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INVITED REVIEW

Microbial influences on severity and sex bias of systemic autoimmunity

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Summary

Commensal microbes have the capacity to affect development and severity of autoimmune diseases. Germ-free (GF) animals have proven to be a fine tool to obtain definitive answers to the queries about the microbial role in these diseases. Moreover, GF and gnotobiotic animals can be used to dissect the complex symptoms and determine which are regulated (enhanced or attenuated) by microbes. These include disease manifestations that are sex biased. Here, we review comparative analyses conducted between GF and Specific-Pathogen Free (SPF) mouse models of autoimmunity. We present data from the B6;NZM-*Sle1*$^{NZM2410/Aeg}$*Sle2*$^{NZM2410/Aeg}$*Sle3*$^{NZM2410/Aeg-}$/LmoJ (B6.NZM) mouse model of systemic lupus erythematosus (SLE) characterized by multiple measurable features. We compared the severity and sex bias of SPF, GF, and ex-GF mice and found variability in the severity and sex bias of some manifestations. Colonization of GF mice with the microbiotas taken from B6.NZM mice housed in two independent institutions variably affected severity and sexual dimorphism of different parameters. Thus, microbes regulate both the severity and sexual dimorphism of select SLE traits. The sensitivity of particular trait to microbial influence can be used to further dissect the mechanisms driving the disease. Our results demonstrate the complexity of the problem and open avenues for further investigations.

KEYWORDS
B6.NZM, germ-free, microbiota, sex bias, SLE, systemic autoimmunity

1 | INTRODUCTION

The microbiota contributes to both essential (nutrition and pathogen colonization resistance) and auxiliary functions (development of immune and other physiological systems) required for the normal existence and fitness of the host. Unraveling these important interactions between the host (both vertebrate and invertebrate) and the commensal microorganisms became possible after the advent of the germ-free technologies.[1–3] Analyses of mice raised in axenic (sterile) and gnotobiotic (with known colonizing microbes) conditions revealed a broad category of functions affected by the microbiota, including behavioral stress responses,[4–6] neurogenesis,[7–9] metabolism,[10–12] intestinal functions and development,[13–15] as well as the immune system,[16–18] highlighting the powerful symbiotic role the microbes assume in the host. The ability to rear animals in germ-free (GF) conditions combined with the high-throughput methods of

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analysis of the composition of microbial communities enabled characterization of the potential functions of individual microbes or their defined consortia in modulating host physiology as well as susceptibility to infections and other types of disease.

The existing definitive evidence of microbial influences across different types of autoimmunity is presented in Table 1, which will be discussed in more detail. We were first to demonstrate the importance of the microbiota and microbiota-triggered innate immune mechanisms in an organ-specific autoimmunity—Type 1 diabetes (T1D)—in the NOD/ShiLtJ (NOD) mouse model.[19] An important finding was that GF mice were susceptible to the disease even more than mice housed at conventional Specific Pathogen-Free (SPF) facilities, indicating that commensals are unlikely to promote disease development but may be used for prevention/attenuation of the disease. Considerable effort was put in the studies of the role of the microbiota in systemic immunity. Systemic lupus erythematosus (SLE) is a complex systemic autoimmune disease that affects multiple organs including kidneys, skin, joints, liver, spleen, lungs, and brain.[20–22] One important characteristic of human SLE is its sex bias; incidence is overwhelmingly shifted towards females.[23,24] SLE is not an exception, and female bias exists in many other diseases.[25–28] The nature of sex bias could be epigenetic, incomplete X chromosome inactivation may lead to aberrant expression of genes involved in autoimmunity,[29] or physiological due to the contribution of estrogens[30] or the lack of protective androgens. At the same time, environmental factors may also contribute. The microbiota is a strong

TABLE 1 Germ-free mouse models of autoimmunity and disease outcome in comparison to Specific Pathogen-Free animals.

Autoimmune conditions	Animal model	Effect of GF environment	Refs.
Increased severity or no effect			
APECED	Aire$^{-/-}$ mice	Severe disease	100
IPEX	Ablation of FoxP3^{+} cells in adult mice	Severe disease	94
Autoimmune gastritis	Aicda$^{-/-}$ mice	No effect	95
T1D	NOD mice	Normal T1D development	87,19
	BB rats	Normal T1D development	88
SLE	MRL-Fas^{lpr} mice	Severe disease. However less severe on “antigen-free” diet	37
	BXD2 mice	No effect	39
Mixed results			
EAE	C57BL/6J mice	Reduction in severity	89
EAU	C57BL/6J mice treated with IRBP peptide	Less severe disease	99
Autoimmune arthritis	K/BxN mice	Attenuated autoantibodies and Th17 cells	97
Rheumatoid arthritis	DA rats treated with collagen	Severe arthritis but no antibody responses	90
SLE	NZB mice	Less severe disease	40
	B6SKG mice	Less severe disease	42
	C57BL/6 mice treated with imiquimod	Less severe disease	43
Protection			
Sialitis	NOD mice	No sialitis	45
Autoimmune arthritis	Il1rn$^{-/-}$ mice	No disease	96
Spondyloarthritis	HLA-B27 mice	No disease in the gut and joints. Skin and genitals affected	46
	SKG	No disease	98
Ankylosing enthesopathy	B10.BR mice	No disease	91,92

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candidate to be a predominant environmental factor. We first noticed that the well-known sex bias in the NOD mouse T1D model was significantly reduced in GF conditions.[19,31,32] Sex prevalence in NOD mice was also dependent male sex hormones.[31] Interestingly, human T1D seems to be sexually unbiased. However, a significant part of T1D patients are diagnosed before puberty excluding the role of the sex hormones in promotion or prevention of the disease. On the other hand, one group of patients that have other glands affected (thyroid, salivary) is diagnosed with Polyglandular Autoimmunity (PGA) and clearly shows the female bias.[33] The role of the microbiota in sex bias in humans is difficult to establish beyond correlation. The use of animal models seems to be the most adequate way to address the issue. Thus, with these observations and considerations in mind, we focused our work on a mouse model of SLE, the B6;NZM-*Sle1*$^{NZM2410/Aeg}$*Sle2*$^{NZM2410/Aeg}$*Sle3*$^{NZM2410/Aeg-}$/LmoJ (B6.NZM) mouse,[34] to unravel the role of the microbiota in both disease development (severity) and its sex bias.

