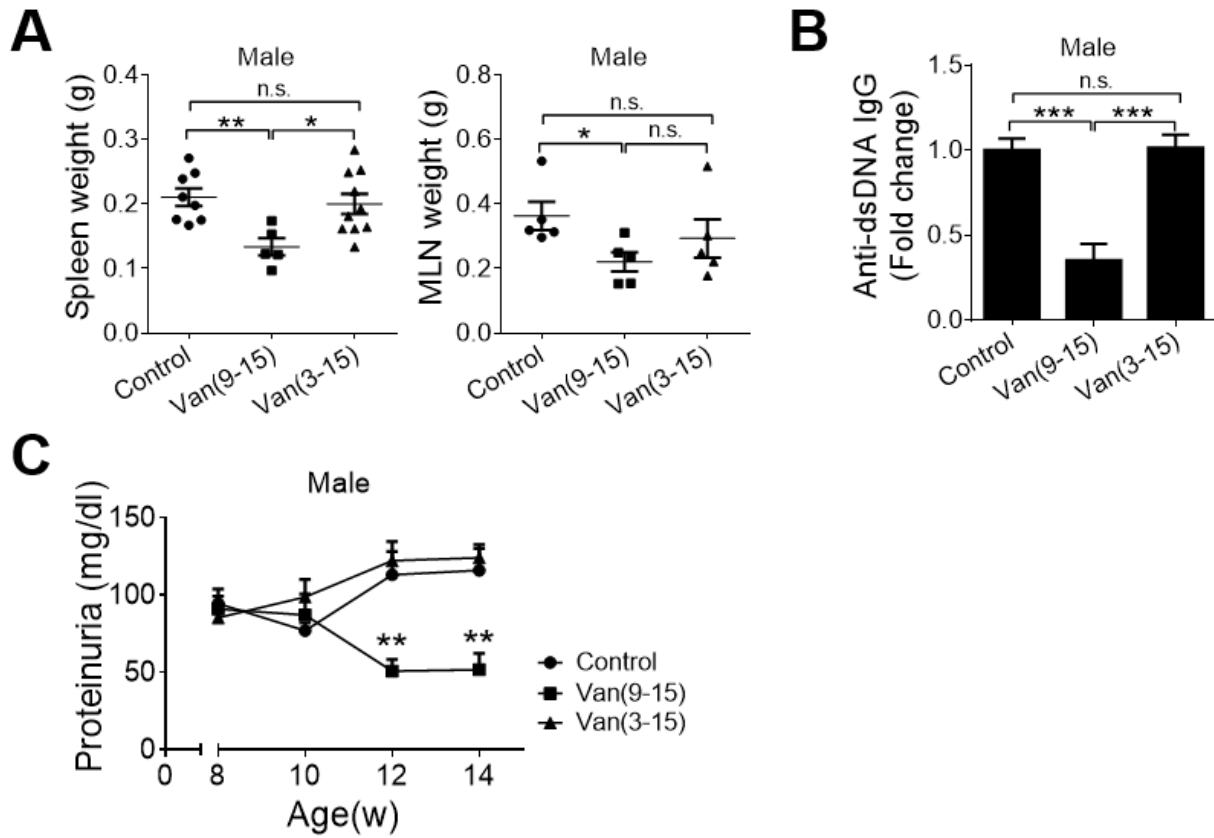
(A) Left: bacterial DNA load in the gut of mice at 7 weeks of age (n=9 per group). Right: bacterial DNA load in the circulation of mice at 8 weeks of age (n=5 per group). (B) Spleen weight (left) and MLN weight (middle) at 15 weeks of age. (C) Serum anti-dsDNA IgG level in mice at 12 and 15 weeks of age (n=5 per group). (D) Level of proteinuria over time (n=5 per group). (E) Renal histopathology at 15 weeks of age. Left: representative PAS-stained kidney sections; bar equals 400  $\mu$ m. Middle: glomerular score. Right: tubulointerstitial score. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

Fig 5.



