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Sex-based differences in microbiota composition and metabolomic profiles associated with altered macrophage function and protection from lupus in BWF1 mice.

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SEX-BASED DIFFERENCES IN MICROBIOTA COMPOSITION AND METABOLOMIC PROFILES ASSOCIATED WITH ALTERED MACROPHAGE FUNCTION AND PROTECTION FROM LUPUS IN BWF1 MICE

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DEDICATION

This Dissertation is dedicated to my parents

Drs. Tim and Julie Harder

and to all the friends and family

who've helped me along the way

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ABSTRACT

SEX-BASED DIFFERENCES IN MICROBIOTA COMPOSITION AND METABOLOMIC PROFILES ASSOCIATED WITH ALTERED MACROPHAGE FUNCTION AND PROTECTION FROM LUPUS IN BWF1 MICE

James W. Harder

January 14, 2021

Systemic lupus erythematosus (SLE) is a complex and an insidious disease that is still not completely understood, and has very few treatment options. Those that are available are ineffective and/or have serious side effects. Both genetic and environmental factors contribute to the susceptibility and resistance to SLE, and understanding the environmental factors underlying this disease could lead to more effective prevention and/or treatment strategies. Like most autoimmune diseases, SLE is much more prevalent in females than males. While there are numerous factors that contribute to this lupus susceptibility, there is increasing evidence that the microbiota can strongly influence lupus progression, and that sex-based differences in microbiota composition and function may play a role in the sex bias (i.e., susceptibility in females and resistance in males) of disease. The NZBxNZW F1 (BWF1) mouse model of lupus has many of the same disease features seen in humans including the sex bias, making it an ideal model for studying sex-based differences in microbiota and how they affect lupus progression.

Previous findings from our laboratory have shown that not only do female and male BWF1 microbiota profiles differ significantly, but male BWF1 microbiota can suppress lupus when cecal contents are transferred into female BWF1 recipients. The overall goal of this dissertation was to identify the players involved and begin to understand the potential mechanisms underlying the suppressive effect of the male microbiota on disease. Three aims were designed to address these issues. The goal of aim 1 was to identify the bacterial populations in the microbiota of female and male BWF1 mice that may either cause or suppress disease. The goal of aim 2 was to analyze the function of female and male microbiota by identifying metabolites that may be involved in the suppression of disease. In aims 1 and 2, we also investigated the impact of androgens on bacterial populations and metabolite profiles, respectively, by comparing intact and castrated male mice. In aim 3, the goal was to investigate the potential immune mechanisms that could underlie the suppression of disease mediated by male microbiota.

The results of the first aim indicate that changes in the abundances of *Bacteroides*, *Clostridium*, and *Alistipes* strongly correlated with the ability of transferred male BWF1 microbiota to suppress disease in female BWF1 recipients. We took advantage of a change in animal facility that had a significant impact on our mouse colony and its microbiota populations, and gave us an opportunity to analyze and compare bacterial populations during periods of time when microbiota transfers had varying results. Specifically, we found that *Bacteroides* (primarily the *Bacteroides acidifaciens* species) abundance was high, *Clostridium* (primarily the *Clostridium leptum* species) abundance was low, and *Alistipes* was present during the periods when transfer of male microbiota was effective at suppressing disease in female BWF1 mice. Conversely, *Bacteroides*

abundance was low, *Clostridium* abundance was high, and *Alistipes* was absent when male microbiota lost that capability. We concluded from our thorough microbiota analyses that a high *Bacteroides*/*Clostridium* ratio in the male microbiota may be a reliable predictor of disease-suppressing capability, since it correlated strongly with disease suppression in female recipients of male microbiota. The second aim of the project used analysis of metabolomic profiles to investigate functional differences in female and male BWF1 microbiota. Differential production of immunomodulatory metabolites is a major mechanism by which the gut microbiota influences the immune system. By measuring the fecal metabolite profiles, we identified phytol as a potential mediator of lupus suppression by male microbiota. Phytol is produced by the microbiota and converted into phytanic acid by host enzymes. Both phytol and phytanic acid were significantly more abundant in intact male than either female or castrated male BWF1 mice. Both phytol and phytanic acid have potent RXR and PPARγ agonist properties, all of which can directly influence many different immune functions. The third aim of this project investigated the differences in female and male macrophage efferocytosis (i.e., phagocytosis of apoptotic cells) efficacy and how phytol and/or phytanic acid could affect this immune function. Deficiencies in efferocytosis, particularly by macrophages, are a major risk factor for SLE because they result in the accumulation of debris that stimulates autoantibody production. We found male BWF1 splenic macrophages were more efficient at efferocytosis than female splenic macrophages, and treatment with phytanic acid *in vitro* or *in vivo* could enhance female splenic macrophage efferocytosis.

Overall, we found that higher *Bacteroides* and lower *Clostridium* abundances correlated with lupus suppression in female BWF1 recipients of male microbiota, and

speculate that this protection could be due, at least in part, to higher levels of phytol and phytanic acid production in males. Furthermore, phytol and phytanic acid produced by males may suppress disease, again at least in part, via enhancement of macrophage efferocytosis. Taken together, these data may provide the basis for a mechanistic understanding of the impact that the microbiota can have on autoimmune diseases such as lupus, and for the development of novel therapies.

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INTRODUCTION

Sex bias in systemic lupus erythematosus (SLE)

The core characteristic of mammalian immune systems is the ability to differentiate between self and non-self-antigens. This self-tolerance is mediated by both central and peripheral mechanisms. Central mechanisms involve removal of autoreactive T and B cells before they fully mature, while peripheral mechanisms involve inducing tolerance to self-antigens to suppress immune responses against non-foreign cells and tissues. When these mechanisms fail and self-tolerance is lost, the result is autoimmunity.

Most autoimmune diseases have a significant sex bias. Women comprise over 80% of autoimmune disease patients in Western countries. For the multi-organ autoimmune disease, systemic lupus erythematosus, also called SLE or lupus, this sex difference is even more pronounced as about 9 in every 10 people affected by SLE are women [1,2]. SLE is characterized by the loss of tolerance to nuclear self-antigens such as double-stranded DNA (dsDNA), single-stranded DNA (ssDNA, dsRNA), single-stranded RNA (ssRNA), histones, small nuclear ribonucleoproteins (snRNPs), and ribosomes. This results in the production of anti-nuclear antibodies, which bind nuclear antigens and form antigen-antibody complexes that are deposited in the joints, vasculature, skin lungs, kidneys, and other organs. These immune complex deposits cause inflammation and immune cell infiltration. Over time this leads to increasing levels of tissue damage that can result in organ failure if untreated. This can manifest as lupus

nephritis, vasculitis, pulmonary hypertension, or stroke, and over 50% of lupus patients develop one or more of these complications [3]. These complications are even more common in male SLE patients, who tend to have more severe and rapid disease progression despite being less likely to develop the disease [4-8]. However, despite lupus nephritis being more common in male SLE patients, the incidence of progression to end-stage renal disease is not different between male and female SLE patients [5,9-13]. Musculoskeletal involvement, on the other hand, seems to be more common in women [14].

There are many mouse models of lupus, and many of them share the same sex bias as the human disease. The models can be divided into two major categories: spontaneous and induced [15]. The most well-established spontaneous mouse model of lupus is the NZBxNZW F1 (BWF1) model. Female BWF1 mice spontaneously develop anti-dsDNA autoantibodies, lymphadenopathy, splenomegaly, and glomerulonephritis in a manner that resembles the progression of disease in humans. Male BWF1 mice rarely develop the disease, and the disease is much milder in them if they do [16]. Androgen depletion of males by castration results in severe disease at a much younger age, indicating that androgens are as critical for the sex bias in BWF1 mice as they are in humans [17]. Backcrossing the BWF1 strain with the NZW strain has given rise to two additional strains that are used as lupus models, NZM2410 and NZM2328 [15]. NZM2410 mice develop lupus-like disease similar to BWF1, however, the pronounced sex bias seen in BWF1 mice is not present in the NZM2410 strain [18,19]. NZM2328, on the other hand, does display a sex bias closer BWF1 mice, with females developing a

more severe form of lupus-like disease at an earlier age, though the penetrance of disease is lower in female NZM2328 mice than in female BWF1 mice [15,20].

The SWRxNZB F1 (SNF1) mouse model of lupus shares the NZB parental strain with the BWF1 model. It spontaneously develops lupus-like disease in a manner very similar to BWF1, producing anti-nuclear autoantibodies that lead to glomerulonephritis [21-23]. Also, like the BWF1 model, the SNF1 has a distinct sex bias. Female SNF1 mice develop disease more often, more severely, and more quickly than male SNF1 mice [21,24,25]. Although interestingly, unlike the BWF1 model where castration causes severe disease and mortality in male mice, a recent study of SNF1 mice found that castration of male mice caused only a slight increase in disease that was not a statistically significant change [26].

Another mouse model of lupus is the MRL/*lpr* model, which lacks a functional *Fas* gene. This results in the accumulation of double negative $CD4^{-}CD8^{-}$ T cells and levels of lymphadenopathy that is not seen in human SLE patients. However, it does develop autoantibodies and glomerulonephritis at a younger age than other lupus mouse models. This occurs in both male and female mice, with no sex bias in incidence [27], though female MRL/*lpr* mice do develop severe nephritis and organ failure a few weeks earlier than male MRL/*lpr* mice [28]. This contrasts with another mouse model, the BXSB/*Yaa* (BXSB) model. The X chromosomes of this strain have a translocated Y chromosome genetic locus that includes the gene for TLR7. Male BXSB mice thus have 2 copies of the locus and consequently develop lymphadenopathy, anti-nuclear autoantibodies, and glomerulonephritis. Female BXSB mice do not develop disease [29].

There are also mouse models of lupus that require specific treatment to develop disease. Pristane-induced lupus models of mice use intraperitoneal injections of the pristane, a naturally occurring hydrocarbon oil that induces chronic inflammation in the peritoneal cavity. This leads to the production of anti-nuclear autoantibodies and glomerulonephritis [30]. The exact responses depend on the strain of mice, and in some strains, such as the SJL strain, the induced disease is more severe in female mice [31,32]

Another class of induced-lupus models uses chronic graft-versus-host disease (cGVHD) to mimic lupus. These models rely on transferring donor lymphocytes into MHC mismatched recipients. Disease is stimulated by the alloreactive donor $CD4^+$ T cells reacting with the MHC class II expressing host B cells resulting in polyclonal activation and the development of autoantibody-producing B cells [15,33]. There are several variations of the cGVHD model based on what strains are used for donors and recipients. Two of the most common are the DBA/2 donor into (C57BL/6 X DBA/2) F1 recipient model (DBA->BDF1) and the C57BL/6.bm12 donor into C57BL/6 recipient model (bm12->B6). While both models result in lupus-like disease, with anti-nuclear autoantibody production and glomerulonephritis, the DBA->BDF1 model exhibits a sex bias where female to female transfer recipients develop more severe renal disease than male to male transfer recipients, but the bm12->B6 model has no sex bias [33-35]. This is due to the fact that the bm12->B6 model only has an MHC II mismatch while the DBA->BDF1 has an MHC I and MHC II mismatch. While the MHC II mismatch drives disease as described above, the MHC I mismatch results in a graft vs host reaction where the donor $CD8^+$ cytotoxic lymphocytes (CTL) recognize the host autoantibody-producing B cells as foreign and eliminate them. This graft vs host $CD8^+$ T cell CTL response is

much stronger when the donor cells are from male mice. This stronger $CD8^+$ T cell CTL has two significant outcomes. First, the reduction of numbers of host B cells results in lower autoantibody production and lower disease. Second, the strong response of the male donor $CD8^+$ T cells results in subsequent contraction of the male donor $CD8^+$ T cell population and lower engraftment of male donor $CD8^+$ T cells. After the initial CTL response is over and the donor $CD8^+$ T cells engraft, they promote disease via an IFN-γ production. Thus, the lower engraftment of $CD8^+$ T cells in male mice leads to lower IFN-γ production and contributes to the milder disease seen in males. Conversely, the lower initial $CD8^+$ T cell CTL responses and subsequent higher engraftment and IFN-γ production in female results in a more severe disease [33,35].

Deficient CD8 CTL activity is also present in other mouse models, including BWF1 mice [36-38], MRL/lpr mice [39,40], and BXSB-Yaa mice [41,42]. Humans prone to SLE also exhibit dysfunctional CD8 T cells and lower cytolytic activity [43-49]. However, how this connects to the sex bias in humans is unclear. While studies have found males have higher CD8 T cell frequencies, some studies have also found PBMCs from women to have higher levels of CD8 cytotoxic activity [50-55]. These seemingly contradictory results may be due to differences in estrogen levels in the women who participated in the studies. Estrogen levels rise and fall during the menstrual cycle, and estrogen has repeatedly been shown to suppress $CD8^+$ T cell CTL activity [56-58]. Further complicating comparisons between male and female patients is the fact that sex differences in levels of CD8 cells change with age [59]. This is interesting, as hormone levels also change with age, and the changes in these levels have been shown to have a significant impact on the incidence and progression of SLE. [60]. Together, these suggest

the possibility that age-related differences in hormone levels could cause age-related differences in $CD8^+$ T cell activity. Since deficiencies in $CD8^+$ T cells have been linked to SLE [43-49], it suggests that the differences in SLE susceptibility at different ages may be mediated by age-dependent hormonal suppression of $CD8^+$ T-cells.

Influence of genetics on the sex bias of SLE

The wide variety of mouse models have benefitted lupus research because SLE is an extremely heterogeneous disease influenced by many genetic, hormonal, and environmental factors. Genome-wide association studies have identified many genetic polymorphisms in genes like ITGAM, CD40, and IRF5 that are linked to susceptibility to SLE [61-67]. However, twin studies have found concordance to be 24-35% in monozygotic twins and 2-4% in dizygotic twins [60,68,69], showing that while SLE has a strong genetic component, genetics alone is insufficient to account for disease development. Similarly, the mechanisms behind the sex bias of SLE are not completely understood, but differences in both sex-linked genes and sex hormones are believed to play major roles.

The mammalian X chromosome contains many genes important for normal immune function [2,70]. Males, who carry only one copy of each X-linked gene, are much more susceptible to genetic immunodeficiencies [71]. In addition, the female's heterozygosity for the X-linked genes allows for a more robust and diverse immune system [72]. Following viral exposure, peripheral mononuclear cells from women produce greater levels of pro-inflammatory factors such as interferon-γ (IFNγ), interferon- and tumor necrosis factor α (TNFα) [59,73]. In healthy controls, macrophages

and neutrophils from female rats and mice have higher phagocytic activity than those from male rats. [74,75]. Studies have found that circulating T-cells from women have a more robust proliferative response after stimulation and higher cytotoxic T-cell activity [76]. Parasitic infections provoke higher levels of T helper 1 (Th1) cytokine production in adult female mice, which may explain their greater resistance to parasites like *Leishmania major* [77]. Females also exhibit higher levels of basal immunoglobulin levels and larger antibody responses [2,78-80]. All of this likely contributes to women's superior survival rates and outcomes after injury due to reduced susceptibility to sepsis and infections [81,82].

However, this hyperreactive immune system does come with a downside, as increased copies of the X chromosome also result in higher incidence of autoimmune diseases in general, and lupus in particular. Men with Klinefelter's syndrome (XXY) have 14-fold higher rates of SLE than XY males [83], and females with XXX karyotypes are 2.5 times more likely to develop the disease than XX females [84]. Conversely, females with Turner syndrome (XO) have a lower prevalence of SLE than XX females [85].

Women and men with Klinefelter's syndrome have a compensatory mechanism to equalize the gene dosage and limit overexpression of X-linked genes. One of the X chromosomes is randomly silenced during female embryogenesis [2]. This process, called X chromosome inactivation, was at first believed to be largely complete and stable, with only a small number of genes not fully suppressed. However, studies since then have shown that 20-30% of X-linked genes are incompletely inactivated and that the level of inactivation varies widely between tissue and cell types [86-89]. This discovery has

opened a whole new avenue for understanding the differences in lupus between males and females.

To investigate the link between incomplete X inactivation and overexpression of lupus-promoting genes, multiple studies have examined T cells from female lupus patients. Studies have found considerable overlap between genes that are incompletely silenced and genes that are upregulated T cells from lupus patients, including CD40L, CXCR3, OGT, miR-98, let-7f-2, miR-188-3p, miR-421 and miR-503 [90-92]. Since XY males, having only one X-chromosome, are not susceptible to increased lupus-promoting X-linked gene expression due to incomplete X-inactivation, incomplete silencing of X-linked genes may contribute to the sex bias of lupus. This phenomenon is not limited to T cells, as a recent study found that in plasmacytoid dendritic cells (pDCs), B cells, and monocytes from healthy women and men with Klinefelter syndrome (XXY), both copies of the X-linked gene encoding Toll-like receptor 7 (TLR7) gene were transcribed resulting in higher expression than in cells from XY men. TLR7 is important for detecting viral RNA and promoting IFNα production, which drives antibody responses to viral infections [93]. However, TLR7 signaling can also be activated by RNA-containing immune complexes associated with advanced SLE [94]. Numerous studies of mouse models of lupus have identified TLR7 as essential for the development of spontaneous germinal centers and the production of autoantibodies [95-97]. This effect of TLR7 has been found to be gene-dosage dependent, with increased TLR expression promoting the development of lupus-like disease [98-100]. Incomplete inactivation of TLR7 has clear implications for the development of SLE. However, a recent study of female BWF1 mice found that TLR7 did not escape X inactivation in B cells and that, while the B cells did

express higher levels of autoimmunity-associated genes, none of them were on the X chromosome. This finding suggests that incomplete X inactivation may not be a major cause of the sex bias in the BWF1 mouse model [101]

Contribution of sex hormones in the sex bias of SLE

The other major mechanism by which sex is thought to affect SLE is via the production of sex hormones. The connection between sex hormones and immune responses is long established. Estrogens generally promote immune responses, while androgens typically suppress them [58,102,103]. There is ample evidence that higher levels of estrogens increase lupus severity.

While estrogens are known to promote immune activation in general, at higher doses they can suppress certain immune responses [102,104,105]. However, importantly for SLE, both low and high concentrations of estrogen stimulate B cells to increase production of immunoglobulins in mice and humans [58,104,106,107]. Consequently, while high estrogen levels can suppress some aspects of disease that are more dependent on T cells, its overall effect is to increase SLE severity and progression [58,108]. Even in mice not prone to autoimmunity, high doses of estrogen increased the numbers of antibody producing B cells, including cells that produced autoantibodies [109]. Estrogen promotes B cell survival and activation, leading to the increased numbers of autoreactive B cells escaping negative selection [106,110]. Estrogen can also inhibit B cell tolerance and induce lupus-like phenotypes in mice [111]. Knocking out the estrogen receptor gene in B cells has an impact on autoantibody production in BWF1 mice [112,113]. Additionally, treating peripheral blood mononuclear cells taken from SLE patients with

estrogen enhanced their production of autoantibodies [114]. Female mice with graft-versus-host induced glomerulonephritis had higher levels of autoantibodies against dsDNA and other nuclear antigens after treatment with 17-β-estradiol (E2), a form of estrogen [71,115].

Estrogen's effects are not limited to B cells. E2 causes dendritic cells (DCs) to upregulate CD40, a costimulatory molecule that is critical for the induction of humoral immunity [116]. A recent study found that proteinuria decreased in BWF1 lupus-prone mice after treatment with a CD40 antagonist antibody [117]. There are several CD40-blocking therapies undergoing clinical trials for SLE and other autoimmune diseases [118]. Another type of dendritic cell, plasmacytoid DCs (pDCs), is a key producer of interferon-α (IFNα) which activates B cells and drives autoantibody production. Lupus immune complexes can activate pDCs via TLR7 and TLR9 and stimulate them to produce IFNα. Studies have found that treating human or mouse pDCs with E2 increases their IFNα production in response to TLR7 and TLR9 signaling [119,120]. Additionally, there is a positive regulatory feedback loop between interferon and estrogen signaling [121].

Androgens, especially testosterone, are strongly immunosuppressive and anti-inflammatory [103]. Men who have androgen deficiencies have higher serum antibody titers, CD4/CD8 T cell ratios, and higher levels of inflammatory cytokines including IL-1β, IL-2, and TNFα [122-125]. Consequently, androgen levels have a significant effect on autoimmune disease. Castration has been found to stimulate or exacerbate autoimmune symptoms in several mouse models of autoimmune diseases, including Experimental Autoimmune Encephalomyelitis (EAE), non-obese diabetic (NOD), and

rheumatoid arthritis [126-128]. In those same mouse models, treatment with androgens ameliorated disease symptoms [103,128-130]. In male rheumatoid arthritis patients, lower levels of testosterone have been found to correlate with disease severity. Also, testosterone supplementation reduces disease severity in postmenopausal men and women with rheumatoid arthritis [131].

Studies with mouse models have repeatedly found that androgens play a protective role in SLE. In a graft-versus-host induced glomerulonephritis mouse model, testosterone treatments decreased proteinuria [132]. In the BWF1 mouse model of lupus, treating disease-prone females with androgens delays disease onset and reduces serum anti-dsDNA titers and other markers of disease [133]. When BWF1 mice were treated with the androgen receptor-blocking drug flutamide, it accelerated mortality in both male and female mice [134]. Similarly, male BWF1 mice that undergo androgen depletion by castration develop more severe disease at an earlier age. Some studies have shown that these male BWF1 mice have a testosterone-dependent splenic $Gr1^{+}CD11b^{+}F4/80^{-}$ neutrophil population that inhibits autoantibody-producing germinal center reactions [135,136]. However, while depleting $Gr1^{+}$ neutrophils in male BWF1 mice increased autoantibody levels, it did not have any impact on kidney disease progression [135]. This finding and the findings of a more recent study that showed neutrophil depletion accelerated both autoantibody production and kidney damage in female BWF1 mice, cast doubt on the $Gr1^{+}$ neutrophils as a significant source of male BWF1 disease resistance [137].

Androgens are protective in humans as well as in mice. Women with active SLE had decreased levels of androgens, including testosterone, androstenedione, and

dehydroepiandrosterone [115,138]. Interestingly, the same has been found for female SLE patients without active disease, suggesting that the low levels are not caused by active disease activity [139,140]. Androgen levels are also lower in male SLE patients [141-144], and a subset of them exhibit hypogonadism [143,145]. Both males and females with SLE have been found to exhibit excessive estradiol hydroxylation, which depletes testosterone and produces estrogens [144]. Similarly, men with Klinefelter syndrome, who naturally have lower androgen levels and higher estrogen levels than XY males, have a higher risk for SLE [146,147]. Since disease in men and women correlates with higher estrogens, female SLE patients were treated with tamoxifen, a drug which competes with estrogens for the estrogen receptor, but the treatment failed to alleviate disease severity [148]. On the other hand, multiple studies have found that treatment with androgens can ameliorate disease activity and lower anti-dsDNA antibody production [149-152]. Clearly, androgens can have a major impact on lupus progression.

Efficacy of current androgen-based treatments and other therapeutics

While androgens are known to inhibit lupus progression, translating these successes in the laboratory into effective clinical treatments has been challenging. Treatment with the testosterone-like anabolic steroid, nandrolone resulted in some patients improving but others saw no benefits [153-155]. Similarly, dehydroepiandrosterone, a weak androgenic steroid, has been shown to have mild benefits in premenopausal patients with non-severe disease, but it has been less beneficial in postmenopausal patients [149,156-162]. While these androgens can alleviate some symptoms of disease, the lack of consistency in benefits and the masculinizing side

effects make these androgens non-viable as frontline treatments [163]. Nevertheless, while directly treating with weak androgens has had little success, understanding androgen's mechanisms of action for immunosuppression may lead to the discovery of targets for treating SLE [164].

In the past few years, therapies that deplete specific cell lineages or block certain cell-cell interactions have been developed. One such therapeutic, Anifrolumab, blocks the IFN-α cell signaling that can drive lupus progression. It has been found to benefit some patients if used early in the course of the disease before active renal involvement, but over half the patients tested did not respond to the treatment [165,166]. Another, Rituximab, is an anti-CD20 antibody that depleted mature B cells, but it was not found to be more effective than standard treatment [167,168]. Belimumab is an antibody that binds the cytokine B-cell activating factor (BAFF) and prevents it from binding to B cells and promoting their maturation. It has been found to be beneficial for patients with severe disease that is not responding to standard treatments, but not for patients with mild to moderate disease [169-171]. Atacicept, a recombinant protein that binds two B-cell maturation-promoting cytokines, B-lymphocyte stimulator (BLyS) and A proliferation-inducing ligand (APRIL), has recently been developed and clinical trials testing its ability to treat SLE are underway. Initial results are promising, as it has been shown to decrease lupus flares [172]. However, atacicept was only effective in a subset of lupus patients, as 40% of patients treated showed no benefit [173,174].

SLE has proved a challenging disease to treat. Between the 1950s and 1990s, the 10-year survival rate for adults increased dramatically from 50% up to 90% [175-177]. In recent decades, though, progress has plateaued. Both survival rates [177,178] and the risk

of end-stage renal disease resulting from lupus nephritis [179] are little different now than they were in the 1990s. The current standard treatments are limited by high rates of failure and toxicity. Corticosteroid therapy, a current first-line treatment, may actually contribute to kidney damage over the long term [178]. Clearly, recent advances in treatment have not succeeded in improving the condition of SLE patients, and there is a dire need for new paradigms of SLE management.

Autoimmunity and the gut microbiota

In recent years, a new paradigm has emerged as we have learned more about the interactions between the microbiota and the immune system. The immune system is designed to detect and respond to foreign entities. The presence of a few thousand bacteria in the blood or peripheral tissues would result in recognition by innate immune cells followed by a strongly inflammatory immune response that would drive a subsequent adaptive immune response [180]. In dramatic contrast, the trillions of bacteria normally present in a healthy human intestine rarely trigger spontaneous pathological inflammatory immune responses or systemic immune responses [181-183]. These commensal bacteria are collectively termed the gut microbiota, and a complex system of interactions with the host immune system is needed to maintain a state of mutualism and homeostasis [181-184]. The past two decades have seen a dramatic increase in our understanding of the importance of the microbiota in health and disease, including how interactions between the host immune system and the gut microbiome are essential for proper immune system development and function [184-187].

Because colonization by commensal organisms begins after birth [188], it is possible to use cesarean sections in a germ-free environment to derive mice that lack any commensal bacteria [189,190]. Experiments comparing these germ-free (GF) mice to genetically identical mice born with a microbiota ("conventional" mice) have greatly enhanced our understanding of how the microbiome affects the host [186]. These studies with germ-free animals have long shown that the absence of gut microbiota leads to serious defects in the development of the spleen, thymus, lymph nodes, and other lymphoid tissue [191,192]. Structures of gut-associated lymphoid tissue (GALTs) are most affected. GF mice have lower frequencies of isolated lymphoid follicles as well as smaller Peyer's patches and mesenteric lymph nodes (mLN) [193-195]. In addition to deficient lymphoid tissue structures, the lack of microbiota in GF mice also causes significant and detrimental changes to immune cell populations. The numbers of αβ and γδ intra-epithelial lymphocytes are considerably lower in GF mice than conventionally colonized animals [196]. The balance between Th1 and Th2 T cells is also affected by the lack of a microbiota [197]. The small intestine lamina propria normally contains a considerable number of IL-17 producing T helper cells. These cells are critical for defense against bacterial and fungal pathogens but are absent in GF mice [198,199]. Similarly, the microbiota also influences the development and V(D)J recombination of lamina propria B cells [200]. These differences in B cell development may contribute to the lower production of IgA antibodies, the primary antibody in protective mucosal immunity, in GF mice [201].

Germ-free pups that have never been directly exposed to bacteria are still affected by the microbiota of their mother. A recent study showed that germ-free pups

whose mothers had been transiently colonized by *E. coli* during pregnancy had increased intestinal group 3 innate lymphoid cells and $F4/80^{+}CD11c^{+}$ monocytes as well as higher production of epithelial antibacterial peptides. This effect was dependent on maternal antibodies, and the study found that maternal IgG facilitated transfer of microbiota-derived compounds to the fetus. It further showed that these changes had functional consequences, as the pups whose mothers were transiently colonized had improved intestinal barrier integrity that was better able to block bacterial translocation [202].

It is important to note that while many immune deficiencies of GF mice can be corrected by colonization with commensals at a later age [197,199,201,203], some defects are the result of the absence of microbiota during a crucial period of development in early life [184,186]. This was demonstrated in a study that compared the intestinal transcriptome profiles of mice born with a microbiota and mice that were born germ-free but colonized with commensals as adults. The transcriptional profiles of jejunal and colonic epithelial cells were significantly different between these two groups, with dramatic differences in genes related to inflammatory responses, immune cell trafficking, and other biological processes [204]. A more recent study identified an age-dependent immune response to bacteria that affects the composition of the microbiota into adulthood. It found that the neonatal mouse gut epithelium expresses the flagellin-sensing Toll-like receptor 5 (TLR5). This expression is age-dependent and transitory. While it is expressed, TLR5-mediated signaling drives a *Reg3g* c-type lectin-dependent immune response that selects against flagellated bacteria. This resulted in a long-term reduction of the presence of flagellated bacteria. Experiments with GF mice showed that the potential for an anti-flagellated bacteria immune response was only present for a short period after

birth, demonstrating that early life represents a crucial time for the establishment of the microbiota and development of the immune system [205].

Multiple studies have shown that changes in the immune system brought about by early-life exposure to microbes have considerable health ramifications. GF mice accumulate higher numbers of invariant natural killer T (iNKT) cells in the lung and colon. This made the mice more vulnerable to oxazolone-induced colitis and ovalbumin-driven allergic asthma. These vulnerabilities could be eliminated if the GF mice were colonized with a conventional microbiota as neonates, but colonization of adult GF mice failed to protect them against disease [206]. GF mice also have abnormally high serum levels of IgE [207]. This makes them more susceptible to severe allergic reactions and systemic anaphylaxis. Colonizing GF mice with a conventional microbiota up to a week after weaning prevents the development of high IgE and protects against allergic reactions, but colonizing adult GF mice does not have the same therapeutic effect [208]. These effects can be seen even in mice that are not completely germ-free. Treating mice with vancomycin, a widely-used antibiotic, can decrease microbiota diversity and cause significant shifts in microbial populations. However, the effect of these changes on health depends on the timing of the treatment. Mice that are treated with vancomycin as neonates and as adults are more susceptible to experimental allergic asthma than mice that only received the antibiotic as adults [209].

These findings in mice parallel findings in humans, as there is a plethora of evidence that immune system interactions with the microbiota at an early age affect the chances of developing immune-mediated diseases later in life. As an infant grows, the diversity of the microbiota increases gradually, but the composition can shift abruptly in

response to health or dietary changes [210,211]. One of the things that can cause a drastic shift is if the child is treated with antibiotics. While the development of antibiotics has been an incredible boon to the fight against infectious diseases, increasing evidence suggests that their use during childhood increases the risk of developing asthma and other autoimmune diseases [184]. Multiple studies have found children that are treated with antibiotics during the first year of life are considerably more likely to develop asthma [212-214]. Another study found that treating pre-school children with macrolide antibiotics resulted in a long-lasting shift in the microbiota, with lower levels of Actinobacteria and higher levels of Bacteroidetes and Proteobacteria. These changes correlated with increased risk of asthma and antibiotic-associated weight gain [215] Similarly, children receiving antibiotic treatment before one year of age are 5 times more likely to develop inflammatory bowel disease [216,217]. Clearly, the long-term adverse effects of antibiotics on the microbiota can increase the chances of immune-mediated diseases. Understanding the mechanisms by which the microbiota affects the immune system will be key to mitigating the long-term side effects of these crucial medicines.

By inhibiting the growth of bacteria, antibiotics cause a change in the bacterial composition of the microbiome. This can potentially lead to an imbalance and/or a loss of diversity that results in the microbiota promoting disease. Bacteria that promote health may be lost or suppressed, and bacteria that promote inflammatory and harmful effects may be gained or expand. A microbiome in this state is said to be in dysbiosis. While childhood treatment with antibiotics offers a dramatic view of the effects of dysbiosis, adults can develop a dysbiotic microbiota as well [184,218,219]. Also, while antibiotics are a common cause of dysbiosis, it can also result from other environmental factors such

as the side effects of other medications, dietary deficiencies, and infectious diseases. Since the 1950s, there has been a significant increase in the prevalence of autoimmune diseases and allergies, and there is considerable evidence this is partly due to changes in microbial exposure [220-222]. Increases in antibiotic treatments, reductions in the frequency of infections, and other environmental changes have led to alterations in the microbiota which in turn have affected the functions of the immune system [222]. Consistent with this hypothesis, dysbiosis has been linked to multiple autoimmune diseases [184,218,219]. Considerable effort has been put into understanding how dysbiosis and autoimmune disease are linked [184]. Understanding the mechanisms that connect dysbiotic microbiota and autoimmune disease could potentially lead to therapies that treat or prevent autoimmune disease by altering the microbiota into a more beneficial state.