2 | COMMENSAL MICROBES REGULATE THE SEVERITY OF DISEASE IN MOUSE MODELS OF AUTOIMMUNE DISORDERS

Utilization of axenic (GF) mouse models has been successful in evaluating the role of the microbiota in pathogenesis of autoimmune disorders. Table 1 lists published studies that conducted comparative analyses of the disease severity in GF and SPF mouse models. These include both organ-specific and systemic autoimmune diseases. GF experiments conducted with different genetic mouse models of SLE showcase the intricate interplay between host genetics and the microbiota underlying the progression of autoimmunity. To date, several mouse models of SLE exist. MRL-Fas^{lpr} strain harbors homozygous premature termination mutant alleles within *Fas* (CD95) termed *lymphoproliferation* (*Fas*^{lpr}) that blocks Fas protein expression[35] and develops spontaneous systemic autoimmunity with symptoms of SLE. These include severe lymphadenopathy, glomerulonephritis, splenomegaly, and increased levels of pathogenic autoantibodies in circulation among others.[36] Fas, which belongs to a tumor necrosis factor receptor (TNF-R) family, is a cell surface death receptor that results in a caspase-mediated apoptotic cell death in response to engagement with the Fas Ligand (FasL). Fas–FasL pathway regulation of cell death is necessary for the maintenance of homeostasis in the immune system, including proper selection of T and B cells. The difference in the severity of autoimmune pathologies was tested in MRL-Fas^{lpr} mice reared in SPF or GF conditions upon exposure to normal diet versus antigen-free diet.[37] It was found that, under the condition of normal diet, GF MRL-Fas^{lpr} mice suffer from similar levels of autoimmunity compared to SPF mice with no significant differences in the degrees of lymphoproliferation, glomerulonephritis, or circulating autoantibody levels,[37] suggesting that the autoimmune pathways implicated in the genetic background of MRL-Fas^{lpr} strain are insensitive to the input from the microbiota. However, GF and SPF mice fed on antigen-free diet displayed differences in *select* categories of parameters, including attenuated levels of C3 deposits in the kidneys of GF mice. This finding suggests that the presence of microbial ligands in the food may compensate for the absence of the live microbiota and contribute to heightening of autoreactive pathways that result in increased kidney pathologies in MRL-Fas^{lpr} model. In BXD2 strain, a recombinant inbred mouse line generated through intercrossing of C57BL/6J and DBA/2J mice,[38] the microbiota did not seem to play a significant role in modulation of the disease severity.[39]

On the other hand, absence of the microbiota in NZB mice was reported to attenuate the overall renal disease score with a reduction in gamma-globulin levels.[40] Similarly, in B6SKG mice, which have a point mutation in *zap70*,[41] the presence of the microbiota exacerbates autoimmune phenotypes.[42] Lastly, GF condition ameliorated overall disease severity and enhanced survival in Toll-like Receptor 7 (TLR7) agonist imiquimod-mediated inducible mouse model of SLE.[43] Together, GF studies conducted using various mouse models of SLE suggested that microbial influence can impact the severity of autoimmunity and that host genetics play a role in the sensitivity to microbial influence on the progression of autoimmunity. In our previous evaluation of the role of the microbiota in autoimmunity,[44] we noted that monogenic diseases are exempt from microbial influences, whereas polygenic diseases are usually susceptible.

Another important issue with making solid conclusions from SPF/GF comparisons is that distinct disease parameters can display differential susceptibility to microbial manipulation. NOD mice develop T1D, but they also develop other organ-specific lesions: thyroiditis and sialitis.[45] That makes the model very close to human condition known as Polyglandular Autoimmunity (PGA) [formerly Autoinflammatory Polyglandular Syndrome, APS 2–5 (APS 1 results from mutations in transcriptional regulator AIRE)].[33] We found that NOD mice reared in GF conditions developed insulitis similar to the counterparts raised in SPF conditions.[45] However, GF NOD mice were protected from sialitis, suggesting the differential involvement of the microbiota in targeting autoreactivity in organ-specific manner.[45] Similarly, in HLA-B27 mouse model of spondyloarthritis, GF mice were spared from autoimmunity in the gut and joints but not in skin or genitals,[46] suggesting that discrete pathophysiological pathways may or may not be affected by microbial stimuli. Overall, these findings warrant detailed investigation into dissecting the complex crosstalk between host genetics and the microbiota and their contribution to autoimmunity.

3 | B6.NZM MOUSE MODEL OF SLE

B6.NZM model is a tricongenic mouse model of spontaneous SLE with three lupus susceptibility loci on chromosome 1, 4, and 7 transferred onto the C57BL/6J background from the lupus-prone NZM strain.[34] This spontaneous model of SLE has multiple hallmarks of human disease.[34,47] We assumed that the existing evidence of the microbiota involvement in SLE pathogenesis and promotion was sufficient to invest in generation of GF B6.NZM

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mice. An intrinsic problem with this strain of mice is its very poor breeding performance precluding conventional way of GF rederivation. However, frozen embryos were available from The Jackson Laboratory, and we used them to implant sterilely into pseudopregnant females of a different strain. As a result, we were able to establish a GF colony for further studies of disease pathology. GF status was routinely checked by polymerase chain reaction amplification of bacterial 16S rRNA genes from fecal DNA samples and by standard cultivation methods. To directly dissect the influence of the microbiota on the pathogenesis of SLE on the B6.NZM genetic background, we measured pathological traits in GF mice for comparative analyses with B6.NZM mice reared in SPF environment. We found that the absence of microbes exacerbated splenomegaly (Figure 1A). Kidney pathology was assessed by immunohistochemistry. Kidney samples embedded in OCT. (Sakura Tissue Tek®, Torrance, CA) were sectioned into 8 μm sections using a cryostat. Slides were fixed in −20°C acetone, dried, and stained with TRITC-α-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA) or FITC-α-mouse C3a antibody (MP Biomedicals, LLC, Irvine, CA) in FACS buffer. The slides were imaged using DMLB microscope (Leica Microsystems, Wetzlar, Germany) equipped with SPOT camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Interestingly, the absence of microbes did not alter the severity of complement C3 deposition in kidneys (Figure 1B), whereas the deposition of Ig was not sensitive to the microbiota (Figure 1C). Presence of anti-nuclear antibodies (ANA) in the serum was tested in samples diluted 1:100 and incubated with HEp-2 slides (Bio-Rad Laboratories, Hercules, CA) followed by staining with TRITC-α-mouse IgG antibody (Jackson ImmunoResearch). ANA levels in GF males were exacerbated compared to their SPF counterparts (Figure 1D).