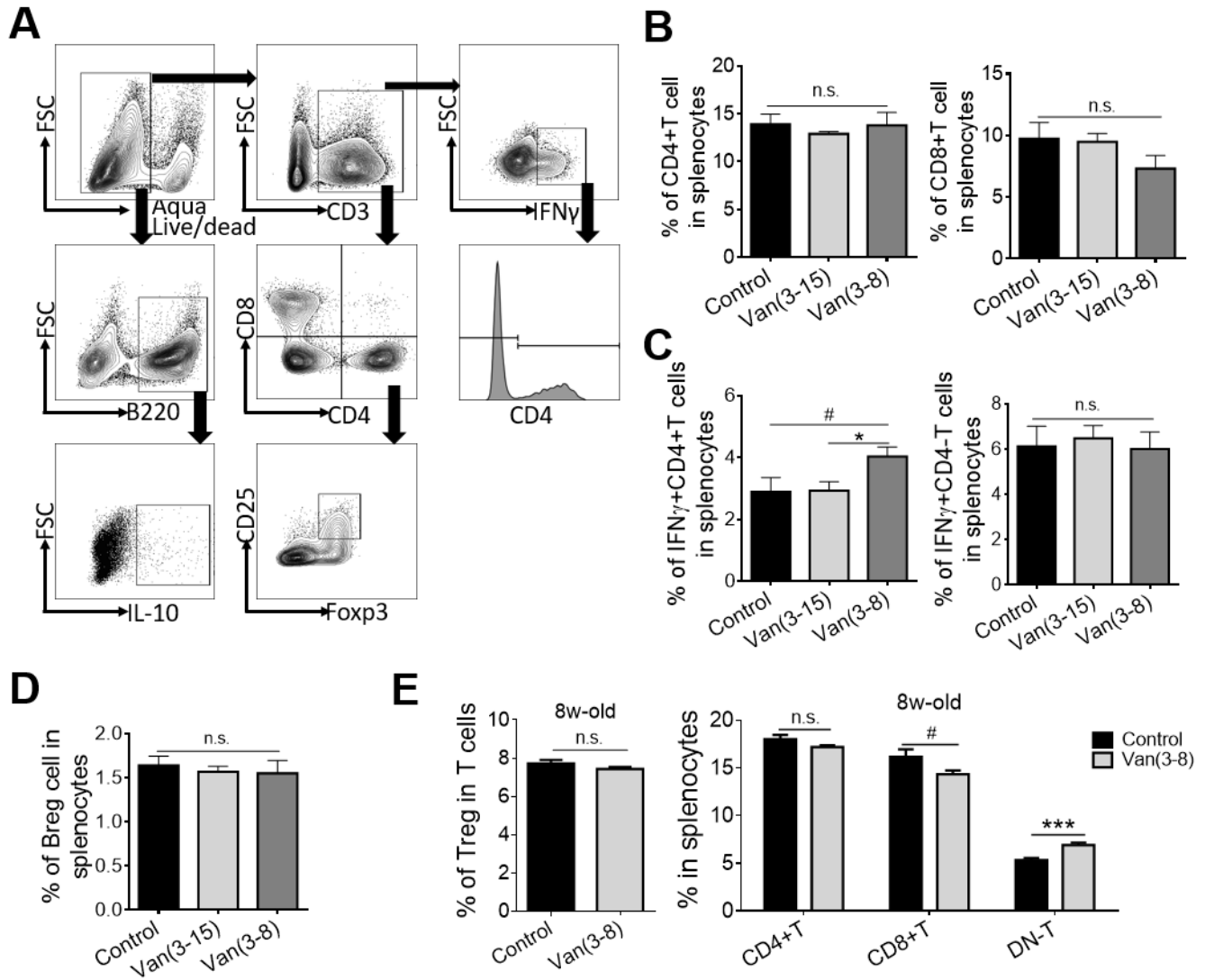
**Fig 5. Bacterial DNA treatment suppressed multiple pro-inflammatory cytokines and increased CD44 expressing CD8<sup>+</sup> T cells.** (A) Serum level of IL-6 at 15 weeks of age (n=5 per group). (B) The percentages of CD4<sup>+</sup>, CD8<sup>+</sup> and DN T cells (left) and the ratio of Treg to IL-17 producing T cells in spleen of mice at 15 weeks of age (n=5 per group). (C) Transcript level of IL-17A gene in the spleen and kidney at 15 weeks of age (n=5 per group). (D) The percentage of IFN $\gamma$  producing CD4<sup>+</sup> T cells in spleen (left) and IFN $\gamma$  level in the serum at 15 weeks of age (n=5 per group). (E) Representative CD44 fluorescence histogram (left) and percentage of CD44<sup>+</sup> cells in CD4<sup>+</sup>, CD8<sup>+</sup> and DN T cells (right) at 15 weeks of age (n=5 per group). (F) The percentage of CD44 positive CD8<sup>+</sup> T cells (right) in the spleen at 15 weeks of age (n=5 per group). (G) Transcript level of Has1, Has2 and Has3 genes in kidney at 15 weeks of age (n=5 per group). #p<0.1, \*p < 0.05, \*\*p < 0.01.