One disease with considerable evidence linking it to the microbiota is inflammatory bowel disease (IBD) [223,224]. IBD, which includes Crohn's disease and ulcerative colitis, is characterized by chronic and relapsing immune-mediated intestinal inflammation that causes abdominal pain, diarrhea and bloody stools [223,224]. The correlation between IBD and dysbiosis is well established [225-235], and treatment with antibiotics can alleviate disease symptoms, indicating that the bacteria do play a causative role in the disease [236-240]. While all of these studies found dysbiosis in IBD patients, the specific alterations in bacterial taxa varied widely [223,225-235]. The most consistently found alteration was an increase in Enterobacteriaceae in IBD patient stools relative to healthy individuals, but even this finding was not universal [223,241-245]. Adherent-invasive strains of the Enterobacteriaceae member, *E. coli* have been found in

the ileal mucosa of Crohn's patients, but no clear cause-and-effect relationship has been established [246]. Multiple studies found lower levels of the Firmicutes member *Faecalibacterium prausnitzii* in the stools of IBD patients [230-235], though studies of mucosal biopsies from IBD patients have not consistently replicated that finding [247,248]. This lack of consistency is likely due to the heterogeneous nature of both the disease and the human microbiota [223,224]. Differences in environment and genetics result in the human microbiota varying widely from person to person and from region to region [223,224]. The more controlled settings of mouse models have shed light on the mechanisms of microbiota-immune system interactions that can drive or suppress IBD. Some bacterial species, including *Bacteroides fragilis*, and assorted *Clostridia* strains, can suppress disease in mice by promoting the development of immunosuppressive regulatory T cells (Tregs) in the intestine. Other bacterial species have been shown to promote disease. Some IBD models induce disease by injecting mice with exogenous inflammatory Th17 cells, which drive inflammation and tissue damage in an interferon gamma (IFNγ) dependent manner [249]. However, these cells will not cause disease in the absence of a microbiota [250,251]. *Helicobacter muridarum*, *Helicobacter hepaticus*, and segmented filamentous bacteria have been identified as being able to stimulate colitis-promoting activities in adoptively transferred Th17 cells [250-252]. These studies show that differences in microbiota composition can affect autoimmune disease progression.

Just as interactions with the microbiota shape the immune system, the actions of the immune system affect the composition of the microbiota. The immune system can produce pathogen specific IgA antibodies to coat and neutralize problematic bacteria in

the intestine. Studies have found that known colitis-promoting pathobionts such as *Prevotellaceae*, *Helicobacter*, and SFB are more likely to be coated in IgA. Transferring IgA-coated bacteria from IBD patients into mice rendered the recipient mice significantly more susceptible to severe DSS-induced colitis [253-255]. Additionally, IgA deficiency is associated with increased risk for ulcerative colitis and Crohn's disease [253] This highlights the complexity of the interactions between the microbiota and the immune system, as a deficiency in the immune system can alter the composition of the microbiota, which in turn can affect the immune system and promote responses that result in disease. Consequently, attempting to determine whether dysbiosis or inflammation was the initial cause of the disease is not an easy task.

The microbiota can also affect the development of autoimmune diseases in tissues outside the intestine. Rheumatoid arthritis (RA) has also been linked to dysbiosis. RA is a chronic autoimmune disease characterized by the production of autoantibodies against collagen and mediate synovial inflammation resulting in destruction of bone cartilage in affected joints. In addition to genetic and environmental factors, the microbiome is believed to influence the pathogenesis of RA [184,256,257]. This systemic disease is associated with periodontitis, a severe infection of the gums, and treatment of periodontitis ameliorates RA disease activity [258-260]. Studies investigating this link have shown that infection with one of the major periodontal pathogens, *Porphyromonas gingivalis*, can drive RA progression. One of the characteristics of RA is the production of antibodies against cyclic citrullinated proteins (CCP), self-antigens present in cartilage. Infection with *P. gingivalis* correlates with increased production of anti-CCP antibodies, because *P. gingivalis* produces an enzyme that citrullinates proteins. Antibodies against

these proteins also bind the self-antigen CCP and thus the immune response against *P. gingivalis* promotes the autoimmune response that causes RA [261-264]. In addition to promoting autoantibody production via molecular mimicry, *P. gingivalis* infection also induces a Th17 response via TLR2 stimulation that further drives RA disease activity [265]. Recently, other periodontitis-causing bacteria, including *Anaerglobus geminatus* and *Aggregatibacter actinomycetemcomitans*, have also been found to promote RA progression via an anti-CCP antibody-dependent mechanism [266,267]. In addition to the oral microbiome, the intestinal microbiome has also been implicated in the pathogenesis of RA. Both individuals at high risk for RA [268] and new-onset RA patients who have not begun treatment [269,270] have increased abundance of intestinal *Prevotella copri*. A recent study used the SKG mouse model of RA to investigate whether *P. copri* could play a causative role. The SKG mouse model of RA contains a point mutation in ZAP-70 that decreases T cell receptor (TCR) signaling, allowing auto-reactive T cells to escape negative selection. This renders SKG mice susceptible to arthritis induction by activation of innate immunity. When treated with innate-immune activating compounds such as the fungal-derived zymosan, SKG mice develop arthritis similar to human RA. However, because disease induction depends on innate immunity, it is affected by the microbiota, and arthritis cannot be induced in germ-free mice that contain no commensals [271,272]. To test whether the presence of *P. copri* would affect the course of disease, microbiota from high-Prevotella-abundance RA patients or from healthy controls was transferred into germ-free SKG mice and the mice were treated with Zymosan to induce arthritis. The mice that received the high-Prevotella microbiota developed significantly more severe arthritis with higher IL-17 responses [270]. A recent study found T cells activated

by *Prevotella* epitopes cross-reacted with the self-antigens GNS and FLNA, suggesting that *P. copri* could potentially drive RA progression by activating autoreactive T cells [273]. However, a study of gut commensal compositions of people from Africa and tropical Asia found that healthy people had a large abundance of *Prevotella* species, including *P. copri*, in their intestines [274]. Similarly, another recent study found *Prevotella histicola* isolated from the intestine of healthy humans decreased disease severity in a collagen-induced mouse model of RA [275]. These seeming contradictions emphasize the challenges of identifying what bacterial species in the human microbiota can promote disease. The context of the microbiota and the other species that it contains will affect the effect of a given species, and even in very similar contexts, closely related species can have opposite effects.

Like rheumatoid arthritis, systemic lupus erythematosus (SLE) is a systemic disease characterized by autoantibody production, and SLE patients also exhibit dysbiosis. Numerous recent studies have found the microbiota of SLE patients differ significantly from those of healthy controls [276-283]. However, while all of these studies found dysbiosis, the differences in specific taxa were significantly less consistent. The only finding that was replicated across multiple studies was an increase in the *Bacteroidetes* phylum and a decrease in *Firmicutes* phylum in SLE patients [277-280,284]. Another recent study confirmed the lower ratio of *Firmicutes*/*Bacteroidetes* in SLE patients and found that treatment with glucocorticoids increased the *Firmicutes*/*Bacteroidetes* ratio and correlated with lower serum levels of inflammatory cytokines [285]. Overall richness, as measured by α-diversity is generally decreased in SLE patients in comparison to healthy controls [276,280,282]. Despite this lack of

consistency, investigations of the microbiota in SLE patients as well as in mouse models of lupus yielded several interesting discoveries that have increased our understanding of how the gut microbiota can affect disease progression.

One study identified *Ruminococcus gnavus* as a potential pathobiont that could contribute to lupus progression. SLE patients have 5-fold greater abundance of *R. gnavus*, and many had antibodies against one or more strains of *R. gnavus*. The level of anti *R. gnavus* antibodies correlated positively with overall autoantibody levels and active nephritis, which could suggest that immune responses against *R. gnavus* may increase disease severity [276].

Another study found that the pathobiont *Enterococcus gallinarum* might drive lupus both in human SLE patients, and the NZWxBXSB F1 (NxB F1) mouse model of lupus. NxB F1 male mice develop lupus due to having a duplicate TLR7 gene, and the study found male mice with disease had *E. gallinarum* in their livers. Liver biopsies from patients with SLE or other autoimmune diseases were more likely to contain *E. gallinarum* than biopsies from healthy patients. This could be due to impaired gut barrier function, which is common in SLE patients and mouse models of lupus. Germ-free B6 mice monocolonized with *E. gallinarum* developed impaired barrier function, *E. gallinarum* translocation to the liver, and the production of antinuclear autoantibodies and pro-inflammatory cytokines. However, *E. gallinarum* did not translocate or induce autoimmunity in conventional B6 mice. When NxB F1 male mice were vaccinated against *E. gallinarum*, serum levels of autoantibodies were reduced and survival was increased, showing that eliminating a specific bacterial species could suppress autoimmunity without suppressing the entire immune system. Among SLE patients, the

presence of *E. gallinarum* in the liver correlated with higher levels of autoantibodies, suggesting that the pathobiont could play a similar role in humans as it does in the NxB F1 mouse model [286].

Another recent study suggested commensal bacteria may promote lupus by expressing an ortholog of the human protein Ro60. Ro60 is a nuclear antigen, and anti-Ro60 antibodies are often among the first to develop in SLE patients [287,288]. There is significant cross-reactivity between human Ro60 and the bacterial Ro60 ortholog. The bacterial Ro60 ortholog is recognized by T cells and B cells from SLE patients that have anti-Ro60 autoantibodies. Some people have commensals that express the Ro60 ortholog in their gut microbiota, suggesting a possible link. Additionally, GF mice monocolonized with a Ro60 ortholog-expressing bacteria developed an anti-Ro60 autoantibody response that led to glomerular deposition of immune complexes. This suggests that an immune response against the bacterial Ro60 ortholog could potentially contribute to the development of lupus. However, almost all of the SLE patients and healthy subjects in the study had Ro60 ortholog-expressing bacteria in their microbiomes. This indicates that the presence of the Ro60 bacteria is not enough to stimulate autoimmunity on its own. Also, Ro60 ortholog-expressing bacteria were found at equal frequency in SLE patients that were positive for anti-Ro60 autoantibodies and SLE patients that were negative for anti-Ro60, showing that even in patients with active autoimmune disease the presence of Ro60 ortholog-expressing bacteria in the gut is not enough to stimulate an anti-Ro60 antibody response. Analysis at the family level did not reveal any significant differences in the composition of the microbiota between the anti-Ro60 positive and negative SLE

patients [277]. Thus, while the possibility of microbiota Ro60 cross-reactivity stimulating lupus development is possible, the events that would lead to this are still undetermined.

Gut microbiota and mouse models of lupus

A recent study found a link between microbiota tryptophan metabolism and lupus in the BWF1-derived triple congenic B6.*Sle*1.*Sle*2.*Sle*3 (S123) mouse strain. Compared to healthy control female B6, S123 microbiota is enriched for *Prevotellaceae*, *Paraprevotella*, and *Lactobacillus*. When adult female S123 microbiota was transferred to GF B6 mice, the B6 mice developed lupus-like symptoms including elevated autoantibodies and mild glomerulonephritis. Fecal metabolite analysis found evidence that microbiota tryptophan metabolism was altered in female S123 mice to favor kynurenine production over serotonin production. Changing the tryptophan content of the diet altered the S123 microbiota composition and impacted disease development; a low tryptophan diet protected against disease, and a high tryptophan diet exacerbated disease. A high tryptophan diet increased the ability of female S123 microbiota to cause autoimmune phenomena in GF female B6 mice. Microbiota from mice fed a low tryptophan diet did not induce autoimmune phenotypes in the GF B6 mice. Altering female S123 microbiota by cohousing with female B6 mice reduced autoantibody production, but glomerulonephritis was unchanged [289]. This study showed that changes in the microbiota caused by changes in diet affected whether the microbiota suppresses or promotes lupus-like disease progression. It also showed that the microbiota in the lupus-prone female S123 mice have altered tryptophan metabolism and that they promote the development of lupus-like symptoms. However, it did not determine whether female

S123 microbiota affected the immune system and disease progression via altered production of tryptophan metabolites. Bacterial associated tryptophan metabolites have been found to have immunomodulatory properties [290-292], and the high kynurenine and low serotonin in the mouse feces matches findings in SLE patient sera [293]. Inhibiting kynurenine production did not protect against disease, so the paper did show higher kynurenine production is not responsible for disease, but additional research into the role the other tryptophan metabolites play could be illuminating.

Diet can also affect the composition of the microbiota in the SNF1 mouse model of lupus. Giving lupus-prone female SNF1 mice acidified water suppressed disease. It delayed the onset of nephritis and decreased the level of antinuclear autoantibodies compared to SNF1 mice on neutral pH water. The composition of the microbiota was significantly changed by the acidified water, and the most significant change was in the *Rikenellaceae* family, which was greatly increased in the SNF1 mice receiving acidified water. Additionally, the level of *Firmicutes* was increased and the level of *Bacteroidetes* was decreased in the acidified water group [294]. This mimics findings from human studies that have found healthy controls to have higher *Firmicutes* and lower *Bacteroidetes* levels than SLE patients [277-280,284]. In the SNF1, the changes correlated with increased intestinal levels of Th17 cells which promote lupus progression, suggesting that the alterations to the microbiota might decrease its Th17-stimulating characteristics [294].

It has been shown that treating MRL/*lpr* mice with antibiotics after disease onset ameliorates disease, indicating a connection between microbiota and disease [295]. A study from the lab of Xin M. Luo investigated this connection and found lupus-prone

MRL/*lpr* mice have significantly lower levels of bacteria from the family *Lactobacillaceae* relative to healthy control MRL mice [296]. A later study from the same lab followed up on this finding, and showed Cecal transfers from MRL mice into MRL/*lpr* mice decreased anti-dsDNA autoantibody production. Weekly gavages with a mixture of strains from the *Lactobacillaceae* genus Lactobacillus not only decreased anti-dsDNA production, they also slowed nephritis onset and increased survival in the MRL/*lpr* mice. In both *Lactobacillus* gavaged and non-gavaged MRL/*lpr* mice, *Lactobacillus reuteri* and an unknown strain were by far the most common bacteria. Treatment with the *Lactobacillus* bacteria corrected the "leaky" gut that Mrl/*lpr* mice normally exhibit. In addition, intestinal IL-10 production was increased and IL-6 was decreased, promoting an anti-inflammatory environment. This resulted in decreased deposition of IgG2a in the kidney and in changing the Treg/Th17 balance in the kidney to skew more towards Tregs [297]. Together, these findings suggest an immunoregulatory role for *Lactobacillus* bacteria in the MRL/*lpr* mouse model. This was suggestion is strengthened by another recent paper that found treating MRL/*lpr* mice with antibiotics before disease onset resulted in dysbiosis characterized by lower levels of *Lactobacillus* and *Bifidobacterium*. These dysbiotic MRL/*lpr* mice developed more severe disease compared to untreated controls [298].

A later paper from the Luo lab, however, found that the opposite was true for the BWF1 mouse model of lupus. It found that in disease-prone female BWF1 mice, the gut microbiota composition changed significantly between the pre-disease and lupus disease stages. However, unlike in the studies of MRL/*lpr* mice, higher *Lactobacillus* abundance in female BWF1 mice was associated with increased loss of renal function and higher

levels of autoantibodies [282]. This suggests that the effect of lactobacillus may depend other factors such as the genetics of the host and the overall composition of the microbiota.

A study from a different lab raised even more questions about the role of *L. reuteri* in lupus. This study found greater abundance of *L. reuteri* in a subset of SLE patients compared to healthy controls. *L. reuteri* was also enriched in the feces of a TLR7-dependent models of lupus, the TLR7.1 transgenic (Tg) mouse strain. Like the BXSB/*Yaa* strain, male TLR7.1 Tg mice spontaneously develop lupus-like disease due to having an extra copy of the TLR7 gene on the Y chromosome. This was also true for their TLR7 agonist-induced model, where C57BL/6 (B6) mice are regularly treated with the TLR7 agonist imiquimod (IMQ) to induce a lupus-like disease. Gavaging imiquimod-treated B6 germ-free or conventional mice with cecal contents from lupus-prone TLR7.1 Tg mice significantly increased the severity of glomerulonephritis, splenomegaly, and other lupus symptoms. Additionally, gavaging TLR 7.1 Tg mice with *L. reuteri* exacerbated the spontaneous lupus-like disease. In direct contrast to what was found in the MRL/*lpr* model, *L. reuteri* gavage worsened gut permeability in the IMQ-induced B6 model. *L reuteri* also translocated. The 'leakiness' induced by *L. reuteri* allowed it to translocate across the intestinal barrier to the mesenteric lymph nodes, spleen, and liver, suggesting this may be involved in its increasing of disease severity [299].

These differences in *Lactobacillus* findings underscore the importance of biological context when investigating the microbiota. While the MRL/*lpr*, BWF1, and TLR7.1 Tg mouse strains all model the same disease, the genetic differences that make them lupus prone are quite different. Given their diversity, it is unsurprising that the

effect of a bacterial taxon would differ between them. This is especially important to consider when considering how microbiota findings in animal models could be applied to human patients. The diversity of human lifestyles translates to significant heterogeneity in microbiota. Obviously, it is not possible to replicate the diversity of human diet and environmental exposures in lab animals, however, sex and sex hormones have a significant effect on the microbiota and yet are often overlooked or ignored in studies.

Sex differences in the gut microbiota and the effect on autoimmune diseases

Of course, one of the major sources of microbiota heterogeneity are the effects of sex and sex hormones. Many studies have found differences in the microbiota between men and women, though the taxa that differ vary and often conflict from study to study [182,300-303]. For example, one study found a higher level of *Bacteroides/Prevotella* in men than women [304], while another found that *Bacteroides* abundance was lower in men than women [300]. While taxa may differ, women do tend to have a higher level of richness and α-diversity than men [305,306]. Not only does host sex affect the microbiota directly, it also affects how other environmental factors affect it. A recent study found that the microbiota in men and women responded differently to dietary changes [301]. Combined with the increasing evidence that the microbiota can impact lupus progression, these differences in microbiota between men and women raise the question of whether sex differences in microbiota might contribute to sex bias in SLE.

There is evidence that the sex of the host changes how the gut microbiota interacts with and influences the immune system. When microbiota from male or female conventional mice is transferred into germ-free mice, the sex of the donor affects how the

immune system of the recipient changes after the transfer. Recipients of female microbiota had higher levels of thymic double-negative T cell precursors than recipients of male microbiota. Male recipients of male microbiota had higher levels of mesenteric RORγt^{+}Foxp3^{+} T cells and IgA production than males who received female microbiota [307]. The effect of these differences has not been explored, but it does show that microbiota has sex-specific immunoregulatory characteristics that can be transferred by microbiota transplantation.

Like many differences between males and females, sex differences in microbiota can be mediated by differences in sex hormones levels. In longitudinal human studies, the changes in hormone production during puberty are associated with significant changes in the composition of the microbiota [308] Changes in female hormones due to oral contraceptive use or bilateral ovariectomy have been found to alter the composition of the gut microbiota in women [305]. Additionally, in males and postmenopausal women, urinary estrogen levels positively correlate with microbiome richness and α-diversity as well as higher abundance of *Clostridia* [309,310]. Serum estrogen levels also correlate with a more diverse microbiota, as women with higher serum levels of estradiol had a more diverse gut microbial composition. Hormonal effects are not limited to estrogen either, as men with higher serum levels of testosterone also exhibit greater richness and α-diversity [311]. Mouse studies have further demonstrated the microbiota interacts with and is affected by these hormones. Orchiectomy and ovariectomy both affect the microbiota composition. Supplementing orchiectomized male mice with testosterone suppressed the effect of castration on microbiota [312,313]. Clearly, sex hormones influence the microbiota.

Some of the effects of sex hormones are likely indirect. As described above, sex hormones have a considerable effect on the immune system, and the immune system exerts a great deal of influence over the composition of the microbiota. However, sex hormones can also interact directly with the microbiota. Estrogens and estrogen-like compounds have been found to promote the proliferation and growth of certain kinds of bacteria [314]. Androgens like testosterone can also influence the metabolism and growth of gut bacteria [315]. In fact, the direct interaction between hormones and microbiota goes both ways, as there is evidence that commensal bacterial metabolic activities can affect the levels of sex hormones of the host. Gut commensals can metabolize estrogen-like dietary compounds into active forms [316,317]. Some commensal bacteria have β-glucuronidase activity that could potentially increase reabsorption of estrogens and androgens in the intestine by converting them from the less active glucuronidated form to the more active and absorbable non-glucuronidated form [309]. Some gut bacteria can also convert testosterone to the more potent dihydrotestosterone [318]. A study comparing germ-free mice that lack a microbiome to conventional mice found that in both male and female mice, GF mice had lower intestinal levels of non-glucuronidated androgens and lower levels of the potent androgen dihydrotestosterone compared to conventionalized mice [319]. This suggests that the gut microbiota may play a physiologically relevant role in the maintenance of androgen signaling. This has been supported by the findings of other studies. Treating mice with a strain of *Lactobacillus reuteri* isolated from human milk increased serum levels of testosterone, reduced age-associated testicular atrophy, and increased spermatogenesis [320,321]. When conventional and GF male mice were compared, it was found that GF mice had delayed

testis development and lower intratesticular testosterone levels, suggesting that the microbiota may influence the development of an organ key to androgen production in males [322]. This indicates that microbiota may also affect the immune system by altering the levels of immunomodulatory sex hormones.

The crosstalk between male sex hormones and the microbiota can impact the progression of autoimmune disease. Unlike most autoimmune diseases, Type 1 Diabetes (T1D) does not exhibit a sex bias in humans. However, this is not the case in the Non-obese diabetic (NOD) model of T1D; female NOD mice and castrated male NOD mice have a much higher incidence of disease than intact male NOD mice. GF NOD mice, conversely, lack a sex bias as both male and female GF NOD mice have high incidence of T1D. The microbiota composition differs significantly between male and female NOD mice. Bacterial strains that were more abundant in male NOD mice were cultured and transferred into GF NOD mice. One of them, an Enterobacteriaceae strain (EntS), was able to suppress disease when transferred into GF male NOD mice, but none tested were able to suppress disease in GF female NOD mice. Conventional male NOD mice normally have higher serum levels of testosterone than GF male NOD mice, but colonizing GF male NOD mice with EntS rescued serum testosterone levels. These increased testosterone levels correlated strongly with disease protection and were not seen in GF female NOD mice colonized with EntS [323]. Taken together these findings suggest that both hormones and microbiota act to suppress disease in male mice and that the microbiota-hormone interactions reinforce this disease-suppressing phenotype. This conclusion was reinforced by another study using the NOD model. It found that transferring in the whole gut microbiota from adult male NOD mice into young female

NOD mice suppressed islet inflammation, reduced autoantibody titers, and increased serum testosterone levels. This protection from disease was testosterone-dependent [324]. This raises the possibility of treating autoimmune diseases by correcting intestinal dysbiosis via microbiota transplantation.

Not all sex differences are mediated by hormones. A study of SNF1 mice found that the lupus-prone female mice had significantly higher numbers of proinflammatory molecules and immune cells than the less-lupus-prone male mice before puberty or disease onset [25]. This indicates that the immune responses that shape the microbiota in these lupus-prone mice are also different even in the absence of differences in sex hormone levels.

A recent study of SNF1 mice found that female SNF1 mice, which are more susceptible to lupus than males, have a different microbiota composition than the male SNF1 mice. When antibiotics were used to deplete the microbiota, glomerulonephritis and autoantibody production were suppressed in female SNF1 mice, but antibiotic depletion had no effect in male SNF1 mice. This certainly suggests that female microbiota could be promoting disease progression. However, transferring female SNF1 microbiota into male SFN1 mice or vice versa failed to significantly affect disease progression, which lessens the overall impact of their findings [325]. A follow-up study by the same lab found that female SNF1 mice have higher levels of B cells producing anti-nuclear antigen reactive IgA in the gut mucosa and higher fecal levels of nuclear autoantigen reactive IgA than male SNF1 mice or healthy controls. Antibiotic depletion of SNF1 female microbiota correlated with a decrease in the levels of those B cells and fecal IgA levels, suggesting a mechanism for the antibiotic-mediated reduction in disease

severity [326]. All of these studies illustrate that the microbiota can have a significant impact on the progression of autoimmune disease, and the fact that female S123 anti-dsDNA titers could be lowered by cohousing with female B6 illustrates the possibility that disease progression could be suppressed by transferring microbiota from healthy individuals into sick individuals of microbiome research [289].

Gut microbiota and immunomodulatory metabolite production

One intriguing area of microbiota studies focuses not on identifying what bacteria species are associated with disease and good health, but what bacterial functions are. Identifying the microbial activities that drive the immune system in beneficial or harmful ways could lead to the development of therapies that seek to mimic or to block those mechanisms. One of the major ways that microbiota can affect the immune system is through the production of immunomodulatory metabolites. In recent years, numerous metabolites have been shown to alter immune activities [327].

Perhaps the most studied class of immunomodulatory metabolites is the short chain fatty acids (SCFAs). This category, which includes acetate, butyrate, and propionate, have potent anti-inflammatory effects. By engaging G protein-coupled receptors and inhibiting histone deacetylases, they promote Treg induction [328], suppress inflammasome activation [329,330], and promote anti-inflammatory activities in macrophages and neutrophils [331,332]. Multiple autoimmune diseases including IBD, Crohn's disease, and spondyloarthritis have been found to be associated with decreased levels of SCFAs and SCFA-producing bacteria [333-336]. Additionally, treatments with SCFAs alleviate symptoms in mouse models of these diseases [334,337-339].

Like the microbiota composition, metabolite production can be altered by changes in diet. The clinical relevance of the diet's effect on metabolites was demonstrated in a recent study that examined the changes in the fecal metabolite profiles of children with Crohn's disease (CD) after they started a prescribed diet that has been shown to alleviate disease. It found that the diet resulted in higher intestinal production of anti-inflammatory SCFAs, and that this correlated strongly with a positive clinical response to the treatment. Unfortunately, this beneficial effect was seen only in children with small/bowel colonic CD and not in those with perianal engagement. Since different areas of the intestine have different microbial populations, this could indicate that the success of the CD treatment depends on which population of microbiota is dysbiotic [340].

Autoimmune diseases of the central nervous system (CNS) can also be affected by intestinal metabolites. One recent study found decreased levels of SCFAs in the serum of long-term multiple sclerosis (MS) patients and that SCFA treatment increased anti-inflammatory IL-10-producing T cells and suppressed disease in a mouse model of experimental autoimmune encephalitis (EAE). However, it also found that depending on the immunological context, SCFAs could also induce inflammatory CD4 T cells that promoted disease [336]. Another recent study of CNS autoimmunity and metabolites emphasized that alterations in metabolite production can significantly affect disease. In a mouse model of EAE, SCFA treatment resulted in increased induction of regulatory T cells in the lamina propria that suppressed disease and reduce nerve damage. However, treatment with long-chain fatty acids not only boosted the expansion of inflammatory Th1 and Th17 cells and accelerated disease, they also decreased the levels of anti-

inflammatory SCFAs [341]. These findings show that alterations in microbiota metabolite production can affect immune cells well outside the intestine.

Another class of immunoregulatory metabolites are tryptophan metabolites. Tryptophan conversion into other compounds primarily happens via one of three pathways, the kynurenine, serotonin, and indole pathways [342]. Microbial metabolism contributes to all three of these pathways, especially the indole pathway, and disruptions of the balance between the pathways have been associated with autoimmune diseases [327]. Indole derivatives activate the Aryl hydrocarbon receptor (AhR), which can inhibit Th17 differentiation and protect against colitis [343,344], and colitis patients have decreased intestinal AhR levels, indicating a possible connection between decreased microbial indole production and disease [345]. A recent study found that the commensal bacteria *P. russelli*, could increase production of an AhR-activating indole derivative that increased goblet cell differentiation and decreased inflammatory signals. This resulted in reduced susceptibility to DSS-induced colitis in mice [346]. Another autoimmune disease that correlates with altered tryptophan metabolism is rheumatoid arthritis, as patients have been found to have decreased indole levels [347]. SLE has also been linked to altered tryptophan metabolism. SLE patients have increased serum levels of kynurenine [348], which can promote the increased cellular metabolism that is characteristic of lymphocytes in lupus [293]. Other studies have found tryptophan itself is decreased in SLE patient serum, indicating that the disease is associated with an altered tryptophan metabolite profile [349,350]. Also, as described above, tryptophan metabolism is altered in the microbiota of lupus-prone female S123 mice, and the microbiota promotes lupus-like disease when transferred into GF B6 mice. The study did not establish whether this

lupus-promoting ability was connected to the differential production of tryptophan metabolites, though [348].

Recent studies have identified histamine as another intestinal metabolite that could potentially affect autoimmune diseases. It has been shown that probiotic and endogenous bacterial species can produce histamine and affect intestinal immune responses [351-353]. Histamine is an effector molecule that can mediate either inflammatory or regulatory responses depending on the type of histamine receptor expressed by the target cell (i.e., H1R, H2R, H3R, H4R). While histamine is classically associated with allergic reactions, microbe-produced histamine has been shown to directly inhibit inflammatory factors in the gut [354-356]. Chronic low-grade intestinal inflammation and defective intestinal barrier function are associated with lupus and a number of autoimmune diseases [297,357], so inhibition of gut inflammation in lupus-prone mice (or humans) via microbe-produced histamine could affect autoimmune disease development and/or progression.

Many anti-inflammatory metabolites have been identified, but some metabolites have pro-inflammatory effects. Trimethylamine (TMA), a choline metabolite, is synthesized exclusively by the microbiota. It is transported into the liver where it is converted to trimethylamine N-oxide (TMAO). TMAO is an inflammatory metabolite that activates the NLRP3 inflammasome [358,359], and higher serum TMAO levels have been linked to several human autoimmune diseases, including primary sclerosing cholangitis [360], psoriatic arthritis [361], and RA [362].

Another class of microbial metabolites are secondary bile acid metabolites. These are synthesized from primary bile metabolites by the microbiota, and they have

been shown to have anti-inflammatory effects. Many secondary bile acids activate the farnesoid X receptor (FXR) [363], which reduces secretion of the pro-inflammatory cytokines IL-17, IFNγ, and TNFα [364]. FXR is also crucial for the development of colonic RORγ^{+} regulatory T cells in mice [365]. Increased FXR stimulation has been found to alleviate dextran sodium sulfate-induced colitis in mice [364], Higher primary bile acid levels and lower secondary bile acid levels have been found in patients with IBD [342]. This suggests that the microbiota's ability to convert primary bile acids into secondary bile acids can affect inflammatory intestinal diseases. Further supporting that conclusion is the finding that microbiota bile acid processing can increase FXR stimulation not only by producing FXR agonists, but also by reducing the levels of a primary bile acid, taurine-conjugated β-muricholic acid (TβMCA) which a natural antagonist of FXR [366]. Together these data show that alterations in the metabolism of primary bile acids by intestinal microbes can significantly affect disease processes. Furthermore, the effects of secondary bile acid production are not limited to the intestine. About 95% of bile acids are reabsorbed by the epithelium of the intestine and carried into the liver through the portal vein system [367]. There they interact with innate immune cells, including liver-resident macrophages via the FXR and G protein-coupled bile acid receptor 1 (GBAR1). Activation of these receptors on innate immune cells contributes to the maintenance of a tolerogenic phenotype and the prevention of disease [363]. Disruption of bile acid homeostasis can lead to inflammation and contributes to the development of nonalcoholic fatty liver disease, cholestatic liver injury, and other diseases [368]. It is not fully understood what alterations in cell populations and functions lead to disease. However, it is clear that alterations in the generation of secondary bile

acid metabolites by the microbiota can affect macrophages and other immune cells in an organ outside the intestine in ways that affect disease progression [363,369]. Like the findings on SCFAs and CNS diseases described above, this finding emphasizes that immune processes outside the intestine can be affected by altered microbiota metabolite production. This raises the question of whether the sex bias of disease in BWF1 mice could be due to sex-based differences in the microbiota's production of immunomodulatory metabolites.

Macrophage efferocytosis and systemic lupus erythematosus

The immune system has numerous mechanisms designed to prevent autoimmune reactions. One of these is efferocytosis, which is the clearance of apoptotic cells by phagocytes in an anti-inflammatory manner [370]. Cell turnover results in the constant generation of apoptotic cells, and these cells contain self-antigens that could potentially stimulate autoimmune responses [371]. To prevent this, apoptotic cells are bound and ingested by phagocytes, primarily macrophages. This prevents the apoptotic cells from interacting with and activating other immune cells. The ingestion of apoptotic cells promotes macrophage production of anti-inflammatory cytokines that dampen any inflammatory responses against the apoptotic debris [370-372]. Because apoptotic cells are normally non-inflammatory, as long as they are swiftly cleared, they are unlikely to provoke an immune response. However, if efferocytosis is impaired or defective, apoptotic cells will accumulate and can undergo secondary necrosis. In this case, the apoptotic cells undergo swelling and lysis, which results in the release of damage-associated molecular patterns. These can act as alarm signals that activate nearby immune

cells and promote immune responses against the self-antigens they contain [373,374].

Defects in efferocytosis are thought to significantly increase susceptibility to SLE and to promote disease progression. SLE patients exhibit accumulation of apoptotic cells in the lymph-node germinal centers, bone marrow, and epidermis [375-377]. This results in immune cells being exposed to nuclear self-antigens in the inflammatory context of cells undergoing secondary necrosis. This stimulates an immune response characterized by production of anti-nuclear autoantibodies (ANAs) against dsDNA, ssDNA, snRNPs, and other nuclear antigens. Since apoptotic cells are constantly generated by cell turnover, additional self-antigens are constantly being generated and promoting disease progression [378]. Additionally, the accumulated apoptotic cells provide ample fodder for the formation of nuclear antigen-ANA immune complexes. These complexes lodge in the kidneys and other organs, leading to the inflammation and kidney damage that is the primary cause of lupus morbidity and mortality [3,379]. This is likely why disease severity in SLE patients is strongly correlated with deficient efferocytosis [375,380]. Things that increase the number of apoptotic cells can also trigger lupus flares. For example, excessive sun exposure can cause lupus flares, as dermal cells undergo apoptosis in response to the damage caused by UV radiation [377]. In mouse models, it has been shown that deficiencies in components important for efferocytosis or signaling proteins that promote efferocytosis lead to the mice developing lupus-like disease spontaneously [381-385]. Clearly, there is a substantial amount of evidence connecting efferocytosis deficiencies and lupus.