To detect microbial influence reflected in changes in cellular populations, we analyzed splenocytes of SPF and GF animals. To analyze T cell activation, splenocytes were stained with the following panel of antibodies in FACS buffer (1%FBS, 0.02% NaN_3, 1XPBS): α-CD4-FITC (eBioscience, San Diego, CA), α-CD8a-PacBlue (Invitrogen, Carlsbad, CA), α-CD62L-PECy7 (BioLegend, San Diego, CA), α-CD44-APCCy7 (BioLegend), α-CD69-PE (Invitrogen) in the presence of Fc-Block (BD Biosciences, Franklin Lakes, NJ). Live lymphocytes were plotted as CD4 versus CD8a to identify $CD4^+$ and $CD8^+$ T cells ($CD4^+CD8^-$ or $CD4^-CD8^+$). $CD4^+$ or $CD8^+$ T cells were plotted as CD44 versus CD62L to determine effector/effector memory T cells ($CD44^{hi}CD62L^{lo}$). $CD8^+$ T cells were plotted as SSC versus CD69 to determine recently activated T cells ($CD69^+$). To analyze B cells, the same samples were stained with the following panel of antibodies:

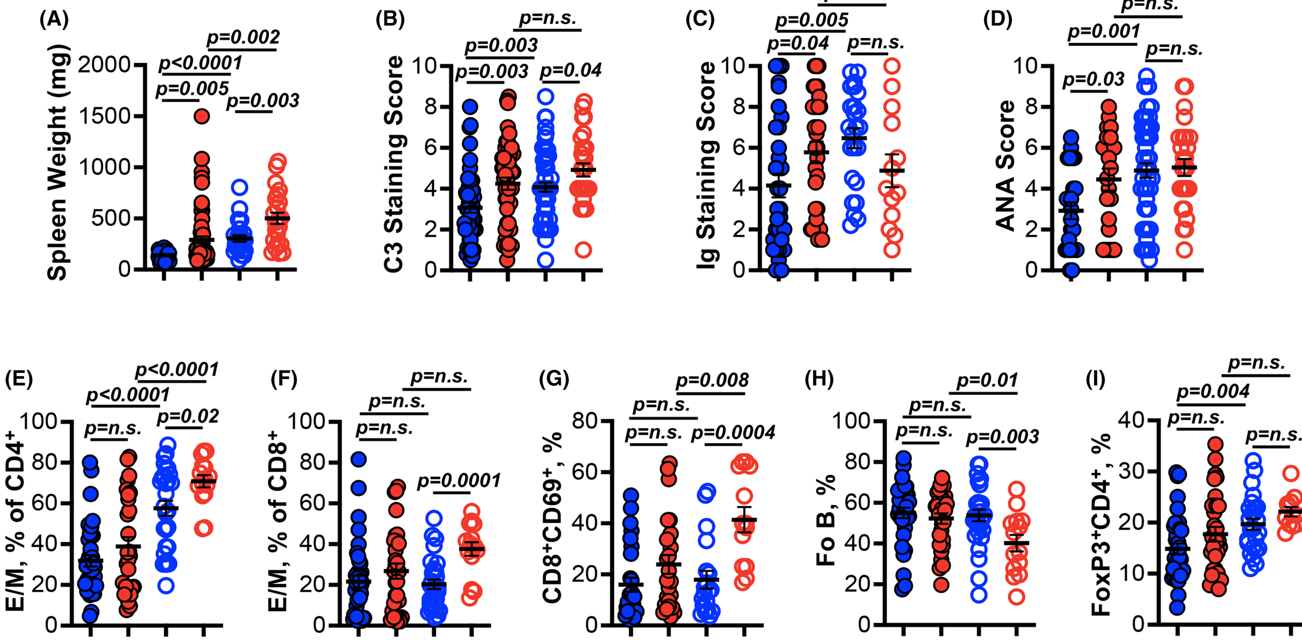

FIGURE 1 Measurements of disease severity and the sex bias of its manifestations in SPF and GF B6.NZM mice. SLE parameters measured in SPF and GF B6.NZM male and female mice. (A–D) spleen weights (A), complement C3 (B) and Ig (C) deposits measured in kidneys via immunofluorescence, and ANA scores (D) analyzed by immunofluorescence on Hep-2 cells. (E–I) FACS analyses conducted on splenocytes to measure the proportions of effector/effector memory (E/M) T cells, follicular B (Fo B cells), and Tregs. E/M $CD4^+$ (E) or $CD8^+$ (F) T cells were defined as $CD4^+CD44^{hi}CD62L^{lo}$ or $CD8^+CD44^{hi}CD62L^{lo}$. Recently activated $CD8^+$ T cells were defined as $CD8^+CD69^+$ T cells (J). $CD4^+$ and $CD8^+$ T cell subsets are shown as percentage of the total $CD4^+$ and $CD8^+$ T cell populations, respectively. Fo B cells (H) were defined as $CD93^-B220^+IgM^{lo}CD23^+$ and shown as percentage of the total $CD93^-B220^+$ B cell population. Tregs (I) are defined as $CD4^+FoxP3^+$ T cells and shown as percentage of the total CD4+ T cell population. Symbols – individual mice. Closed blue – SPF males. Closed red – SPF females. Open blue – GF males. Open red – GF females. Mean ± sem. Significance was calculated using an unpaired *t*-test. All mice were 8–10 months old.

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α-B220-APCCy7 (BioLegend), α-CD93-APC (BioLegend), α-CD23-PECy7 (BioLegend), α-IgM-eF450 (Invitrogen) in the presence of Fc-Block. Live lymphocytes were plotted as CD93 versus B220 to determine $CD93^-B220^+$ cells ($CD93^-B220^+$) that were plotted as IgM versus CD23 to determine follicular (Fo) B cells ($CD93^-B220^+IgM^{lo}CD23^{hi}$). To analyze Tregs, the same samples were stained with the following panel of antibodies: α-CD4-PacBlue (BioLegend) and α-Foxp3-PECy7 (Invitrogen). Live lymphocytes gated on $CD4^+$ T cells were plotted as SSC versus FoxP3 to identify Tregs ($FoxP3^+$). For intracellular staining, cells were permeabilized with Foxp3/Transcription factor staining buffer (Invitrogen), and dead cells were excluded by Zombie Aqua (BioLegend). Sample collection was conducted using Fortessa flow cytometers (Becton, Dickinson & Company, Franklin Lakes, NJ). Data analysis was done using *FlowJo*™ software (Becton, Dickinson & Company).

Evidence was found for differential influence of commensals on activated T cell populations. For example, frequencies of $CD4^+$ effector/effector memory (E/M) T cells were significantly decreased in the presence of commensals (Figure 1E). However, frequencies of $CD8^+$ E/MT cells remained largely comparable between SPF and GF animals (Figure 1F). In addition, commensals negatively influenced the frequencies of $CD8^+CD69^+$ T cells and Fo B cells (Figure 1G,H), whereas $FoxP3^+CD4^+$ Tregs were enhanced in SPF mice (Figure 1I).

Taken together, our data reveal an important point that in B6.NZM mouse model of SLE, only select disease traits display sensitivity to microbiota-mediated regulation of disease severity. These results resemble microbial influence on the development of T1D: successful establishment of the disease in the absence of microbes and differential influence of them on distinct features of the disease.