Fig S1.



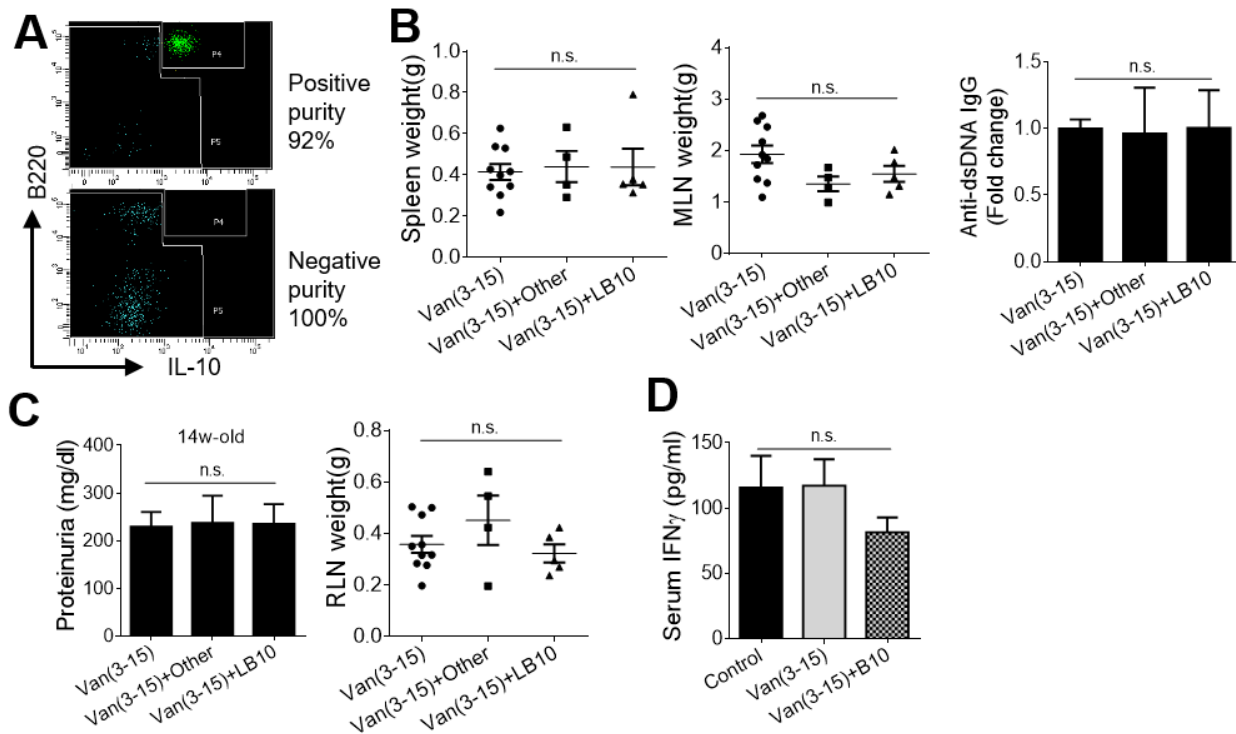
**Fig S1.** (A) Spleen and MLN weights to body weight ratio (%) of mice at 15 weeks of age. (B) The anti-dsDNA IgG level in mouse serum at 15 weeks of age ( $n \geq 5$  per group). (C) Level of proteinuria over time ( $n \geq 5$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Fig S2.