Macrophages isolated from patients with SLE are deficient in apoptotic cell phagocytosis, indicating that they have an intrinsic defect in efferocytic activity [386]. As

macrophages are the cell type primarily responsible for efferocytosis, this defect is likely a major contributor to the accumulation of apoptotic cells [387-390]. Like SLE patients, peritoneal macrophages from lupus-prone female BWF1 mice also have deficient efferocytic activity compared to healthy female controls [391]. Also, in comparison to healthy female controls, female BWF1 macrophages are also deficient in their ability to produce anti-inflammatory cytokines in response to apoptotic cell exposure [392]. These parallels in deficient macrophage function between SLE patients and female BWF1 female mice make the BWF1 mouse model of lupus a good choice for investigating the factors affecting defective macrophage efferocytosis. Also, while female BWF1 mice have deficient macrophage efferocytosis compared to female mice of other strains, no one has investigated whether disease resistant male BWF1 mice share this defect. Previous studies of non-disease-prone rats and mice have found female macrophages had higher phagocytic activity than those from males [74,75]. Determining whether the same sex-based difference exists in BWF1 mice or if macrophages from disease-resistant male BWF1 have higher efferocytic activity could advance our understanding of the source of the lupus sex bias in BWF1 mice.

The clear links between deficient macrophage efferocytosis and lupus progression makes improving macrophage efferocytosis a promising therapeutic target. There are many pathways and genes involved in efferocytosis regulation, providing many possible ways of stimulating efferocytic activity. Three of the major pathways responsible for promoting efferocytosis are the LXR, PPARγ and PPARδ pathways [381,393-400].

PPARδ knockout (KO) mice develop lupus-like autoimmune kidney disease and their macrophages have decreased efferocytic activity. Conversely, PPARδ agonists improve macrophage efferocytosis. This is due to the decreased production of opsonins like C1qb, a component of the C1q trimeric complex that binds phosphatidylserine on apoptotic cells and the C1q receptor on macrophages, bridging the two and facilitating macrophage uptake of the apoptotic cell [400]. In humans, C1q deficiency drastically increases lupus susceptibility; over 90% of C1q-deficient people develop lupus [401-403].

PPARγ signaling can also increase expression of C1qb, as well as C1qa and C1qc [404]. As with PPARδ, PPARγ insufficient mice develop SLE-like disease due to increased Th17 polarization that drives autoantibody production and lymphoproliferation [383]. Treating macrophages with a PPARγ agonist increases their ability to phagocytose apoptotic cells [395]. Another pro-efferocytic opsonin upregulated by PPARγ is milk fat globule-EGF factor 8 (MFG-zE8). Like C1q, MFG-E8 binds phosphatidylserine and a macrophage surface receptor to promote apoptotic cell ingestion [404], and decreased MFG-E8 expression inhibits efferocytosis [405]. In addition to opsonins, PPARγ signaling also upregulates expression of the receptors AXL, MERTK, and CD36 [393,406]. AXL and MERTK bind phosphatidylserine-targeting opsonins Protein S or GAS6, and knocking down either of them decreases macrophage efferocytic activity [378,379]. CD36 binds calreticulin, an eat-me signal that can either be displayed by an apoptotic cell or secreted by macrophages to bind asialoglycans on the apoptotic cell membrane [407]. Blocking PPARγ leads to downregulation of CD36 in macrophages and

decreased efferocytosis [393]. Conversely, increased CD36 expression correlates with increased efferocytosis [408].

Like PPARγ, LXR signaling promotes the expression of many pro-efferocytic genes, including the receptors MERTK and CD36 [381,409,410]. Also, like PPARγ knockout mice, LXR knockout mice develop ANAs and glomerulonephritis, and macrophage efferocytic activity can be increased by an LXR agonist [381,399]. In addition to MERTK and CD36, LXR stimulation can also upregulate TIMD4 [411,412]. Unlike MERTK or CD36, TIMD4 can bind phosphatidylserine directly and promote apoptotic cell phagocytosis without an opsonin [385,412,413]. Knocking out TIMD4 leads to decreased efferocytosis and promotes autoimmunity [385]. Not all efferocytosis genes are directly involved in binding and ingesting apoptotic cells. The phagocytosing of apoptotic cells means ingesting a large amount of cholesterol. Excess free cholesterol could potentially damage the cell membrane, but the LXR-regulated cholesterol pump ABCA1 flushes out the excess cholesterol allowing the efferocytic macrophage to remain functional [414,415].

All three of these pathways have one thing in common. LXR, PPARγ, and PPARδ all form a permissive heterodimer with RXR. This means that an RXR agonist would activate all three of these pathways [416]. Unsurprisingly macrophage lineage-specific deletion of RXR resulted in defective macrophage clearance of apoptotic cells and lupus-like autoimmune kidney disease, while treatment with an RXR agonist significantly increased macrophage efferocytic activity [394]. Clearly, differences in the stimulation of any one of these pathways could potentially contribute to the deficient macrophage efferocytosis seen in lupus-prone female mice.

MATERIALS AND METHODS

Animals and surgeries

Six-week-old female New Zealand Black (NZB) and male New Zealand White (NZW) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). The NZB and NZW mice were bred in our animal facility to produce the NZBxNZW F1 (BWF1) mice. Sixteen-week-old female BALB/c mice were purchased from the Jackson Laboratory, and used in the RNA sequencing (RNA-seq) study. C57/BL mice were bred in our own facility. Mice were maintained under specific pathogen-free conditions in our animal facilities following the guidelines stipulated by the University of Louisville Institutional Animal Care and Use Committee.

For castration, pre-pubertal (24-day-old) male BWF1 mice were anesthetized with isoflurane, the scrotum was incised, the vas deferens and spermatic vessels were ligated, the testes and epididymis were removed, and the skin was sutured. Sham castration was done on age-matched males by incising the scrotum and then suturing the skin. Analgesic (ketoprofen) was administered during the procedure and 24 hours after the procedure.

For flutamide pellet treatment, pre-pubertal (24-day-old) male BWF1 mice were anesthetized and a time-release pellet containing vehicle or the androgen receptor blocker flutamide was implanted subcutaneously in their upper back. (Innovative Research of America, Sarasota, FL; #NA-152). The flutamide-containing pellet released 1.5 g of flutamide over 90 days (16.7 mg dose/day).

Reagent descriptions

Super complete media (SCM) is RPMI 1640 (Sigma, Burlington, MA; #R7509) supplemented with 1000 U/mL penicillin/streptomycin, 2 mM L-glutamine (Corning, Corning, NY; #25-005-CI), 1% Non-essential amino acids (Thermo Fisher, Waltham, MA; #11150-060), 1 mM Sodium Pyruvate (Thermo-Fisher, #SH30239.01), 10 mM HEPES (Corning #25-060-CI), and 10% fetal calf serum (FCS) (R&D Systems, Minneapolis, MN; #S11150).

Clostridium histolyticum Collagenase type VIII and DNase were purchased from Sigma C2139, d4527)

The monoclonal antibodies anti-CD16/32, anti-CD11b-PE, anti-F4/80-APC, anti-CD11b-BV421, anti-F4/80-APC, anti-CD36-PECy7, anti-TIMD4-PE, anti-Thy1-APC/Cy7 were purchased from Biolegend (San Diego, CA; #101301, 101208, 123115, 101251, 102615, 130005, 105328). The monoclonal antibodies anti-rabbit IgG-PE, anti-MERTK-PE/Cy7 were purchased from Thermo Fisher (#12-4739-81, 25-5751-82). The monoclonal antibody anti-LRP1 was purchased from Abcam (Cambridge, UK; #Ab92544). The monoclonal antibody anti-CD36 was purchased from BD Biosciences (Franklin Lakes, NJ; #552544)

Cell-trace Violet^{TM} was purchased from Thermo Fisher (#C34557)

The RXR inhibitor HX531, the PPARγ inhibitor GW9662, the LXR inhibitor, GSK2033, and the PPARδ inhibitor GSK0660 were purchased from Sigma (#SML2170, M6191, SML1617, G5797).

Microbiota composition analysis

Bacterial DNA was extracted from fecal samples using QIAamp PowerFecal DNA Kits (Qiagen 12830-50). Extracted DNA was sent to the Genome Technology Access Center at Washington University. There they sequenced 9 hypervariable regions of the 16S rRNA gene and the multi-amplicon data was used to create species-level taxonomic profiles of the microbiota [417]. In our lab, we used QIIME2 to calculate Bray-Curtis dissimilarity matrices for the species-level microbiota compositions and to perform Principal Coordinate Analysis (PCoA). The intra- and inter-group Bray-Curtis beta diversity distances were compared using Permutational multivariate analysis of variance (PERMANOVA) pairwise comparisons [418]. PCoAs were visualized using EMPeror [419]. The inter-group differences in the abundances of specific taxa were analyzed with two-way ANOVAs with corrections for multiple comparisons.

Metabolomic composition analysis

Three different sets of fecal samples were sent to our collaborators in the Biochemistry department (Metabolomic Experiments #1,2,3). Our collaborators extracted polar metabolites and analyzed them using a variety of methods. For Metabolomic Experiment #1, the samples were derivatized with N-methyl-N-trimethylsilytrifluoroacetamide (MSTFA) and then two-dimensional gas chromatography mass spectrometry (GC×GC-MS) was used to analyze metabolite composition and abundance. For Metabolomic Experiment #2, the samples were analyzed with both GC×GC-MS with MSTFA derivatization and parallel two-dimensional liquid chromatography mass spectrometry (2DLC-MS/MS) in both negative and positive

ionization modes. For Metabolomic Experiment #3, the most recent and comprehensive analysis, samples were analyzed also analyzed by GC×GC-MS with N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) derivatization as well as the methods used in Metabolomic Experiment #2 [420]. We used partial least squares-discriminant analysis was performed to compare the metabolomic profile differences between the groups. Pairwise two-tailed *t*-tests were used to compare metabolite abundances between the groups. Quantitative pathway enrichment analysis was performed using Metaboanalyst (version 4.0). Intensities of metabolites identified with significant difference between intact male and female mice or between intact male and castrated male mice were analyzed by the program. The data were auto-scaled and matched against the KEGG pathway library for *Mus musculus*. The "GlobalTest" and "Betweenness centrality" algorithms were used for pathway enrichment and topology, respectively [421].

Phytanic acid microsphere production and feeding

Poly(lactic-co-glycolic acid) microspheres were made by our collaborators at TherapyX Inc (Buffalo, NY). Phytanic acid was encapsulated by using a modification of the solvent evaporation technique at 10 mg phytanic acid per 1 g of microspheres [422,423]. Vehicle microspheres were not loaded with any compound. *In vitro* validation of phytanic acid release was done by suspending 100 mg of microspheres (containing a predicted 1 mg of phytanic acid) in 1 mL of phosphate-buffered saline (PBS). At 24, 48, and 72 hours the microspheres were spun down and the supernatant was collected and replaced. Subsequent analysis of the collected supernatants via high pressure liquid chromatography (HPLC) confirmed that the microspheres were releasing phytanic acid

and continued to do so for at least 72 hours **(Fig. 1)**. For each feeding, microspheres were resuspended via mechanical agitation with a miniature pestle, and the resulting solution was delivered via oral gavage using a blunt ended 22 gauge feeding needle. Each dose consisted of 80 μg phytanic acid loaded in 2 mg of microspheres suspended in 250 μL of PBS.

For the experiment determining the effect of oral administration of phytanic acid on splenic macrophage efferocytic activity, female BWF1 mice received 3 doses of phytanic acid-containing microspheres a week starting at 12 weeks of age and the spleens were harvested at 16 weeks of age. For the experiment determining the effect of oral administration of phytanic acid on splenic macrophage CD36 expression, female mice were fed vehicle or phytanic acid-containing microspheres at the doses described above. The mice were fed every other day for two weeks, then spleens were harvested and splenic macrophage CD36 expression was measured by flow cytometry as described below.

Cecal microbiota transfer

The cecal contents of a 16-week-old male or female BWF1 mice were harvested and suspended in 12.5 mL of 0.03% L-cysteine in PBS. The suspension was vortexed to suspend cecal contents and then loaded into a syringe. Mice were fed 250 μL of suspension via oral gavage using a blunt ended 22 gauge feeding needle. Cecal feedings were performed at 4 weeks of age, 5 weeks of age, and every 4 weeks thereafter.

Preparation of adherent and sorted spleen cells

For all experiments, splenic macrophages were isolated and processed in the following manner. Spleens were harvested from adult mice (12-16 weeks old) and placed in petri dishes, and mechanically diced. The diced spleens were placed in a 10 mL solution of 2 mg/mL *Clostridium histolyticum* Collagenase type VIII (Sigma #C2139), 10 KU/mL DNase (Sigma #d4527), and 10% FCS in HBSS. Spleens were digested in this solution for 30 minutes at 37^{O}C. EDTA was added (final concentration 0.2 mM) and the solution was incubated at room temperature for 5 minutes to stop the digestion. The spleen fragments were placed in a 70-micron mesh boat, mechanically pressed, and rinsed to create a single cell suspension. The cells were washed twice with FACS buffer (2% FCS in HBSS), and then resuspended in 5 mL RBC lysis buffer (Biolegend #420301). After 3 minutes in the lysis buffer, the cells were washed 2 times with FACS buffer.

For sorting macrophages, splenocytes that were disassociated as described above were suspended in FACS buffer (2% FCS in HBSS) at 50x10^{6} cells/100 μL in 1.5 mL siliconized Eppendorf tubes. Nonspecific binding to FcγRs was blocked by incubating the splenocytes with anti-CD16/32 antibody (Biolegend #101301) at room temperature for 5 minutes. They were then labeled with anti-CD11b-PE and anti-F4/80-APC (Biolegend #101208, 123115) for 20 minutes at 4^{O}C. The splenocytes were washed twice and then resuspended in FACS buffer at 40x10^{6} cells / 1 mL. A BD FACS Aria III cell sorter was used to sort CD11b^{+}F4/80^{+} splenocytes that were then used in the experiments described below.

For the adherent macrophage experiments, splenocytes were prepared as described above, then the cells were counted, and resuspended in super complete media

(SCM) and $40x10^6$ splenocytes in 3mL of SCM were placed in a 60 mm cell culture dish and incubated at 37 ^{O}C in a 5% CO2 incubator for 2 hours to select for adherent splenocytes. After 2 hours the dishes were gently but thoroughly washed 3 times with room temperature HBSS to remove the nonadherent cells, taking care that the cells did not become dry at any point. Two mL of SCM were added and these adherent splenocytes were then used for the experiments described below.

RNA sequencing and transcriptome analysis

Individual spleens from 16-week-old female BALB/c, male BWF1, and female BWF1 mice (n=3) were digested and prepared as described above. Splenocytes were counted, and $5x10^6$ cells were lysed and RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Hilden, Germany; #74134). The rRNA was depleted with the NEBNext rRNA Depletion Kit (New England Biolabs, Ipswich, MA; #E6350). Libraries were prepared using the NEBNext Ultra II RNA Library Prep Kit (NEB #E7775) and the NEBNext Multiplex Oligos for Illumina Index primers sets 1 & 2 (NEB #E7335, E7500) according to the manufacturer's directions. Size, purity, and semi-quantitation was done using an Agilent Bioanalyzer^{TM} (Santa Clara, CA) and the Agilent DNA HS Kit. Samples were diluted to 10 nM aliquots in 10mM TrisHCl, pH 8.5 supplemented with 0.1% Tween20 buffer. The wide range of concentrations amongst samples resulted in total volume of diluted samples varying from 10-50 µL. A pooled 10 nM library was made by taking 3 µL of each sample and combining them in a new LowBind 1.5 mL micro centrifuge tube for a total volume of 27 µL. The library was then diluted to 4 nM and denatured by mixing with diluted NaOH and incubating at room temperature for 5

minutes before neutralizing with 200 mM Tris-HCl, pH 7. The reaction mixture was diluted to 1.8 pM using the pre-chilled HT1 Buffer. Next, 13 µL of denatured 1.8 pM PhiX control was added to 1287 µL of denatured 1.8 pM library to a total volume of 1.3 mL. The library was loaded onto the reagent cartridge, and the library was sequenced was done on an Illumina NextSeq 500^{TM} using the NextSeq 500/550 75 Cycle High Output Kit v2.5 (Illumina, San Diego, CA #20024906). Quality control, adapter trimming, and aligning to the *Mus musculus* genome were done using FastQC, Trimmomatic, and STAR (version 2.6), respectively by the KBRIN Bioinformatics Core at the University of Louisville. Reads were normalized using the Relative Log Expression method and filtered to exclude genes with fewer than 20 counts among samples. Differential expression analysis was done with DESeq2 using a *P*-value cutoff ≤ 0.05, a *q*-value cutoff ≤ 0.05, and $\log_2$Fold Change > 0 [424].

To identify pathways enriched in healthy controls, genes upregulated in female BALB/c or male BWF1 mice compared to female BWF1 mice were functionally classified using the Gene Ontology:Biological Processes (GO:BP) database [425,426]. PANTHER (version 14) was then used to identify biological processes (*q*-value cutoff < 0.05 and fold enrichment cutoff ≥ 2) that were significantly enriched in the healthy controls relative to the lupus-prone female BWF1 mice [427-430]. The list of differentially regulated genes was also analyzed using *Metacore* from *Clarivate Analytics* to perform a transcription factor enrichment analysis, identifying transcription factors whose target genes were enriched in our set of differentially expressed genes. Statistical comparisons were done using Fisher's exact test with an FDR multiple comparison correction of 0.05. Clustvis software was used for both Principal component analysis (PCA) and cluster

analysis with heatmaps [431]. PCA of the transcriptomes was performed using singular value decomposition with imputation method. The normalized read counts of all genes differently expressed between at least two of the three groups were analyzed using hierarchical clustering and heatmap analysis. Column and row clustering distances were calculated as the Pearson correlation subtracted from one, and the linkage method used was average distance of all possible pairs.

Measurement of transcript levels by qPCR

Macrophages were sorted as described above, and RNA was extracted using an RNeasy Micro Kit (Qiagen #74004). Reverse transcription was done using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher #4368814). PowerUp ™ SYBR™ Green Master Mix (Thermo Fisher #A25742) and IDT PrimeTime qPCR primers were used to assay gene expression of murine LRP1 (Mm.PT.58.45834660 IDT), AXL (Mm.PT.58.11506780), C1QA (Mm.PT.58.5375735), and Mfge8 (Mm.PT.58.9107639) using ACTB (Mm.PT.39a.22214843.g) as the reference gene. The ΔCT for each gene was calculated using the reference gene, and Student's t-tests were used to assess statistical differences between experimental groups. The $\Delta\Delta C_T$ was calculated for each gene relative to the average ΔC_T of the control group and the fold difference relative to the control group was calculated as $2^{-\Delta\Delta CT}$ for each sample [432].

Analysis of surface marker expression in splenic macrophages by flow cytometry

Splenocytes from individual mice were prepared as described above. Nonspecific binding to FcγRs was blocked as described above for the sorting protocol and then the samples were labeled with one of two panels consisting of different combinations of antibodies. For panel 1, anti-LRP1 (rabbit IgG, Abcam Ab92544), anti-CD11b-BV421, anti-F4/80-APC, and anti-CD36-PECy7 (Biolegend 101251, 123115, and 102615) for 20 minutes at 4^{O}C. The samples were washed twice and then incubated with anti-rabbit IgG-PE (Thermo Fisher 12-4739-81) for 30 minutes at 4^{O}C. For panel 2, anti-TIMD4-PE (Biolegend 130005) and anti-MERTK-PE/Cy7 (Biolegend 130005) were used instead of anti-LRP1 and anti-CD36 and the second labeling step was skipped. The samples were washed twice and then resuspended in 300 μL FACS buffer and analyzed on a BD Fortessa^{TM} for geometric mean fluorescence intensity (MFI) of LRP1, CD36, MERTK, and TIMD4 on splenic macrophages, ($CD11b^{+}F4/80^{+}$). Fluorescence-minus-one (FMO) controls were used. Analysis of all flow cytometry data in this paper was done using FlowJo^{TM} software and statistical comparisons were done using Student's t-tests.

Assay for phagocytosis of apoptotic thymocytes (efferocytosis)

For adherent macrophage efferocytosis assays, spleens from individual mice were processed and plates of adherent splenocytes were prepared as described above. Next, $16x10^{6}$ Cell-trace Violet^{TM} (CTV)-labeled apoptotic thymocytes, prepared as described below, were suspended in 1 mL of SCM and added to the adherent macrophage plates. For each experiment, an extra adherent macrophage dish was made. No thymocytes were added to that dish and it served as a negative control. The dishes were gently swirled to

mix the cells and then were placed in a 37^{O}C, 5% CO_2 incubator for 1 hour. After 1 hour, the dishes were gently but thoroughly washed 3 times with room temperature HBSS to remove nonadherent thymocytes. Next, they were put on ice for 10 minutes to loosen the adherent cells, and then scraped and rinsed to harvest the adherent cells, which were transferred to 1.5 mL siliconized Eppendorf tubes. The samples were spun down at 3200 RPM for 3 minutes and then resuspended in 100 μL of FACS buffer. Nonspecific binding to FcγRs was blocked with anti-CD16/32 antibody (Biolegend 101301) as described above. The samples were then labeled with anti-CD11b-PE, anti-F4/80-APC, and anti-Thy1-APC/Cy7 (Biolegend 101208, 123115, and 105328) for 20 minutes at 4^{O}C. The samples were washed twice and then resuspended in 300 μL FACS buffer and analyzed on a BD Fortessa^{TM} for the percentage of splenic macrophages, (F4/80^{+}CD11b^{+}) that were positive for the CTV label, indicating that they were in the process of phagocytosis apoptotic thymocytes. The well that received no thymocytes served as a negative control.

CTV-labeled apoptotic thymocytes were prepared in the following manner. Thymi were harvested from 4 to 8-week-old C57/BL mice, placed in a 70-micron mesh boat, mechanically pressed, and rinsed with FACS buffer to create a single cell suspension. This was spun down at 1500 RPM for 5 minutes, then resuspended in 3 mL RBC lysis buffer (Biolegend 420301). After 3 minutes, the suspension was washed 2 times with FACS buffer and counted, then resuspended in SCM+β-ME (SCM with 0.1% β-mercapto-ethanol (Sigma #M3148)) at 20x10^{6} cells/mL. An equal volume of 2 μM dexamethasone (Sigma #D4902) in SCM was prepared and added to the thymocyte suspension, placed in a petri dish, and then placed in a 37^{O}C, 5% CO_2 incubator for 16 hours. After 16 hours, the suspension was washed 2 times with PBS and then

resuspended in room-temperature PBS to 40x10^6 cells/mL in a 15 mL conical, and the cells were labeled with Cell-trace Violet™ (Thermo Fisher #C34557). A 2 μM solution of CTV in room-temperature PBS solution was prepared by adding 0.4 μL of 5 mM stock per 1 mL of room temperature PBS and mixing thoroughly. Immediately after making the CTV solution, an equivalent volume was added to the thymocyte suspension for a final concentration of 1 μM and the mixture was vortexed thoroughly, then incubated at room temperature in the dark for 20 minutes. The cells were washed 3 times with FACs buffer, counted, and resuspended in SCM at $8x10^6$ cells/mL. To verify the thymocytes were apoptotic and CTV-labeled, a 100 μL aliquot was taken, 5 μL of AnnexinV-FITC (Biolegend 640945), and 5 μL of 7-AAD (BD Biosciences 559925) was added and the aliquot was incubated at room temperature in the dark for 15 minutes. The aliquot was then diluted to 300 μL with FACS buffer and analyzed in a BD Fortessa™ for apoptosis (AnnexinV and 7-AAD) and CTV label.

For assays of sorted splenic macrophage efferocytic activity, spleens from individual mice were digested and macrophages were sorted as described above and resuspended in SCM. For each sample, 200,000 (0.5 mL) of the sorted macrophages were put in a separate well of a 24 well plate. CTV-labeled apoptotic thymocytes were prepared as described above and to each experimental well, $2x10^6$ CTV-labeled apoptotic thymocytes in 0.25 mL of SCM were added. An additional control well had macrophages but no thymocytes. The plate was gently swirled to mix the cells and then was placed in a 37^OC, 5% CO_2 incubator for 1 hour. The plate was then placed on ice for 5 minutes, and the wells were scraped and rinsed to harvest the cells, which were transferred to 1.5 mL siliconized Eppendorf tubes. Samples were then labeled for F4/80, CD11b, and Thy1 and

analyzed for the percentage of splenic macrophages ($F4/80^{+}CD11b^{+}$) that were positive for the CTV label, as described above.

Assays of the effect of phytanic acid treatment on splenic macrophages

For analysis of the effect of phytanic acid treatment on splenic macrophage marker expression, spleens from individual mice were digested and processed as described above, and suspended in super complete media (SCM). SCM is RPMI 1640 (Sigma E#7509) supplemented with 1000 U/mL penicillin/streptomycin, 2 mM L-glutamine (Corning #25-005-CI), 1% Non-essential amino acids (Gibco #11150-060), 1 mM Sodium Pyruvate (HyClone #SH30239.01), 10 mM HEPES (Corning #25-060-CI), and 10% FCS. Two $5x10^{6}$ cell aliquots from each sample were put in the wells of a 24-well plate, and either phytanic acid (final concentration = 50 μM) or vehicle (ethanol, final concentration = 0.06% v/v) was added. The plates were placed in a $37^{O}C$, 5% CO_2 incubator for 24 hours. Next, the plates were placed on ice for 5 minutes, and then the wells were scraped and rinsed to harvest the cells. Staining for macrophage markers and analysis was done as described above.

For the experiments examining the effect of phytanic acid on adherent female BWF1 macrophage efferocytosis, the adherent macrophages were prepared as described before, with the exception that spleens were pooled, and after the 2-hour incubation and removal of non-adherent cells, SCM containing 50 μM phytanic acid was added to the dish, and the cells were placed back in the incubator for an additional 24 hours. After 24 hours, the phagocytosis assay proceeded as described above.

For the experiments examining the effect of phytanic acid on sorted BWF1 macrophage efferocytosis, spleens were pooled and digested and macrophages were sorted as described above. Sorted macrophages were then resuspended in SCM containing, vehicle (ethanol, final concentration = 0.06% v/v), 50 μM phytanic acid (Cayman 90360), a nuclear receptor inhibitor. Macrophages were divided into 200,000 cells/0.5 mL aliquots and placed in a 24 well plate and incubated at 37^{O}C at 5% CO_2 for 48 hours. After 48 hours, the phagocytosis assay proceeded as described above.

Assays for effect of nuclear receptor inhibition on efferocytic activity

Spleens from adult female BWF1 mice were pooled and digested as described above. Macrophages were sorted, split into two groups, and resuspended in SCM containing, vehicle (ethanol, final concentration = 0.06% v/v), 50 μM phytanic acid (Cayman 90360), a nuclear receptor inhibitor. Macrophages were divided into 200,000 cells/0.5 mL aliquots and placed in a 24 well plate and incubated at 37^{O}C at 5% CO_2 for 48 hours. After 48 hours, the phagocytosis assay proceeded as described above. The following concentrations of nuclear receptor inhibitors were used: The RXR inhibitor, HX531 (Sigma SML2170), was used at a concentration of 10 μM. The PPARγ inhibitor, GW9662 (Sigma M6191), was used at a concentration of 10 μM. The LXR inhibitor, GSK2033 (Sigma SML1617), was used at a concentration of 5 μM. The PPARδ inhibitor, GSK0660 (Sigma G5797), was used at a concentration of 10 μM.

Assays for the effect of CD36 inhibition on macrophage efferocytic activity

The CD36 neutralizing antibody was validated with a titration experiment where macrophages were sorted as described above and treated with 0.5, 1, or 2 μg/mL of anti-CD36 for 30 minutes (Clone CRF D-2712, BD Biosciences #552544) [372]. Macrophages were then cultured with CTV-labeled apoptotic thymocytes for one hour and the efferocytosis assay proceeded as described above. A dose-dependent reduction in efferocytosis after anti-CD36 treatment was observed **(Fig. 2)**

For the phytanic acid and anti-CD36 co-treatment experiment, sorted macrophages were treated for 48 hours with 50 μM phytanic acid. Macrophages were then treated with 2 μg/mL of anti-CD36 for 30 minutes. CTV-labeled apoptotic thymocytes were then added for one hour and the efferocytosis assay proceeded as described above.

Statistical analyses

Statistical analysis of disease scores was performed using two-way ANOVA and two-tailed Student's *t*-tests. Statistical analyses of incidence and survival curves were performed using the Log rank (Mantel-Cox) test. For Intra- and inter-group microbiota Bray-Curtis beta diversity distances were compared using PERMANOVA statistical analyses [418]. The inter-group differences in the abundances of specific taxa were analyzed with two-way ANOVAs with corrections for multiple comparisons. For comparisons of metabolite abundance, metabolites were filtered for those that had an abundance change of over 20% between two groups and were present in more than 75% of the samples for at least one group. Student's *t*-tests with sample permutation were used

to determine if the abundance change was significant. Grubbs' test was used to detect and remove outliers. For comparisons of gene expression as measured by flow cytometry or qPCR, Student's *t*-tests were used. For comparisons of efferocytic activity as measured by fold change %CTV^+ macrophages, statistical comparisons between groups were performed using Student's t-tests if only two groups were compared. If three or more groups were compared, then one-way ANOVAs with multiple comparisons were performed, using the Tukey post-analysis method to correct for multiple comparisons. For comparing expression of genes by qPCR or flow cytometry, Student's *t*-tests were used to assess statistical differences between experimental groups.

GraphPad Prism 9 (Sand Diego, CA) was used for all statistical analysis unless stated otherwise. All error bars shown are +/- standard error of the mean. A cutoff of 0.05 was used for significance for analyses, and significance was indicated as follows: $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$

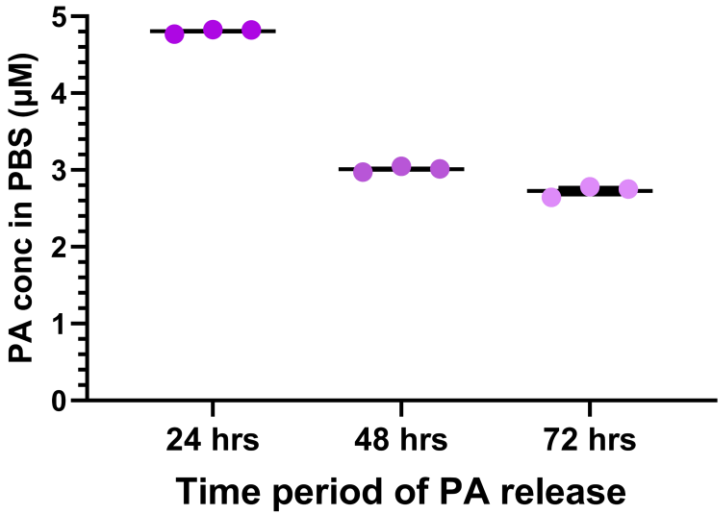


Figure 1. Validation of Phytanic acid release by microspheres. Phytanic acid was encapsulated in poly(lactic-co-glycolic acid) microspheres at 10 mg phytanic acid per 1 g of microspheres. Release of phytanic acid was assayed by suspending 100 mg of microspheres in 1 mL of PBS and incubated at 37 ^{O}C. At 24, 48, and 72 hours the microspheres were spun down and the supernatant was collected and replaced. Phytanic acid levels in the supernatants were measured by HPLC (n=3).

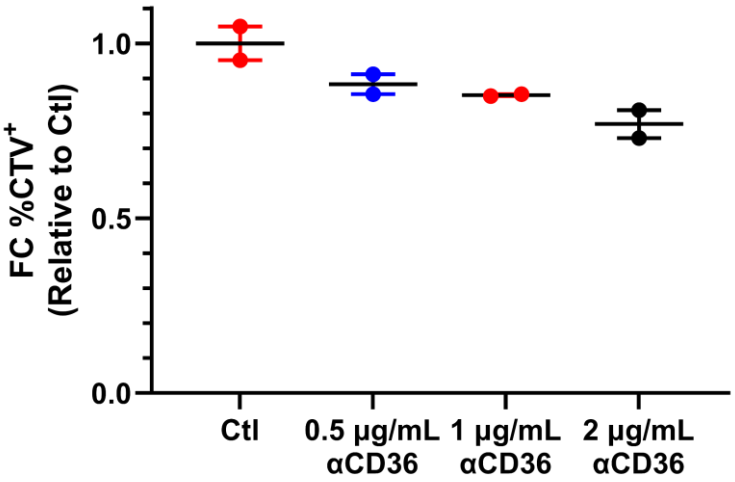


Figure 2 Blocking CD36 with a neutralizing antibody decreases splenic macrophage phagocytosis in a dose-dependent manner. Splenocytes from 16-week-old female BWF1 mice were collected and sorted for $CD11b^{+}F4/80^{+}$ cells by FACS. Sorted cells were cultured without (Ctl) or with varying concentrations of blocking anti-CD36 antibody for 30 mins then phagocytosis of apoptotic cells was assayed by culture with CellTraceTM Violet (CTV)-labeled apoptotic thymocytes for one hour followed by analysis of percentages of $CD11b^{+}F4/80^{+}$ cells that were CTV^{+}. Data are reported as fold change (FC) in $\%CTV^{+}$ cells (n=2).

SPECIFIC AIM 1

Sex-based differences in gut microbiota composition associated with protection from lupus

Introduction

Like many autoimmune diseases, systemic lupus erythematosus (SLE) has a significant sex bias, with women comprising over 90% of SLE patients with autoimmune diseases [1,2]. This sex bias is also present in the NZBxNZWF1 (BWF1) mouse model of lupus. Female mice develop severe disease early in life, often having elevated autoantibodies at 16 weeks of age and beginning to develop glomerulonephritis between 20 to 25 weeks of age; virtually all female mice die of this disease by 50 weeks of age. Male BWF1 mice on the other hand only rarely develop a mild form of disease late in life [16]. Male resistance to disease is androgen dependent, as males who undergo androgen depletion by castration develop more severe disease at a younger age [17]. This is also similar to what is seen in humans, as female and male SLE patients have decreased levels of androgens, and treatment with androgens can suppress disease activity [115,138,141-144,149-152]. The BWF1 mouse strain is an excellent model of lupus particularly for the study of mechanisms underlying the sex bias in lupus.