4 | COMMENSALS ARE INVOLVED IN SEX BIAS IN AUTOIMMUNITY

The variations in the degree of sex bias in type 1 diabetes (T1D) of NOD/ShiLtJ (NOD) mice dependent on housing conditions of different institutions were appreciated a while ago.[19,31] On average, NOD mice reared in SPF conditions display a clear sex bias with female-to-male incidence ratio close to 3:1 over the course of 30 weeks with female incidence around 75% and male incidence around 25%. The absence of the microbiota results in the loss of protection from T1D usually present in NOD males, while female T1D incidence is not altered by the GF condition.[31,32] As a result, GF males and females no longer display sex bias and have similar overall incidence of T1D (~80%). This finding indicates that insulitis is modulated by microbes in a male-specific fashion. Our work also revealed the importance of the crosstalk between male sex hormones androgens and the microbiota in protecting males from T1D in NOD mice.[31] NOD mice also develop sialitis as a characteristic autoimmune pathology. Whereas in contrast to insulitis, autoimmune sialitis in NOD mice was dependent on the presence of the commensal organisms in females, their absence did not alter the low degree of sialitis in males.[33] Thus, microbes regulate distinct autoimmune pathologies in a sex-dependent fashion.

Considering the apparent liaison between sex-specific factors and microbes in development of organ-specific autoimmunity in NOD mice, we sought to determine the extent of sexual dimorphism across multiple pathophysiological SLE parameters in B6.NZM mice housed under our own SPF conditions in comparison to GF conditions. In SPF conditions, we detected significantly increased severity in females compared to males in splenomegaly, C3 and Ig immune complex deposition in kidneys, and ANA levels, suggesting sex-dependent enhancement of lymphocyte proliferation and autoantibody production (Figure 1A–D). However, that could be equally interpreted as a loss of severity due to male sex-related factors. We also performed comparative flow cytometric analyses on the profiles of T cells and B cells in B6.NZM males and females. Interestingly, no sex-dependent difference was found in the frequencies of $CD4^+$ E/MT cells, $CD8^+$ E/MT cells, recently activated $CD8^+$ T cells, follicular B cells (Fo B), and $FoxP3^+CD4^+$ regulatory T cells (Tregs) (Figure 1E–H). Collectively, the data indicate that not all but select autoimmune parameters display increased sensitivity to regulations by sex-dependent factors in B6.NZM mice. This conclusion, however, was made using mice housed in a particular SPF facility. We have previously addressed the issue of host's genetic effects on the commensal microbial composition.[48] As a part of that study, we compared colonization of several GF mouse strains with different input microbiotas. It was clear that the input defined the output microbiota in mice of the same genetic background. It seems to be trivial and predictable, but it emphasizes the importance of comparing a variety of inputs to be able to generalize microbial effects (or attribute them to specific lineages present in each input).

Comparative analyses in GF animals revealed the influence of commensals on regulation of sex bias at a more granular level. Although splenomegaly remained significantly more penetrant in GF females compared to GF males, the absence of microbes exacerbated the phenotype in both males and females (Figure 1A). GF males also had higher levels of C3 and Ig immune complex deposition in kidneys and ANA compared to SPF male counterparts, indicating attenuating microbial influence on pathways involved in autoantibody production acting in a male-specific manner (Figure 1B–D). Furthermore, GF animals lost sex bias in the levels of Ig immune complex deposits in kidneys and ANAs in systemic circulation (Figure 1C,D). Interestingly, unlike B6.NZM mice raised in SPF condition, GF B6.NZM mice developed sexually dimorphic display of several T and B cell profiles, including $CD4^+$ E/MT cells, $CD8^+$ E/MT cells, recently activated $CD8^+$ T cells, and Fo B cells (Figure 1E–H). On the other hand, frequency of $FoxP3^+CD4^+$ Tregs was reduced in SPF males compared to GF males but not in females.

The summary of the results described in the previous two sections is shown in Table 2. The significance of microbiota and sex of the animals for development of the SLE features was analyzed using 2-way ANOVA. Interestingly, the presence of the microbiota *reduced* the values of most parameters except the percentages of Tregs. This is not surprising, as GF mice tend to have fewer Tregs.[49] It is also

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TABLE 2 Two-way ANOVA analysis of the significance of microbiota and sex of the animals for development of the systemic lupus erythematosus features in Specific-Pathogen Free versus Germ-free B6.NZM mice.

	SPF versus GF	
Parameters	**Microbiota's influence**	**Sex bias**
Splenomegaly	<0.0001	<0.0001
C3	0.003	0.0004
Ig	0.26	0.98
ANA	0.005	0.06
E/M CD4$^+$	<0.0001	0.01
E/M CD8$^+$	0.16	0.001
CD8$^+$CD69$^+$	0.01	<0.0001
Fo B	0.03	0.008
Treg	0.001	0.06

important to note that only select SLE traits in B6.NZM mice have sensitivity to microbiota-mediated regulation of disease severity and of its sex bias.

5 | DISEASE SEVERITY AND SEX BIAS DEPEND ON THE COMPOSITION OF COMMENSAL COMMUNITIES

While microbial effects on modulation of disease severity and sex bias have been established in multiple studies using a variety of mouse models of autoimmunity (Table 1), identification of the disease-affecting microbes was not that simple. Our own work in NOD mice was aimed to dissect the abilities of different microbial species in triggering disease and sex bias.[31] To this end, we colonized GF animals with microbial species as distinct as *VSL3* probiotic mix of *Lacobacillaceae* and *Bifidobacteria*, members of *Enterobacteriacae*, and segmented filamentous bacteria (SFB).[31] Male and female progenies of colonized gnotobiotic females were observed for development of insulitis and T1D incidence. Male mice colonized with *VSL3* were not different from GF counterparts in terms of insulitis development. At the same time, male mice colonized with enterobacterium 'Similar to *Escherichia* and *Shigella*' (SECS) developed reduced levels of insulitis compared to females and GF males. The effects of SFB colonization only manifested in males: insulitis was attenuated compared to GF males, whereas females showed no difference in insulitis or T1D incidence with or without SFB colonization. Thus, the gnotobiotic approaches utilized in NOD mice revealed that bacteria differ in their ability to influence disease severity and sex bias in T1D. More importantly, bacteria with diverse cell wall structure, localization in the gut, and phylogeny were capable to elicit similar results. Furthermore, no bacteria accelerating the disease development stronger than observed in GF NOD mice have been found. That questions the whole idea that bacteria can promote T1D and suggests that the differences in microbial composition between susceptible and resistant humans could be explained by the lack of protective microbes in the former and by their presence in the latter. This concept fits well with the results of a large survey of T1D-prone and genetically similar populations in adjacent geographical regions with different environments.[50]

