Impact of Gut Microbiota on Gender Bias in Intestinal Pro-inflammatory Immune Phenotype and Systemic Autoimmune Progression in Lupus-prone SNF1 mice

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1. Abstract

The risk of developing systemic lupus erythematosus (SLE) is about 9 times higher among women compared to men. While sex hormone dependent events that contribute to this gender bias have been widely investigated, our recent report showed a potential link between immune response initiated in the gut mucosa at juvenile age (sex hormone independent) and SLE susceptibility. Here, using (SWRxNZB) F1 (SNF1) mouse model of spontaneous lupus, we show that gut microbiota contributes to a pro-inflammatory immune response in the intestine and autoimmune progression, primarily in females, leading to an associated gender bias. We found that gut microbiota composition in male and female littermates are significantly different only at adult age and depletion of gut microbes using broad-spectrum antibiotics causes suppression of autoimmune progression in females, but not in males. In agreement, microbiota depletion suppressed the pro-inflammatory cytokine response of gut mucosa in both juvenile and adult females. In male SNF1 mice, on the other hand, orchidectomy (castration) caused changes in the composition of gut microbiota and a modest acceleration of autoimmune progression. Nevertheless, cecum microbiota transplantation studies failed to show superior protection of females from autoimmunity by androgen-influenced gut microbiota compared to that of females and castrated males. Overall, our work shows that microbiota-dependent pro-inflammatory immune response in the gut mucosa of females initiated at juvenile ages and androgen-dependent modest protection of males contribute to gender differences in the intestinal immune phenotype and systemic autoimmune progression in lupus-prone SNF1 mice.

Key words: systemic lupus erythematosus, gut mucosa, gut microbiota, testosterone, autoimmunity, gender bias, inflammation.

2. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the production of auto-antibodies against nuclear material and these immune complexes lead to glomerulonephritis and kidney failure [1]. Disease arises when abnormally functioning B lymphocytes produce auto-antibodies to DNA and nuclear proteins, resulting in immune complexes that cause damage to the tissue. While the triggers are not known, it is generally believed that SLE is caused by a combination of genetic and environmental factors [1-3]. Dietary factors can also have a profound impact on the gut and systemic immune responses [4-8]. Recent studies that used human samples and rodent models have shown that gut microbiota composition influences the rate of disease progression and the overall disease outcome [9-13]. In addition to direct effects on the systemic and gut immune cell functions, dietary factors can change the composition of gut microbiota and affect autoimmune outcomes [10, 14, 15]. Recent reports from our laboratory demonstrated that minor dietary deviations such as changes in the pH of drinking water alter the composition of gut microbiota and incidence of SLE as well as type 1 diabetes (T1D) in mouse models [13, 16].

Importantly, there is a predisposition for SLE in women with a prevalence ratio of about 9:1 over men [17-19]. Numerous studies have suggested that sex hormones, estrogen in particular, can contribute to the onset and development of disease activities associated with SLE. Estrogen is reported to have inductive effects on autoimmune-related immune responses such as production of antibody and pro-inflammatory cytokines [17, 19-21]. Studies using the non-obese diabetic (NOD) mouse model of T1D have shown a role for testosterone-influenced microbiota, independent of estrogen-mediated effects, in determining the gender bias of autoimmune diseases [22, 23]. These reports suggested a strong involvement of gut microbial factors in

determining gender disparity in systemic autoimmunity. Although it has been shown that lupus-susceptible GF mice develop disease [24, 25], it is not known if gut microbiota affects gut immune phenotype and modulates disease outcomes differently in males and females under lupus susceptibility. While the contribution of testosterone influenced microbiota in protecting male NOD mice from T1D has been shown [22, 23], such studies in the context of lupus autoimmunity have not been reported. Although MRL/lpr mice showed gender specific differences in gut microbiota at adult ages close to disease onset [26], whether these differences are hormone driven, or can impact intestinal immune phenotype and autoimmune progression are not known.

Our recent study that used lupus-prone (SWRxNZB)F1 (SNF1) mice, for the first time, showed the potential contribution of immune response initiated in the gut mucosa in lupus associated gender bias [27]. We also showed that pro-inflammatory immune response in the gut mucosa of lupus-prone females can be detected as early as at juvenile age, an age at which there is no sex hormone produced and much before the appearance of systemic autoimmunity. In another report, we showed that change in the pH of drinking water provided to this mouse model has a profound effect not only on the composition of gut microbiota and the type of intestinal immune response, but also on the disease progression [13]. These observations suggest a potential role for gut microbiota composition and mucosal immunity in shaping the gender bias in systemic autoimmunity in lupus-prone background. Here we show that gut microbiota composition in lupus-prone SNF1 mice, particularly in littermates, are different at adult ages, but not at juvenile age. Interestingly, depletion of gut microbiota starting at juvenile age not only suppressed the inflammatory phenotype, but also ameliorated autoantibody production and disease incidence in females. While castration of male SNF1 mice resulted in altered gut microbiota and modest

increase in autoantibody production and lupus incidence, androgen influenced gut microbiota did not alter the disease outcomes in female SNF1 mice compared to microbiota from females and castrated males. Overall, these observations show that gender dependent juvenile age onset and progressive increase in intestinal inflammatory immune response and adult age systemic autoantibody production in lupus-prone females are gut mucosa-microbiota interaction dependent, but may not be influenced by the gender dependent microbiota composition. Further, androgen dependent modest protection of lupus-prone males from autoimmunity at adult ages also contributes to this gender bias.

3. Materials and Methods

3.1 Mice. SWR, NZB mice, and C57BL/6 (B6) mice were purchased from the Jackson Laboratory (Bar Harbor, Maine) and housed under SPF conditions at the animal facilities of Medical University of South Carolina (MUSC). (SWRxNZB)F1 (SNF1) hybrids were generated at the SPF facility of MUSC by crossing SWR females with NZB males. In some experiments, male SNF1 mice were subjected to orchidectomy (castration) or mock surgery after anesthesia with ketamine and xylozine combination. In some experiments, mice were given a cocktail of broad spectrum antibiotics as described in our recent report [28] to deplete gut microbiota. Depletion of gut microbiota was confirmed by culture of fecal pellet suspension on brain heart infusion agar plates under aerobic and anaerobic conditions as described before [28]. Urine and tail vein blood samples were collected at different time-points to detect proteinuria and autoantibodies. All animal experiments were performed according to ethical principles and guidelines were approved by the institutional animal care committee of MUSC.

3.2 Proteinuria. Urine samples collected weekly were tested for protein levels by Bradford assay (BioRad) against bovine serum albumin standards. Proteinuria was scored as follows; 0: 0-1mg/ml, 1: 1-2mg/ml, 2: 2-5 mg/ml, 3: 5-10mg/ml and 4: ≥ 10mg/ml. Mice that showed proteinuria >5 mg/ml were considered to have severe nephritis.

3.3 ELISA. Levels of antibodies against nucleohistone and dsDNA in mouse sera were determined by ELISA as described in our recent reports [13, 27]. Briefly, 1.0μg/well of nucleohistone (Sigma-Aldrich) or dsDNA from calf thymus (Sigma-Aldrich) was coated as antigen, overnight, onto ELISA plate wells. Serial dilutions of the sera were made and total IgG

and IgG isotypes against these antigens were detected using HRP-conjugated respective anti-mouse immunoglobulin isotype antibodies (Sigma-Aldrich, eBioscience and Invitrogen).

3.4 Quantitative PCR. RNA was extracted from 2-cm pieces of the distal ileum or 1cm pieces of distal colon using Isol-RNA Lysis Reagent (5prime) according to manufacturer's instructions. cDNA was prepared from RNA using first strand synthesis kit (Invitrogen) and PCR was performed using SYBR-green master-mix (BioRad) and target-specific custom-made primer sets. A CFX96 Touch real time PCR machine (BioRad) was used and relative expression of each factor was calculated by the 2-ΔCT cycle threshold method against β-actin control.