Sex differences in the commensal bacteria in the intestine could potentially contribute to the sex bias of lupus in humans and in lupus-prone mice. A complex

network of interactions exists between these bacteria, called the gut microbiota, and the immune system. These interactions shape both microbiota composition and immune responses and they are vital for the proper development and function of the immune system [181-187]. When the microbiota is disrupted or imbalanced, it is said to be in dysbiosis. A dysbiotic microbiota can have a pro-inflammatory effect on the immune system and promote the development of autoimmune diseases [184,218,219].

Many autoimmune diseases have been linked to dysbiotic microbiota. Patients with inflammatory bowel disease (IBD) have been found to have dysbiotic microbiota [225-235]. No clear causal links between alterations in bacterial taxa and disease have been established in human IBD, but mouse models have shed some light on possible mechanisms. *Bacteroides fragilis* and some *Clostridia* strains have been shown to suppress IBD in mice by promoting Treg induction [433-435]. On the other hand, *Helicobacter muridarum*, *Helicobacter hepaticus*, and segmented filamentous bacteria can exacerbate disease by promoting pro-inflammatory activity in Th17 cells [250-252].

Non-intestinal autoimmune diseases such as rheumatoid arthritis can also be affected by the microbiota [184,256,257]. SLE is a systemic disease that has been linked to dysbiotic microbiota. The microbiota of SLE patients differ significantly from healthy controls [276-283]. While the specific taxa altered in SLE patients vary from study to study, their findings have still increased our understanding of how the gut microbiota can affect disease progression. For example, *Ruminococcus gnavus* abundance was five times higher in SLE patients, and higher levels of antibodies against *R. gnavus* correlated with overall antibody levels and kidney damage [276]. This suggests that *R. gnavus* may stimulate immune responses that increase SLE activity. Another study found SLE

patients with *Enterococcus gallinarum* in the liver had higher levels of autoantibodies. In further experiments using the lupus-prone NxB F1 mouse strain, *E. gallinarum* stimulated disease by translocating across the intestinal barrier to the liver and stimulating pro-inflammatory cytokine production [286]. Lupus-prone B6.Sle1.Sle2.Sle3 mice were also found to exhibit dysbiosis, with higher levels of *Prevotellaceae*, *Paraprevotella*, and *Lactobacillus* compared to healthy controls. Cohousing the B6.Sle1.Sle2.Sle3 mice with the healthy control mice reduced autoantibody production, suggesting that the differences in microbiota could affect processes involved in disease [293]. A study of the MRL/lpr lupus mouse model had a similar finding, as cecal transfers from healthy control MRL mice decreased autoantibody production [297]. The MRL/*lpr* study found that *Lactobacillus*, which was enriched in healthy controls, protected against disease, but studies in SLE patients and BWF1 mice found that higher *Lactobacillus* was associated with more severe lupus [282,297,299]. This demonstrates that the effect of a given taxa of microbiota is highly dependent on the overall context of the microbiota.

Sex and sex hormone levels also significantly affect the microbiota, suggesting that sex-based differences in microbiota function could potentially contribute to the sex bias of lupus [182,300-303,315]. Recently, two studies found evidence to support this theory. In the NOD mouse model of type 1 diabetes (T1D), female mice are more susceptible and male mice are more resistant to disease. However, the studies found that male mice possessed a distinct microbiota that conferred this protection in an androgen-dependent manner [323,324]. Also, a recently published paper on the SWRxNZB F1 (SNF1) mouse model of lupus found differences in microbiota composition between

intact male, androgen-depleted male, and female mice. Kidney disease wasn't affected by these sex-based microbiota changes, but it also wasn't affected by androgen depletion (i.e., castration), suggesting that their model may not have been effective at investigating sex differences [325]. Nevertheless, these studies suggest that sex-based differences in microbiota could influence immune functions associated with lupus progression.

Previous studies have found microbiota from healthy control female mice can suppress disease in lupus-prone female mice, but none have shown the same effect between male and female mice of a lupus-prone strain. The goal of the present study was to investigate differences in the gut microbiota composition in female and male BWF1 mice and their impact on disease. We have shown that transferring microbiota from disease-resistant BWF1 mice into female BWF1 mice delays disease onset and improves survival. However, changes in mouse facilities resulted in a period of time where the transfer of male microbiota was no longer protective. The protective phenotype was eventually restored, and we have used this opportunity to compare the sex differences in the microbiota between periods when male microbiota was protective and periods when it was not, with the goal of identifying what male-enriched species are associated with protection against lupus.

Results

Disease susceptibility and microbiota composition in the BWF1 mouse model of lupus

The findings of recent studies suggested sex differences in the microbiota might contribute to the lupus sex bias in BWF1 mice [16,323,324]. To investigate this, our lab

first examined microbiota composition in female and male BWF1 mice as well as confirmed that disease incidence and progression in our colony is accelerated and more severe in female than male BWF1 mice as has been previously reported [16,17]. As expected, the incidence and level of glomerulonephritis severity were significantly higher in females, and survival was significantly decreased **(Fig. 3A-C)**. We analyzed the bacterial composition of feces from female and male BWF1 mice to compare the intestinal microbiota. We used Bray-Curtis analysis of beta-diversity to analyze how different the microbiota composition of each sample was from the other samples. The principal coordinate analysis (PCoA) visualization of this analysis showed that the microbiota profiles formed two distinct groups based on sex **(Fig. 4A)**. Permutational multivariate analysis of variance (PERMANOVA) of the beta-diversity values confirmed the female and male BWF1 gut microbiota profiles were significantly different **(Fig. 4B)**. This showed that in addition sex-based differences in disease, there were also sex-based differences in the microbiota in BWF1 mice. As with human SLE patients, androgens suppress disease in BWF1 mice, and depleting androgens in male BWF1 mice makes them more susceptible to disease [133,134]. Castrated male BWF1 mice have significantly increased incidence and severity of glomerulonephritis and a dramatic decrease in survival **(Fig. 5;** [420]**)**. Comparison of intact and castrated male BWF1 microbiota showed that androgen depletion caused a major shift in the microbiota profile **(Fig. 6)**. These results showed that lupus-susceptible female and castrated male BWF1 mice have divergent microbiota profiles from lupus-resistant intact male BWF1 mice.

In order to investigate whether the sex-based differences in the microbiota could affect lupus progression, our laboratory carried out microbiota transfer experiments,

where BWF1 mice were fed cecal contents from female or male BWF1 mice **(Fig. 7A)**. Since dysbiotic microbiomes have been linked to autoimmune diseases [184,218,219], our lab tested whether the female BWF1 microbiota could promote lupus progression in male recipients. Transfer of cecal contents from female BWF1 mice did not increase disease in male BWF1 mice (data not shown). Interestingly, when the opposite experiment was performed, and male microbiota was transferred via cecal contents to female BWF1 recipients, the transfer of male microbiota significantly delayed disease onset and improved survival in female BWF1 mice **(Fig. 7B-D).** Our lab has also found that this protective effect is androgen dependent, as shown in experiments where microbiota from castrated male mice transferred into female mice had no protective effect on disease incidence or progression, or survival (data not shown). Taken together, these data indicate that female and male gut microbiota differ in composition and the male microbiota may contain bacterial populations that can protect against lupus.

Alterations in mouse facilities/animal husbandry procedures resulted in changes in the ability of male BWF1 microbiota to suppress lupus progression in female BWF1 mice and in microbiota composition.

The disease progression, and initial microbiota analysis and transfer experiments in BWF1 mice described above were performed in our animal facility in the A Tower building on the University of Louisville campus from 2014 through late 2016. However, in January 2017, due to circumstances beyond our control, our mouse colony was moved to the barrier animal facility in the Center for Translational Research Building (CTRB). We first monitored disease in female and male BWF1 mice produced in the CTRB

facility, and found no differences in disease kinetics, severity, or incidence, or mortality in either sex compared to our colony in the A Tower facility, i.e., female BWF1 mice exhibited accelerated and severe glomerulonephritis and increased mortality by comparison to male BWF1 mice (data not shown). We also conducted experiments to confirm that male microbiota transferred into female BWF1 mice delayed disease, and decreased severity and mortality in CTRB as we had found in the A Tower. We conducted three microbiota transfer experiments between January 2017 and Fall 2018 (designated the "CTRB Early" period), but were unable to replicate the protective phenotype that we had seen in the A Tower. We found no difference in the onset and progression of glomerulonephritis between female BWF1 mice receiving male microbiota and those receiving female microbiota **(Fig. 8A,B)**. Survival was also not different between these two groups of recipients **(Fig. 8C)**. A Bray-Curtis analysis comparing microbiota compositions in feces collected from female and male BWF1 mice from the A Tower and the CTRB Early period showed that the microbiota compositions differed between the A Tower and the CTRB Early mouse colonies **(Fig. 9A).** Analysis of Bray-Curtis beta-diversity distances between female and male profiles from the CTRB Early colony found that they were only slightly statistically different (**Fig. 9B;** p=0.043) by comparison to the differences found between female and male microbiota composition found in the A Tower **(Fig. 4;** p=0.001**)**. This suggests that not only did the microbiota profiles differ between the A Tower and the CTRB Early period, but also that the CTRB Early female and male microbiota compositions are less different from each other than female and male compositions were in the A Tower facility.

To attempt to recover our protective transfer phenotype, several changes were made in Fall 2018 to the animal husbandry of our mice in the CTRB facility that included a change in animal room (we did not actually have a choice in this, since we were moved out of the room that eventually became part of the new germ-free facility), a change in disinfecting protocols used by our animal care technician, and a change in water source, from either autoclaved tap water or water purified by sterile filtration to water purified by reverse osmosis (RO) then autoclaved directly in the water bottles. Although we cannot conclude with any certainty which if any of these changes had an effect, we have found that since these changes were implemented, transfers of male microbiota again consistently suppress disease in female BWF1 recipients. In microbiota transfer experiments performed during this more recent time period in the CTRB (designated "CTRB Recent" and covering the period of 2019 to present), disease onset is significantly delayed and incidence is decreased, and survival is increased in female BWF1 recipients of male microbiota compared to recipients of female microbiota **(Fig. 10)**. Additionally, Bray-Curtis analysis of beta-diversity shows that the female and male microbiota profiles form distinct groups based on sex **(Fig. 11A)**, and are again highly significantly different **(Fig. 11B; p=0.001)**. Taken together, our preliminary analysis strongly suggests that the microbiota populations were altered after the move from the A Tower (A Tower - 2014-2016) to the CTRB (CTRB Early - 2017-2018) and then again after the modification of the animal husbandry practices in the CTRB (CTRB Recent - 2019-present) and these changes correlated with changes in the protective phenotype we observed in our male microbiota transfer experiments. These observations presented us with the opportunity to

explore whether and which sex-based differences in commensal abundance at the genus/species level are associated with protection from disease.

The change in mouse facilities/animal husbandry procedures resulted in genus and species level changes in the male and female BWF1 microbiota

We first analyzed female and male BWF1 microbiota down to the genus and species levels in feces collected shortly before the move from the A Tower (2016) and in the months following the move to the CTRB (CTRB Early - 2017-2018). Our findings showed that the change in facilities had a dramatic and destabilizing impact on the composition of the microbiota. In the A Tower, *Bacteroides* abundance, in general, was high (~20-30%) and significantly higher in adult male BWF1 mice than in adult female BWF1 mice **(Fig. 12A)**. However, this was not the case in the CTRB Early experiments. In three of the CTRB Early experiments, *Bacteroides* levels were almost undetectable in both female and male BWF1 mice **(Fig. 13A,C,E)**. Moreover, the levels of *Clostridium* abundance in the CTRB Early experiments (~40-60%) were three to four times greater than was seen in the A Tower **(**~15-20%; **Fig. 12A; 13A,C,E)**. In a fourth CTRB Early experiment, *Bacteroides* was present, but was actually higher in female than male BWF1 mice, and *Clostridium* was still considerably higher than had been seen in the A Tower **(Fig. 12A; 14A)**. This experiment (CTRB Early Exp 4) took place four months after the first CTRB Early experiment (Exp 1) and two months after CTRB Early Experiments 2 and 3, so the wildly disparate levels of *Bacteroides* abundance between CTRB Early Experiments 1-3 and CTRB Early Experiment 4 could indicate that the microbiota composition of the mice was still in flux, although it should be noted that the *Clostridium*

levels remained very high in all four CTRB Early experiments. The microbiota analysis also identified another genus, *Alistipes* (*Alistipes putredinis* and *Alistipes timonensis*), that was present in A Tower, but was undetectable in the CTRB Early experiments **(Fig. 12-14)**. Species analysis of the microbiota showed that *Bacteroides acidifaciens* and *Clostridium leptum* were the predominant *Bacteroides* and *Clostridium* species, respectively. These species, when present, exhibited the same patterns as their genera in both the A Tower and CTRB Early experiments **(Fig. 12B; 13B,D,F; 14B)**. Clearly the change in facilities and the loss of the ability of male microbiota to suppress lupus coincided with significant alterations, both decreases and increases, in the relative abundances of bacteria in the female and male mice.

Mice microbiota compositions are affected by how their drinking water is sterilized.

During the CTRB Early period, experiments were conducted to evaluate the effect of different drinking water sources on the microbiota. Female and male BWF1 mice received either tap water autoclaved in bottles (Autoclaved Tap H2O; this was the same water that was provided to mice in the A Tower) or sterile filtered (but not de-ionized) water (Filtered H_2O) from birth through the end of the experiment. Feces were collected and mice were monitored for disease incidence and severity, and survival. There were no differences in disease or mortality for either the female or male mice compared to the A Tower (data not shown). A side-by-side comparison shows there were subtle differences in genus/species abundance that were dependent on the water source. This experiment showed that changes in environmental (e.g., water source) and genetic factors (e.g., sex) affect different bacterial taxa differently. *Lachnoanaerobaculum* abundance (primarily,

the *L. umeaense* species) was affected by the water source, but less so by sex. Conversely, *Lactobacillus* abundance (primarily the *L. gasseri* and *johnsonii* species) was affected by the sex of the mice but not the water source. *Clostridium* abundance was affected by the water source and by the sex at the genus level **(Fig. 15A)**, but the predominant species, *C. leptum*, was only affected by the water source **(Fig. 15B)**. These data highlight the complexities and challenges of studying the gut microbiota and how changes in mouse facility conditions can alter microbiota compositions.

During the CTRB Recent period, *Bacteroides* and *Clostridium* abundances returned to levels similar to the A Tower

As described above, we had found that the loss of the protective phenotype of the male microbiota during the CTRB Early period (2017-2018) corresponded to decreases or absence of both *Bacteroides* and *Alistipes* abundances and/or an increased abundance of *Clostridium*, in general, by comparison to the A Tower. We next turned to the in-depth analysis of the microbiota in the feces collected from mice during the CTRB Recent period (2019-2020) when the protective transfer phenotype was re-established in the CTRB **(Fig. 10)**. We found that the CTRB Recent period coincided with another shift of the microbiota composition to one that more resembled that seen in the A Tower. In two experiments (CTRB Recent Experiments 1 & 2) conducted during the CTRB Recent time period, *Bacteroides* **(Fig. 16A,C)**, particularly the *Bacteroides acidifaciens* species **(Fig. 16B,D)**, is once again present and at high levels, and was found at consistently greater abundance in male compared to female BWF1 mice. Also, the levels of *Clostridium* **(Fig. 16A,C)**, again particularly *C. leptum* **(Fig. 16B,D)**, are back down to levels at or even

below (~5-10%) those seen in the A Tower in both female and male BWF1 mice compared to the considerably elevated levels (40-60%) found during the CTRB Early period. Additionally, *Alistipes*, which had been present in the A Tower, but undetectable in the CTRB Early experiments **(Fig. 12; 13; 14)**, was detected in all of the CTRB Recent experiments **(Fig. 16)**. The same species of *Alistipes* that were predominant in the A Tower, *A. putredinis* and *A. timonensis*, were also predominant in the CTRB Recent experiments. **(Fig. 12B; 16B,D)**.

Interestingly, the high levels of *Bacteroides* (and also the *B. acidifaciens* species) found in male BWF1 mice appeared to be androgen-dependent. In experiments also conducted during the CTRB Recent period, we found that when androgen was depleted by castration, the abundance of *Bacteroides* was significantly decreased, although still present at detectable levels. In addition, as was found in the experiments comparing microbiota composition in female and male BWF1 mice described above, both castrated and intact male mice had relatively low *Clostridium* levels in comparison to the CTRB Early period and also had detectable levels of *Alistipes*. **(Fig. 17)**.

In summary, *Bacteroides* was consistently present and levels of *Bacteroides* abundance were consistently higher in males compared to females during both the A Tower and CTRB Recent periods (when the protective transfer experiments were working) and this was mostly due to differences in the *B. acidifaciens* species. Conversely, during the CTRB early period (when the protective transfer experiments were not working), *Bacteroides* levels were either barely detectable in female and male BWF1 mice or significantly lower in females compared to males. Furthermore, *Clostridium* (primarily *C. leptum*) levels were much higher during the CTRB Early

period than either the A Tower and CTRB Recent periods. While the same *Alistipes* species, *A. timonensis* and *A. putredinis* were predominant in mice during the A Tower and CTRB Recent periods, no members of the genus Alistipes were detectable in mice during the CTRB Early period. The shift in microbiota profiles that coincided with the change in our mouse facility from the A Tower to the CTRB had reverted during the CTRB recent period to be more similar to the profiles seen in the A Tower.

Protection from disease by male to female microbiota transfer correlates with elevated levels of *Bacteroides* in the recipients

The fact that male BWF1 microbiota can be protective suggests that the bacteria that are more abundant in male BWF1 mice may be acting to suppress disease. The only taxa consistently enriched in male BWF1 mice during the A Tower and CTRB Recent periods, periods during which the male microbiota protected against disease **(Fig. 7; 10)**, were the genus Bacteroides and in particular, the species *Bacteroides acidifaciens* **(Fig. 12; 16)**. This makes *Bacteroides* a possible candidate for a mediator of male microbiota disease suppression. However, if increased *Bacteroides* does promote protection against disease, then suppression of disease in female recipients of male microbiota should correlate with the presence of high levels of *Bacteroides* (received via transfer of cecal contents). To evaluate this possibility, we performed an in-depth analysis of microbiota data from microbiota transfer experiments conducted during the CTRB Early period (when transfer was not protective) and the CTRB Recent period (when transfer mediated protection was reestablished). We saw several of the same differences in microbiota abundances between the CTRB Early and Recent microbiota transfer experiments that we

saw between the CTRB Early and Recent experiments comparing female and male BWF1 mice. In the CTRB Early cecal transfer experiment, where male microbiota failed to protect **(Fig. 8)**, *Bacteroides* levels (which were predominantly the *Bacteroides acidifaciens* species) were actually high in all of the groups, but they were lower in female recipients of male microbiota compared to recipients of female microbiota **(Fig. 18)**. However, similar to the microbiota profiles female and male mice in the four CTRB Early experiments described above **(Fig. 13; 14)**, all of the groups had very high levels of *Clostridium* (30-40%), and undetectable levels of *Alistipes* **(Fig. 18)**. In contrast, analysis of a microbiota transfer experiment from the CTRB Recent period found that recipients of male microbiota had significantly higher *Bacteroides* abundances compared to the female BWF1 recipients of female microbiota **(Fig. 19A)**. The levels of *Clostridium* were considerably lower for all groups (10-20%) in comparison to the levels found in CTRB Early transfer experiment (30-40%) **(Fig. 18A; 19A)**. Furthermore, unlike the CTRB Early period when *Alistipes* was completely absent **(Fig. 13; 14)**, *Alistipes* was detected in all recipients during the CTRB Recent period and the same species were predominant as in the A Tower female and male mice (*A*. *timonensis* and *A*. *putredinis*). **(Fig. 19B)**. This microbiota profile in the CTRB Recent cecal transfer experiment was highly stable, as we tested the microbiota composition again 4 weeks later and found the same higher levels of *Bacteroides* in the recipients of male microbiota as well as lower levels of *Clostridium* and detectable *Alistipes* in all groups **(Fig. 19C)**. Taken together, these data show that higher *Bacteroides* and lower *Clostridium* levels consistently correlated with delay and decrease in severity of disease and increase in survival. Additionally, species-level analysis of the CTRB Early and CTRB Recent transfer experiments showed that as

in the other experiments, *Bacteroides acidifaciens* and *Clostridium leptum* comprised almost all of the *Bacteroides* and *Clostridium* present, respectively **(Fig. 19B,D)**.

In conclusion, during periods when transfers of male microbiota were shown to suppress lupus in female recipients (A Tower and CTRB Recent), *Bacteroides* was consistently present and more abundant in male than female BWF1 mice **(Figs. 16; 20A)**. *Bacteroides* also correlated with protection in the microbiota transfer experiments, as female recipients of male microbiota that were protected from disease (CTRB Recent) had elevated levels of *Bacteroides* compared to female recipients of female microbiota **(Figs. 19; 21)**. This suggests that *Bacteroides* may play a role in suppressing lupus progression. Failure to protect also correlated with high abundance of *Clostridium* in both female and male mice and in the microbiota transfer recipients during the CTRB Early period **(Figs. 13; 14; 18)** compared to the A Tower **(Fig. 12)** and CTRB Recent Experiments **(Figs. 16; 19; 20; 21)**. The fact that higher levels of *Clostridium* correlate with a failure of the male microbiota to protect against disease raises the possibility that an overabundance of *Clostridium* might interfere with the disease-suppressing activities of other bacteria, and that the ratio of *Bacteroides* to *Clostridium* may be critical. We calculated the *Bacteroides/Clostridium* ratio, and found that during periods when the male microbiota was protective the *Bacteroides*/*Clostridium* ratio was higher in male than female BWF1 mice. **(Fig. 22A)**. Additionally, when we compared the CTRB Early and Recent transfer experiments, the *Bacteroides*/*Clostridium* ratio differed little in the female recipients of female microbiota, but for recipients of male microbiota, the *Bacteroides*/*Clostridium* ratio was much higher in the CTRB Recent transfer experiment **(Fig. 22B)**. In addition, *Alistipes* was present in BWF1 mouse microbiota during periods

(i.e., A Tower and CTRB Recent) when the male microbiota protected against disease when transferred into female recipient, and absent during the period when the male microbiota failed to protect (i.e., CTRB Early). Taken together, these data suggest that there might be some type of cooperation or collaboration occurring between several bacterial species in the gut community that confer protection that can only occur when other species are in low abundance.

Discussion

One new paradigm in autoimmune disease that has emerged in recent years is the ability of the microbiota to significantly affect immune functions. The intestinal microbiota is significantly affected by immune activities and it can significantly affect the immune system via a variety of mechanisms [218,436]. When a microbiome has been disrupted, it is said to be in dysbiosis, and patients with autoimmune diseases have been found to exhibit dysbiotic microbiomes [184,256,257,276-280,282,283] as well as altered metabolite production [333-336,348,360-362]. This includes SLE, as many studies have found the intestinal microbiota of SLE patients differs significantly from those of healthy controls [276-283,437]. The microbiota of lupus prone mouse strains like B6.*Sle1*.*Sle2*.*Sle3* and BWF1 has also been found to differ from sex-matched healthy control strains [282,289]. However, despite the fact that BWF1 mice exhibit a similar sex bias as human SLE patients [16], the microbiota of female and male BWF1 mice had not been compared until our lab found that lupus-susceptible female BWF1 mice and lupus-resistant male BWF1 mice had divergent microbiota compositions (manuscript in preparation). Our lab has also found that transferring adult male BWF1 microbiota into

female BWF1 mice suppressed the progression of lupus (manuscript in preparation). Our lab has repeated the finding that transfer of male microbiota suppresses disease in several experiments, and we have since performed new microbiota analysis experiments confirming that female and male BWF1 microbiota composition differs. The initial experiments were performed during the period of 2014 to 2016, while our mouse colony was located in the A Tower. At the end of 2016, however, we moved our mouse colony to a different facility on the University of Louisville campus, the barrier facility located in the CTR Building (CTRB), and we observed that, in experiments begun after that point, transferring male microbiota to female BWF1 mice no longer suppressed disease. This period, which we have termed the CTRB Early period, lasted from early 2017 to 2018. When we compared female and male BWF1 microbiota from the A Tower period (2014-2016) and the CTRB Early period (2017-2018), we saw a clear difference between the two periods. Additionally, while the female and male BWF1 microbiota from the CTRB Early period were still significantly different, the degree of significance was considerably less than had been seen in the A Tower. Later, following changes in mouse facilities and animal husbandry procedures in the CTRB facility, the protective ability of male microbiota was restored. During this later period, termed the CTRB Recent period (2019-present), the male and female microbiota profiles were again highly significantly different. **Figure 23** provides a timeline of microbiota composition, microbiota transfer results and animal facility/husbandry changes. Comparison of CTRB Early Experiments 2 and 3, which were contemporaneous but received water that had been purified using different methods, showed that sterilization using filtering or autoclaving techniques had some effect on the abundance of microbiota taxa illustrating the sensitivity of the

microbiota to environmental changes. In the time between the CTRB Early and CTRB Recent periods, one of the changes we made was a switch to water that was purified by reverse osmosis (RO) and also autoclaved. This and other changes that were made to the animal housing and husbandry in the CTRB very likely have had a significant impact on the microbiota composition, including making it more stable.

Our first aim centered around investigating what changes in microbiota composition correlated with the loss of the protective ability of the male microbiota to suppress disease in female recipients. During the A tower period, *Bacteroides* (predominantly *B*. *acidifaciens*) abundance was significantly higher in male than female BWF1 mice. This is similar to what has been found in patients with autoimmune rheumatoid arthritis. Multiple studies have shown that healthy controls have significantly higher abundance of *Bacteroides* than rheumatoid arthritis patients [269,438]. However, in the CTRB Early experiments, male BWF1 mice did not have higher *Bacteroides* abundance, and in fact, in CTRB Early Experiments 1, 2, and 3, *Bacteroides* levels were almost undetectable, although in CTRB Early Experiment 4, *Bacteroides* was higher in female than male BWF1 mice. The samples from CTRB Early Experiment 1 were collected 2 months before the samples for CTRB Early Experiments 2 and 3 were collected and 4 months before the samples from CTRB Early Experiment 4. For the levels of *Bacteroides* to change so drastically in mice from the same colony in such a short span of time suggests that the microbiota profiles were in a state of flux. Together with the differences found between the A Tower and CTRB Early microbiota profiles, the data indicate that moving the mouse colony and setting up at the new facility resulted in significant changes to the microbiota. Another change that highlighted the differences

between the A Tower and CTRB Early mice was the disappearance of the entire *Alistipes* genus. Whereas in the A Tower the *Alistipes* abundance was ~10-20%, it was completely undetectable in the microbiota of all CTRB Early experiments. Additionally, in the CTRB Early Experiments, 30-40% of all bacteria were from the *Clostridium* genus (predominantly *C. leptum*), which was 2 to 3 times higher than in the A Tower mice. This suggests a possible explanation for why high *Bacteroides* in female mice in CTRB Early Experiment 4 did not correlate with protection against disease. It is possible that high levels of *Clostridium* may promote lupus progression and/or block any beneficial effect of high *Bacteroides*. There is some evidence in the literature supporting this possibility. A longitudinal analysis of SLE patient gut microbiota found that *C. leptum* was enriched in SLE patient gut microbiota compared to healthy controls, and that after treatment that successfully reduced disease activity, *C. leptum* abundance was decreased [439]. Furthermore, in a recent study, bacteria from the class Clostridia, which includes *Clostridium*, were able to suppress retinoic acid synthesis in the intestine [440]. Retinoic acid is important for the induction of tolerance-promoting T regulatory cells (Tregs) [441], and SLE is associated with lower levels of Tregs [442]. This suggests that an overabundance of *Clostridia* could potentially increase susceptibility to lupus by suppressing retinoic acid-dependent Treg induction. Further study will be required to determine if *Clostridium* is playing a similar role in the BWF1 mice.

Analysis of the female and male microbiota compositions in the CTRB Recent period (when the male microbiota was again able to suppress disease in female recipients) strengthened the association of high *Bacteroides* and low *Clostridium* with protection from disease. In both CTRB Recent female vs male experiments, *Bacteroides*

is higher in male than female *BWF1* mice, and *Clostridium* abundance is very low (~5-10%). The two genera have the same predominant species, *B*. *acidifaciens* and *C*. *leptum*, as the A Tower, further supporting the similarities between the CTRB Recent and A Tower microbiota profiles. Additionally, analysis of a CTRB Recent intact vs castrated male experiment found lupus-resistant intact male BWF1 mice had higher *Bacteroides*, and specifically *B*. *acidifaciens*, abundance than the lupus-susceptible castrated male BWF1 mice, which further supports the connection between higher *Bacteroides* and disease resistance. The finding that *B*. *acidifaciens* is the predominant *Bacteroides* species is interesting, as *B*. *acidifaciens* has been associated with anti-inflammatory phenotypes. A previous study found that in the non-obese diabetic (NOD) mouse model, a therapeutic diet increased both the survival rate and *B*. *acidifaciens* abundance. The increase in *B*. *acidifaciens* correlated with changes in multiple changes in immune parameters that shifted systemic immunity towards a more anti-inflammatory profile [443]. Similarly, in a study examining the colitis-suppressing activities of a *Lactobacillus* probiotic, it was found that treatment with the *Lactobacillus* resulted in a massive increase in *B*. *acidifaciens* abundance, and this correlated with suppression of colitic inflammation and restoration of gut homeostasis [444]. This supports the theory that increased abundance of *B*. *acidifaciens* could suppress the inflammatory immune responses seen in lupus.

Alistipes, which had been absent in CTRB Early experiments, was present in all of CTRB Recent experiments, and the same species (*A*. *putredinis* and *A*. *timonensis*) were predominant as in the A Tower. This further supports the conclusion that the return of the protective effect of male to female microbiota transfers coincides with a shift of the

microbiota taxa abundances to become more similar to that seen in the A Tower. It also suggests that the presence of *Alistipes* could potentially be required for the protective effect of the male microbiota.

It is also worth noting that the samples from CTRB Recent Experiments 1 and 2 were collected about 2 months apart, similar to the length of time separating CTRB Early Experiments 2 and 3 from CTRB Early Experiment 4. However, while *Bacteroides* went from being undetectable in CTRB Early Experiments 2 and 3 to up to 30-40% abundance in CTRB Early Experiment 4, the *Bacteroides* abundances of CTRB Recent Experiments 1 and 2 varied far less. This suggests that the *Bacteroides* population during the CTRB Recent time period is more stable than it was during the CTRB Early time period.

Further evidence of the correlation of high *Bacteroides* and low *Clostridium* with protection from disease was found when the microbiota profiles of CTRB Early and CTRB Recent cecal transfer experiments were compared. In the CTRB Recent transfer experiment, the female and male BWF1 recipients of male BWF1 microbiota had higher *Bacteroides* abundance compared to female recipients of female microbiota, and this higher level of *Bacteroides* corresponded with suppression of disease. This fits with previous studies that have found adoptive transfer of *Bacteroides* suppresses inflammatory diseases. One study showed that transferring *Bacteroides vulgatas* into IL-2 knockout mice protected them against *Escherichia coli*-triggered colitis [445]. In another study, *Bacteroides fragilis* transfer conferred resistance to EAE [446]. More recently, a study found that administering *B. acidifaciens* to mice fed a high fat diet protected them against obesity and the development of obesity-derived inflammation [447]. Taken together with our findings, these data suggest species within the Bacteroides

genus can suppress inflammatory diseases.

However, female recipients of female microbiota in the CTRB Early transfer experiment were not protected from disease, despite having higher *Bacteroides* abundance than recipients of male microbiota. This could potentially be because, as with the female vs male experiments, the CTRB Early transfer experiment had substantially higher levels of *Clostridium* than the CTRB Recent transfer experiments. A comparison of the *Bacteroides/Clostridium* ratios suggested that the combination of high *Bacteroides* with low *Clostridium* may be key to the protective effect of male microbiota. Also, like the male vs female experiments, *Alistipes* was absent in the CTRB Early and present in the CTRB Recent cecal transfer experiments supporting the idea that *Alistipes* and *Bacteroides* could potentially be acting in concert. Findings like this not only could potentially lead to the development of new lupus therapies, but could also inform the use of current treatments. For example, the current frontline treatment for lupus is corticosteroids, but a recent study comparing SLE patients who had and had not undergone glucocorticoid treatment found glucocorticoid treatment was associated with decreased *Bacteroides* abundance [285]. If further research showed that higher *Bacteroides* abundance was beneficial for human SLE patients, the effects of a therapy on the microbiota might need be considered when designing a treatment plan.

Taken together, our results emphasize that no single taxon can be considered in isolation, and that examination of the entire microbiological profile is essential for studies of the microbiota. For our data, male microbiota suppression of disease does not track with any one taxon, but with a combination of high *Bacteroides*, low *Clostridium* and the presence of *Alistipes*.