However, the story could be different for the SLE. The search for specific microbes affecting SLE in animal models and in humans is rather intense. Global differences in microbial composition such as Firmicutes/Bacteroidetes ratio were detected in (SWR×NZB) F_1 (SNF1) model.[51] More precise identification of perpetrator lineages is more difficult. In TLR7.1 Tg-expressing mice (spontaneous transgenic model) and mice treated with TLR7 agonist imiquimod (inducible lupus mouse model),[43,52] antibiotics treatment, rederivation into GF conditions of TLR7.1 Tg, or imiquimod treatment of GF mice resulted in amelioration of various SLE manifestations, including splenomegaly, glomerulonephritis, and proteinuria, leading to enhanced survival in comparison to mice with intact microbiota. Further high-throughput 16s rRNA analysis of the gut microbial composition in TLR7.1 Tg and imiquimod models identified a commensal *Lactobacillus reuteri* to be enriched compared to control mice.[43] Subsequently, the authors demonstrated that diet enriched in resistant starch that requires fermentation by commensals in the distal gut was able to significantly suppress *L. reuteri* colonization in the ileum and attenuate lupus-associated mortality. Fermentation of resistant starch by the gut microbiota is known to yield short-chain fatty acid metabolites including acetate, propionate, and butyrate.[53] Not surprisingly, the gut microbiota from mice fed on diet rich in resistant starch revealed expansion of *Clostridiales*, a taxon able to ferment resistant starch into short-chain fatty acid metabolites. At the same time, *Lactobacill* were found to be beneficial in MRL^{lpr},[54,55] (NZB×NZW)F_1,[56] and (SWR×NZB)F_1[51] models. Transfers of *Lactobacillus* species led to reduction of proteinuria, autoantibodies, and kidney damage correlated with increased Treg presence in MRL^{lpr} mice[55] and worked similarly in (NZB×NZW)F_1 model.[56] Interestingly, *L. reuteri* (and *L. casei*) reduced the morbidity in (NZB×NZW)F_1 mice, whereas *L. planarum* was neutral. Whereas *L. reuteri* DSM17509 was used in this study, Zegarra-Ruiz et al.,[43] utilized *L. reuteri* strain SP-C2-NAJ0070 in their TLR7.1 transgenic model. Obviously, different species of the same genus or even strains of the same species can have very different properties. That may well explain the differences in the results. The principal differences in the mechanisms of disease development could also be responsible for opposing effects of similar microbes. In such cases, several approaches could be used: comparison of bacterial genomes and identification of potentially important pathways; derivation of mutant microbes and cross-testing in different SLE models; and derivation of susceptible mouse strains into GF conditions and measuring the effects of introduced deletions and insertions.

The link between the microbiota and the pathogenesis of autoimmune disorders has also been claimed in humans. By nature, most of these studies are correlative and do not prove causation. In recent years, several studies have reported differences in the composition of the gut microbiota in patients with SLE compared to healthy

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controls.[57–59] One study[58] performed a comparative analysis of fecal microbiota composition in 300 SLE patients in Asturias, Spain, and healthy controls. The SLE patient group displayed a characteristically higher relative abundance of *Bacteroidetes* and lower relative ratio of *Firmicutes*/*Bacteroidetes* despite variability in the range of clinical symptoms in the patients included in the study. Another comparative study conducted using SLE patients from Spain and China reported similar findings, in which both groups of patients had relative reduction in *Firmicutes* and enrichment of *Bacteroidetes* compared to healthy controls.[59] Similar findings between multiple cohorts of patients from distant geographical areas suggest that genetics and environmental factors such as diet may have less contribution to pathways resulting in the relative increase in *Bacteroidetes* and decrease in *Firmicutes*. Such differences, however, are very broad and make no easy way to identification of the exact perpetrators. One way to partially bypass the correlative nature of human studies is to follow microbial communities in patients longitudinally before and after treatment. A *Clostridium* species abundance was found to be reduced after anti-inflammatory treatment[60] in the same patients. However, a number of other bacteria unrelated phylogenetically followed the same trend. Another approach would be to perform longitudinal studies in the populations with a high prevalence of SLE. This approach has been utilized in the studies of T1D, where groups of high-risk children have been observed for over a decade.[61] It is more difficult to do for SLE, but there are groups with high incidence of SLE, such as Gullah people of the Lowcountry region of the US southern Atlantic coast. Longitudinal studies should reveal whether SLE-specific features of the microbiota predate the disease onset or are the consequence of the systemic dysregulation of the immune system.

Some groups found similarities in SLE-related microbial alterations in patients and animal models. Mice with pristane-induced lupus and human patients had similar five taxa with an increased abundance.[62] Another example of potential coalescence of commensal influence in animals and humans is a gut commensal, *Enterococcus gallinarum*.[63] It was shown to translocate across the gut epithelial layer in (NZW×BXSB)F_1 mice that develop kidney autoimmunity from aberrant TLR7 signaling likely in response to endogenous retrovirus glycoprotein 70 (ERV gp70).[63–65] Translocation of microbiota was found in various anatomical sites including the mesenteric lymph nodes, liver, and spleen. Importantly, *E. gallinarum* was also found in livers of the human SLE patients.[63]

Translocation due to disturbed intestinal barrier could be an important contributor to microbial stimulation of SLE pathology. Equally important is the ability of microbes to produce active metabolites or modify dietary and host's metabolites. For example, the control of tryptophan metabolism has been found to influence SLE.[63,66] The ability of *E. gallinarum* to generate bacterial aryl hydrocarbon receptor (AhR) ligands from tryptophan-derived indoles[63] and the resulting induction of Th17 cells, T follicular helper (Tfh) cells, and autoantibody production[63,67–69] were linked to pathogenic properties of *E. gallinarum* in (NZW x BXSB)F_1 background. Commensal bacteria represent a significant source of AhR ligands,[70] and a variety of commensals have been shown to utilize tryptophan as a source of energy, resulting in the production of various AhR ligands. For example, several species of the *Lactobacillus* genus have been described to metabolize tryptophan and generate indole-3-acetic acid, tryptamine, and 3-methyl indole, all of which can act as AhR ligands.[71–75] *E. faecalis* has also been described as an AhR ligand producer.[76] The understanding of how *E. gallinarum*, among other gut commensals that reside within the same anatomical area, preferentially translocates to peripheral organs during the breach of intestinal barrier integrity would further our understanding of the relationship that allows certain bacterial species to acquire properties of a pathobiont. Choi et al.[66] identified a dietary tryptophan dose-dependent, microbiota-mediated change in distribution of tryptophan metabolites including an increase in kynurenine in B6.NZM mice compared to B6 counterparts. In B6.NZM model, translocation of bacteria from the gut to peripheral organs was not found to be a significant contributor to autoimmunity development, but tryptophan-high diet resulted in the exacerbation of autoimmune phenotypes. The autoimmunity-promoting effects of tryptophan-high diet were recapitulated in B6 GF mice colonized with fecal microbiota of SPF B6.NZM[30] mice fed on tryptophan-high diet, reinforcing the contribution of the microbiota in triggering autoimmunity in B6.NZM mice. Metabolomic studies identified microbiota-dependent difference in tryptophan catabolism in B6.NZM mice compared to B6 controls.[66] In B6.NZM mice, T cell-intrinsic activation of mTOR metabolism in response to tryptophan and microbial tryptamine, as well as kynurenine-stimulated interferon gamma production were found.[66]