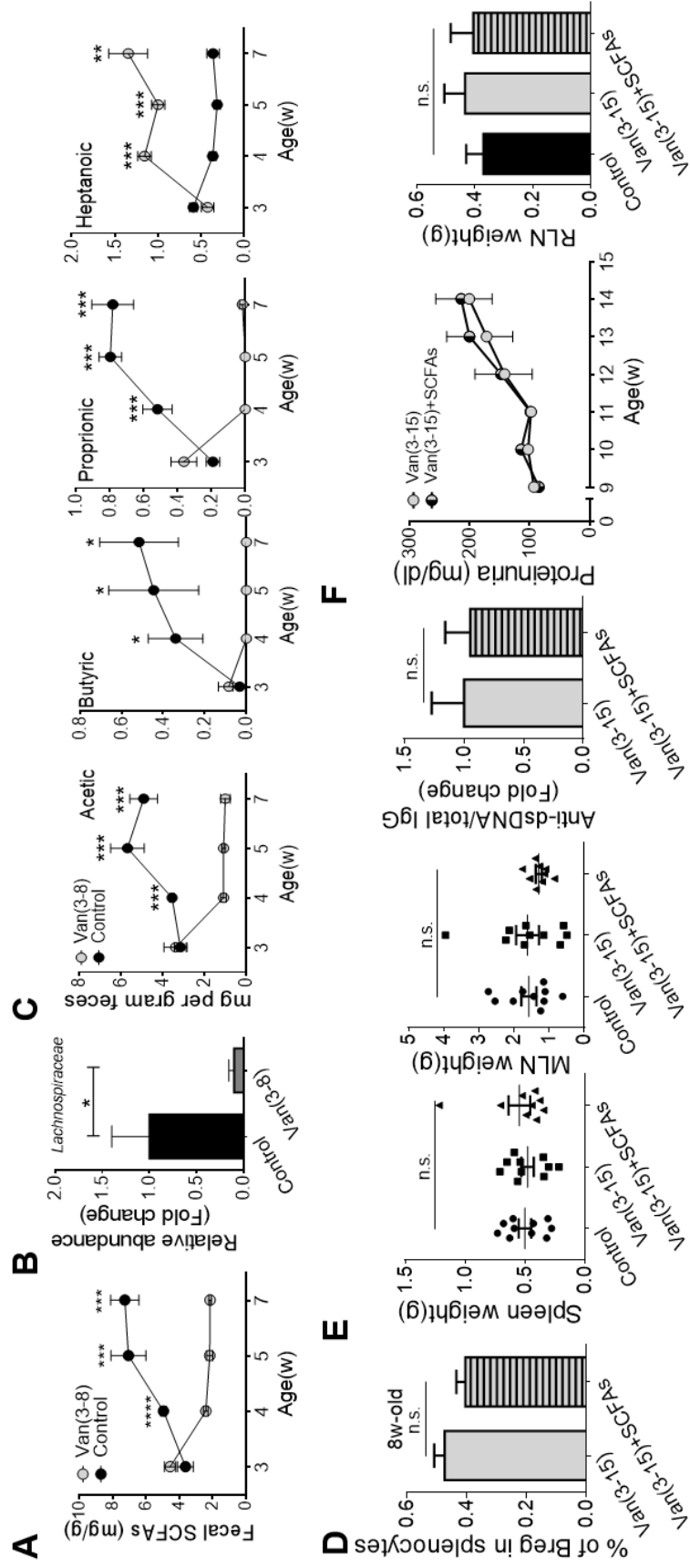
**Fig S2.** (A) Gating strategy for the FACS analysis of IL-10<sup>+</sup> Breg, IFN $\gamma$ <sup>+</sup> T cells, DN T cells, and CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells. (B) The percentage of CD4<sup>+</sup> (left) and CD8<sup>+</sup> (right) T cells in mouse spleen at 15 weeks of age (n $\geq$ 5 per group). (C) The percentage of IFN $\gamma$  producing CD4<sup>+</sup> (left) and CD4<sup>-</sup> (right) T cells in mouse spleen 15 weeks of age (n $\geq$ 5 per group). (D) The percentage of IL-10<sup>+</sup> Breg cells in mouse spleen at 15 weeks of age (n $\geq$ 5 per group). (E) The percentage of Treg cells (left) and CD4<sup>+</sup>, CD8<sup>+</sup> and DN T cells (right) in mouse spleen at 8 weeks of age (n=10 per group). #p<0.1, \*p < 0.05, \*\*\*p < 0.001.