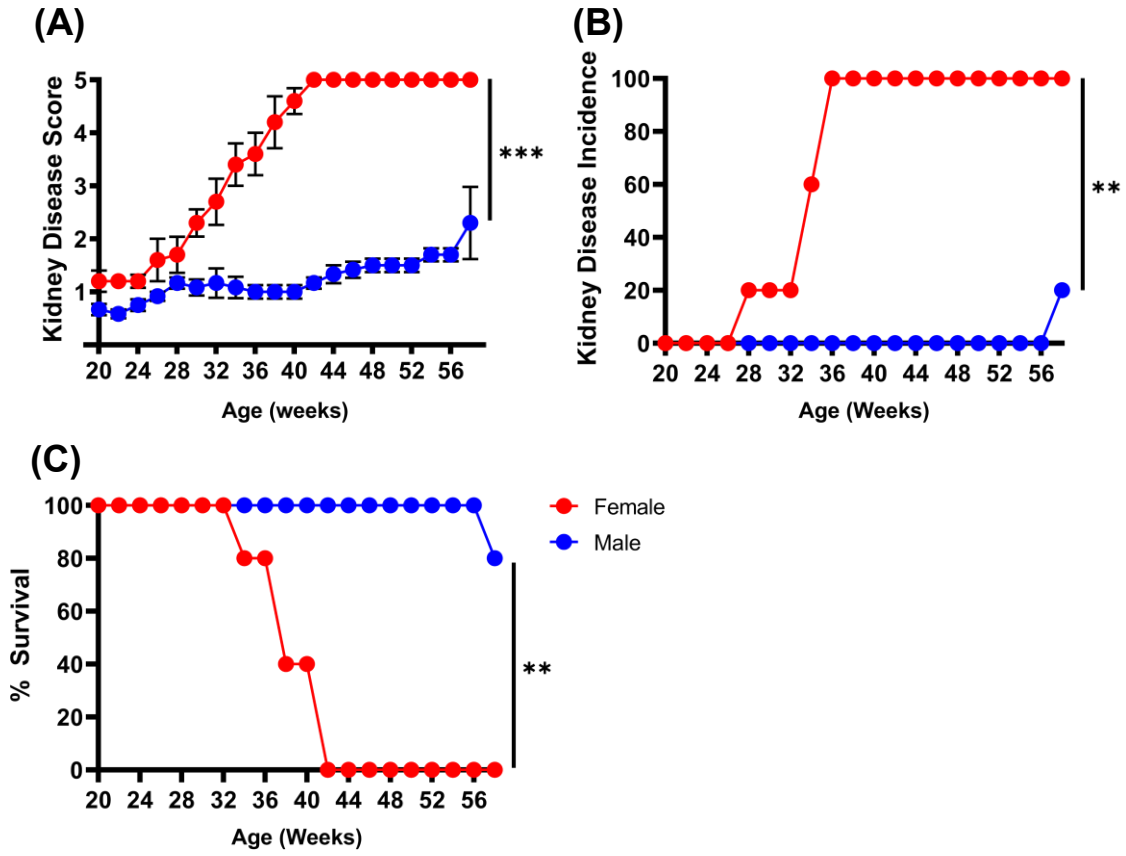


Figure 3. Female, but not male, NZBxNZWF1 (BWF1) mice develop severe glomerulonephritis spontaneously. Female and male BWF1 mice were monitored for kidney disease (glomerulonephritis) bi-weekly by measuring proteinuria in the urine. Proteinuria was scored on a scale of 1-5 with a score of 5 indicating death (n=5). **(A)** Kidney disease score **(B)** Incidence of kidney disease (% mice with a proteinuria score of ≥3 for two consecutive biweekly readings). **(C)** Survival curve. Kidney disease scores were compared with two-way ANOVAs, and incidence and survival curves were compared using the Log rank (Mantel-Cox) test. *$p<0.05$, **$p<0.01$, ***$p<0.001$

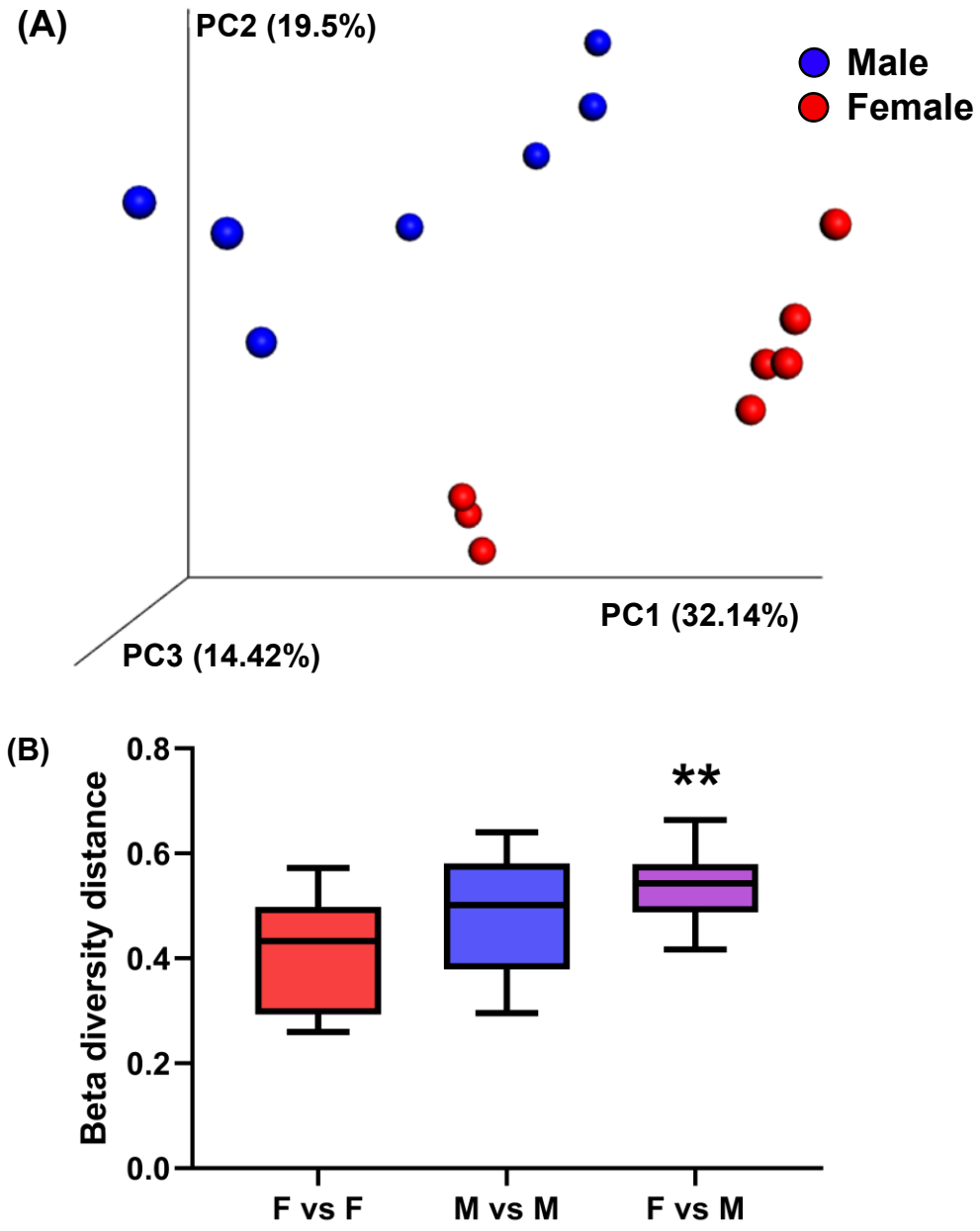


Figure 4. Adult female and male BWF1 mice have significantly different microbiota compositions. Feces were collected from 16-week-old female **(F)** and male (M) BWF1 mice during the A Tower period (2014-16) and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. **(A)** Principal coordinate analysis (PCoA) plot of female and male BWF1 microbiota beta diversity using the Bray-Curtis dissimilarity metric. Each symbol represents one fecal sample. **(B)** Female and male microbiota beta diversity distances were compared with the permutational multivariate analysis of variance (PERMANOVA) pairwise statistical test (n=7). (p=0.001)

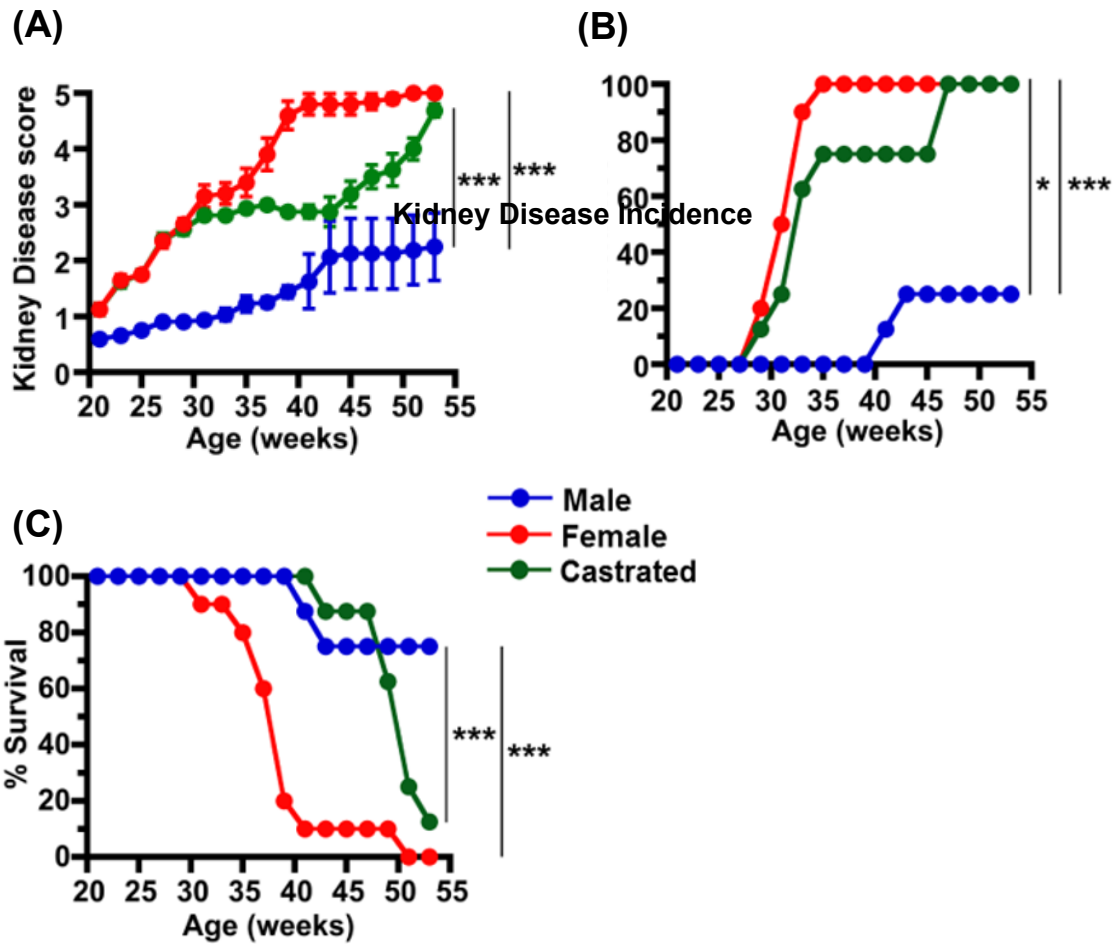


Figure 5. Androgen depletion increases incidence and time of onset of kidney disease and decreases survival in male BWF1 mice. Female, intact male, and castrated (at ~ 24-days of age) male BWF1 mice were monitored for kidney disease (glomerulonephritis) bi-weekly by measuring proteinuria in the urine. Proteinuria was scored on a scale of 1-5 with a score of 5 indicating death (n=8) [420]. **(A)** Kidney disease score. **(B)** Incidence of kidney disease (% mice with a proteinuria score of ≥3 for two consecutive biweekly readings). **(C)** Survival curve. Kidney disease scores were compared with two-way ANOVAs, and incidence and survival curves were compared using the Log rank (Mantel-Cox) test [420]. *p<0.05, **p<0.01, ***p<0.001

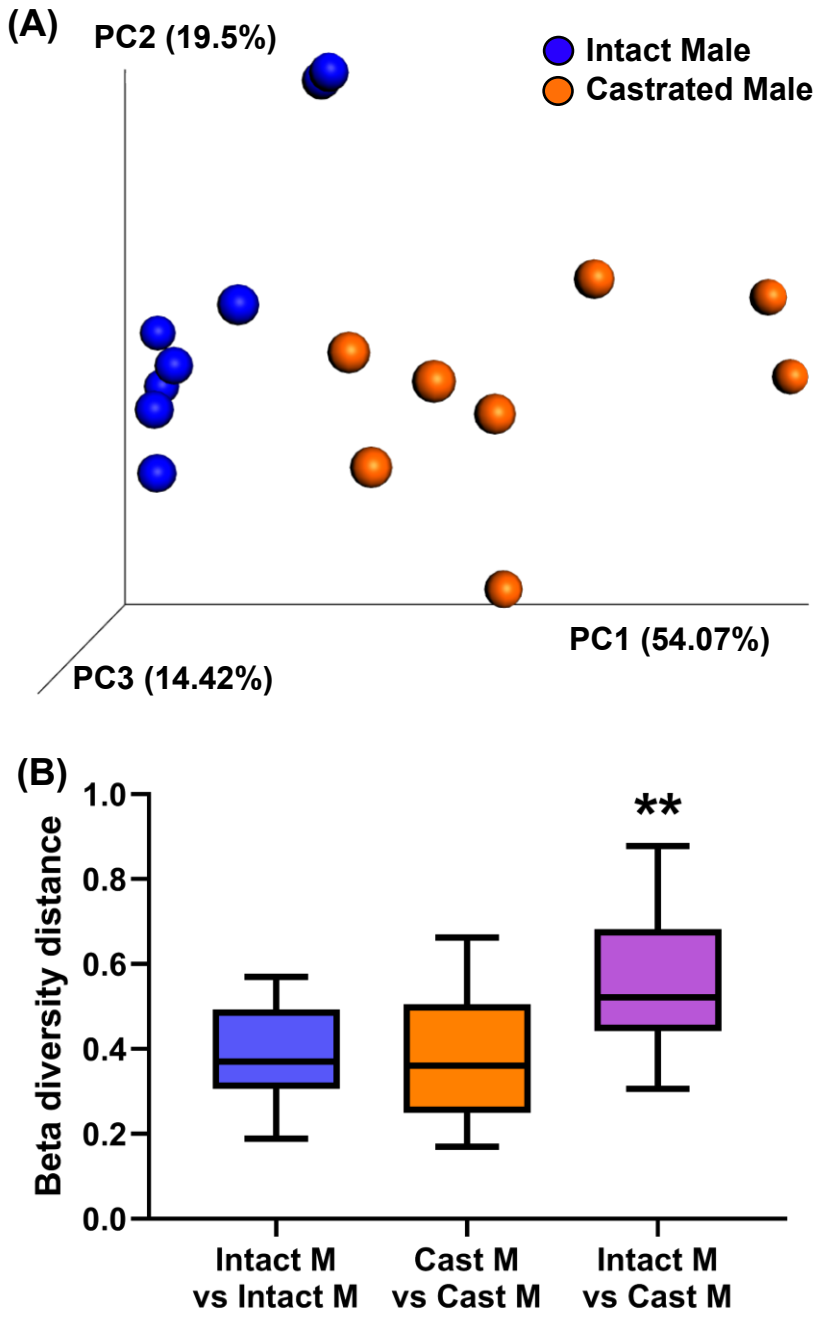


Figure 6. Adult intact and castrated male BWF1 mice have significantly different microbiota compositions. Feces were collected from 16-week-old intact (Intact M) and castrated (at ~24 days of age; Cast M) BWF1 mice and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. **(A)** Principal coordinate analysis (PCoA) plot of intact and castrated male BWF1 microbiota beta diversity using the Bray-Curtis dissimilarity metric. **(B)** Intact and castrated male microbiota beta diversity distances were compared with the permutational multivariate analysis of variance (PERMANOVA) pairwise statistical test (n=8). (p=0.0015)

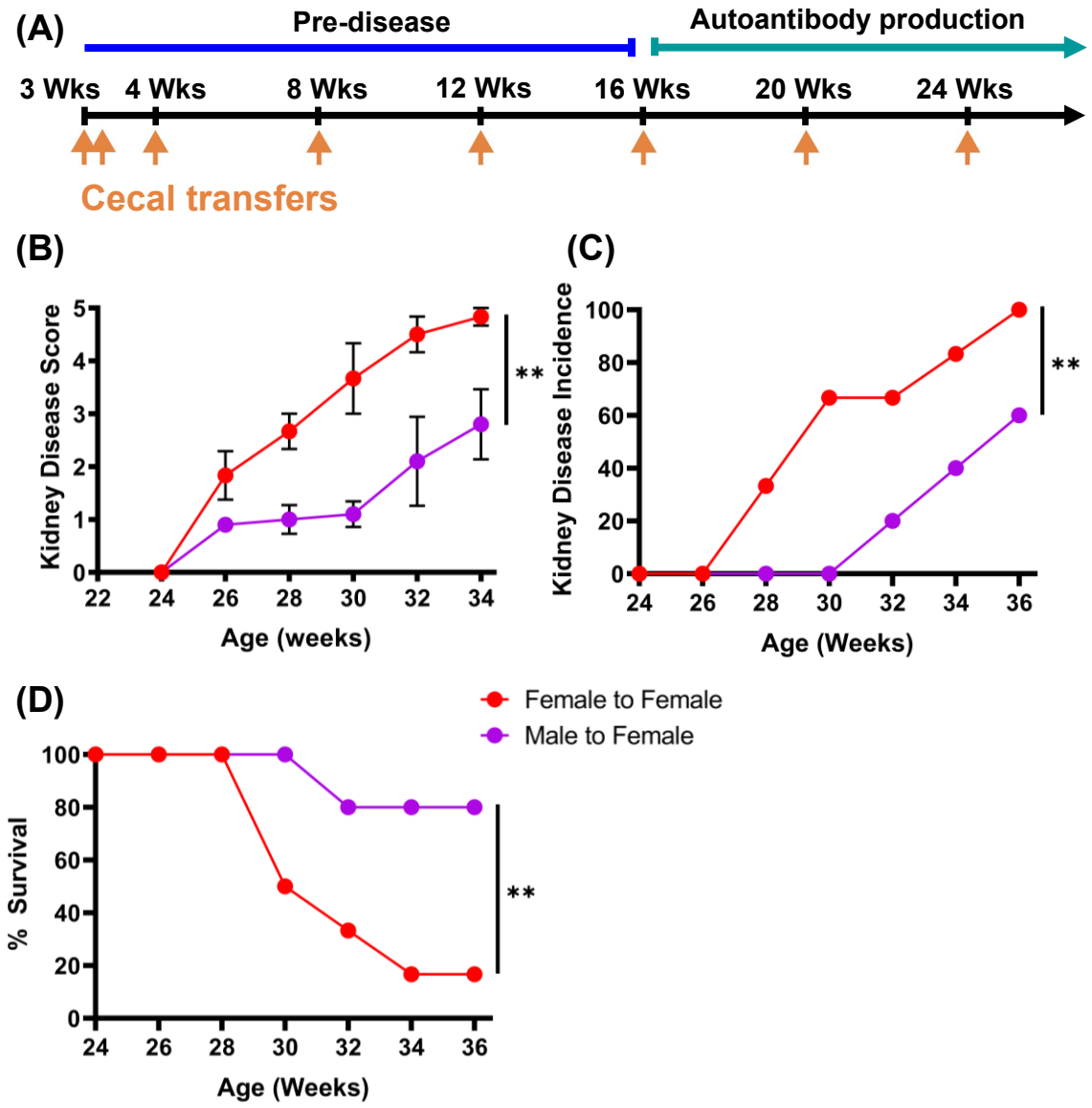


Figure 7. Transfer of male microbiota significantly suppresses kidney disease and enhances survival in female BWF1 mice. Female BWF1 mice were fed cecal contents (i.e., microbiota) via gavage from 16-week-old female or male BWF1 mice. Female-to-female (F-F, n=6) and male-to-female (M-F, n=5) cecal transfer recipients were monitored for kidney disease (glomerulonephritis) bi-weekly by measuring proteinuria in the urine. Proteinuria was scored on a scale of 1-5 with a score of 5 indicating death. Experiments were conducted during the A Tower period (2014-16). **(A)** Cecal transfer regimen. **(B)** Kidney disease score. **(C)** Incidence of kidney disease (% mice with a proteinuria score of ≥3 for two consecutive biweekly readings). **(D)** Survival curve. Kidney disease scores were compared with two-way ANOVAs, and incidence and survival curves were compared using the Log rank (Mantel-Cox) test. $^{**}p<0.01$

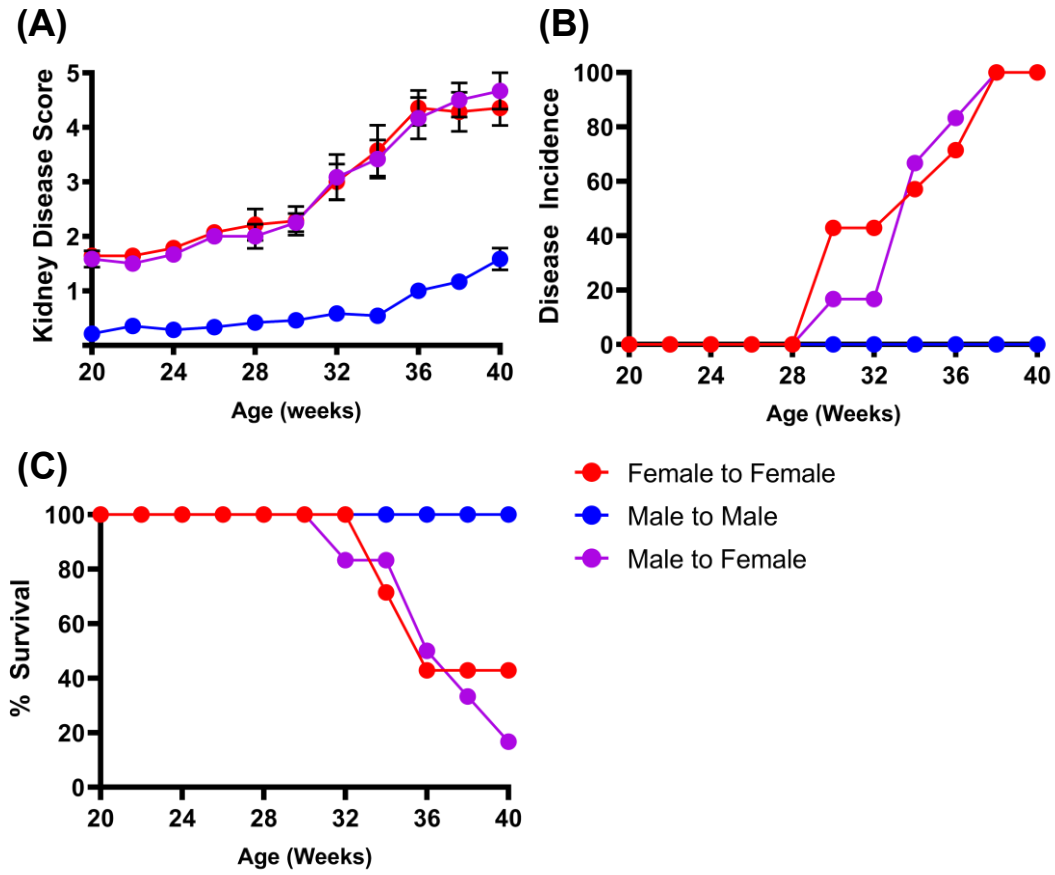


Figure 8. Transfer of male microbiota did not suppress kidney disease or enhance survival in female BWF1 mice during the CRTB Early period. Female BWF1 mice were fed cecal contents (i.e., microbiota) via gavage from 16-week-old female or male BWF1 mice. Female-to-female (F-F) and male-to-female (M-F) cecal transfer recipients were monitored for kidney disease (glomerulonephritis) bi-weekly by measuring proteinuria in the urine. Proteinuria was scored on a scale of 1-5 with a score of 5 indicating death (n=6). Experiments were conducted during the CTRB Early period (2017-18). **(A)** Kidney disease score. **(B)** Incidence of kidney disease (% mice with a proteinuria score of ≥3 for two consecutive biweekly readings). **(C)** Survival curve. Kidney disease scores were compared with two-way ANOVAs, and incidence and survival curves were compared using the Log rank (Mantel-Cox) test.

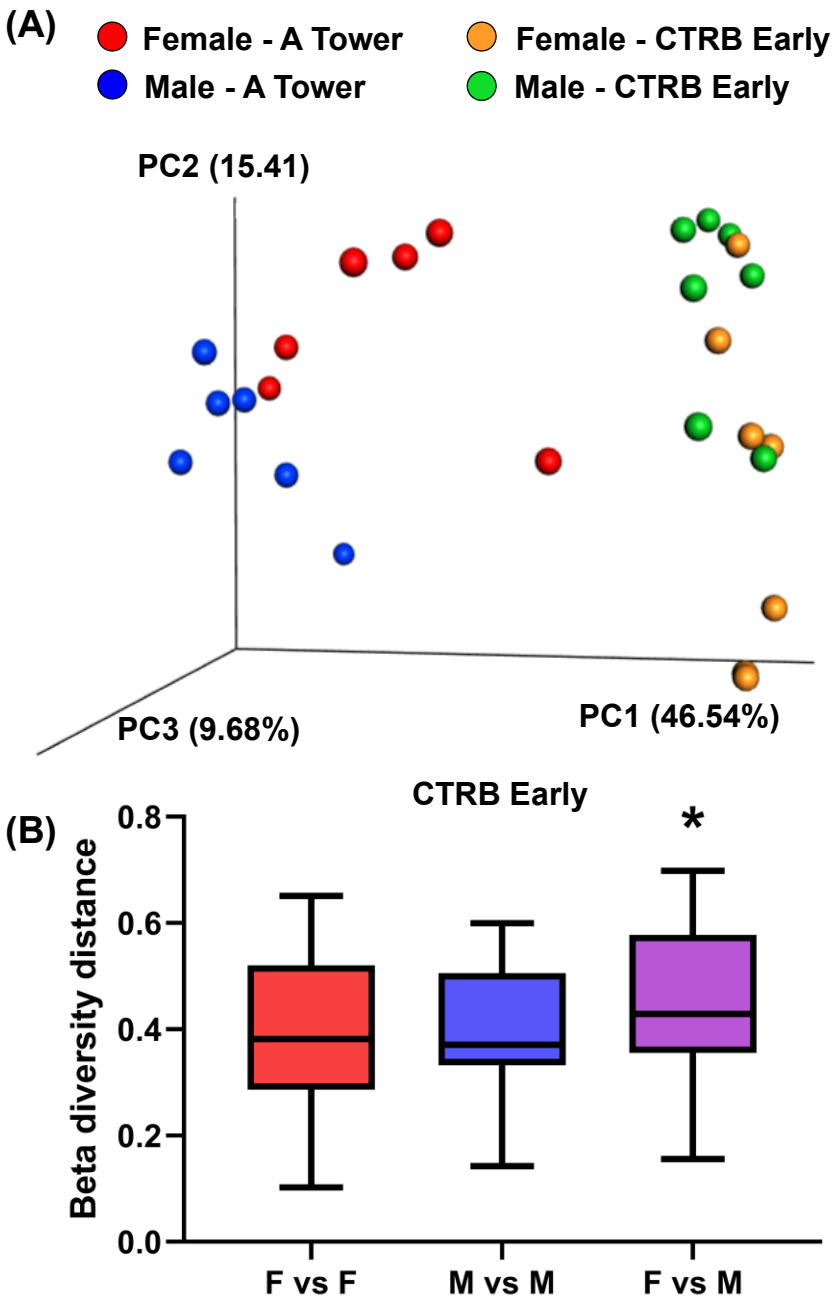


Figure 9. Female and male microbiota compositions differ between the A Tower and CTRB Early periods. Feces were collected from 16-week-old female and male BWF1 mice during either the A Tower (2014-16; n=6) or the CTRB Early (2017-18; n=7) periods, and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. **(A)** Principal coordinate analysis (PCoA) plot of female and male BWF1 microbiota beta diversity using the Bray-Curtis dissimilarity metric. Each symbol represents one fecal sample. **(B)** Female and male microbiota beta diversity distances for samples collected during the CTRB Early period were compared with the permutational multivariate analysis of variance (PERMANOVA) pairwise statistical test (p = 0.043).

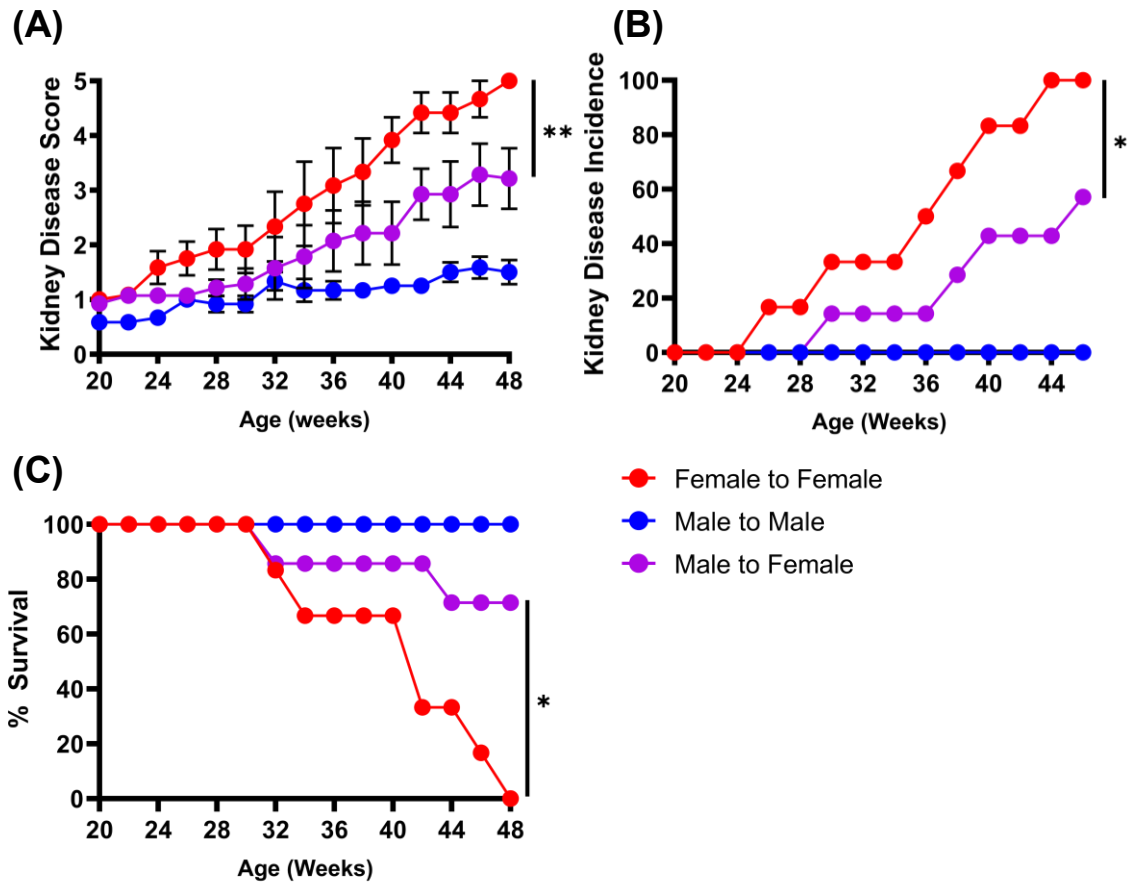


Figure 10. Transfer of male microbiota suppressed kidney disease and enhanced survival in female BWF1 mice in during the CRTB Recent period. Female and male BWF1 mice were fed cecal contents (i.e., microbiota) via gavage from 16-week-old female or male BWF1 mice. Female BWF1 mice were fed cecal contents from adult male or female BWF1 mice. Female-to-female (F-F), male-to-female (M-F), and male-to-male (M-M) cecal transfer recipients were monitored for kidney disease bi-weekly by measuring proteinuria in the urine on a scale of 1-5 with a score of 5 indicating death (n=6). Experiments were conducted during the CTRB Recent period (2019-present). **(A)** Kidney disease score. **(B)** Incidence of kidney disease (% mice with a proteinuria score of ≥3 for two consecutive biweekly readings). **(C)** Survival curve. Kidney disease scores were compared with two-way ANOVAs, and incidence and survival curves were compared using the Log rank (Mantel-Cox) test. *$p<0.05$, **$p<0.01$

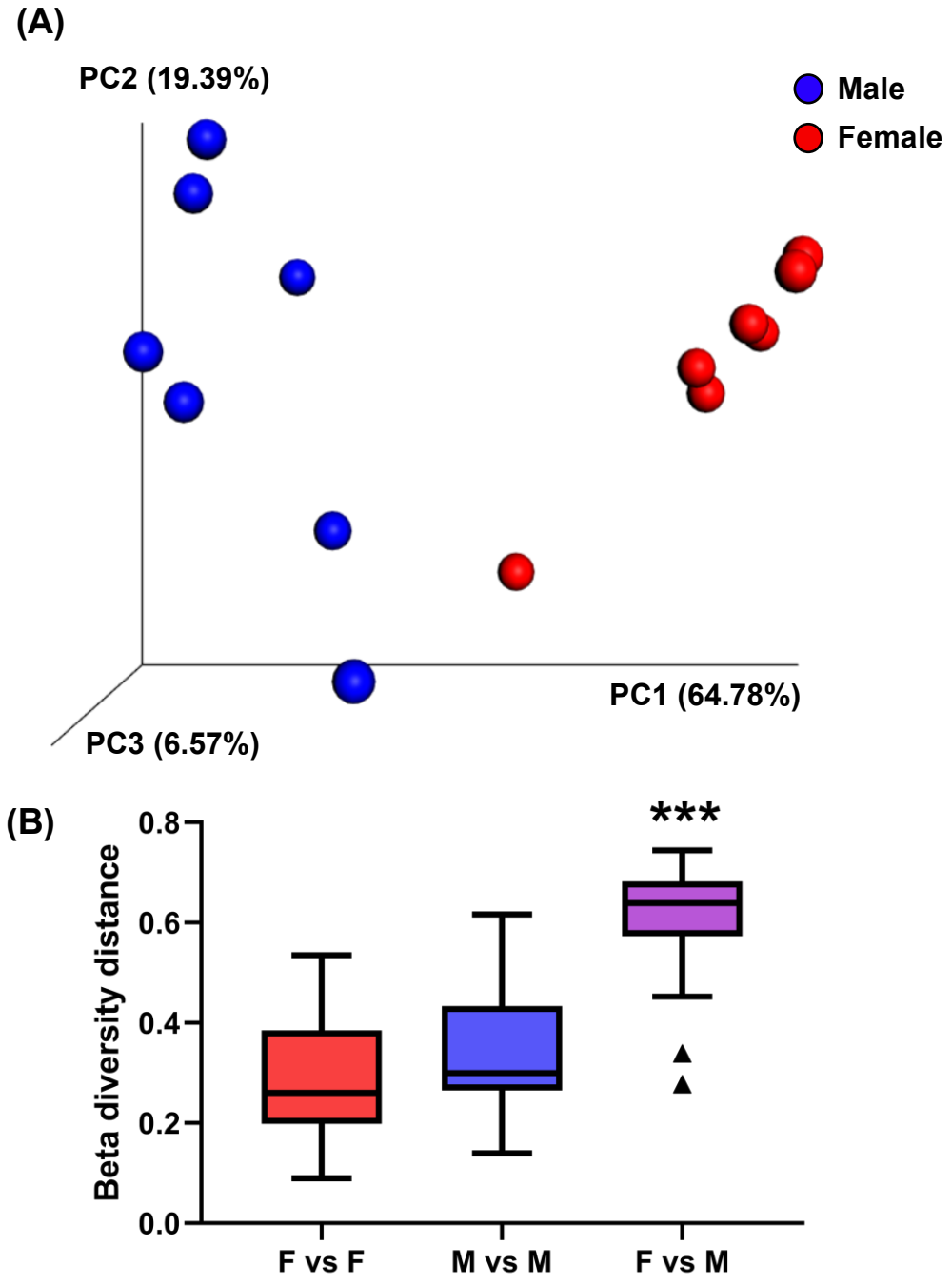
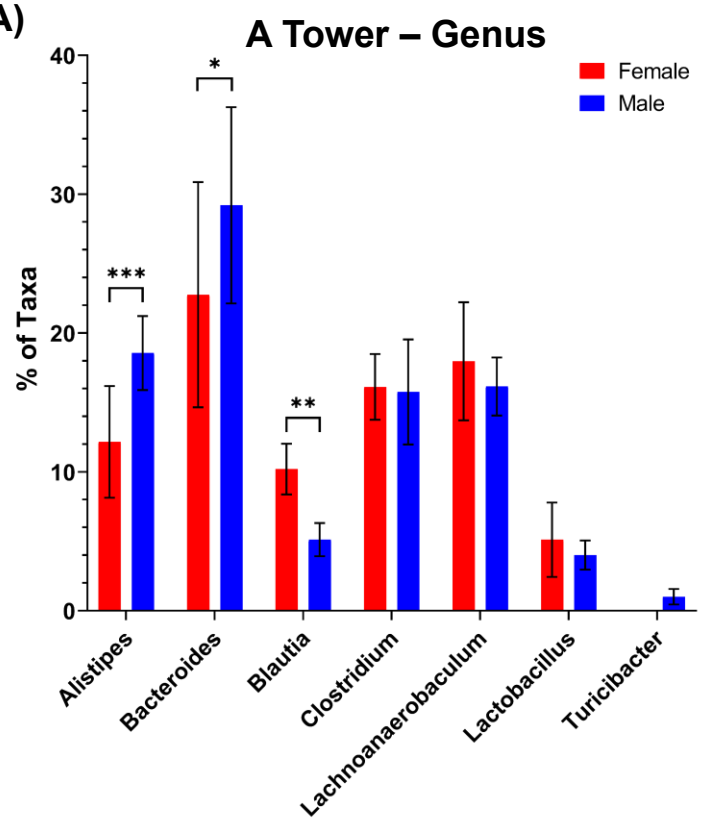


Figure 11. Female and male BWF1 mice have highly significantly different microbiota compositions during the CTRB Recent period. Feces were collected from 16-week-old female and male BWF1 mice during the CTRB Early (2019-present) period and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition (n=7). **(A)** Principal coordinate analysis (PCoA) plot of adult male and female BWF1 fecal microbiota beta diversity using the Bray-Curtis dissimilarity metric. **(B)** Female and male microbiota beta diversity distances were compared with the permutational multivariate analysis of variance (PERMANOVA) pairwise statistical test. Outliers are indicated as triangles. ($p = 0.001$).