3.5 16S rRNA gene targeted sequencing and bacterial community profiling. Total DNA was prepared from fresh fecal pellets of individual mice for bacterial community profiling as described in our recent reports [13, 16, 28]. Briefly, DNA in samples was amplified by PCR using 16S rRNA gene-targeted primer sets to assess the bacterial levels. V3-V4 region of 16S rRNA gene sequencing was performed using Illumina MiSeq platform at MUSC genomic center. The sequencing reads were fed in to the Metagenomics application of BaseSpace (Illumina) for performing taxonomic classification using an Illumina-curated version of the GreenGenes taxonomic database which provided raw classification output at multiple taxonomic levels. The sequences were also fed into QIIME open reference operational taxonomic units (OTU) picking pipeline [29] using QIIME pre-processing application of BaseSpace. The OTUs were compiled to different taxonomical levels based upon the percentage identity to GreenGenes reference sequences (i.e. >97% identity) and the percentage values of sequences within each sample that map to respective taxonomical levels were calculated. The OTUs were also normalized and used for metagenomes prediction of Kyoto Encyclopedia of Genes and Genomes (KEGG) orthologs employing PICRUSt as described before [26, 30-32]. The predictions were summarized to

multiple levels and functional categories as well as microbial communities at different taxonomical levels were compared among different groups. Statistical Analysis of Metagenomic Profile Package (STAMP) [31] or web-based MicrobomeAnalyst application [33] were employed for analyzing OTU.Biom table and PICRUSt data for visualization and statistical analyses where appropriate.

3.6 Microbiota transfer. Transfer microbiota was performed as described in our recent reports [13, 16]. Briefly, homogenous suspensions of cecum contents were made in chilled 0.1M bicarbonate buffer and administered to recipients by oral gavage. Suspension from one cecum was given to 5 mice. This process was repeated for three consecutive days using fresh preparations of microbes from additional donors. The recipients were tested for proteinuria and auto-antibody levels as described above.

3.8 Multiplex assay / suspension bead array. Immune cells were enriched from collagenase digested intestinal tissues by Percoll gradient centrifugation and culture supernatants were tested for cytokine levels. Luminex multiplex assay based suspension bead array (SBA) was carried out using magnetic bead based cytokine/chemokine 26-plex or IFN-α and IFN-β 2-plex panels from eBiosciences and/or Invitrogen. Assay plates were read and concentrations were calculated using FlexMap3D instrument and xPONENT software (Luminex Corporation) at MUSC's immune monitoring and discovery core.

3.7 Statistical analysis. Proteinuria curves were analyzed using log-rank method (http://bioinf.wehi.edu.au/software/russell/logrank/) and the proteinuria scores were analyzed using Chi-square test. Two-tailed *t*-test or Mann Whitney test was also employed to calculate *p*-values where indicated. A p value of ≤ 0.05 was considered statistically significant. GraphPad

Prism was used for calculating statistical significance. For some 16S rDNA data, Welch's two-sided t-test was employed and the *p*-values were corrected from multiple tests using the Benjamini and Hochberg approach using STAMP or MicrobiomeAnalyst applications.

4. Results

4.1 Intestinal immune phenotype in SNF1 mice. SNF1 mice that develop lupus symptoms and proteinuria spontaneously and show gender bias in disease incidence similar to human SLE patients [34, 35] have widely been used for understanding the disease etiology. Recently, we reported that significant amounts of circulating autoantibodies against nucleohistone and dsDNA are detectable in SNF1 mice at 16 weeks of age and severe nephritis indicated by high proteinuria after 22 weeks of age [13, 27]. We also showed that about 80% of the female and 20% of the male SNF1 mice develop severe nephritis within 32 weeks. Previous studies have shown that sex hormones may play a critical role in determining gender bias in the lupus disease outcomes in humans and mouse models [17, 19-21]. Interestingly, we found that small intestinal mucosa of female SNF1 mice, as compared to their male counterparts, harbor profoundly higher frequencies of activated B cells including plasma cells and Th17 and Th9 cytokine expressing T cells, as well as express high levels of pro-inflammatory factors as early as at juvenile age (4 weeks), at which there is no sex hormones produced, and an amplified levels of these factors at 16 weeks of age [27].

Here we determined both mRNA and/or protein levels of these key pro-inflammatory cytokines factors in both large and small intestines of pre-nephritic 4 or 16 week old male and female littermates of SNF1 mice. Quantitative PCR analysis shows that most pro-inflammatory factors

including *Il6*, *Il4*, *Il9*, *Il17*, *Il21, Ifna and Ifnb* are expressed at higher levels in both colon and ileum of female SNF1 mice as compared to their age-matched male counterparts (Fig. 1A and Supplemental Fig. 1), and lupus-resistant B6 mice (not shown). Significantly higher *Ifng* expression was detected primarily in the large intestine of the SNF females. Further, the expression levels of endosomal TLRs, which are implicated in SLE (*Tlr7*, *Tlr8* and *Tlr9*) [36-40], are also high in both large and small intestines of SNF females. Importantly, significant differences in the expression levels of most pro-inflammatory factors were detectable as early as 4 weeks of age (juvenile) and a robust increase in the expression levels of these factors in the small and large intestines were detected at 16 weeks. To further validate the observations on inflammatory immune phenotype of lupus-prone female intestine, immune cells were enriched from colon, cultured for 48 h in the absence of added stimuli, supernatants were tested for the levels of spontaneously secreted cytokines. Figure 1B shows that the amounts of spontaneously released cytokines were significantly higher in females compared to males. Overall, in conjunction with our recent report showing activated T and B cells in the gut mucosa of lupus-prone female mice [27], these observations confirm that pro-inflammatory immune phenotype of gut mucosa in lupus-prone female mice is initiated, sex hormone independently, at juvenile age.

4.2 Gut microbiota composition in lupus-prone SNF1 male and female mice are different at adult ages. Differences in the intestinal immune phenotype in male and female SNF1 mice suggested a role for gut microbiota in this gender bias. The influence of sex hormone-dependent gut microbiota in T1D gender bias in NOD mice has been reported before [22, 23]. However, not only that such studies in the context of lupus autoimmunity have not been reported, but also it is not known if gut microbiota in juvenile lupus-prone males and females are different and if it contributes to the pro-inflammatory immune phenotype of lupus-prone female intestine. Our

initial studies used age-matched, but not littermate controlled, mice to determine the composition of fecal microbiota in younger (4 week old) and older (24 week old) SNF1 males and females. Principal component (PC) analysis of 16S rRNA gene sequences showed profound gender specific clustering only by adult mice, but not juveniles (Supplemental Fig 2A). Accordingly, profound differences in the abundances of microbial communities were observed only in 24 week old male and female mice (not shown). Further, SNF1 females at 24 weeks, the age at which severe nephritis begins to appear, but not 4 week old juvenile females, showed relatively lower gut microbial diversity compared to male counterparts (Supplemental Fig. 2B).

Although these observations are consistent with the previous studies employing T1D model [22, 23], to unambiguously validate our observations in the lupus-prone model, fecal pellets from 8 individually housed littermates (4 males and 4 females) of SNF1 mice were collected at 4 weeks and 16 weeks of age and subjected to microbial community profiling. As shown in Fig. 2A, PC analysis found gender specific separation of gut microbiota diversity only with 16 week samples, but not with the samples from 4 week old juvenile mice. Further, as shown in Fig. 2B, fecal microbial diversity was significantly different only in adult mice. Compilation of OTUs to different taxonomical levels showed that fecal microbiota in 16 week old females had significantly lower abundance of Verrucomicrobia phyla members and is relatively richer, albeit not significant statistically, in Bacteroidetes phyla members (Fig. 2C and Supplemental Fig. 3). Interestingly, a significant increase in Bacteroidetes abundance was observed in females at 16 weeks of age compared to 4 weeks (Supplemental Fig. 4A). Since Fig. 2A showed gender dependent clustering only with samples from 16 week old mice, these samples were compared for abundances of microbial communities at the genus level. As observed in Fig. 2D, compared to littermate males, samples from females at 16 weeks of age had profoundly higher abundance

of Bacteroides and Parabacteroides genus members, and lower Dysgonomonas genus members of Bacteroidetes phylum. Further, significant differences in large number of minor communities were observed between 16 week samples from males and females. As observed in Supplemental Fig. 4B, significant age-dependent differences in the abundance of multiple microbial communities were observed in both males and females when samples collected at 4 weeks were compared to that of 16 weeks of age.