**Fig S3.**



**Fig S3.** Groups: Van(3-15)+Other: van(3-15) mice received adoptive transfer of cells excluded Breg at the age of 6 and 7 weeks old. Van(3-15)+LB10: van(3-15) mice received adoptive transfer of IL-10 producing Breg cells at the age of 11 and 12 weeks old. (A) Cell purity test post IL-10<sup>+</sup> Breg isolation. (B) Spleen weight (left) and MLN weight (middle) to body weight ratio (%) and anti-dsDNA IgG level in mouse serum (right) at 15 weeks of age (n $\geq$ 4 per group). (C) Left: proteinuria level at 14 weeks of age (n $\geq$ 4 per group). Right: RLN weight at 15 weeks of age. (D) Serum IFN $\gamma$  level at 15 weeks of age (n $\geq$ 8 per group).

Fig S4.

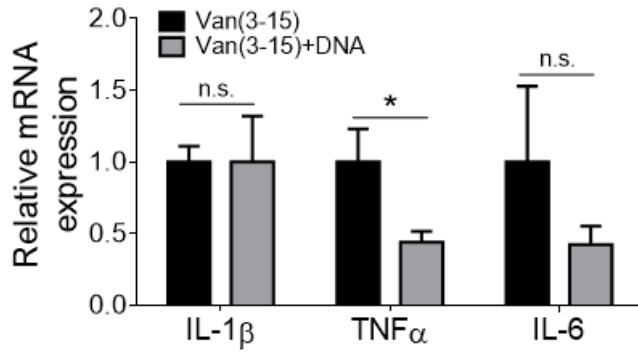




**Fig S4.** (A) The level of total SCFAs in feces of mice over time at early age ( $n \geq 4$  per group). (B) Relative abundance of *Lachnospiraceae* in feces of mice at 7 weeks of age ( $n=9$  per group). (C) The levels of acetic, butyric, propionic and heptanoic acids in feces of mice at early ages ( $n \geq 4$  per group). (D) The percentage of Breg cells in spleen at 8 weeks of age ( $n=5$  per group). (E) Spleen weight (left) and MLN weight (middle) at 15 weeks of age and anti-dsDNA IgG to total IgG ratio (right) in the serum of mice at 12 weeks of age ( $n \geq 9$  per group). (F) Left: level of proteinuria over time; Right: the weight of RLN ( $n \geq 9$  per group). \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

# Fig S5.

## A



**Fig S5.** (A) Transcript level of IL-1 $\beta$ , TNF $\alpha$  and IL-6 genes in kidney at 15 weeks of age (n=5 per group). \*p < 0.05.

## CHAPTER VI

### Discussion and Future Directions

The gut microbiota differs in SLE patients and lupus-prone mouse models compared to their respective controls, but it is in debate whether these differences are causative factors affecting the pathogenesis of SLE, or they are just the consequences of progressive inflammation during active lupus disease. This is an extremely interesting question that we seek to answer. However, an experiment comparing disease outcomes between MRL/*lpr* mice housed under germ-free vs. conventional conditions showed no significant changes. This suggests that the gut microbiota might not play any meaningful role in regulating lupus disease. We interpret the germ-free mouse results differently. We believe that there are both beneficial and pathogenic bacteria co-existing in the gut microbiota and that complete depletion of all these bacteria could result in neutralization of the opposing effects from the two types of gut bacteria.

To test the hypothesis that both beneficial and pathogenic bacteria co-exist, we analyzed the gut microbiota of MRL/*lpr* mice and observed a depletion of Lactobacilli but an increase of Clostridium species (*Lachnospiraceae*) together with increased bacterial diversity compared to age-matched healthy controls. We thus determined whether Lactobacilli and Clostridia were beneficial and pathogenic bacteria that attenuate and exacerbate lupus disease, respectively. For Lactobacilli, we added them back to the gut of MRL/*lpr* mice, which significantly ameliorated lupus-like disease. For Clostridia, we removed them through vancomycin treatment during active disease, which resulted in disease relief. These results support our hypothesis and suggest Lactobacilli and Clostridia as “good” and “bad” bacteria, respectively, in MRL/*lpr* mice. They also highlight supplementation of Lactobacilli as a potential

lupus disease prevention and treatment strategy. The beneficial effects of Lactobacilli were only observed in female and castrated male mice, suggesting a role for sex differences. Additional studies are required to test the therapeutic effects of Lactobacilli in other lupus-prone mouse models, which may or may not lack *Lactobacillus* spp. in their gut microbiota. Moreover, no mouse model can completely reflect the disease in SLE patients. In particular, the human gut microbiota is quite different compared to the mouse gut microbiota. Thus, while *Lactobacillus*-containing probiotics are generally considered safe, we are still far away from recommending the supplementation to SLE patients.