Having established that pathophysiology of specific SLE parameters could be influenced by the microbiota in B6.NZM mice, we asked whether different microbial composition could be responsible for the regulation of severity and sex bias of select SLE parameters. The breeding performance of B6.NZM is still poor in GF conditions making it difficult to perform any kind of high-throughput experiments, but sufficient to test a few distinct options. We hypothesized that colonization with different microbial communities would result in variations in the severity and sex bias of autoimmune traits in B6.NZM mice. To test this hypothesis, we conducted a colonization study in which GF animals housed at the University of Chicago (UC) were orally gavaged with cecal contents from donor mice raised in two independent institutions – UC and University of Florida, Gainesville (UF). As neonatal exposure to microbes is critical for the establishment of a stable microbial community,[77] we chose to perform analysis of G_1 progeny of the colonized females. Colonized mice were analyzed for various SLE traits in comparison with GF B6.NZM mice of similar age (around 7 months). Sex bias was still present in splenomegaly (in UC microbiota group) and levels of C3 immune complex deposits in kidneys of B6.NZM G_1 mice colonized with the UC microbiota and UF microbiota (Figure 2A,B). In this experiment, sex bias in the level of Ig immune complex deposits in kidneys was more pronounced in G_1 mice colonized with the UF microbiota, suggesting that distinct members of the microbial community may impose differential regulation of sex bias of Ig immune complex deposition

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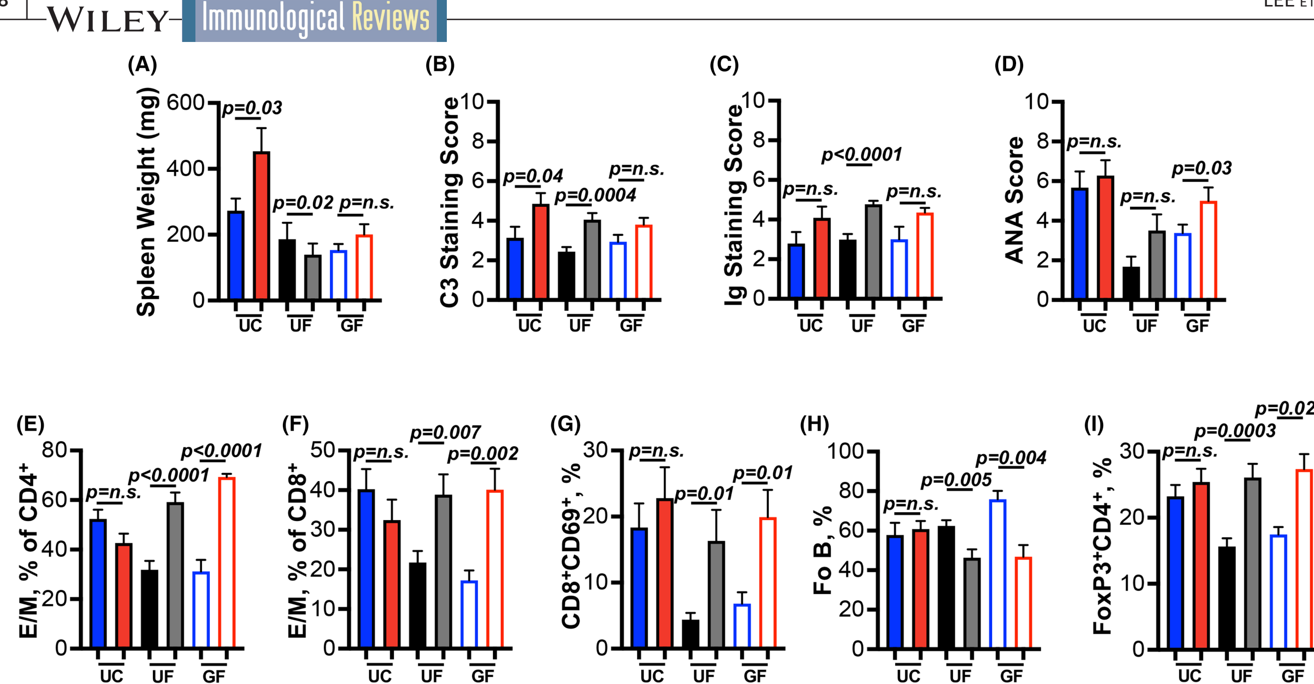

FIGURE 2 Comparative analysis of disease severity and sex bias in GF B6.NZM mice colonized with the microbiota taken from mice in two independent institutions. Cecal contents from University of Chicago (UC) or University of Florida (UF) donor mice were gavaged into GF female mice and they were bred and transferred their microbiota to the progeny (G_1). (A–I) SLE parameters measured in SPF (G_1) and GF B6.NZM male and female mice. (A–D) spleen weights (A), complement C3 (B) and Ig (C) deposits measured in kidneys via immunofluorescence, and ANA scores (D) analyzed by immunofluorescence on Hep-2 cells. (E–I) FACS analyses conducted on splenocytes to measure the proportions of effector/effector memory (E/M) T cells, follicular B (Fo B cells), and Tregs. E/M CD4$^+$ (E) or CD8$^+$ (F) T cells were defined as CD4$^+$CD44^{hi}CD62L^{lo} or CD8$^+$CD44^{hi}CD62L^{lo}. Recently activated CD8$^+$ T cells were defined as CD8$^+$CD69$^+$ T cells (G). CD4$^+$ and CD8$^+$ T cell subsets are shown as percentage of the total CD4$^+$ and CD8$^+$ T cell populations, respectively. Fo B cells (H) were defined as CD93$^-$B220$^+$IgM^{lo}CD23$^+$ and shown as percentage of the total CD93$^-$B220$^+$ B cell population. Tregs (I) are defined as CD4$^+$FoxP3$^+$ T cells and shown as percentage of the total CD4+ T cell population. UC colonization: closed blue – G_1 males, n = 10; closed red – G_1 females, n = 10. UF colonization: black – G_1 males, n = 11; gray – G_1 females, n = 8; GF: open blue – GF males, n = minimum of 5 mice; open red – GF females, n = minimum of 5 mice. Mean ± sem. Significance was calculated using an unpaired *t*-test. All mice were 7 months old.