We then employed PICRUSt application for OTUs based *in silico* prediction of metabolic functions that may be associated with intestinal pro-inflammatory immune phenotype and lupus disease process in lupus-prone females. The PICRUSt analysis of data from 16-week old males and females showed that a variety of metabolic pathways such as glycan degradation and glycosphingolipid biosynthesis pathways, and nitrogen, amino sugar, nucleotide sugar, galactose, starch and sucrose metabolism pathways are overrepresented in females at 16 weeks of age (Fig. 2E). On the other hand, pathways involved in secondary metabolite synthesis and degradation, fatty acid, glycerophospholipid and lysine metabolism are underrepresented in 16 week old females compared to male counterparts. Although further analysis and additional studies are needed to understand if these metabolic pathways contribute to modulation of intestinal immune response and disease progression or suppression, these observations further support the notion that microbial communities are functionally different in lupus-prone adult males and females.

4.3 Gut microbiota depletion suppresses autoimmune progression in female SNF1 mice. Although we observed pro-inflammatory immune phenotype of female SNF1 mouse intestine as early as juvenile age, significant differences in the overall gut microbiota composition between males and females was detected only at adult ages. Nevertheless, we examined if microbiota depletion impacts systemic autoimmunity and disease incidence in these mice. Both male and

female SNF1 mice were given a broad-spectrum antibiotic cocktail in drinking water, starting immediately after weaning to deplete the gut microbiota (Supplemental Fig. 5) and monitored for proteinuria and serum autoantibody levels. As shown in Fig. 3A, as compared to mice with intact gut microbiota, severe nephritis was significantly delayed in majority of the female SNF1 mice that were given antibiotics. While about 80% of control females developed severe nephritis by 31 weeks of age, only about 40% of the microbiota deplete mice showed similar degree of disease severity. Intriguingly, microbiota depletion had no impact on the disease progression in male SNF1 mice. Fig. 3B which shows differences in the degree of proteinuria in antibiotic treated and control mice further support the observation that the overall disease progression was suppressed significantly in females, but not in male SNF1 mice, upon gut microbiota depletion. In addition, determination of serum autoantibodies against dsDNA and nucleohistone revealed significantly lower autoantibody production in microbiota depleted female SNF1 mice at 24 and 30 weeks of age compared to their control counterparts (Fig. 3C). On the other hand, autoantibody responsess were comparable in microbiota depleted and control males.

4.4 Microbiota depletion suppresses intestinal pro-inflammatory immune phenotype in female SNF1 mice. Since depletion of gut microbiota resulted in slower autoimmune progression in females, intestinal immune phenotype of mice with intact and depleted gut microbiota were determined in cohorts of young (5 weeks; pre-puberty) and adult (16 week) aged mice. As observed in Fig. 4A, the expression levels of pro-inflammatory cytokines in the colon of microbiota depleted females were significantly lower compared to control females, irrespective of the age. On the other hand, expression of *Il10*, an immune regulatory cytokine, was not diminished in microbiota depleted younger mice and significantly higher in microbiota depleted older mice compared to their counterparts with intact microbiota. Further, the levels of cytokine

secretion by intestinal immune cells showed correlation with that of mRNA levels (Fig. 4B). Interestingly, *Tlr7, Tlr8, and Tlr9* expression levels in the colon of microbiota depleted females were not significantly different from that of control mice. Further, in agreement with proteinuria and autoantibody results of Fig. 3, expression levels of pro-inflammatory cytokines and TLRs in the colon were not significantly different in microbiota depleted and control SNF1 males (not shown).

4.5 Castration of male SNF1 mice causes changes in gut microbiota composition. Our observations (Supplemental Fig. 4) show that both male and female SNF1 mice present age dependent changes in gut microbiota composition. However, age-dependent β-diversity clustering of 16S rRNA gene sequences (Fig. 2A) and impact of microbiota depletion (Figs. 3 & 4) were observed only with females. This suggests that gut microbes contribute to higher disease incidence in females. Of note, previous reports have shown androgen dependent changes in the gut microbiota in NOD mouse model of T1D at adult ages [22, 23] and resistance to disease compared to female counterparts. Therefore, we examined if androgen has an influence on gut microbiota in lupus-prone male SNF1 mice. Juvenile mice were castrated to curtail androgen production (Supplemental Fig. 6), and examined for changes in fecal microbiota composition. PC analysis graphs (Fig. 5A) show that castrated and mock-surgery control mice showed better clustering at adult age (post-castration) compared to juvenile age (pre-castration). Although difference in microbial alpha diversity indices was not statistically significant different in castrated and control mice (Fig. 5B), lower abundances of Bacteroidetes and Verrucomicrobia and higher abundances of Proteobacteria and Firmicutes phyla were observed in castrated males compared to controls (Fig. 5C). Figure 5D shows that the abundance of multiple minor microbial communities (those with <1% of all identified sequences) at genus level were significantly

different in castrated and non-castrated SNF1 males. Overall, these observations show that castrated and non-castrated male SNF1 mice have different microbiota compositions, and as reported by others in NOD mice [22, 23], testosterone may be contributing to these differences.

4.6 Castration of male SNF1 mice results in modestly accelerated autoimmune progression. Since depletion of gut microbiota had no impact on autoimmune progression in male SNF1 mice (Fig. 3) and castration appeared to influence the gut microbiota composition in them (Fig. 5), we examined if autoimmune progression and disease incidence in SNF1 males are impacted by low testosterone levels due to castration. Castrated and mock-surgery control male mice were monitored for proteinuria for up to 36 weeks of age. As observed in Fig. 6, although not significant statistically, castrated male SNF1 mice showed relatively earlier onset of nephritis, higher severity of nephritis as indicated by urinary protein levels, higher disease incidence, and higher anti-nucleohistone and anti-dsDNA antibody responses compared to controls. These observations along with the observation that castrated and non-castrated mice show differences in gut microbiota suggest that androgen may directly or indirectly (through gut microbiota) contribute to the lower susceptibility of male SNF1 mice to systemic autoimmunity. Since microbiota depletion did not impact disease incidence in male SNF1 mice (Fig. 3), microbiota transfer experiment was carried out to determine if androgen influenced microbiota can affect disease progression. Puberty aged (6 weeks old) SNF1 females received cecum microbial preparations from 20 week old castrated male, mock surgery control mice or age matched females by oral gavage and were monitored for proteinuria and autoantibody levels. As shown in Fig. 6D, female SNF1 mice that received cecum microbial preparations from all three adult groups (castrated male, mock surgery control mice or female mice) showed comparable disease progression and proteinuria severity. Further, the levels of anti-dsDNA and anti-nucleohistone

antibody levels were comparable in these groups of mice (Supplemental Fig. 7). Of note, albeit not significant statistically, all microbiota recipient groups of mice showed relatively slower, but comparable, disease progression relative to control females. These observations show that, as reported by others in the past [41, 42], androgen promotes protection of SNF1 males from lupus, perhaps microbiota independently.

5. Discussion

Gender bias is prevalent in many major autoimmune diseases including SLE [43-45]. The influence of sex hormones on lupus autoimmunity in general and gender bias in particular has been extensively studied. While estrogen has been linked to both the cause and pathology of disease [42, 46, 47], testosterone has generally been found to be protective [41, 42]. Interestingly, recent studies using SPF and germ-free (GF) NOD mice have linked the sex-hormone, primarily androgen,-influenced gut microbes and their metabolites to gender bias in T1D [22, 23]. It has been shown that, similar to T1D mouse model, SLE mouse models in GF background do develop disease, suggesting that gut microbes are not required for autoantibody response in these mice [24, 25]. However, the role of gut microbiota in gender bias associated with lupus has not yet been fully realized. In this regard, our recent report [27] showed, for the first time, that the immune phenotype of gut mucosa is significantly different in lupus-prone male and female SNF1 mice. Pro-inflammatory immune phenotype of gut mucosa appears in female mice at juvenile age, much before the production of sex hormones. Further, gut mucosa of female SNF1 mice carry higher number of plasma cells compared to their male counterparts even at juvenile age. These observations indicated that immune response initiated in the gut mucosa may be involved in the initiation and perpetuation of systemic autoimmunity and

determining lupus associated gender bias. Here, in the current study, we show significant influence of gut microbiota on intestinal pro-inflammatory immune phenotype and systemic autoimmune progression in females, but not in males. Further, we show that while androgen has an influence on gut microbiota composition in lupus model as reported in T1D model by others [24, 25], it appears to promote microbiota-independent modest protection from lupus autoimmunity.