(A)


A Tower – Genus
Female
Male
% of Taxa
40
30
20
10
0
***
*
**
Alistipes
Bacteroides
Blautia
Clostridium
Lachnoanaerobaculum
Lactobacillus
Turicibacter

(B)

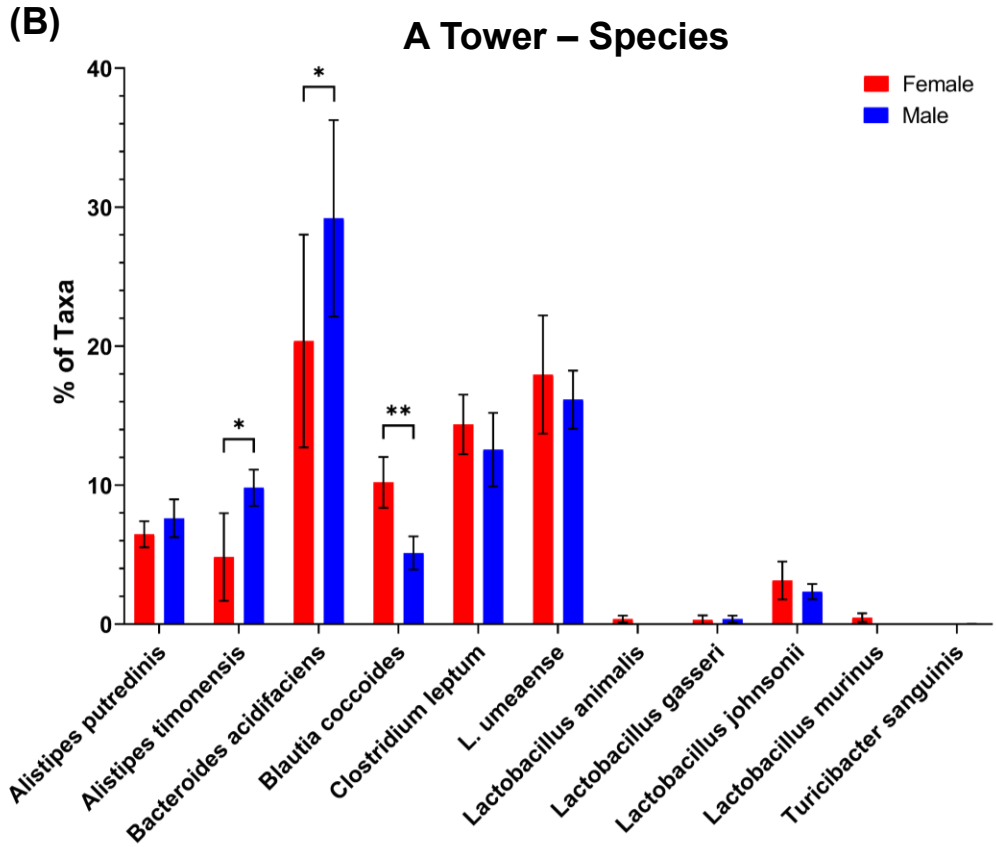
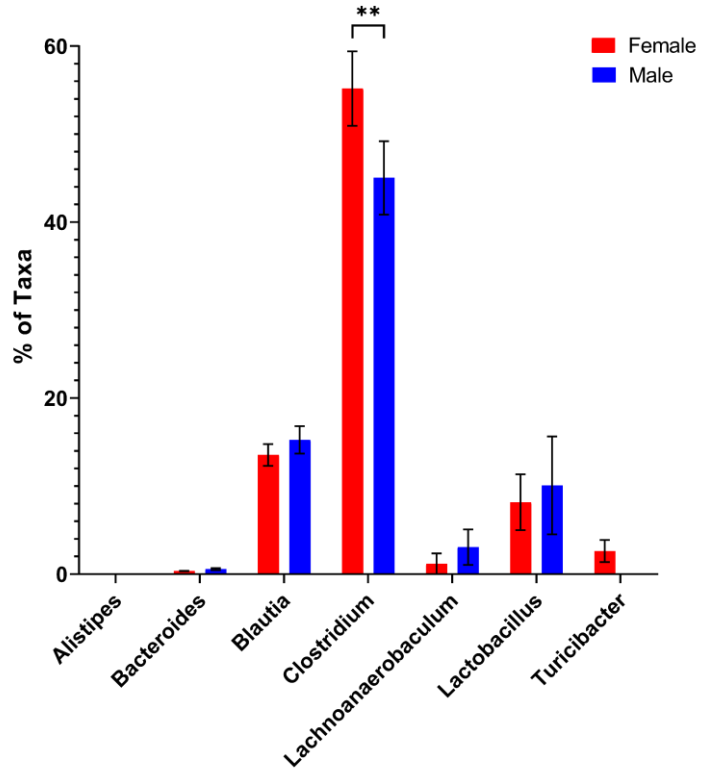

A Tower – Species
Female
Male
% of Taxa
40
30
20
10
0
*
*
**
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
L. umeaense
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Turicibacter sanguinis

Figure 12. Abundance of *Bacteroides* was higher in adult male than female BWF1 mice during the A Tower period. Feces were collected from 16-week-old female and male BWF1 mice during the A Tower period (2016) and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition (n=7). Abundances were compared with One-way ANOVAs. **(A)** Genus level microbiota from the A Tower – Female vs Male experiment genus (n=6). **(B)** Species level microbiota from the A Tower – Female vs Male experiment species (n=6). *p<0.05, **p<0.01, ***p<0.001

(A)


CTRB Early – Experiment 1 – Genus
**
Female
Male
% of Taxa
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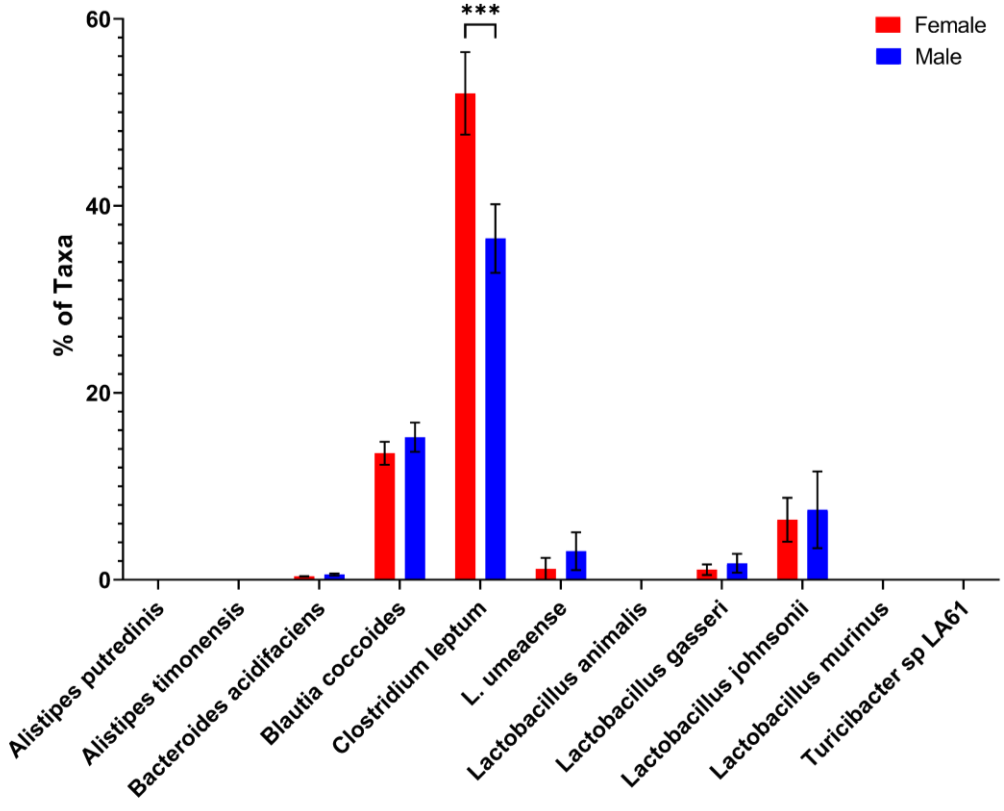
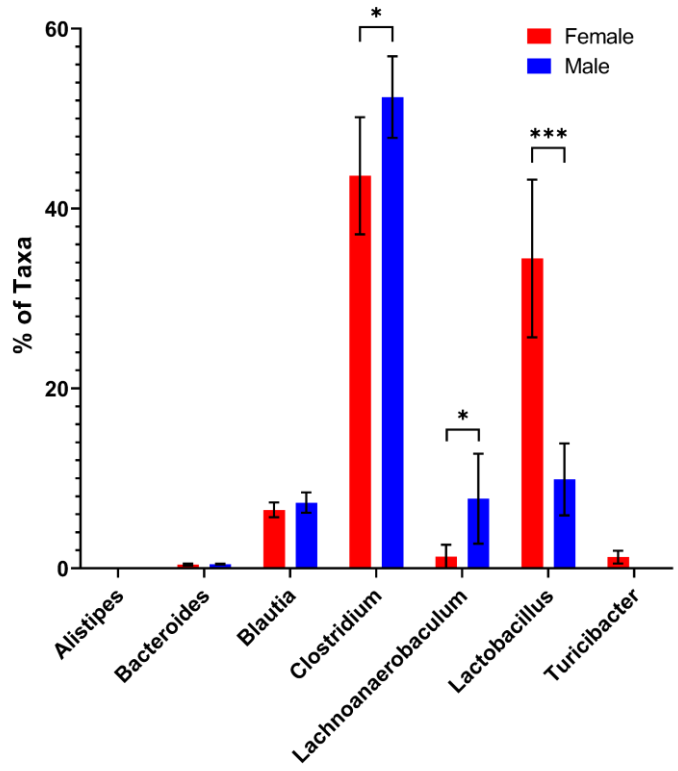
40
20
0
Alistipes
Bacteroides
Blautia
Clostridium
Lachnoanaerobaculum
Lactobacillus
Turicibacter

(B)


CTRB Early – Experiment 1 – Species
***
Female
Male
% of Taxa
60
40
20
0
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
L. umeaense
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Turicibacter sp LA61

(C)


CTRB Early – Experiment 2 – Genus
Female
Male
% of Taxa
60
40
20
0
*
***
*
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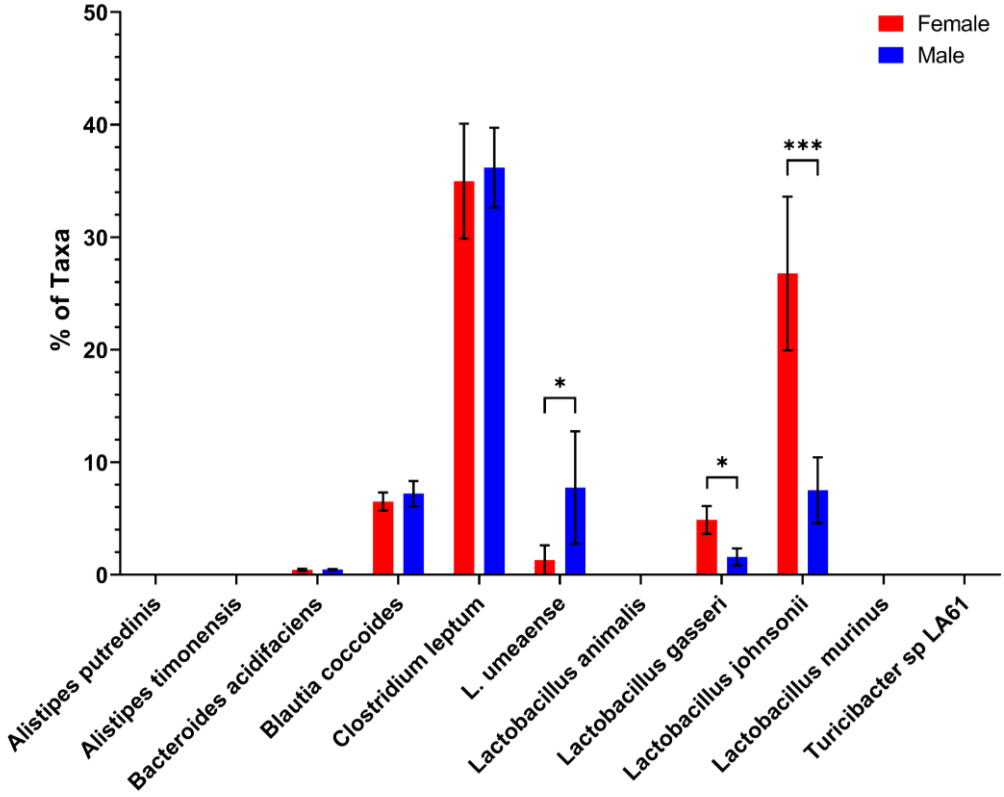
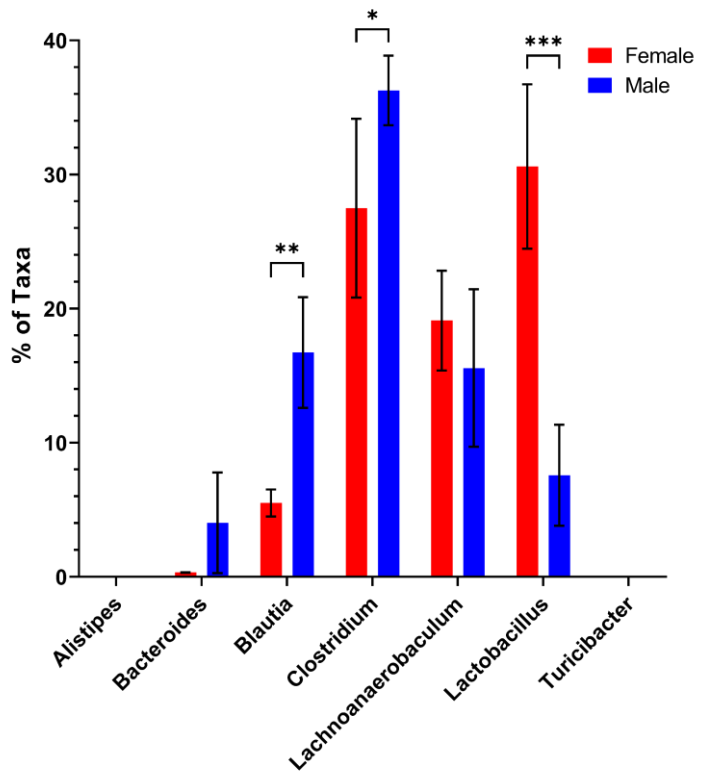
Bacteroides
Blautia
Clostridium
Lachnoanaerobaculum
Lactobacillus
Turicibacter

(D)


CTRB Early – Experiment 2 – Species
Female
Male
% of Taxa
50
40
30
20
10
0
*
*
***
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
L. umeaense
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Turicibacter sp LA61

(E)


CTRB Early – Experiment 3 – Genus
Female
Male
% of Taxa
0
10
20
30
40
*
**
***
Alistipes
Bacteroides
Blautia
Clostridium
Lachnoanaerobaculum
Lactobacillus
Turicibacter

(F)

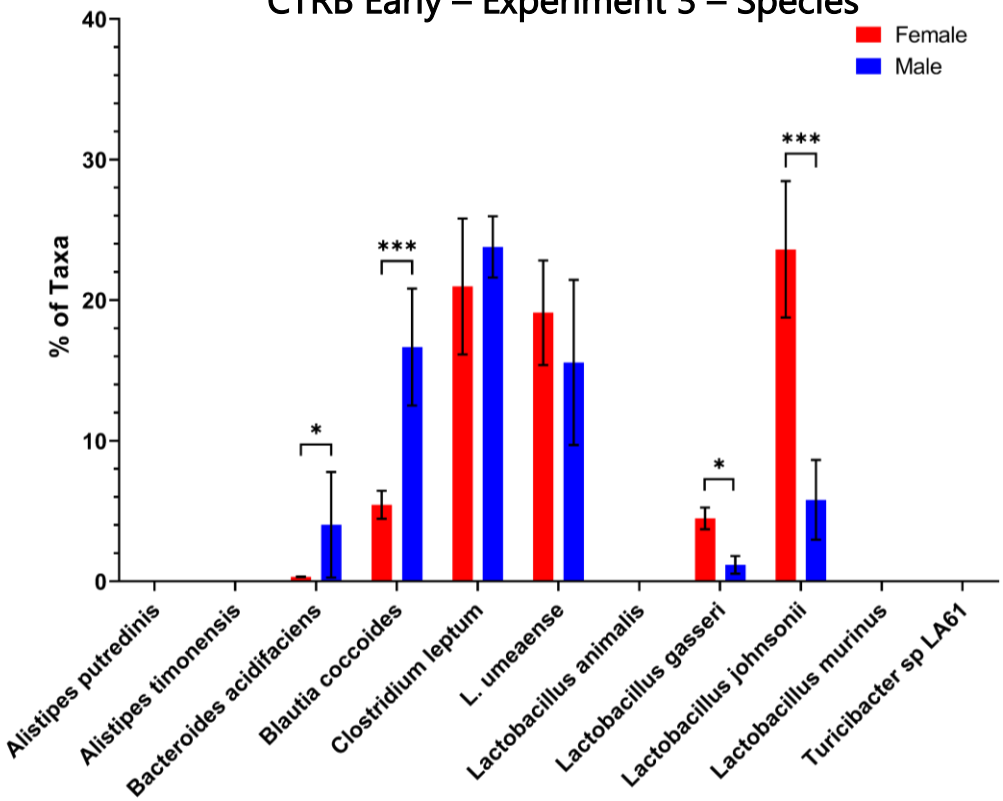
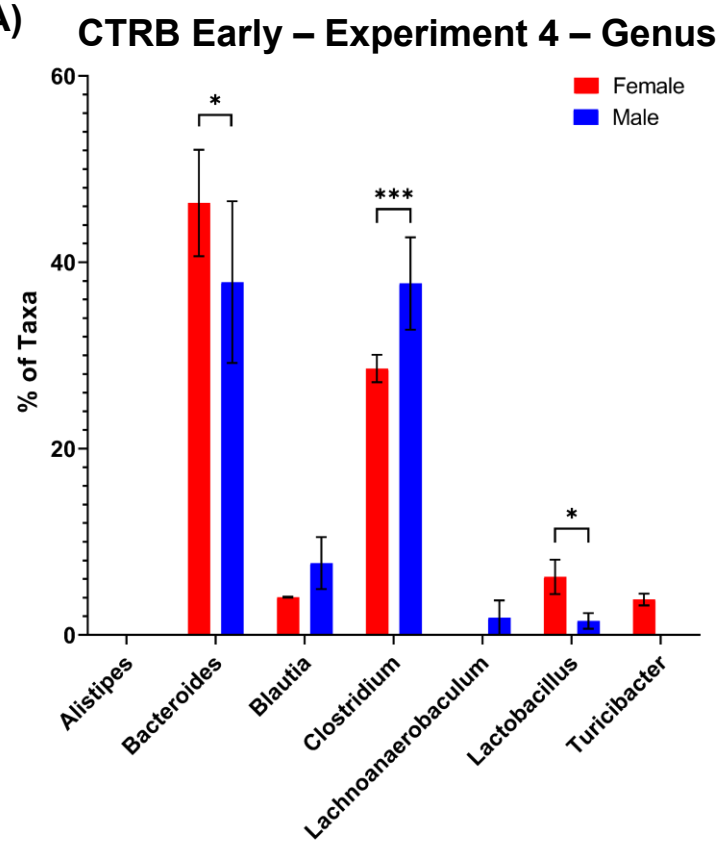

CTRB Early – Experiment 3 – Species
Female
Male
% of Taxa
0
10
20
30
40
*
***
*
***
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
L. umeaense
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Turicibacter sp LA61

Figure 13. *Bacteroides* is virtually absent and abundance of *Clostridium* is very high in both females and males in most CTRB Early Experiments. Feces were collected from 16-week-old female and male BWF1 mice during the CTRB Early (2017-18) period and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. Abundances were compared with One-way ANOVAs. **(A)** Genus level microbiota from Experiment 1 (n=8). **(B)** Species level microbiota from Experiment 1 (n=8). **(C)** Genus level microbiota from Experiment 2 (n=7). **(D)** Species level microbiota from Experiment 2 (n=7). **(E)** Genus level microbiota from Experiment 3; (n=7). **(F)** Species level microbiota from Experiment 3 (n=7). *$p<0.05$, **$p<0.01$, ***$p<0.001$

(A)


CTRB Early – Experiment 4 – Genus
Female
Male
% of Taxa
60
40
20
0
*
***
*
Alistipes
Bacteroides
Blautia
Clostridium
Lachnoanaerobaculum
Lactobacillus
Turicibacter

(B)

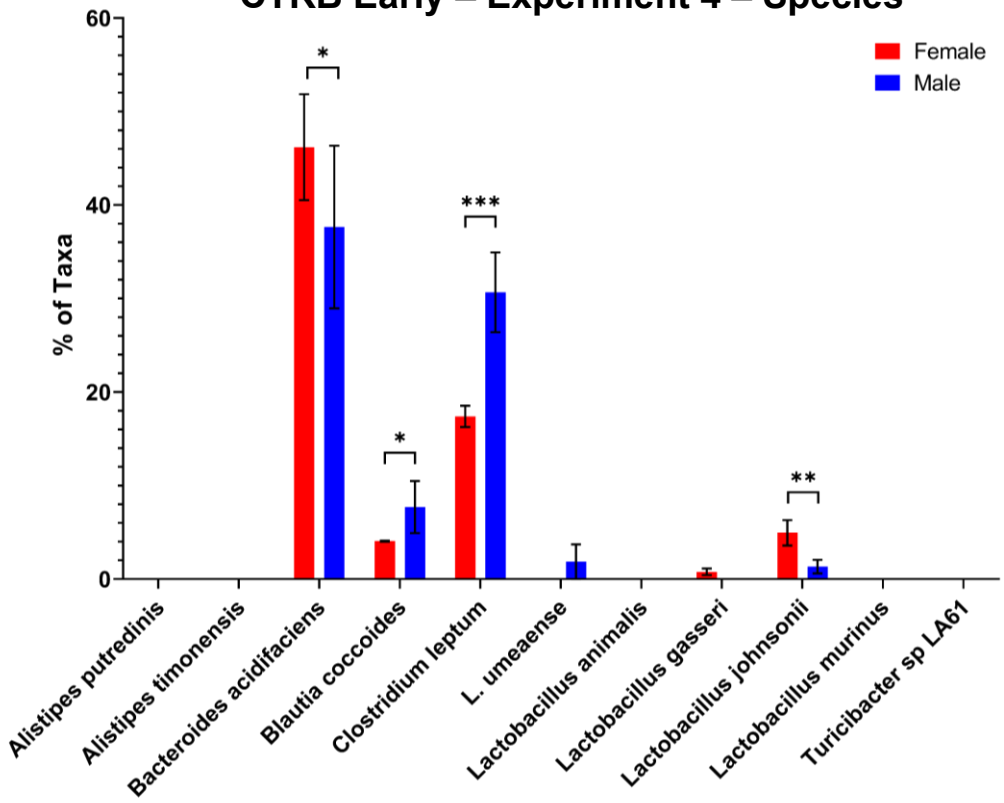

CTRB Early – Experiment 4 – Species
Female
Male
% of Taxa
60
40
20
0
*
***
*
**
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
L. umeaense
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Turicibacter sp LA61

Figure 14. In the final CTRB Early, Experiment 4, unlike in other CTRB early experiments, abundance of *Bacteroides* was high, while abundance of *Clostridium* also continued to be high in both female and male adult BWF1 mice. Feces were collected from 16-week-old female and male BWF1 mice during the CTRB Early (2017-18) period and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition (n=6). Abundances were compared with One-way ANOVAs. **(A)** Genus level microbiota from Experiment 4. **(B)** Species level microbiota from Experiment 4. *p<0.05, **p<0.01, ***p<0.001

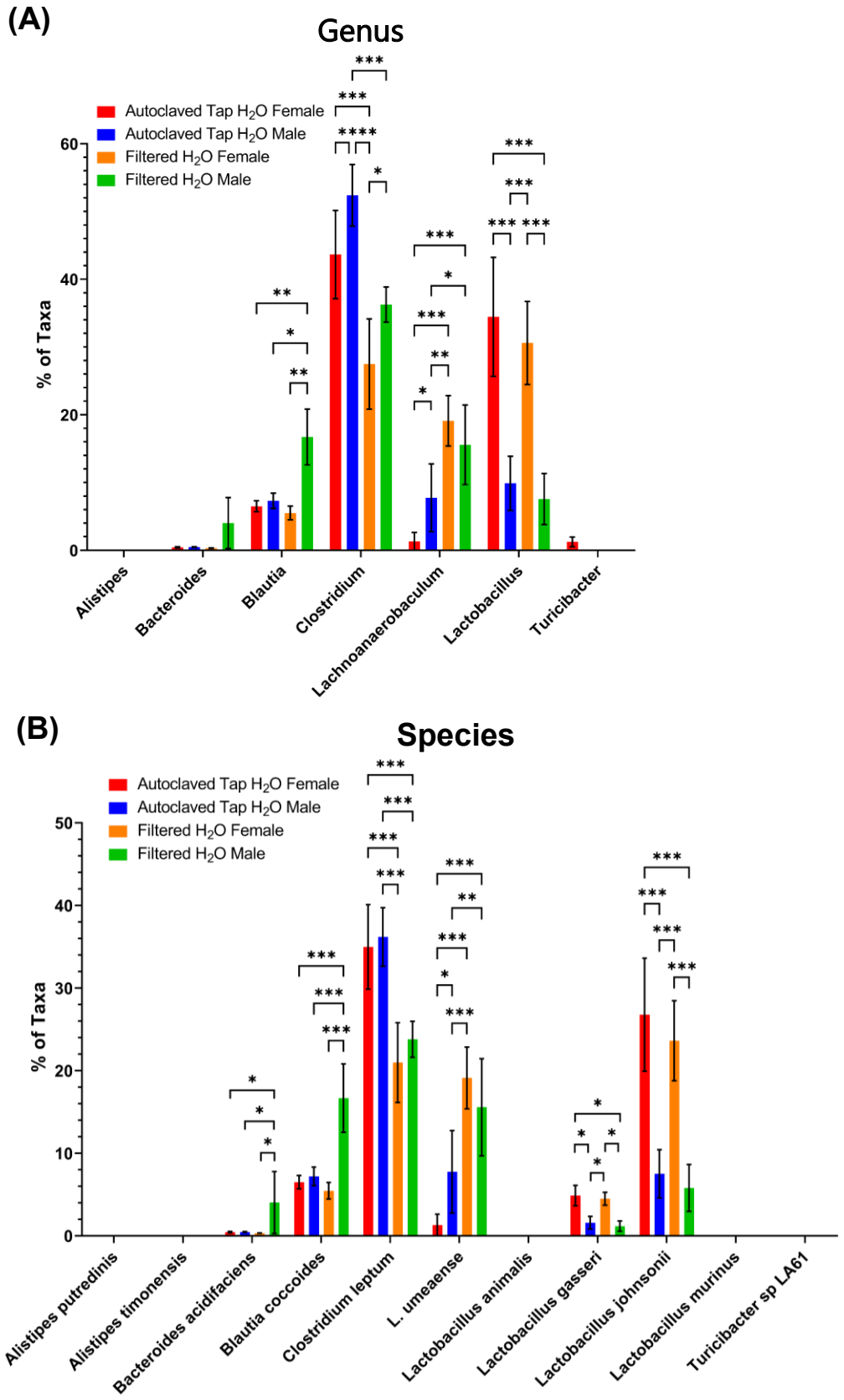
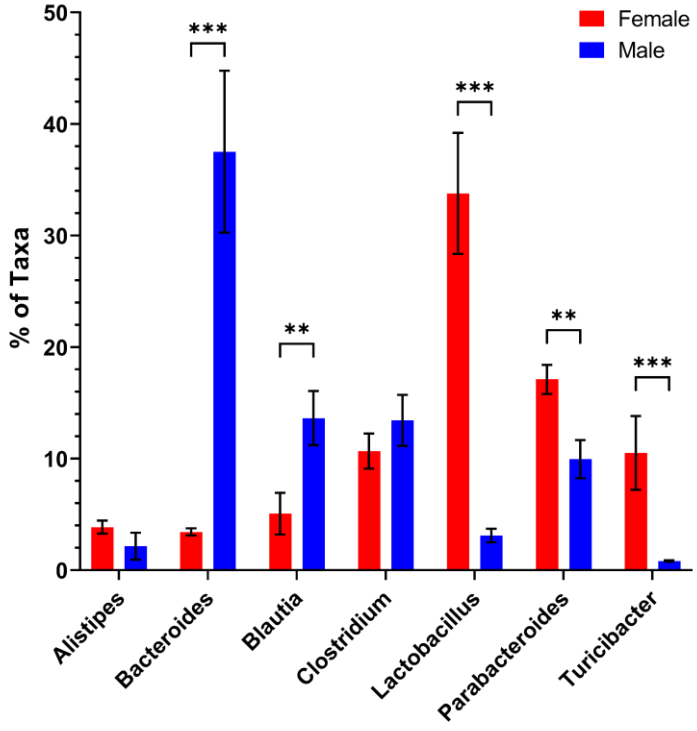
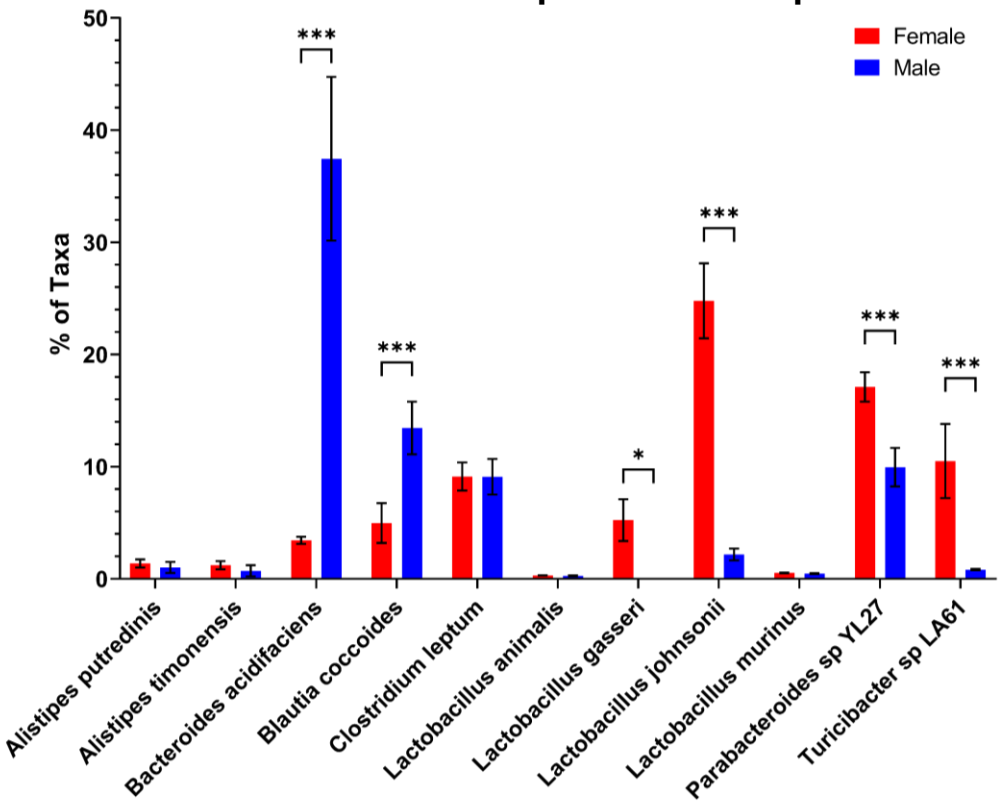