Unlike the amelioration of lupus in MRL/*lpr* mice treated with vancomycin post disease onset (9 to 15 weeks of age), when the microbiota removal was initiated early, the disease parameters exhibited no change (3 to 15 weeks of age) or even exacerbation (3 to 8 weeks of age). Further analysis revealed that the removal of gut microbiota also removed Breg cells and that the regulatory function of microbiota-induced Breg cells was time-dependent and only effective at young age. Known as a ligand for TLR9, CpG-DNA can induce the development of B cells and promote IL-10 production in B cells originated from different lupus-prone mouse models. Moreover, lupus manifestations in mice with B-cell depletion or TLR9-deficiency were very similar—both exhibited reduced survival and more severe disease. In contrast, blocking B cells with a neutralizing antibody in mature mice led to dramatic amelioration of lupus disease. Therefore, I hypothesize that inhibition of Breg development early in life contributes to lupus disease progression in B cell- or TLR9-deficient mice. To test the hypothesis, I propose the following experiments: 1) Add Breg cells back to the lupus-prone mice with pan B-cell depletion at early age and determine the attenuation of lupus; 2) Block TLR9 with an antagonist at different ages (3 to 8 weeks of age vs. 3 to 15 weeks of age vs. 9 to 15

weeks of age) and examine differences in disease outcomes; 3) Restore TLR9<sup>+</sup> B cells in TLR9-knockout MRL/lpr mice and test if the restoration attenuates the exacerbated disease phenotype. With these experiments, we will have a better understanding of lupus pathogenesis and in particular the pathway involving bacterial DNA, TLR9, and Breg cells.

In the MRL/lpr mice, when oral vancomycin was given at post disease stage, we observed significant amelioration on disease outcome. A similar beneficial effect was also reported in another lupus-prone model – (NZW×BXSb)F<sub>1</sub> hybrid mouse. Notably, oral neomycin treatments didn't result in any the disease attenuation either of the two mouse models. Not like neomycin which targets gram-negative bacteria, the vancomycin mainly targets gram-positive bacteria. This indicates that the removal of gram-positive bacteria, but not gram-negative bacteria, is probably the reason for lupus-like disease attenuation in mouse. However, in human study, the protective role of antibiotics in SLE hasn't been observed yet. What's more, some cases showed that intravenous vancomycin administration even lead to lupus flare in SLE patients. Nephrotoxicity is a major adverse effect of vancomycin and it may explain the exacerbated disease condition. However, vancomycin is not absorbed in GI tract. Thus it seems much valuable to monitor those SLE patients who have oral vancomycin treatment against gastrointestinal infections.

We found that female MRL/lpr mice possessed a “leaky” gut during disease progression. Microbial translocation across the intestinal epithelium has been hypothesized as an initiator of inflammation in remote tissues such as the kidney. Several recent reports have shown that probiotics can reverse the leaky gut by enhancing the production of barrier-forming tight junction proteins. Some prebiotics have also been observed to protect the gut epithelium, but whether the effects are gut microbiota-dependent is not clear. I am particularly interested in

studying the involvement of gut leakiness in lupus disease development. However, this research field is still in its infancy and we do not have an appropriate mouse model. Therefore, I propose to generate intestinal epithelial cell-specific knockouts of tight junction proteins as novel mouse models of the leaky gut. In addition, I am interested in examining the effects of oral prebiotics, which can strengthen barrier-forming tight junction protein expression, in regulating autoimmunity in lupus-prone mice. While this dissertation is focused on lupus-prone mice, my goal is to understand the mechanisms governing the role of gut microbiota in the pathogenesis of SLE in both mouse and human. Ultimately, I hope that the results of my studies can be translated to clinical use and benefit patients suffering from this devastating disease.