Expression profiling of intestines from lupus prone male and female SNF1 mice demonstrated that both colon and ileum expresses higher levels of pro-inflammatory cytokines as well as TLRs as early as juvenile age as compared to male counterparts. Endosome TLRs such as TLR7, TLR8 and TLR9 are widely implicated in lupus [36-39] and many of the therapeutic agents that are already being used in the clinic do target endosome pathway [48]. Pro-inflammatory cytokine factors such as IFN-α, IFN-β, IL-17 and IL9 have also been linked to pathogenesis associated with SLE [36, 39, 49-52]. Importantly, signaling through endosomal TLRs triggers production of pro-inflammatory cytokines such as type 1 IFNs. Intriguingly, compared to female SNF1 mice with intact gut microbiota, lower expression of only pro-inflammatory cytokines, but not the immune regulatory cytokine IL10 or endosomal TLRs, were observed in pre-puberty and adult mice upon depletion of gut microbiota. Moreover, microbiota depletion associated suppression of pro-inflammatory cytokines in the intestinal mucosa occurs mainly in females, but not in males. This is intriguing because the composition of gut microbiota in male and female SNF1 mice are comparable at younger ages. Nevertheless, these microbiota dependent features of gut mucosa of male and female SNF1 mice explain why systemic autoimmunity and the disease incidence are significantly suppressed only in females upon microbiota depletion.

Our observations, consistent with previous reports [24, 25, 53, 54], that gut microbiota compositions in SNF1 male and female littermates are significantly different only at adult age, but not juvenile age, do argue against the notion that differences in the composition of gut microbiota could be responsible for the pro-inflammatory immune phenotype of juvenile SNF1 female intestine. Nevertheless, microbiota depletion did result in diminished expression of pro-inflammatory cytokines in the intestine of not only adult, but also the juvenile SNF1 females. Although studies using GF lupus-prone mice are needed to further evaluate the true impact of microbiota on intestinal immune phenotype, our observation raises the possibility that the magnitude/dosage of microbiota-gut mucosa interaction, rather than the microbiota composition, is different in male and female SNF1 mice. Higher expression levels of microbiota interacting receptors in the gut mucosa could contribute to higher expression of pro-inflammatory factors in female intestine and disease associated gender bias. A likely example, based on our qPCR data, is that higher levels of TLRs, such as TLR7, TLR8 and TLR9, in the gut mucosa of lupus-prone females could facilitate higher dose interaction with gut microbiota, resulting in more pronounced inflammatory cytokine response. In this regard, previous studies have shown that TLR7 and TLR8 stimulation promotes significantly higher pro-inflammatory cytokine response in females than in males [36-39]. A recent study using mouse models has shown that dosage of X-linked *Tlr8* plays a major role in the higher incidence of SLE in females [39]. Another study using samples from lupus patients has demonstrated that TLR7 ligands induce higher IFN-α production in females [36]. Of note, the fact that the expression of pro-inflammatory cytokines is much higher in adult females compared to prepubescent SNF1 females suggest a progressive increase in the recruitment and/or activation of immune cells in the gut mucosa causing a vicious cycle of pro-inflammatory responses in lupus-prone females.

Lack of significant impact of microbiota depletion on gut immune phenotype and autoimmune progression in male SNF1 mice is intriguing. One possible explanation is that under normal circumstances, unlike in lupus-prone females, low dose gut mucosa-microbiota interaction produces a modest cytokine response in control mice with intact microbiota and, as reported by other [41, 42], testosterone alone may have a disease protective effect in lupus. Although previous reports have shown that testosterone influenced gut microbiota protects NOD mice from T1D [24, 25], our microbiota transplant studies suggest that modest increase in disease susceptibility in castrated male SNF1 mice could be due to the lack of testosterone, but may not be dependent on microbiota. Of note, castration of male SNF1 mice, as reported by others in NOD mice [24, 25], did cause changes in the overall composition of gut microbiota, primarily by altering the abundances of minor communities.

Overall, our study, for the first time, demonstrates distinct roles for gut microbiota in lupus-prone female and male mice contributing to gender bias in the disease outcomes. In females, gut microbiota appears to have composition-independent, dose-dependent interaction, role in promoting intestinal pro-inflammatory immune response and eventual systemic autoimmunity. On the other hand, lower pro-inflammatory cytokine production in the intestine and lower autoantibody production and disease incidence in SNF1 males compared to females appear to be primarily sex hormone dependent. These observations also suggest that the mechanisms associated with the gender bias in lupus model may be different from that in NOD mouse model of T1D, which is primarily due to protection of males from the disease by testosterone influenced gut microbiota [24, 25]. Hence, comprehensive studies using GF mice are needed in the future to delineate the microbiota dependent and independent mechanisms of gender bias in lupus autoimmunity.

Figure legends

Fig. 1: *Intestinal immune phenotype in SNF1 mice.* A) cDNA preparations from the distal colon of 4 and 16 week old lupus-prone male and female SNF1 mice were subjected to real-time quantitative PCR to assess the expression levels of key cytokines and endosomal TLRs. Expression levels of individual factors were calculated against the β-actin values and values from 4 male and 4 female SNF1 mouse littermates (tested individually) were used for generating the heat-map. Statistical analysis by two-sided *t*-test. Distal ileum from male and female littermates were analyzed similarly and shown in supplemental Fig. 1. **B)** Immune cells were enriched from collagenase digested single cell suspension of ileum and colon by Percoll gradient centrifugation, equal number of enriched cells ($3x10^5$/well in 150 μl medium) from each tissue were cultured for 48 h, and the spent media were subjected to SBA assay to detect the levels of spontaneously released cytokines. Mean±SD values (4 male and 4 female SNF1 mouse littermates; tested individually) are shown. Statistical analysis by two-sided Mann Whitney test. These experiments were repeated at least once using 4 mice/group and obtained similar results.

Fig. 2: *Gut microbiota composition in lupus-prone SNF1 male and female mice.* Four male and 4 female SNF1 mouse littermates were individually housed upon weaning at 3 weeks of age, fresh fecal pellets were collected at 4 and 16 weeks of age, DNA preparations were subjected to 16S rRNA gene (V3/V4 region) -targeted sequencing using the Illumina MiSeq platform. The OTUs that were compiled to different taxonomical level based upon the percentage identity to reference sequences (i.e. >97% identity) and the percentage values of sequences within each sample that map to specific phylum and genus were calculated employing 16S Metagenomics application of Illumina BaseSpace hub. OTU.Biom tables were also generated from the sequencing data employing QIIME preprocessing application of BaseSpace. **A)** OTU.Biom table data were used

for generating principal component analysis (PCA) plots of samples representing β-diversity (Bray Curtis distance). **B)** α-diversity (Chao1)/species richness comparison of all 4 groups. **C)** Relative abundances of 16S rRNA gene sequences in fecal samples of individual mice at phyla level. **D)** Mean relative abundances of sequences representing major and minor microbial communities (>0.1 % of total sequences) at genus level. **E)** Normalized OTU.biom table was used for predicting gene functional categories using PICRUSt application and selected level 3 categories of KEGG pathway with statistically significant differences (between 16 week males and 16 week females) are shown. Statistical analyses were done employing two-sided Welch's *t-test* and the *p*-values were FDR corrected using Benjamini and Hochberg approach. Additional analyses of this sequencing data are shown in Supplemental Figs. 3 & 4.

Fig. 3: *Impact of gut microbiota depletion on autoimmune progression.* Male and female SNF1 mice were maintained on regular drinking water or drinking water containing broad-spectrum antibiotic cocktail starting at 3 weeks of age. Urine and serum samples collected from individual mice at different time-points were examined for proteinuria and auto-antibody levels. **A)** Protein levels in urine samples were quantified by Bradford assay. Percentage of mice with severe nephritis as indicated by high proteinuria (≥5mg/ml) is shown. *p*-values by log-rank test. **B)** Severity of proteinuria/nephritis in different groups of mice at different time points is shown. Nephritis severity was scored based on urinary protein levels as follows; 0: 0-1mg/ml, 1:1-2mg/ml, 2:2-5 mg/ml, 3: 5-10mg/ml and 4: ≥ 10mg/ml. *p*-values by 2-sided Chi-Square test. **C)** Serum levels of total IgG and IgG2a antibodies specific to nucleohistone and dsDNA were assessed by ELISA for samples collected at 24 and 30 weeks of age. Mean ±SD of OD values of samples (1:1000 dilution) from 10 mice/group for controls and 12 mice/group for antibiotic treated mice are shown. *p*-values by two-sided Mann Whitney test.