(A)
Genus
Autoclaved Tap H2O Female
Autoclaved Tap H2O Male
Filtered H2O Female
Filtered H2O Male
% of Taxa
0
20
40
60
Alistipes
Bacteroides
Blautia
Clostridium
Lachnoanaerobaculum
Lactobacillus
Turicibacter
(B)
Species
Autoclaved Tap H2O Female
Autoclaved Tap H2O Male
Filtered H2O Female
Filtered H2O Male
% of Taxa
0
10
20
30
40
50
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
L. umeaense
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Turicibacter sp LA61

Figure 15. Both sex and drinking water source affect microbiota compositions. Feces were collected from 16-week-old female and male BWF1 mice during the CTRB Early (2017-18) period and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition (n=7). Mice received either autoclaved tap H2O (also see Experiment 2 in **Figure 13C,D**) or filtered H2O (also see Experiment 3 in **Figure 13E,F**) drinking water for the entire period of the experiment. Abundances were compared with One-way ANOVAs. **(A)** Genus level microbiota; **(B)** Species level microbiota. *p<0.05, **p<0.01, ***p<0.001

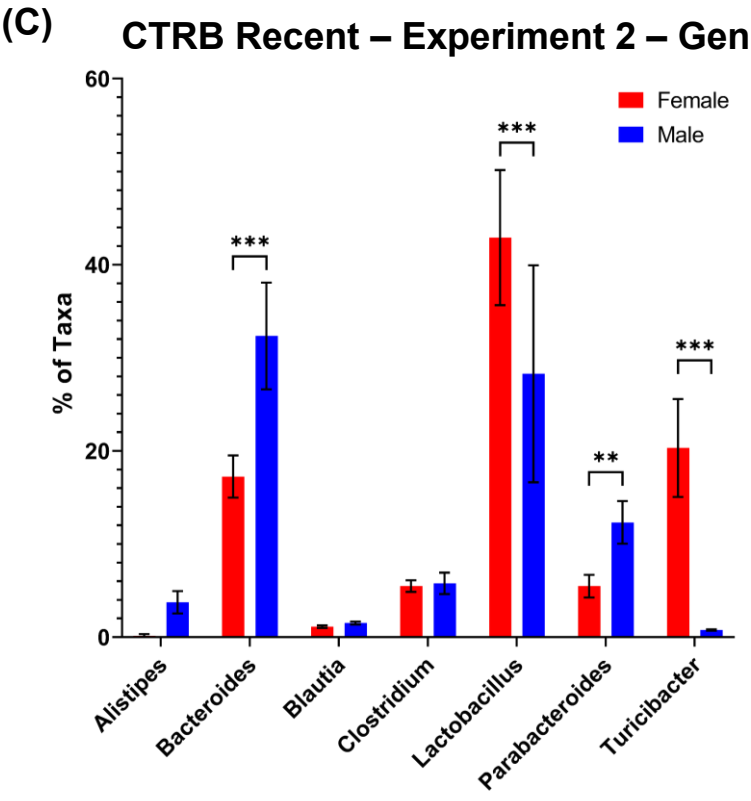
(A) CTRB Recent – Experiment 1 – Genus



(B) CTRB Recent – Experiment 1 – Species



(C)


CTRB Recent – Experiment 2 – Genus
Female
Male
% of Taxa
60
40
20
0
***
***
**
***
Alistipes
Bacteroides
Blautia
Clostridium
Lactobacillus
Parabacteroides
Turicibacter

(D)

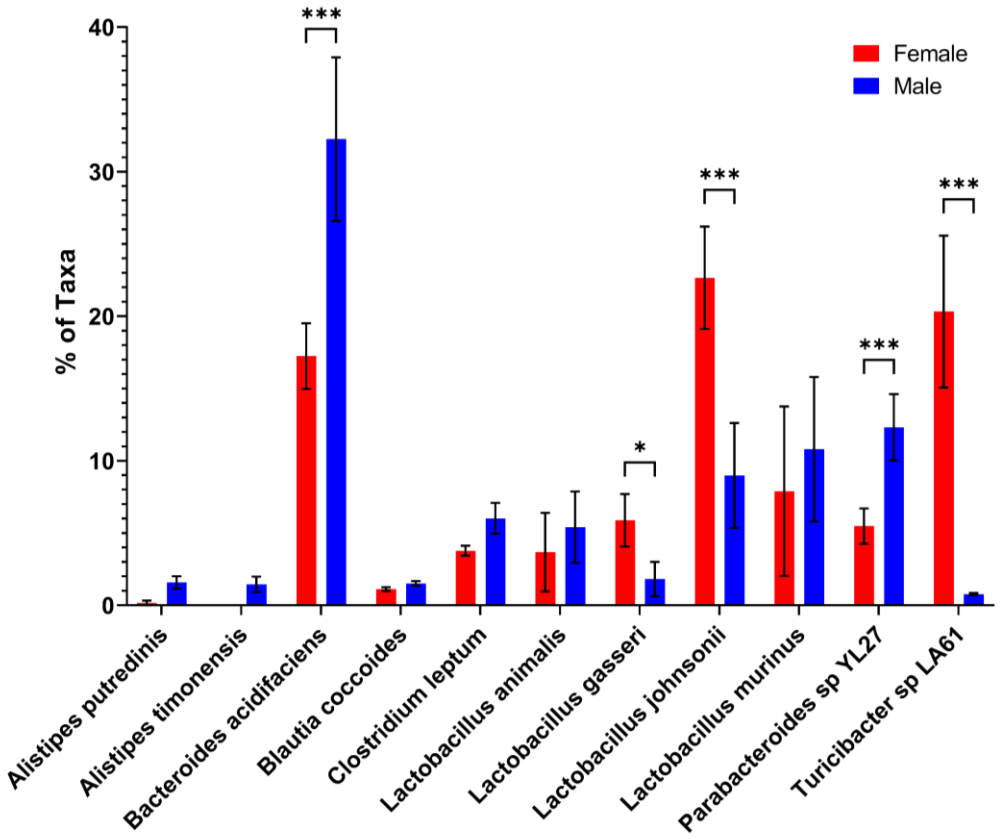
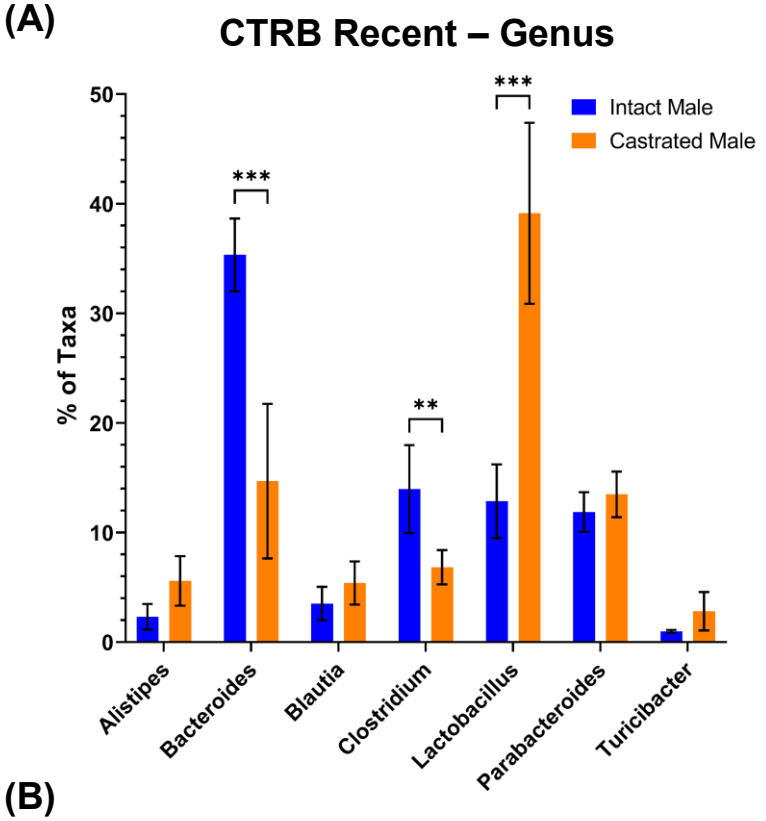

CTRB Recent – Experiment 2 – Species
Female
Male
% of Taxa
40
30
20
10
0
***
*
***
***
***
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Parabacteroides sp YL27
Turicibacter sp LA61

Figure 16. Abundance of *Bacteroides* was considerably higher in adult male than female BWF1 mice, and abundance of *Clostridium* levels was low in CTRB Recent experiments. Feces were collected from 16-week-old female and male BWF1 mice during the CTRB Recent (2019-present) period and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. Abundances were compared with One-way ANOVAs. **(A)** Genus level microbiota from Experiment 1 (n=7). **(B)** Species level microbiota from Experiment 1 (n=7). **(C)** Genus level microbiota from Experiment 2 (n=6). **(D)** Species level microbiota from Experiment 2 (n=6). $^{**}p<0.01$, $^{***}p<0.001$

(A)


CTRB Recent – Genus
Intact Male
Castrated Male
% of Taxa
0
10
20
30
40
50
***
***
**
Alistipes
Bacteroides
Blautia
Clostridium
Lactobacillus
Parabacteroides
Turicibacter

(B)

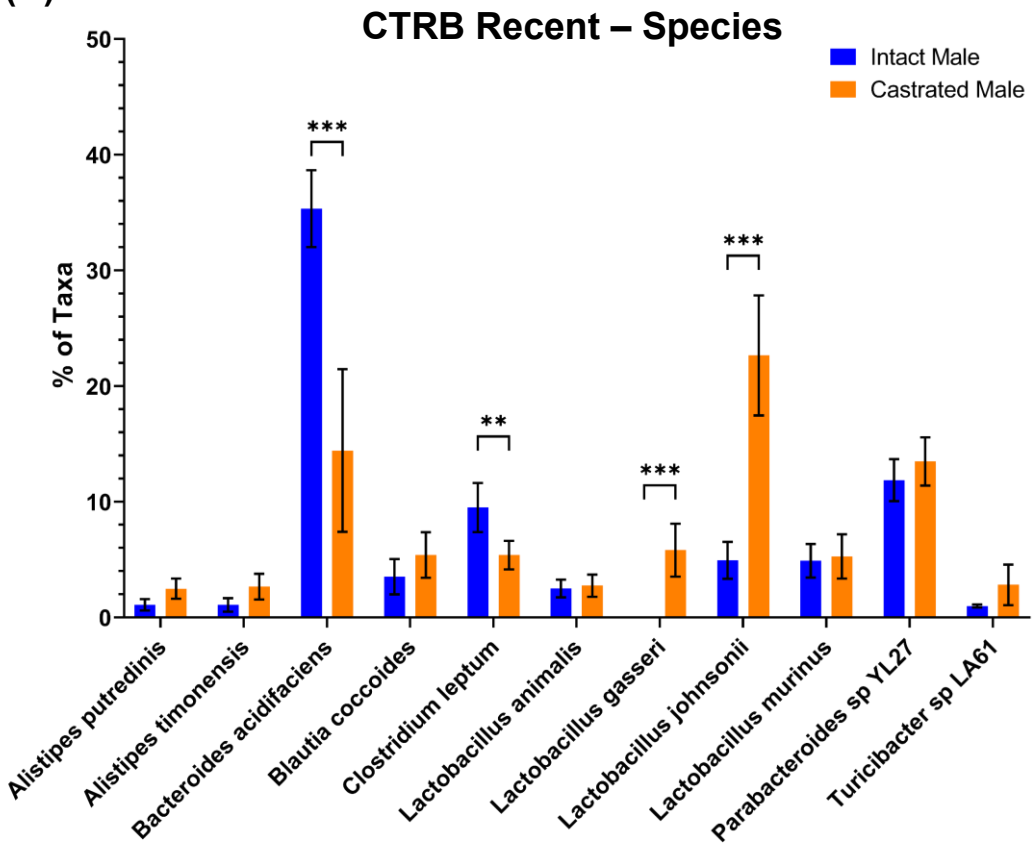
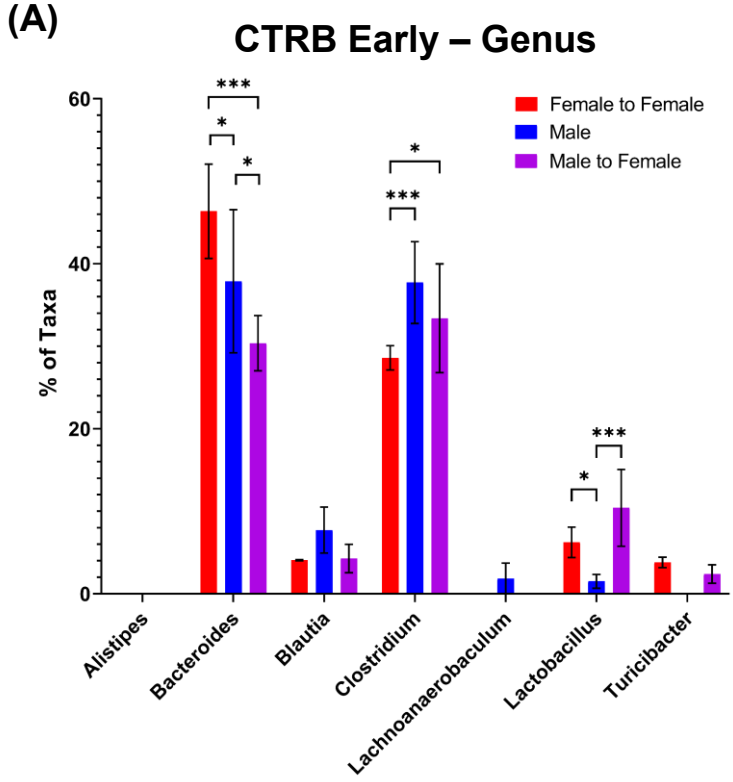

CTRB Recent – Species
Intact Male
Castrated Male
% of Taxa
0
10
20
30
40
50
***
**
***
***
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Parabacteroides sp YL27
Turicibacter sp LA61

Figure 17. Androgen depletion through castration reduces *Bacteroides* abundance. Feces were collected from 16-week-old intact and castrated (at ~24 days of age) BWF1 mice during the CTRB Recent period and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition (n=8). Abundances were compared with One-way ANOVAs. **(A)** Genus level microbiota; **(B)** Species level microbiota. ***p<0.01*, ****p*<0.001

(A)


CTRB Early – Genus
Female to Female
Male
Male to Female
% of Taxa
60
40
20
0
Alistipes
Bacteroides
Blautia
Clostridium
Lachnoanaerobaculum
Lactobacillus
Turicibacter

(B)

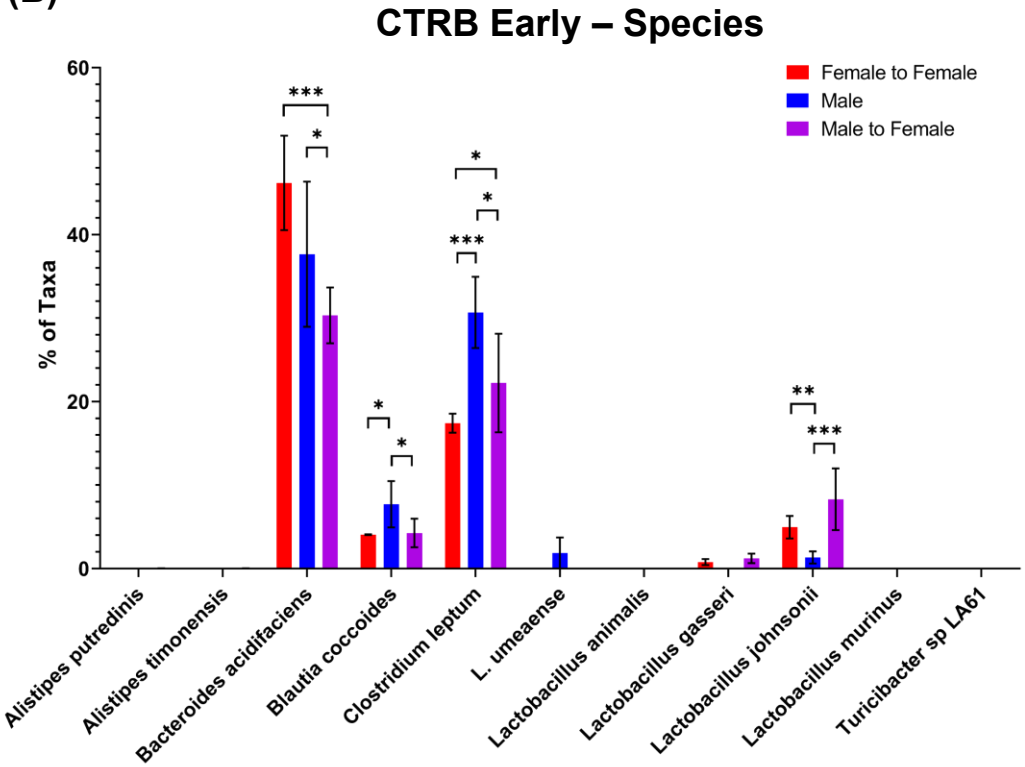
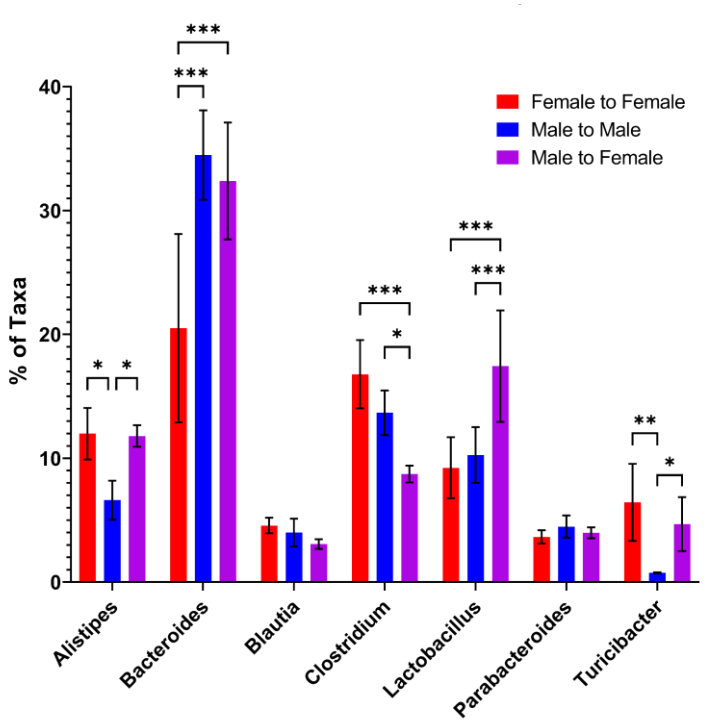
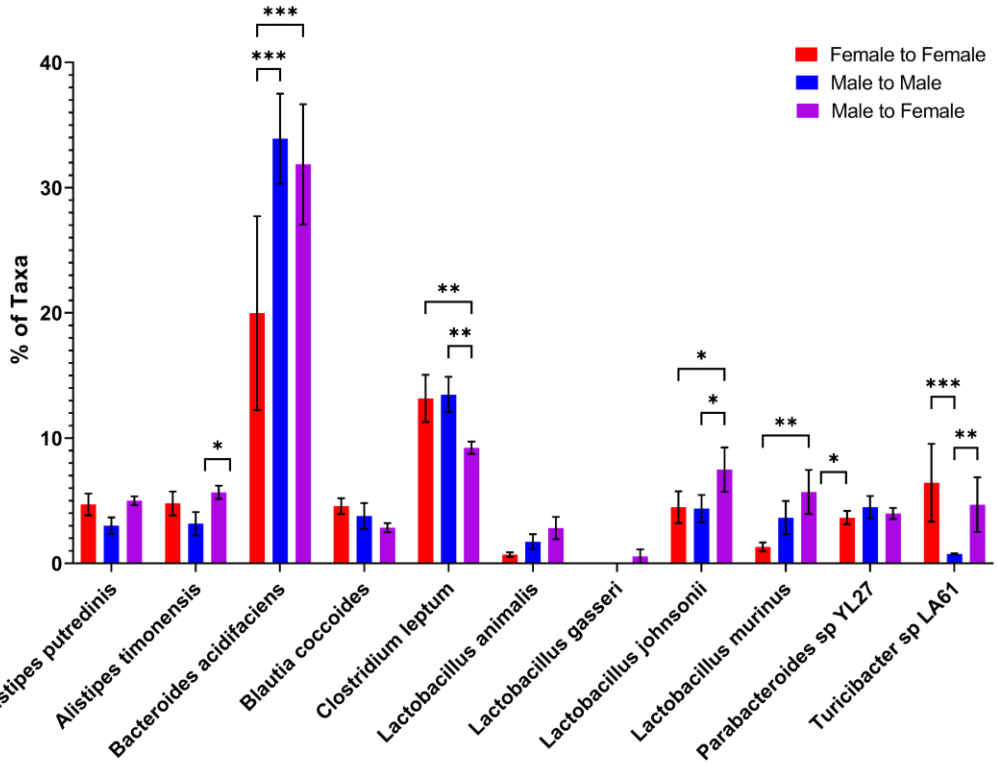

CTRB Early – Species
Female to Female
Male
Male to Female
% of Taxa
60
40
20
0
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
L. umeaense
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Turicibacter sp LA61

Figure 18. In CTRB Early microbiota transfer experiments, *Bacteroides* was present but abundance was lower in the Male to Female transfers and *Clostridium* was high in all experimental groups. During the CTRB Early (2017-18) period, female BWF1 mice were fed cecal contents (i.e., microbiota) from 16-week-old female (Female-to-Female) or male (Male-to-Female) BWF1 mice via gavage. Feces were collected from 16-week-old cecal transfer recipients (4 weeks after cecal transfer), and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. Abundances were compared with One-way ANOVAs (n=6). **(A)** Genus level microbiota; **(B)** Species level microbiota. *p<0.05, **p<0.01, ***p<0.001

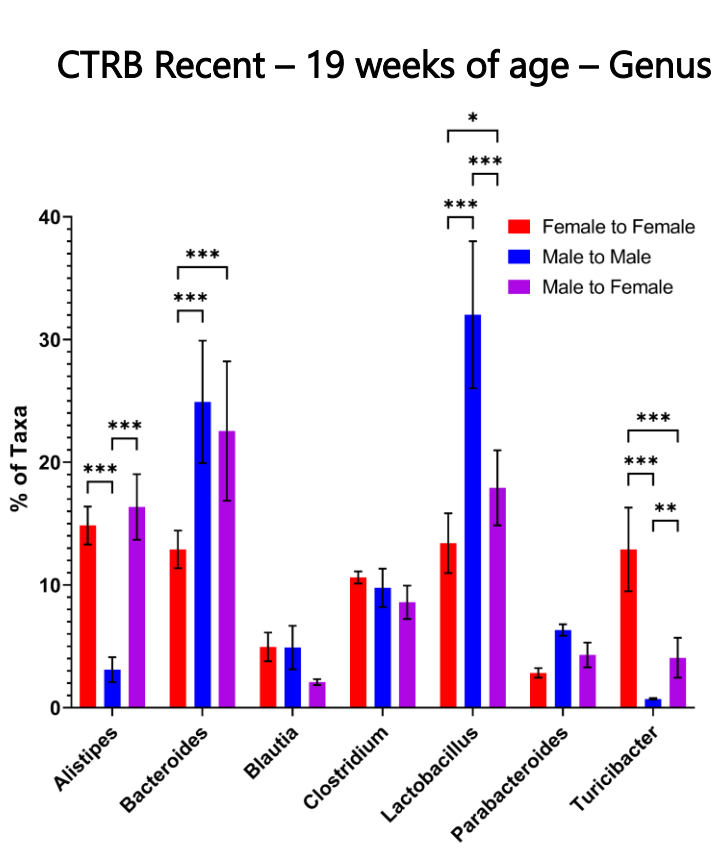
(A)


CTRB Recent – 15 weeks of age – Genus
Female to Female
Male to Male
Male to Female
% of Taxa
0
10
20
30
40
Alistipes
Bacteroides
Blautia
Clostridium
Lactobacillus
Parabacteroides
Turicibacter

(B)


CTRB Recent – 15 weeks of age – Species
Female to Female
Male to Male
Male to Female
% of Taxa
0
10
20
30
40
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Parabacteroides sp YL27
Turicibacter sp LA61

(C)


CTRB Recent – 19 weeks of age – Genus
Female to Female
Male to Male
Male to Female
% of Taxa
0
10
20
30
40
Alistipes
Bacteroides
Blautia
Clostridium
Lactobacillus
Parabacteroides
Turicibacter

(D)

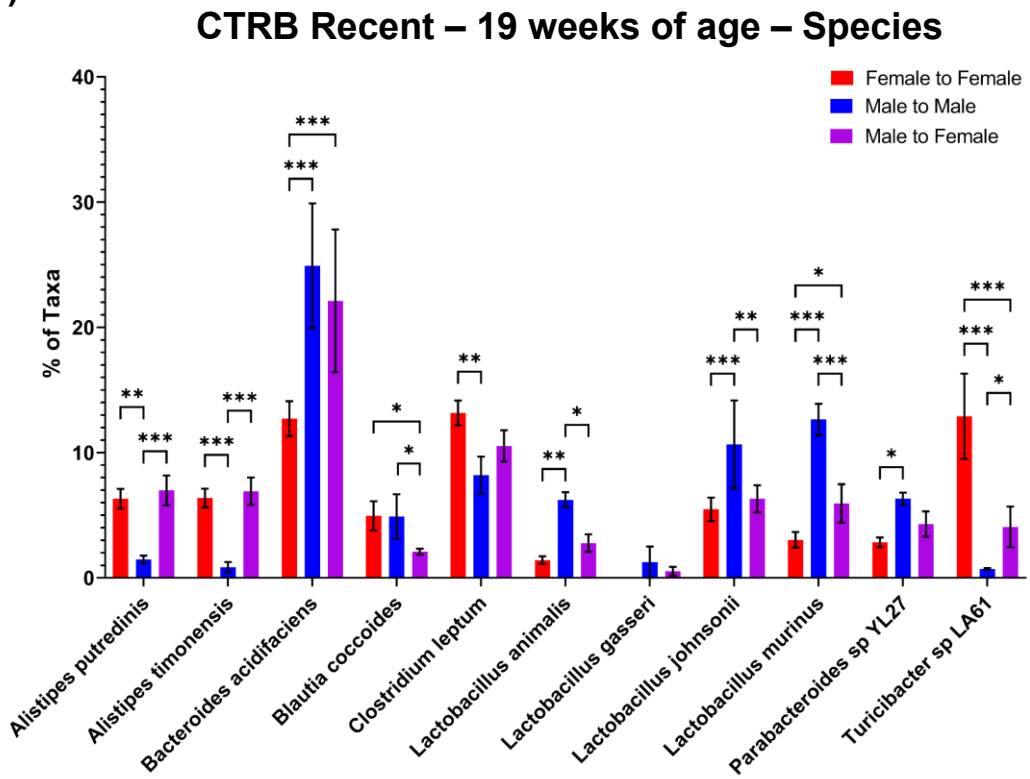
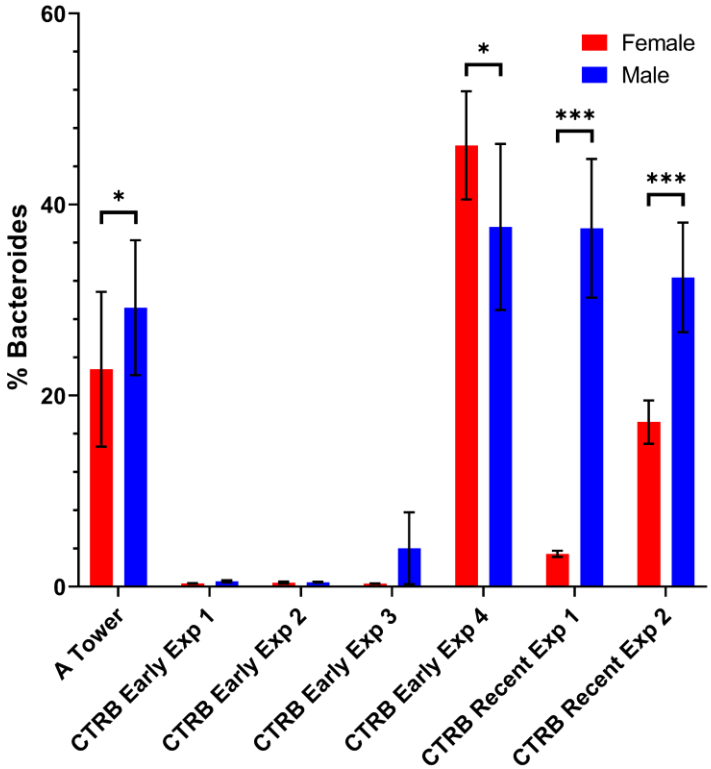

CTRB Recent – 19 weeks of age – Species
Female to Female
Male to Male
Male to Female
% of Taxa
0
10
20
30
40
Alistipes putredinis
Alistipes timonensis
Bacteroides acidifaciens
Blautia coccoides
Clostridium leptum
Lactobacillus animalis
Lactobacillus gasseri
Lactobacillus johnsonii
Lactobacillus murinus
Parabacteroides sp YL27
Turicibacter sp LA61

Figure 19. In CTRB Recent microbiota transfer experiments, abundance of *Bacteroides* was higher in the recipients of male microbiota, and abundance of *Clostridium* was low at two different time-points. During the CTRB Recent (2019 - present) period, female BWF1 mice were fed cecal contents (i.e., microbiota) from 16-week-old female (Female-to-Female) or male (Male-to-Female) BWF1 mice via gavage. Male mice (Male to Male) were fed male cecal contents as a control. Feces were collected from 16-week-old cecal transfer recipients and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. Abundances were compared with One-way ANOVAs (n=6). **(A)** Genus level microbiota in feces collected at 15 weeks of age (4 weeks after cecal transfer); **(B)** Species level microbiota in feces collected at 15 weeks of age (4 weeks after cecal transfer); **(C)** Genus level microbiota in feces collected at 19 weeks of age (4 weeks after cecal transfer); **(D)** Species level microbiota in feces collected at 19 weeks of age (4 weeks after cecal transfer). *p<0.05, **$p<0.01$, ***p<0.001

(A)


60
40
20
0
% Bacteroides
Female
Male
*
*
***
***
A Tower
CTRB Early Exp 1
CTRB Early Exp 2
CTRB Early Exp 3
CTRB Early Exp 4
CTRB Recent Exp 1
CTRB Recent Exp 2

(B)