TABLE 3 Two-way ANOVA analysis of the significance of microbiota and sex of the animals for development of the systemic lupus erythematosus features in University of Chicago versus Germ-free and University of Florida versus Germ-free B6.NZM mice.

Colonization	UC versus GF		UF versus GF	
Parameters	Microbiota's Influence	Sex Bias	Microbiota's Influence	Sex Bias
Splenomegaly	0.0002	0.01	0.69	0.99
C3	0.18	0.008	0.69	0.0003
Ig	0.63	0.01	0.54	<0.0001
ANA	0.02	0.15	0.02	0.01
E/M CD4$^+$	0.51	0.001	0.24	<0.0001
E/M CD8$^+$	0.15	0.16	0.69	<0.0001
CD8$^+$CD69$^+$	0.09	0.04	0.34	0.0003
Fo B	0.73	0.04	0.12	<0.0001
Treg	0.43	0.02	0.46	<0.0001

(Figure 2C). Whereas colonization with both microbiotas did not result in the restoration of sex bias in ANA levels seen in SPF B6.NZM mice, colonization with UF microbiota led to significant reduction in circulating levels of ANAs compared to SPF mice as well as mice colonized with the UC microbiota (Figure 2D). This data demonstrates that ANA represents an SLE trait that is sensitive to regulation by microbes, and different members of the microbial community can influence the pathogenesis of ANA production in varying manner. A differential influence on sex bias dependent on specific microbial members became more obvious in profiles of splenic T cells and B cells: only G_1 mice colonized with UF microbiota, but not G_1 mice colonized with the UC, exhibited strong sex bias in the frequencies of CD4$^+$ E/M T cells, CD8$^+$ E/M T cells, recently activated CD8$^+$ T cells, Fo B cells, and Tregs (Figure 2E–I). In conclusion, the data reveal that

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microbes do participate in regulating both the severity and sexual dimorphism of select SLE traits. To add to the complexity, different microbial members may control the microbial sensitive autoimmune parameters in distinct manners, resulting in varying dependence of SLE traits on the composition of the microbiota. The summary of the results described in this section is shown in Table 3. There, the significance of microbiota and sex of the animals for development of the SLE features was analyzed using 2-way ANOVA. There is a visible difference in the influence of the presence of the microbiota on the tested parameters; it is much less than obtained by comparison of in-house SPF mice with GF animals (Figure 1 and Table 2). There could be several reasons for such discrepancy. First, mice in the colonization experiment were slightly younger (7 months). Interestingly, the severity of SLE parameters increased with age in GF mice (compare Figures 1 and *2*), whereas some features (e.g., splenomegaly) in ex-GF mice colonized with the UC microbiota were stronger at 7 months of age and remained at the same level in SPF mice at 10 months of age. It would be important to appreciate whether the influence of microbes works throughout the full development of the disease or just at its inception. Second, the GF mice used in colonization experiment were rederived from frozen embryos, whereas our SPF colony was started with mice purchased from The Jackson Laboratory, where they could have undergone some genetic changes during maintenance. The importance of the genetic factors is difficult to underestimate. Finally, it could be as trivial as the loss of some microbial lineages during the physical transfer.

To compare the microbial diversity between the UC microbiota and the UF microbiota, we performed a 16 s rRNA gene sequencing of the cecal contents of donor mice as well as of the G_1 population. The V4 region of 16-s rRNA genes was PCR amplified with barcoded dual-index primers. Illumina libraries were generated using Qiagen QIASeq 1-stpe amplicon kit (QIGAEN), followed by sequencing using the Illumina MiSeq platform (Illumina, San Diego, CA) with a 2×250 Paired End reads. Microbiome amplicon read data was processed using Qiime pipeline[78] and count data was rarified using the *phyloseq* R library.[79] Microbiome 16S amplicon read data was demultiplexed using DADA2[80] and samples were rarified to an equal depth of 1103. Features were taxonomically mapped using a classifier that has been pretrained on GreenGenes[81] database with 99% operational taxonomic units. Alpha and Beta diversity metrics were calculated using Shannon's Entropy and the Bray–Curtis Dissimilarity metric, respectively. Beta diversity was visualized using principal coordinate analysis and clustering significance was calculated using a multivariate ADONIS test considering sex, generation, and site. Compositional plots were constructed at family level, where features with a median relative abundance less than 0.025 were grouped into a single category. All preprocessing and analyses were performed using Qiime2 and the *phyloseq* R package, and visualizations were generated using the *ggplot2* R library with R version 4.2.2.

Principal component analysis (PCoA) (Figure 3A) clearly distinguished groups of mice colonized with the two microbiotas. That was expected since the donors came from two independent colonies. The differences between the two groups were explained by different ratios of major bacterial families (Figure 3B). Interestingly, families with low representation (<2.5%) contributed significantly to the diversity between the two inputs. It is quite possible that the most important microbes could be attributed to the group of minoritarians. At this point, it would be difficult to attribute observed changes in SLE parameters to specific microbial lineages. That would require a significant additional effort to be proven by future colonization experiments.

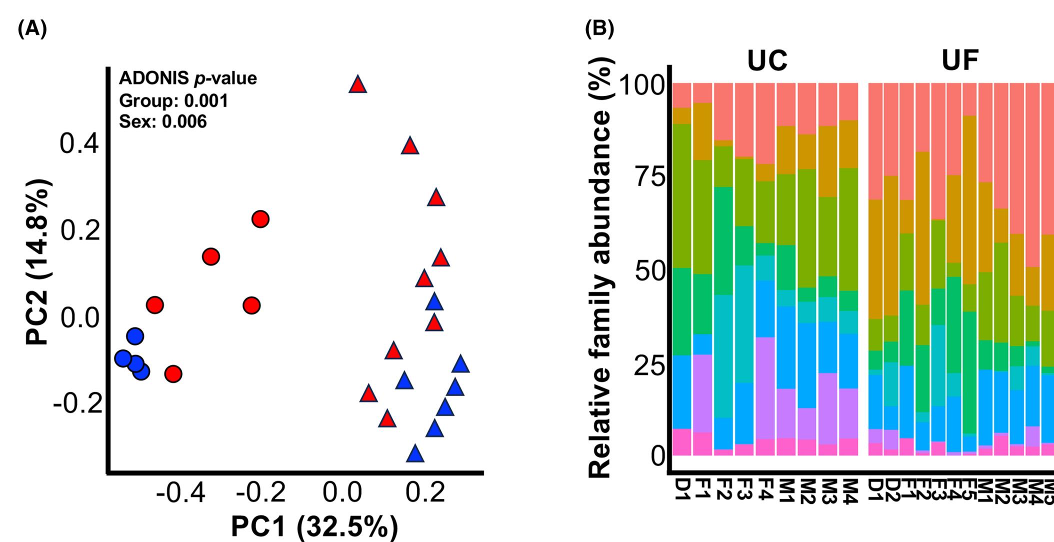

FIGURE 3 Comparative analysis of the microbiotas from G_1 B6.NZM mice colonized with two independent B6.NZM microbial communities. Cecal contents from University of Chicago (UC) or University of Florida (UF) donor mice (D) were gavaged into GF female mice. They were bred and transferred their microbiota to the progeny (G_1). Both males and females were used for the microbiome analysis. (A) A principal coordinate analysis (PCoA) plot generated using Bray–Curtis dissimilarity. Males – blue, females – red. UC colonization – circles, UF colonization – triangles. (B) Compositional plot constructed at family level. Features with a median relative abundance less 0.025 at the family level were grouped into a single category. D – donor females, M – G_1 males, F – G_1 females.