Fig. 4: *Impact of microbiota depletion on intestinal pro-inflammatory immune phenotype.* A) cDNA preparations from the distal colon of 5 week and 16 week old control and antibiotic cocktail (Abx) treated female SNF1 mice were subjected to real-time quantitative PCR to assess the expression levels of key cytokines and endosomal TLRs. Relative expression levels of individual factors were calculated against the β-actin values and values from a total of 5 control and 5 antibiotic treated females (tested individually) were used for generating the heat-map. **B)** Immune cells were enriched from collagenase digested single cell suspension of whole ileum and colon of 16 week old mice (4 mice/group) by Percoll gradient centrifugation, equal number of cells ($3x10^5$/well in 150 μl medium) from each tissue were cultured for 48 h and the spent media were subjected to SBA assay to detect spontaneously released cytokines. Statistical analyses by two-sided Mann Whitney test. These experiments were repeated at least once using 3 mice/group and similar results were obtained.

Fig. 5: *Impact of castration of SNF1 males on gut microbiota.* Four week old SNF1 males were subjected to orchidectomy or mock-surgery (3 mice/group), fecal pellets were collected from individual mice before the surgery (pre-surgery) and at 20 weeks of age (post-surgery). DNA prepared from these fecal samples were subjected to 16S rRNA gene-targeted sequencing and analysis as described for Fig. 2. **A)** OTU.Biom table data were used for generating principal component analysis (PCA) plots. **B)** Relative abundances of 16S rRNA gene sequences in fecal samples of individual mice collected pre- and post- surgery at phyla level. **C**) Mean relative abundances of sequences compiled to genus level in post-surgery (20 week) samples are shown. Statistical analyses were done employing two-sided Welch's *t*-test and FDR corrected using Benjamini and Hochberg approach.

Fig. 6: Impact of Castration of SNF1 males on lupus autoimmunity. A) Four week old male SNF1 littermates were subjected to orchidectomy or mock-surgery (10 mice/group) and monitored for disease progression as detailed for Fig. 3. **A)** Protein levels in urine samples were quantified by Bradford assay. Percentage of mice with severe nephritis as indicated by high proteinuria. *p*-values by log-rank test. **B)** Severity of nephritis in different groups of mice at different time points. Nephritis severity was scored based on urinary protein levels. *p*-values by 2-sided Chi-Square test. **C)** Serum levels of IgG antibodies against nucleohistone and dsDNA were assessed by ELISA using samples collected at 28 weeks of age. Mean ±SD of OD values of samples from 10 mice/group. *p*-values by two-sided Mann Whitney test. **D)** Cecum microbial preparations from 20 week old indicated groups of mice were given to 6 week old female SNF1 mice (5 recipients or control mice/group) and monitored for disease progression, and percentage of mice with severe nephritis as indicated by high proteinuria is shown. *p*-values by log-rank test. **E)** Severity of nephritis in different groups of mice at 28 weeks of age is shown. *p*-values by 2-sided Chi-Square test. *not statistically significant. Serum levels of IgG antibodies against nucleohistone and dsDNA assessed by ELISA are shown in supplemental Fig. 7.

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Conflict of Interest statement: Authors do not have any conflict(s) of interest to disclose.

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Figure 1

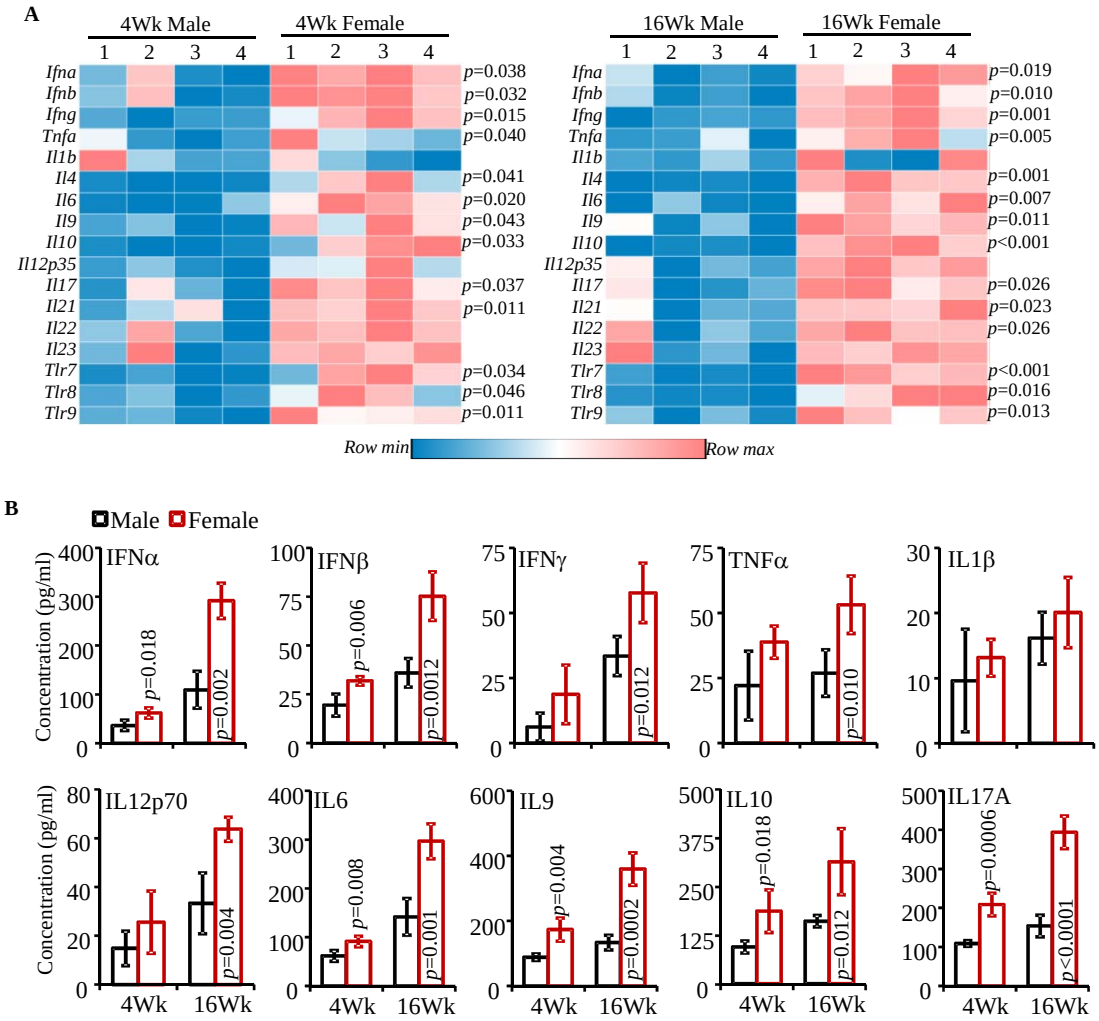

A
4Wk Male
4Wk Female
16Wk Male
16Wk Female
1 2 3 4
Ifna
Ifnb
Ifng
Tnfa
Il1b
Il4
Il6
Il9
Il10
Il12p35
Il17
Il21
Il22
Il23
Tlr7
Tlr8
Tlr9
p=0.038
p=0.032
p=0.015
p=0.040
p=0.041
p=0.020
p=0.043
p=0.033
p=0.037
p=0.011
p=0.034
p=0.046
p=0.011
p=0.019
p=0.010
p=0.001
p=0.005
p=0.001
p=0.007
p=0.011
p<0.001
p=0.026
p=0.023
p=0.026
p<0.001
p=0.016
p=0.013
Row min
Row max
B
Male
Female
Concentration (pg/ml)
IFNα
IFNβ
IFNγ
TNFα
IL1β
IL12p70
IL6
IL9
IL10
IL17A
p=0.018
p=0.002
p=0.006
p=0.0012
p=0.012
p=0.010
p=0.004
p=0.008
p=0.001
p=0.004
p=0.0002
p=0.018
p=0.012
p=0.0006
p<0.0001
4Wk
16Wk

Figure 2

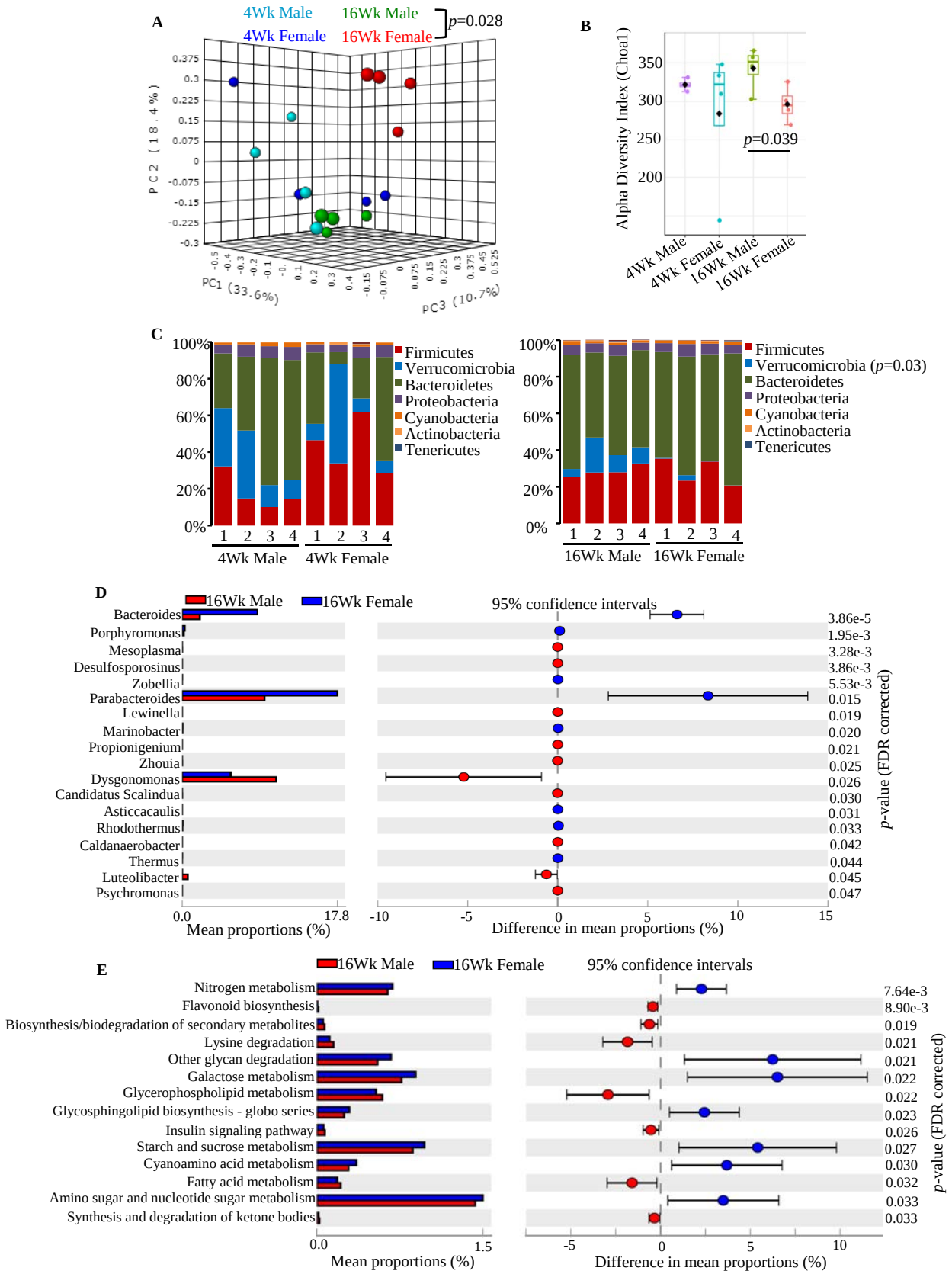

A
4Wk Male
16Wk Male
4Wk Female
16Wk Female
p=0.028
PC2 (18.4%)
PC1 (33.6%)
PC3 (10.7%)
B
Alpha Diversity Index (Chao1)
p=0.039
4Wk Male
4Wk Female
16Wk Male
16Wk Female
C
Firmicutes
Verrucomicrobia
Bacteroidetes
Proteobacteria
Cyanobacteria
Actinobacteria
Tenericutes
Verrucomicrobia (p=0.03)
4Wk Male
4Wk Female
16Wk Male
16Wk Female
D
16Wk Male
16Wk Female
95% confidence intervals
Bacteroides
Porphyromonas
Mesoplasma
Desulfosporosinus
Zobellia
Parabacteroides
Lewinella
Marinobacter
Propionigenium
Zhouia
Dysgonomonas
Candidatus Scalindua
Asticcacaulis
Rhodothermus
Caldanaerobacter
Thermus
Luteolibacter
Psychromonas
p-value (FDR corrected)
Mean proportions (%)
Difference in mean proportions (%)
E
16Wk Male
16Wk Female
95% confidence intervals
Nitrogen metabolism
Flavonoid biosynthesis
Biosynthesis/biodegradation of secondary metabolites
Lysine degradation
Other glycan degradation
Galactose metabolism
Glycerophospholipid metabolism
Glycosphingolipid biosynthesis - globo series
Insulin signaling pathway
Starch and sucrose metabolism
Cyanoamino acid metabolism
Fatty acid metabolism
Amino sugar and nucleotide sugar metabolism
Synthesis and degradation of ketone bodies
p-value (FDR corrected)
Mean proportions (%)
Difference in mean proportions (%)