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6 | CONCLUSIONS

There is no doubt that commensal microbes influence the development and severity of autoimmunity.[19,44,77,82] Comparison of disease progression in SPF and GF animals has been a useful tool for determination of microbial contribution[16–19,31,32,83] (Table 1). Commensal microbes contribute to not only disease severity but to a well-known phenomenon of sexual dimorphism of autoimmune disorders.[19,31,32] Genetically identical SPF mice reared in different institutions are exposed to different microbial consortia, and therefore different microbial species may impose a spectrum of effects over progression of organ-specific autoimmunity like T1D. We extended our research towards systemic immunity and explored microbial regulation of disease severity and sexual dimorphism using B6.NZM model of SLE. It was also chosen because it was reported to develop various SLE traits with an increased penetrance in females over males.[47] Interestingly, the severity of the disease at our University of Chicago colony was lesser compared to previously reported,[47] indicating that environmental influences likely affected the course of the disease. It needs to be pointed out that the poor breeding performance of B6.NZM mice may lead inadvertently to expansion of the progeny of select breeders potentially resulting in genetic bottlenecks. This possibility should not be ignored. Regardless of that, sexual dimorphism was still present in some of the disease traits (Figure 1). This finding is similar to a report that demonstrated sex bias of selective SLE parameters in (NZM2410xC57BL/6)F_2 mice housed under SPF conditions.[84] To test the hypothesis that the microbiota serves as a key player in modulation of disease severity and sexual dimorphism in B6.NZM mice, we rederived B6.NZM mice into GF conditions. These mice came from early embryo freeze at The Jackson Laboratory suggesting that their genetics were closest to originally published.[34] Interestingly, two parameters that had sex bias—splenomegaly, which reflects the intensity of cell proliferation, and complement deposits in the kidneys—were independent of the presence of the microbiota. Thus, the sex bias of these traits must be driven by microbe-independent mechanisms based on hormonal influences or incomplete X chromosome inactivation.[85,86]

Recolonization of GF animals is a powerful tool for the analysis of the role of microbiota in disease control. In fact, two independent inputs affected measured SLE parameters differently (Figure 2). The inputs were analyzed, and the two input communities were quite distinct, and some bacterial lineages were identified as different as well (Figure 3).

The results of these experiments are summarized in Tables 2 and *3* and in the overall summary in Figure 4. The take-home messages are the following:

a. Disease progression is sensitive to the input microbiota.
b. Most disease features are attenuated by microbial colonization, except for the reduction of Treg population in GF mice. These observations could be connected.

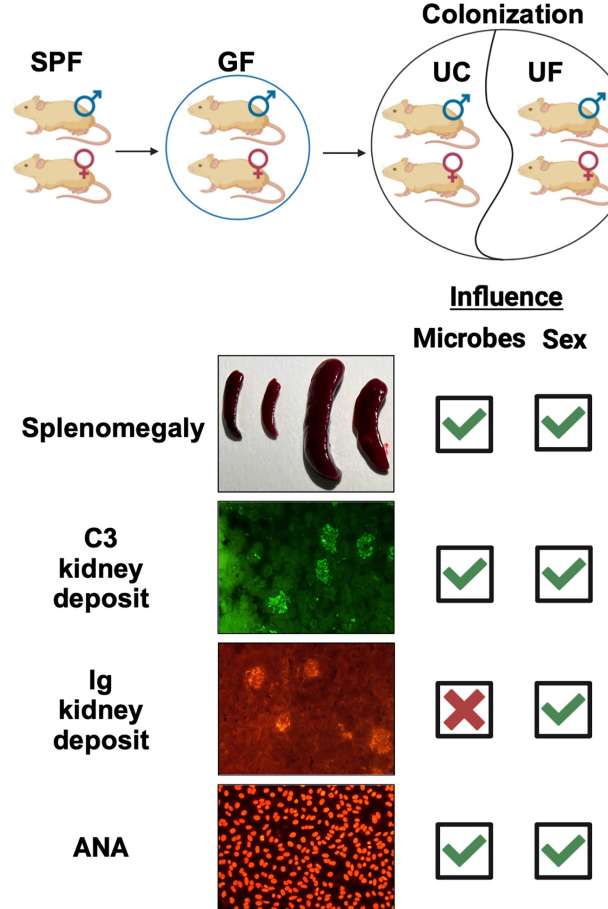

FIGURE 4 Summary figure. In B6.NZM mouse model of SLE, GF mice are compared to SPF, and ex-GF mice colonized with different sources of B6.NZM SPF microbiota. The shown features of the disease are influenced by the microbiota and the sex of animals. Germ-free mice do not require microbes to develop the symptoms. In most cases, the microbiota attenuated disease severity, and not all features were affected to the same extent by different microbial inputs. Such variability points at importance of the composition of the microbial consortia for the outcomes of the SLE development.

c. Some parameters (such as splenomegaly and kidney C3 deposits) are not sensitive to microbial influence and, thus, must have a strong genetic component.
d. Sex bias of the disease is influenced by commensal microbes.
e. Some critical microbial lineages can be lost in transfer experiments, as microbe-dependent sexually biased features were not transferred from UC SPF mice to GF mice with colonization with the UC SPF microbiota.

The varying degree of microbial involvement across different parameters in the SLE of B6.NZM mice echoes the findings in other models of autoimmunity[19,37,39,40,42,43,45,46,87–100] (see Table 1). It remains to be determined whether certain microbial interactions can act as a driver or a rheostat in regulation of sex bias and disease

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severity in autoimmune conditions that affect the host in a systemic fashion. If so, the identity of such microbes and the metabolic and signaling employed by them to exert such effects need to be pursued.

Overall, our findings open new avenues to the studies of the mechanisms behind manifestations that must be driven by host genetics and not by the environment. It would be fair to say that GF experiments reveal the diseases' '*tabula rasa*', which is used by microbes to etch their scripts for the pathogenesis in affected individuals.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

Data available on request from the authors. Sequencing data deposited under accession number PRJNA1095922.

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