Figure 3

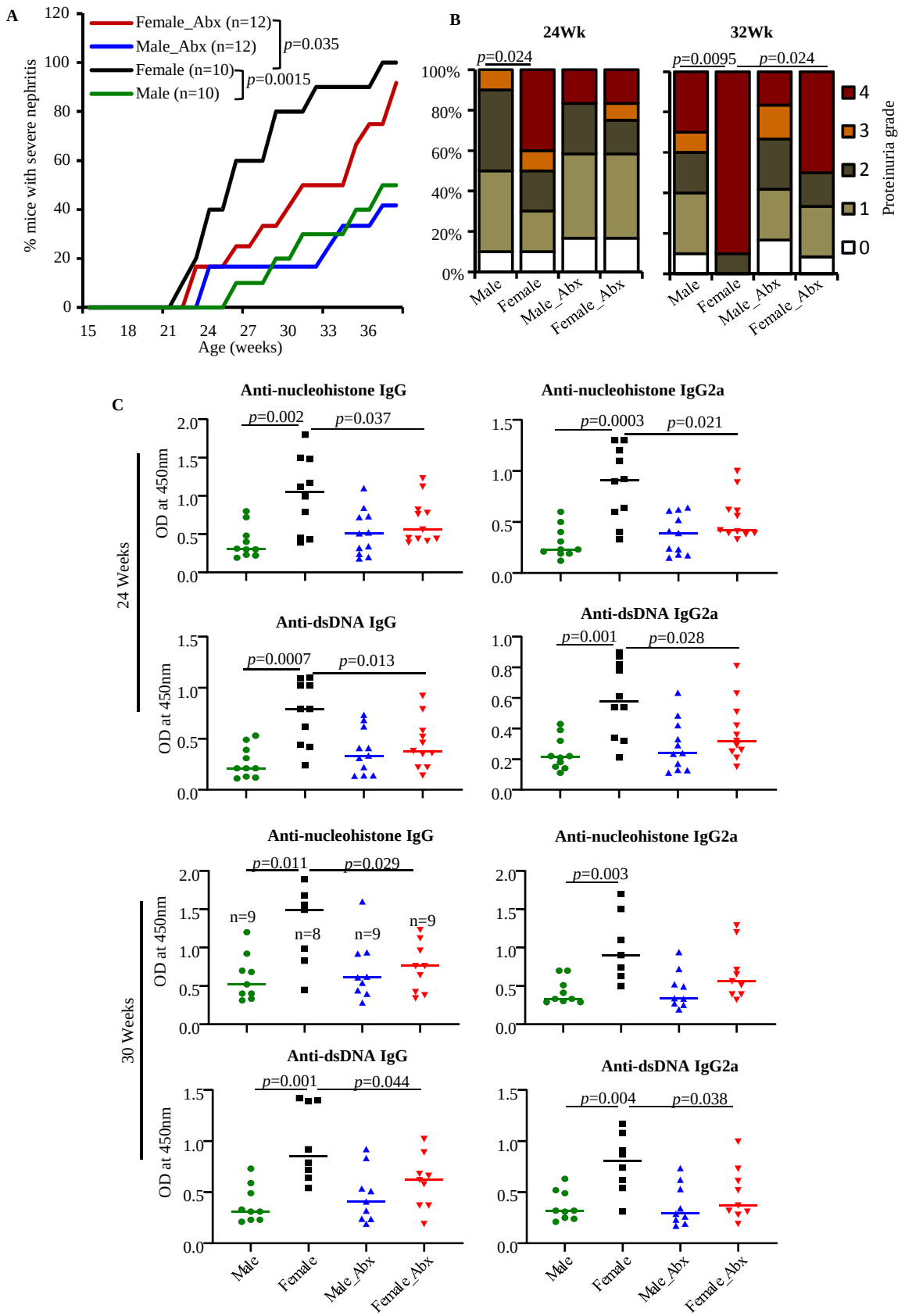

A
Female_Abx (n=12)
Male_Abx (n=12)
Female (n=10)
Male (n=10)
p=0.035
p=0.0015
% mice with severe nephritis
Age (weeks)
B
24Wk
32Wk
p=0.024
p=0.0095
p=0.024
Proteinuria grade
Male
Female
Male_Abx
Female_Abx
C
24 Weeks
30 Weeks
Anti-nucleohistone IgG
Anti-nucleohistone IgG2a
Anti-dsDNA IgG
Anti-dsDNA IgG2a
OD at 450nm
p=0.002
p=0.037
p=0.0003
p=0.021
p=0.0007
p=0.013
p=0.001
p=0.028
p=0.011
p=0.029
p=0.003
p=0.001
p=0.044
p=0.004
p=0.038
n=9
n=8
n=9
n=9

Figure 4

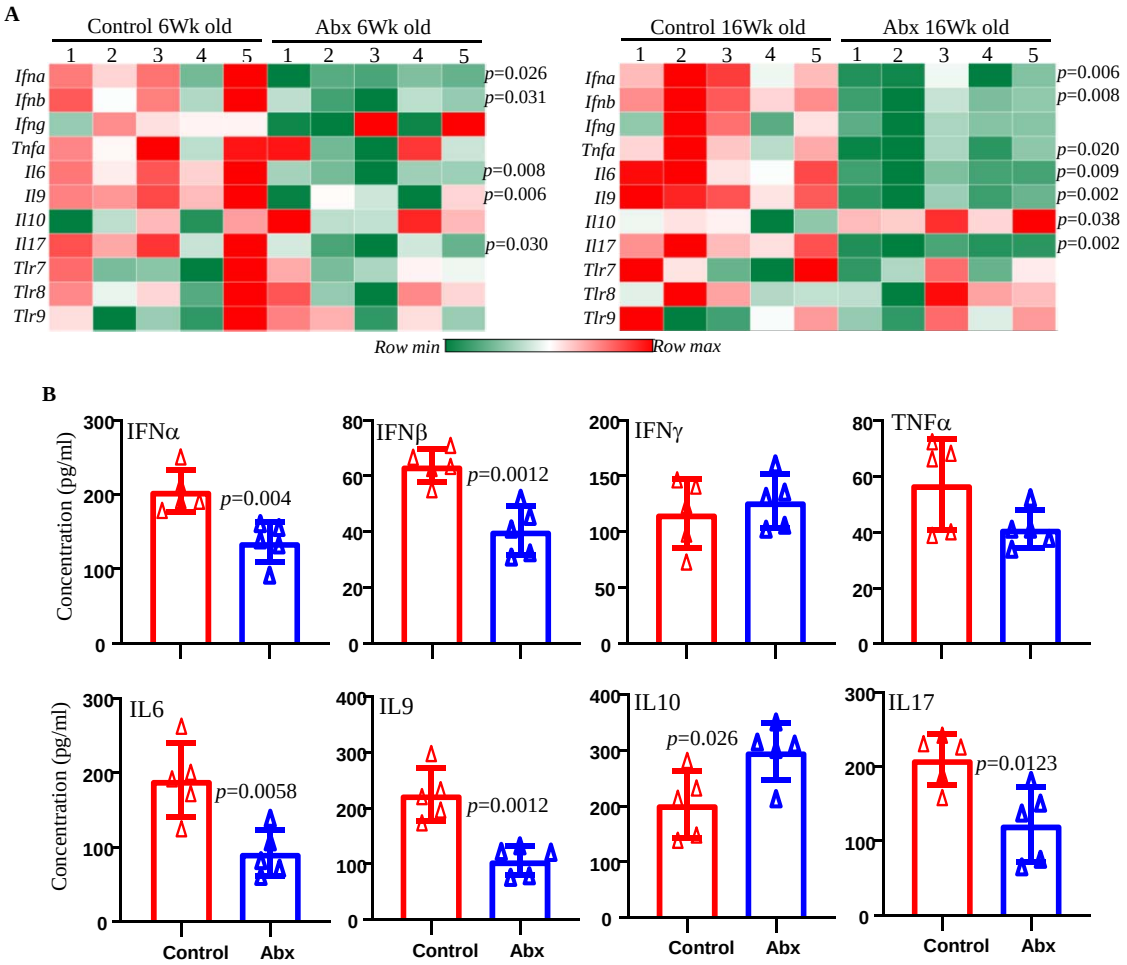

A
Control 6Wk old
Abx 6Wk old
Control 16Wk old
Abx 16Wk old
1 2 3 4 5
Ifna
Ifnb
Ifng
Tnfa
Il6
Il9
Il10
Il17
Tlr7
Tlr8
Tlr9
p=0.026
p=0.031
p=0.008
p=0.006
p=0.030
p=0.006
p=0.008
p=0.020
p=0.009
p=0.002
p=0.038
p=0.002
Row min
Row max
B
Concentration (pg/ml)
IFNα
p=0.004
IFNβ
p=0.0012
IFNγ
TNFα
IL6
p=0.0058
IL9
p=0.0012
IL10
p=0.026
IL17
p=0.0123
Control
Abx

Figure 5

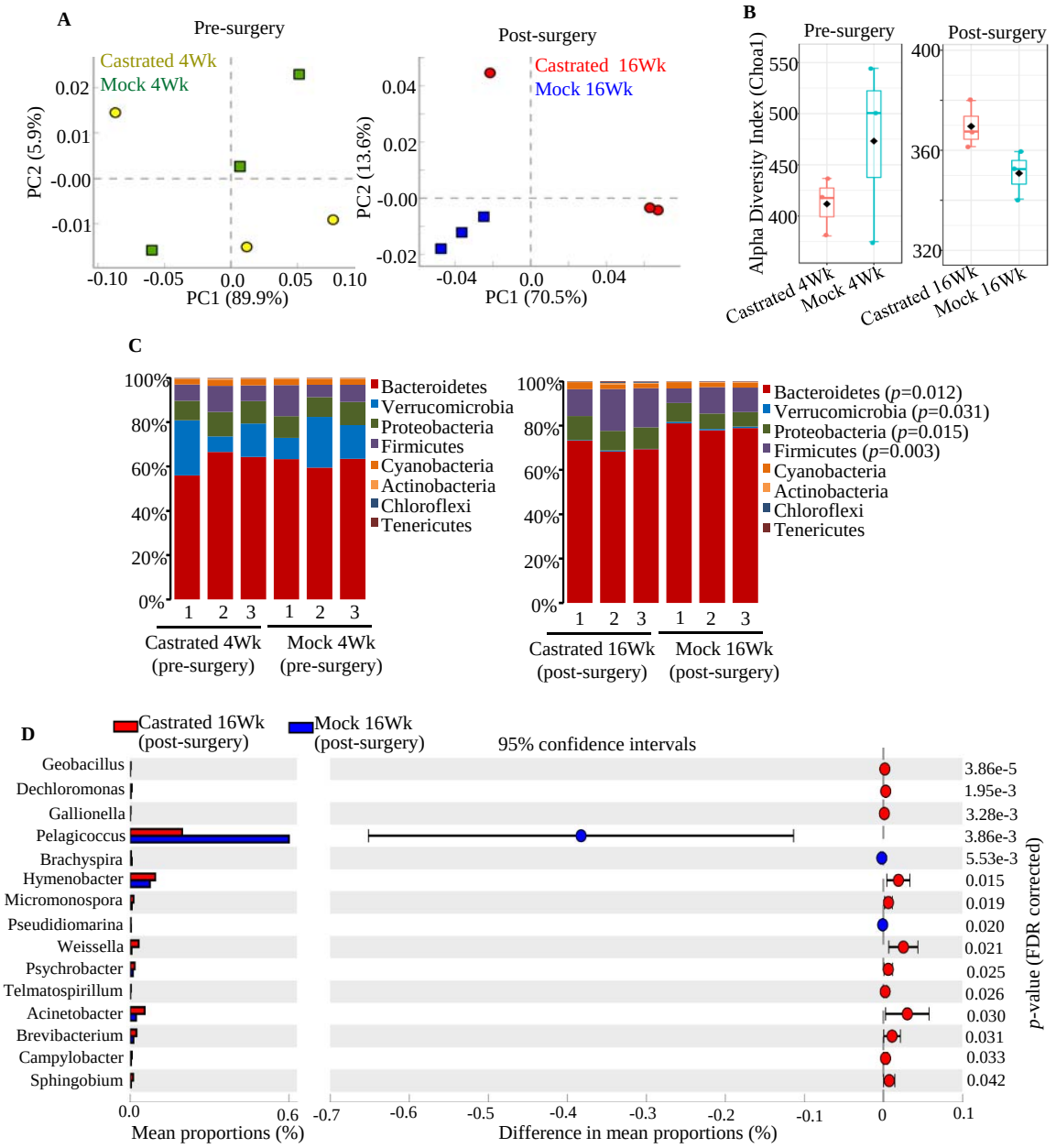
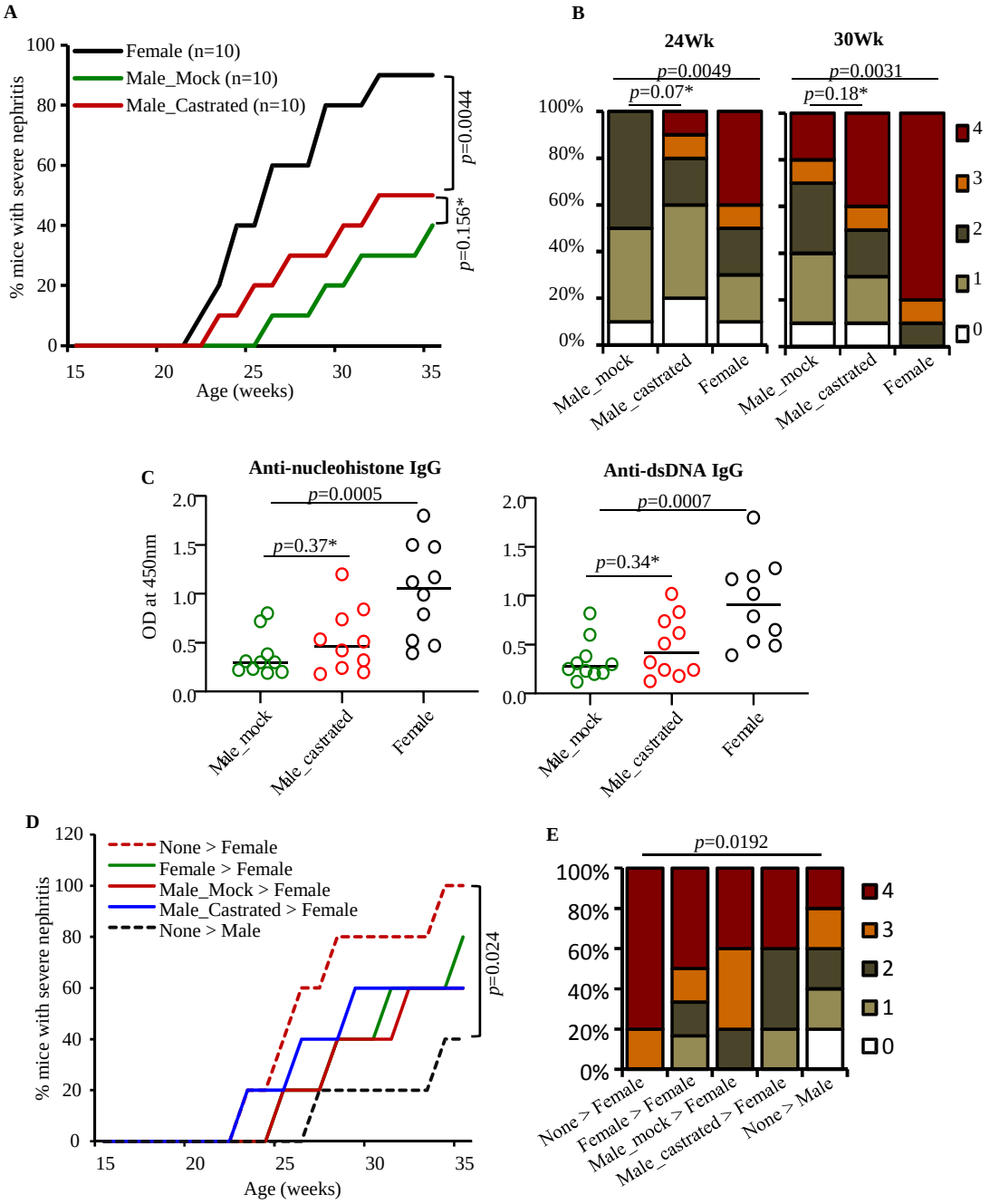

A
Pre-surgery
Castrated 4Wk
Mock 4Wk
PC2 (5.9%)
PC1 (89.9%)
Post-surgery
Castrated 16Wk
Mock 16Wk
PC2 (13.6%)
PC1 (70.5%)
B
Pre-surgery
Post-surgery
Alpha Diversity Index (Chao1)
Castrated 4Wk
Mock 4Wk
Castrated 16Wk
Mock 16Wk
C
Bacteroidetes
Verrucomicrobia
Proteobacteria
Firmicutes
Cyanobacteria
Actinobacteria
Chloroflexi
Tenericutes
Castrated 4Wk (pre-surgery)
Mock 4Wk (pre-surgery)
Bacteroidetes (p=0.012)
Verrucomicrobia (p=0.031)
Proteobacteria (p=0.015)
Firmicutes (p=0.003)
Castrated 16Wk (post-surgery)
Mock 16Wk (post-surgery)
D
Castrated 16Wk (post-surgery)
Mock 16Wk (post-surgery)
95% confidence intervals
Geobacillus
Dechloromonas
Gallionella
Pelagicoccus
Brachyspira
Hymenobacter
Micromonospora
Pseudidiomarina
Weissella
Psychrobacter
Telmatospirillum
Acinetobacter
Brevibacterium
Campylobacter
Sphingobium
3.86e-5
1.95e-3
3.28e-3
3.86e-3
5.53e-3
0.015
0.019
0.020
0.021
0.025
0.026
0.030
0.031
0.033
0.042
p-value (FDR corrected)
Mean proportions (%)
Difference in mean proportions (%)

Figure 6


A
Female (n=10)
Male_Mock (n=10)
Male_Castrated (n=10)
% mice with severe nephritis
Age (weeks)
p=0.0044
p=0.156*
B
24Wk
30Wk
p=0.0049
p=0.0031
p=0.07*
p=0.18*
Male_mock
Male_castrated
Female
C
Anti-nucleohistone IgG
Anti-dsDNA IgG
OD at 450nm
p=0.0005
p=0.37*
p=0.0007
p=0.34*
D
None > Female
Female > Female
Male_Mock > Female
Male_Castrated > Female
None > Male
p=0.024
E
p=0.0192
None > Female
Female > Female
Male_mock > Female
Male_castrated > Female
None > Male