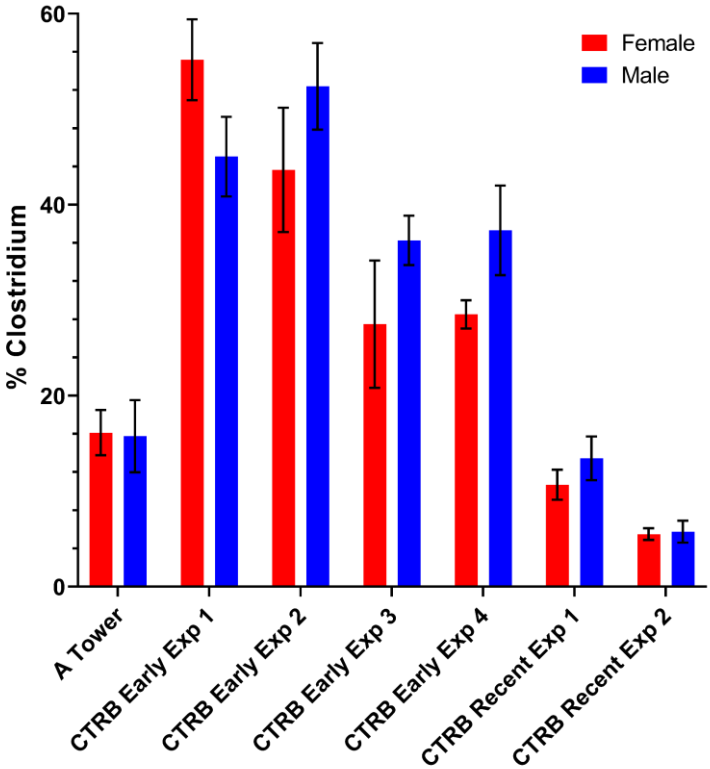
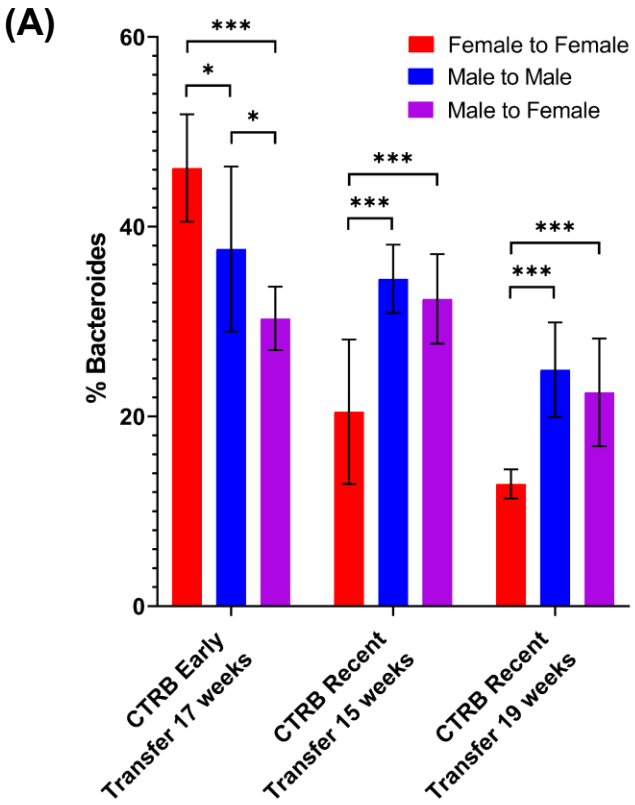

60
40
20
0
% Clostridium
Female
Male
A Tower
CTRB Early Exp 1
CTRB Early Exp 2
CTRB Early Exp 3
CTRB Early Exp 4
CTRB Recent Exp 1
CTRB Recent Exp 2

Figure 20. Comparison of *Bacteroides* and *Clostridium* abundance data in female and male BWF1 mice from experiments conducted during the A Tower, CTRB Early, and CTRB Recent periods. Feces were collected from female and male BWF1 mice during the A Tower (2014-16), CTRB Early (2017-2018), and CTRB Recent (2019-present) periods and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. Abundances were compared with One-way ANOVAs. Data were compiled from experiments shown in **Figures 12, 13, 14** and **16**. **(A)** Compilation of data for *Bacteroides* abundances in females and males; **(B)** Compilation of data for *Clostridium* abundances in females and males. *$p<0.05$, ***$p<0.001$


(A)
Female to Female
Male to Male
Male to Female
% Bacteroides
60
40
20
0
***
*
*
***
***
***
***
CTRB Early Transfer 17 weeks
CTRB Recent Transfer 15 weeks
CTRB Recent Transfer 19 weeks

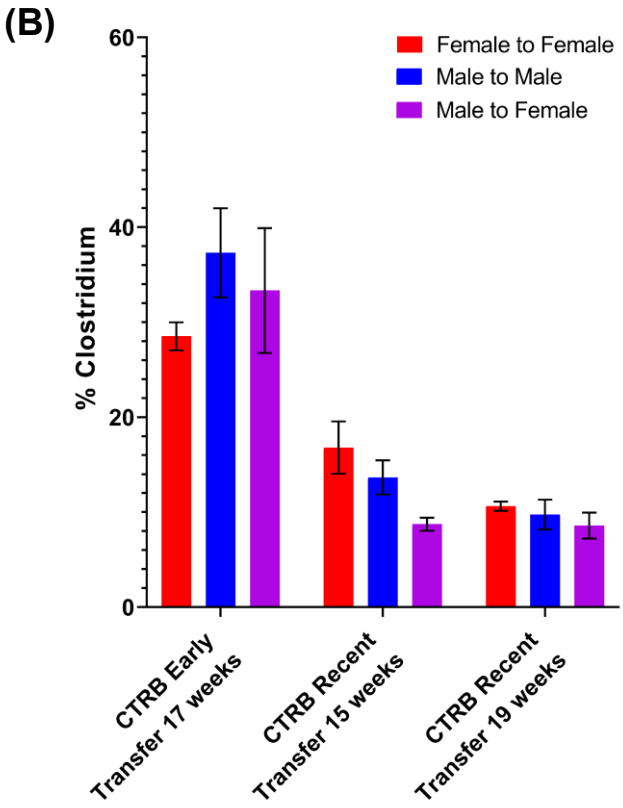

(B)
Female to Female
Male to Male
Male to Female
% Clostridium
60
40
20
0
CTRB Early Transfer 17 weeks
CTRB Recent Transfer 15 weeks
CTRB Recent Transfer 19 weeks

Figure 21. Comparison of *Bacteroides* and *Clostridium* abundance data in cecal transfer experiments conducted during the CTRB Early and CTRB Recent periods. During the CTRB Early (2017-2018) and CTRB Recent (2019-present) periods, female BWF1 mice were fed cecal contents (i.e., microbiota) from 16-week-old female (Female-to-Female) or male (Male-to-Female) BWF1 mice via gavage. Feces were collected from 16-week-old cecal transfer recipients (4 weeks after cecal transfer), and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. Abundances were compared with One-way ANOVAs. Data were compiled from experiments shown in **Figures 18** and **19**. **(A)** Compilation of data for *Bacteroides* abundances in cecal recipients; **(B)** Compilation of data for *Clostridium* abundances in cecal recipients. *$p<0.05$, ***$p<0.001$

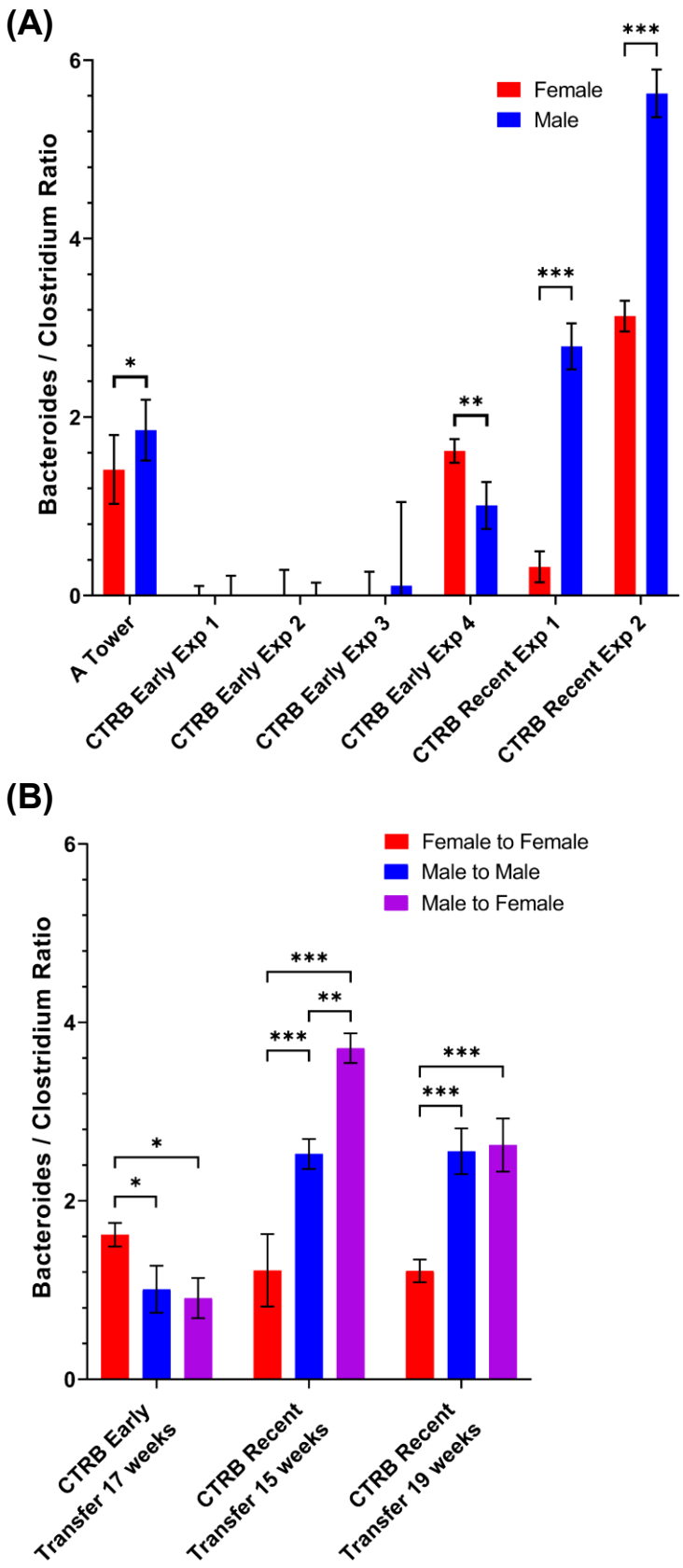

(A)
Bacteroides / Clostridium Ratio
Female
Male
A Tower
CTRB Early Exp 1
CTRB Early Exp 2
CTRB Early Exp 3
CTRB Early Exp 4
CTRB Recent Exp 1
CTRB Recent Exp 2
(B)
Bacteroides / Clostridium Ratio
Female to Female
Male to Male
Male to Female
CTRB Early Transfer 17 weeks
CTRB Recent Transfer 15 weeks
CTRB Recent Transfer 19 weeks

Figure 22. Ratios between Bacteroides and Clostridium abundance in female vs male and cecal transfer experiments. Fecal samples from **(A)** female and male collected during the A Tower, CTRB Early (2017-2018) and CTRB Recent (2019-present) periods and **(B)** cecal transfer recipients collected during the CTRB Early (2017-2018) and CTRB Recent (2019-present) periods and bacterial DNA was extracted. The 9 hypervariable regions of the 16S rRNA gene were sequenced and used to determine microbiota taxonomic composition. The *Bacteroides*/*Clostridium* ratios were calculated from the data shown in **Figure 20** for the female and male comparisons and **Figure 21** for the cecal transfer comparisons by dividing average *Bacteroides* abundance by average *Clostridium* abundance. **(A)** *Bacteroides/Clostridium* ratios for the female to male comparisons; **(B)** *Bacteroides/Clostridium* ratios for the cecal recipient comparisons. *$p<0.05$, **$p<0.01$, ***$p<0.001$

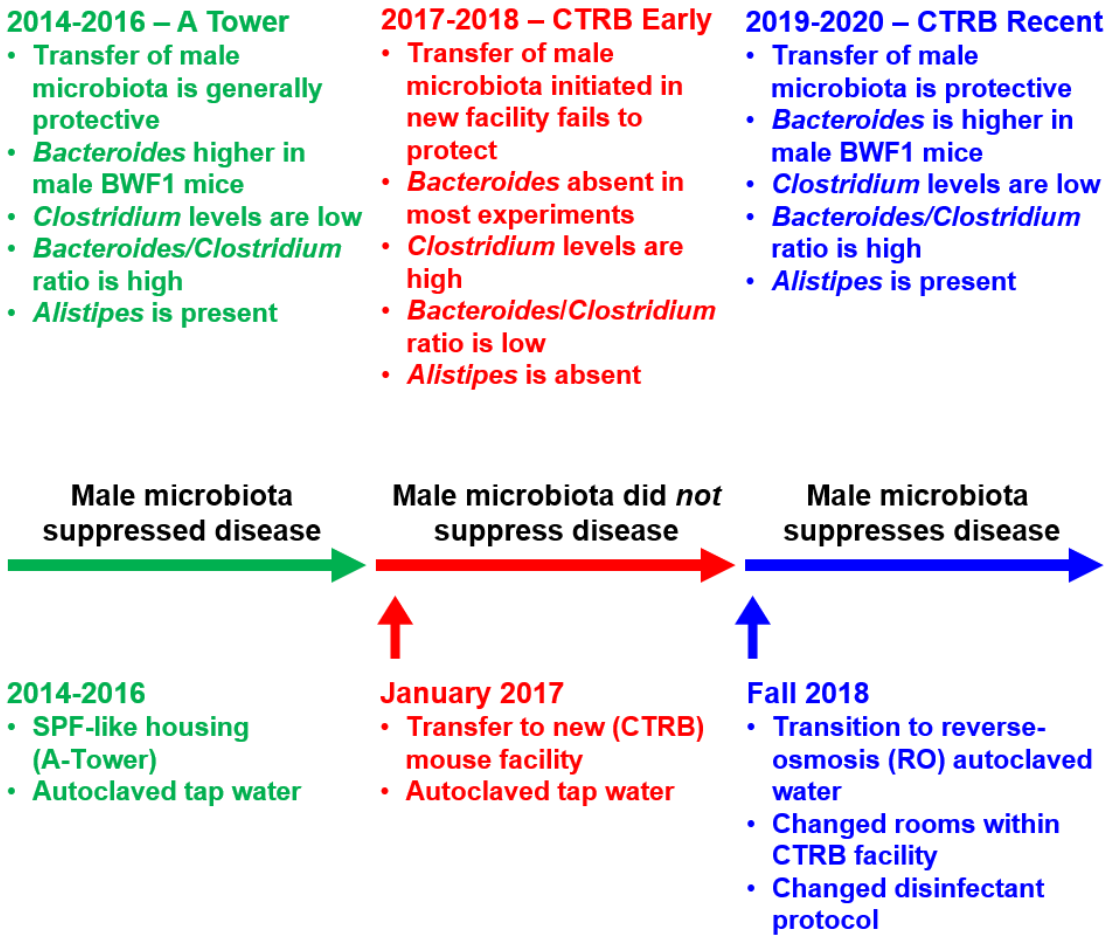


Figure 23. Timeline of facility, disease phenotype, and microbiota composition changes.

SPECIFIC AIM 2

Comparison of metabolomic profiles in lupus-prone versus lupus-resistant BWF1 mice

Introduction

Lupus exhibits a considerable sex bias with women having a much higher incidence [1,2], and sex hormone levels can significantly affect lupus progression [115,138,141-144,149-152]. Additionally, l*upus has* been linked to dysbiotic microbiomes in both human SLE patients and in mouse models of lupus [276-283,286,293,297,299]. Alterations in the microbiome have been shown to affect lupus progression [282,297,299], and microbiome composition can be affected by sex and sex hormones [182,300-303,315]. Consequently, sex-based differences in the microbiota have been proposed as a potential contributor to the sex bias of lupus. This is supported by recent studies showing that sex-based microbiota differences contributed to the sex bias in the NOD mouse model of type 1 diabetes [323,324]. As described above, we have found differences in microbiota composition between female and male BWF1 mice and between intact and castrated male BWF1 mice **(Fig. 4; 6)**. Furthermore, we have found that transfer of male microbiota to female recipients protects them from disease **(Fig. 7; 10)**. The mechanisms underlying this protection are currently not known.

One way that the gut microbiota can affect immune response is by producing immunomodulatory metabolites. These come in many different classes and have been shown to be able to affect the progression of autoimmune diseases [327]. Short chain fatty acids (SCFAs) including acetate, butyrate, and propionate, are one class that have been repeatedly shown to promote anti-inflammatory immune activities in dendritic cells, macrophages, and neutrophils [328-332]. Low levels of SCFAs and SCFA-producing bacteria are associated with multiple auto-inflammatory diseases including IBD, Crohn's disease, and spondyloarthritis [333-336], and SCFAs can ameliorate symptoms in mouse models of auto-inflammatory diseases [334,337-339]. SFCAs can also affect diseases outside the intestine. Multiple sclerosis patients have lower levels of SCFAs in their serum [448] and treating a mouse model of experimental autoimmune encephalomyelitis (EAE) with SCFAs reduces disease severity [341].

Not all gut metabolites have anti-inflammatory effects. Trimethylamine (TMA) is a choline metabolite synthesized exclusively by the microbiota. It is transported into the liver where it is converted to trimethylamine N-oxide (TMAO). Unlike SCFAs, TMAO promotes inflammatory responses. It activates the NLRP3 inflammasome [358,359], and higher serum TMAO levels have been linked to several human autoimmune diseases, including primary sclerosing cholangitis [360], psoriatic arthritis [361], and RA [362].

Another category of gut metabolites are secondary bile acid metabolites which are synthesized from primary bile metabolites by the microbiota. Like SCFAs, they have been shown to have anti-inflammatory effects. Disruption of secondary bile acid production can contribute to the development of IBD, nonalcoholic fatty liver disease, cholestatic liver injury, and other diseases [342,363,368]. As with the studies on SCFAs

and described above, these studies emphasize that immune processes both inside and outside the intestine can be affected by altered microbiota metabolite production.

In the current study, we have found that the fecal metabolomic profiles of female and male BWF1 mice differ. Androgen depletion via castration also causes a shift in metabolite composition. In three separate experiments, we found phytol, a gut metabolite with RXR and PPARγ agonist activities, to be more abundant in male than female BWF1 feces. In the most recent and comprehensive analysis, phytol was also elevated in intact vs castrated male BWF1 feces [420], and phytol's derivative, phytanic acid which is a very potent RXR and PPARγ agonist [449-453], was detected in intact male BWF1 feces, but not female or castrated male BWF1 feces. This indicates that lupus-resistant male BWF1 mice and lupus-susceptible female and castrated male BWF1 mice have differences in intestinal metabolites that may be linked to the differences in microbiota profiles we have found and possibly to the ability of the male microbiota to suppress disease.

Results

Lupus-prone female and castrated male BWF1 mice have significantly different gut metabolite profiles from lupus-resistant male BWF1 mice

Differences in microbiota composition between female and male, and between intact and castrated male mice such as we have found could lead to differential production of gut metabolites that could have an impact on the immune response and/or disease. To evaluate this possibility, we compared the intestinal metabolomic profiles of adult lupus-prone female and lupus-resistant male BWF1 mice by analyzing fecal

metabolite compositions. Metabolites were extracted from the feces, derivatized with N-methyl-N-trimethylsilytrifluoroacetamide (MSTFA), and analyzed using two-dimensional gas chromatography mass spectrometry (GC×GC-MS). Retention time and mass spectra of compounds detected were compared to those of known metabolites to determine the composition and abundance of gut metabolites **(Fig. 24A; Table 1)**. Partial least squares-discriminant analysis (PLS-DA) was performed to visualize the differences between female and male metabolite profiles. We observed a clear sex-based difference in metabolite profiles **(Fig. 24B)**. In the time since that initial experiment (Metabolomic Experiment #1), our collaborators have upgraded their metabolite detection platforms, and this experiment was repeated. In the second experiment, (Metabolomic Experiment #2), in addition to the analysis using GC×GC-MS with MSFTA derivatization, metabolite composition was analyzed using two-dimensional liquid chromatography mass spectrometry (2DLC-MS/MS) with either negative or positive ionization. This expanded the range of metabolites that could be detected, since 2DLC-MS/MS is more efficient at detecting less polar metabolites. Combined with improvements to the GC×GC-MS reference database, Experiment #2 identified 76 metabolites significantly different between female and male BWF1 mice **(Table 2)**, compared with 16 in Experiment #1 **(Table 1)**. The ionization mode, which is the charge metabolites are given before entering the detector, also influences which metabolites can be detected. Positive ionization allows the detection of a greater range of compounds, but negative ionization lowers background noise and is useful for detecting low-abundance metabolites [454]. In a third experiment (Metabolomic Experiment #3), we compared the metabolomic profiles of lupus-resistant intact male BWF1 mice not only to lupus-prone female mice, but also

to lupus-prone castrated male BWF1 mice. In Metabolomic Experiment #3, the samples were analyzed via four different methods: GC×GC-MS *with* either MSTFA or N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) derivatization, and two-dimensional liquid chromatography mass spectrometry (2DLC-MS/MS) with either negative or positive ionization **(Tables 3-5)**. The use of multiple derivatization reagents further increased the range of metabolites that could be detected. The derivatization reagent MSTFA is more efficient at facilitating detection of larger metabolites, while MTBSTFA is more efficient at facilitating detection of smaller metabolites [455]. Using multiple methods therefore increases the number and types of metabolites detected. For all four of the methodologies, PLS-DA analysis indicated that there were differences between the intact male, castrated male, and female BWF1 metabolomic profiles **(Fig. 25)**, strongly suggesting that both sex and sex hormone levels affect the composition of intestinal metabolites. In addition, there was considerable overlap between the metabolites that differed between the female and intact male BWF1 mice and the metabolites that differed significantly between the castrated and intact male BWF1 mice. Side-by-side comparison of the fold changes in these sets of metabolites showed that not only did they overlap, but the direction of the fold change compared to the male mice was often the same **(Fig. 26)**. These data suggest that the male sex hormones, androgens, have a significant impact on metabolomic profiles. In addition, quantitative pathway enrichment analysis of the two sets of significantly different metabolites showed several pathways were significantly altered in intact male BWF1 mice compared to both female and castrated male BWF1 mice **(Fig. 27)**. This suggests that lupus-resistant intact male

BWF1 may have functional metabolic differences from lupus-susceptible female and castrated male BWF1 mice.

Specific immunomodulatory metabolites are increased in lupus-resistant intact male BWF1 mice compared to lupus-susceptible female and castrated male BWF1 mice.

Differences in the production of microbial metabolites with immunomodulatory properties are known to affect the progression of autoimmune diseases [327]. We studied the known properties of the metabolites that were significantly different between female and male BWF1 mice to determine whether any could potentially affect the development and/or progression of disease. Pathway analysis of Metabolomic Experiment #3 identified histidine metabolism as being significantly altered both between the female and male BWF1 mice, and the intact and castrated male BWF1 mice **(Fig. 27)**. In this pathway, histidine is converted into histamine, a molecule with potent effector activities. 2DLC-MS/MS with positive ionization (2DLC-MS/MS (+)) showed that compared to female and castrated male BWF1 mice, intact males had lower levels of the histamine precursor, histidine **(Fig. 28A)**, and higher levels of histamine **(Fig. 28B)**, and the histamine breakdown product, methylimidazoleacetic acid **(Fig. 28C)**. This suggests that intestinal histamine production may be higher in intact male than female or castrated male BWF1 mice. The gut microbiota is a known producer of histamine, so the higher production in intact males could be due to the altered microbiota profiles seen in male BWF1 mice [351-353]. This theory is supported by our previous results from positive ionization 2DLC-MS/MS (2DLC-MS/MS(+)) analysis in Experiment #2, which also found histamine was more abundant in male than female BWF1 mice **(Fig. 28D)**. In this

prior analysis, neither histidine or methylimidazoleacetic acid were detected, but this is likely due to improvements in metabolite detection ability and reference databases between Experiments #2 and #3.

Many findings of significant metabolite differences between female and male BWF1 feces in one experiment were not repeated in other experiments. For example, butyrate and niacin, both well-documented immunomodulators, were both found to be significantly higher in male than female BWF1 mice in Experiment #2 **(Fig. 29A,B, Table 2)**. However, in Experiment #3, although both were detected, neither of these metabolites was found to be significantly different between male and female BWF1 mice **(Fig. 29C,D)**. Neither butyrate nor niacin was detected in Experiment #1, but this could be due to the more limited detection capabilities of the platform at that time. This inconsistency in findings between experiments is a major obstacle in the study of metabolomics.

However, there was one metabolite that was consistently higher in male than female BWF1 mice across all three metabolomic experiments. This metabolite, phytol, was more abundant in lupus-resistant male than lupus-prone female BWF1 mice in all three analyses **(Fig. 30)**. Phytol is an acyclic diterpene alcohol which is produced from the breakdown of chlorophyll and has known RXR and PPARγ agonist activity, [449,450,456], and there is evidence in animal models that signaling through RXR and PPARγ receptors can, in general, alter immune responses in ways that could potentially suppress lupus [394,457-459]. This correlation between lower phytol levels and lupus susceptibility was also found in lupus-prone castrated male BWF1 mice which also have lower abundance of phytol than intact male BWF1 mice and similar levels as female

BWF1 mice **(Fig. 31A)**. Phytol is produced exclusively by the gut microbiota [460-462], but once absorbed by the intestine, host enzymes convert it to phytanic acid, which has even more potent RXR and PPARγ agonist activity than phytol, and is more easily transported in esterified form through the circulatory system [449-453,463]. For the most recent experiment, Metabolite Experiment #3, improvements in detection capabilities and reference databases allowed us to detect phytanic acid. We found high levels of phytanic acid in feces from intact male mice compared to feces from female and castrated male BWF1 mice, and in fact, no phytanic acid was detected in female and castrated male feces **(Fig. 31B)**. Taken together, the data show that both phytol and its derivative, phytanic acid, are more abundant and/or only present in intact male BWF1 mice. The known RXR and PPARγ agonist activity of these metabolites raises the question of whether this could contribute to the protective capability of the male microbiota and whether one or both of these molecules could be used as a potential therapeutic for the treatment of lupus. lupus.

Discussion

While our bacterial taxa findings are promising, one of the challenges in microbiota research is the fact that the effects of bacterial taxa are context dependent. What microbiota are associated with a disease can vary between mouse strains. For example, low abundance of *Lactobacillus* is associated with increased lupus symptoms in the MRL/*lpr* mouse model [297,298], but in the BWF1 mouse model of lupus, high abundance of *Lactobacillus* is associated with more severe renal disease [282]. Mouse models and human patients can also differ in the bacteria that are associated with disease,

as a study found that, unlike MRL/*lpr* mice, *Lactobacillus* was elevated in SLE patients compared to healthy controls.

Due to this complexity, it is useful to use multiple approaches when studying the relationship between microbiota and disease. In addition to using 16S rRNA sequencing to determine the bacterial species that make up the BWF1 microbiota, we also used metabolomic analyses to identify the fecal metabolite profiles. This approach allowed us to investigate differences in gut metabolism between lupus-susceptible female and lupus-resistant male BWF1 mice. The influence of the gut microbiota on the host is likely mediated in part via the metabolites they produce [464]. Our aim was to characterize the gut metabolomic profiles of disease susceptible female and disease-resistant male BWF1 mice. To this end, we performed three metabolomic experiments (Metabolomic Experiments #1, 2, & 3) analyzing female and male BWF1 fecal metabolites. The platforms used to analyze the fecal metabolites increased from analysis to analysis, so each analysis was more thorough than the last. While Experiment #1 only used GC×GC-MS with MSTFA derivatization, Experiment #2 also used 2DLC-MS/MS, which is more efficient at detecting less polar metabolites. In Experiment #3, GC×GC-MS with MTBSTFA derivatization was added, which is more effective at detecting small metabolites. For Experiment #3, castrated male BWF1 feces were analyzed in addition to the male and female BWF1 feces, and PLS-DA visualization of their metabolomic profiles showed that for all four platforms, the profiles of the three groups were divergent. This matches our findings that lupus-resistant intact male mice have significantly different microbiota compositions compared to both female and castrated male BWF1 mice. Furthermore, a side-by-side comparison of metabolites significantly

different between intact male compared to female or castrated male BWF1 mice, shows considerable overlap between the two sets, suggesting that androgens may have considerable influence on metabolite production. This fits with the findings of previous studies, which have shown that androgen levels affect the microbiota [323,324,465,466]. Also, androgens are known to suppress lupus progression [103,122-125], and alterations of production of immunomodulatory metabolites can influence autoimmune diseases [464]. In this context therefore, our findings suggest that androgens could potentially suppress lupus by altering microbiota metabolite production in addition to their direct effects on immune cells.

The increased coverage of metabolites in Metabolomic Experiment #3 also enhanced our ability to identify differentially regulated metabolic pathways that could be relevant to the development and/or progression of lupus. We therefore used the metabolites with significant abundance difference between groups for pathway analysis. The metabolic pathway analysis showed that the differences in pathways between castrated and male mice closely resembled the differences between female and male mice. Of the 41 pathways differentially regulated in castrated vs intact male mice, 28 were also differentially regulated in female vs male mice **(Tables 6,7)**. Thus, the data showed that depleting androgens in castrated male mice causes shifts in intestinal metabolites toward a female-like phenotype that correlates with an increase in susceptibility of the castrated male mice to lupus.

Androgen does not account for all biological differences between male and female mice. The changes in the 28 common pathways differentially regulated in both the castrated vs intact male analysis and the female vs male analysis of Metabolomic

Experiment #3 could be due to androgen depletion or due to other sources of sex differences (i.e., Y chromosome effects). This is evident from the fact that there were 43 metabolites significantly different between castrated male mice and female mice **(Table 5)**. To narrow our pathway analysis to identify metabolic pathways that are dependent on androgen, we performed a pathway analysis on the set of metabolites that were significantly different between both castrated and intact male mice as well as between female and male mice, but were not significantly different between female and castrated male mice (i.e., metabolites present in both **Tables 3** and **4** but not in **Table 5**). We found that after removing the metabolites that were significantly different between female and castrated male mice, 18 of the 28 common pathways remained **(Table 8)**. These pathways are the most likely to be different as a result of androgen depletion.

The significance of the pathway analysis is that it identifies potential areas of study for how the differences in intestinal metabolite production could affect lupus progression. One of the pathways differentially affected in Experiment #3 in both female and castrated male mice compared with intact male mice was histidine metabolism. Histidine is a precursor to histamine, an effector molecule that can mediate either inflammatory or regulatory responses depending on the target cell and type(s) of histamine receptor expressed. Recent studies have found that probiotic and endogenous bacterial species could produce histamine and affect intestinal immune responses [351-353]. In Experiment #3, both castrated and female mice had lower levels of histamine and higher levels of the precursor, histidine than intact male mice. Additionally, castrated and female mice had lower levels of methylimidazoleacetic acid (methylimid), which is the end-product of histamine breakdown and is used as an indicator of histamine production

[467]. While histidine and methylimidazoleacetic acid were not detected in Experiment #2, likely due to the lower range of detection and poorer reference database, histamine was detected and was higher in male than female BWF1 feces. Therefore, our results clearly show intact male mice have a higher production of histamine in the gut that correlates with their lower susceptibility to lupus. However, whether altered histamine metabolism is simply a consequence of lupus or if it actually plays a role in the progression of disease has not been established. Gut-generated histamine could potentially affect the development or progression of lupus via local effects on the intestine. While histamine is classically associated with allergic reactions, microbe-produced histamine has been shown to directly inhibit inflammatory factors in the gut [354-356]. Because lupus is associated with low-grade intestinal inflammation and the "leaky gut" that this causes [297,357], inhibition of gut inflammation in lupus-prone mice (or humans) via microbe-produced histamine could potentially affect the development and/or progression of the disease. Additionally, suppressing intestinal inflammation could also inhibit disease by promoting the ability of intestinal DCs to induce immunosuppressive Tregs in the mesenteric lymph node (mLN) since inflammatory impairs this ability in intestinal DCs [468-475]. Multiple studies have found links between lupus and decreases in regulatory T cell frequency or function in both humans and mouse models [476-482], so this could clearly impact the progression of lupus in BWF1 mice. Bacterial histamine can also directly inhibit intestinal DC production of TNFα [483], an inflammatory cytokine that suppresses DC induction of Tregs in the mLN [475,484]**.** In summary, the higher levels of histamine in intact male intestine could

affect the development and/or progression of lupus via indirect or direct effects on DCs that increase Treg induction by DCs in the mLN.

However, while the finding of elevated histamine in male BWF1 mice repeated in Experiments #2 and #3, many metabolites did not. For example, niacin and butyrate are both known to induce Tregs, and therefore the finding in Experiment #2 that they were more abundant in male than female BWF1 feces was very interesting. However, in Experiment #3, both were detected but neither were significantly different between female and male BWF1 mice. This inconsistency between experiments has been a major challenge in our metabolomic studies. Therefore, it is very significant that a metabolite, phytol, was more abundant in male than female BWF1 mice in all three experiments and was higher in intact than castrated male BWF1 mice in Experiment #3. Phytol is an acyclic diterpene alcohol produced by the breakdown of chlorophyll by bacteria in the gut, and it can bind and activate the RXR and PPARγ nuclear receptors [449,450,456]. Once phytol is absorbed, by the host, it is broken down into phytanic acid by the liver and other tissues [485]. Phytanic acid has even more potent PPARγ and RXR agonist activity compared to its parent compound, phytol, and it is more readily transported throughout the body in an esterified form [449-453,463]. In Experiment #3, improvements in metabolite detection allowed us to test phytanic acid levels, and we found that while male BWF1 feces had significant levels, no phytanic acid was detected in female or castrated male BWF1 feces. Increased levels of phytanic acid could potentially affect lupus progression in a number of ways, since RXR and PPARγ stimulation promote a number of immune processes that could potentially suppress lupus autoimmunity [383,400,457,459,486]. One possibility is that phytol and or phytanic acid could enhance

CD103 positive dendritic cell ($CD103^+$DC) production of retinoic acid (RA), which would enhance their induction of immunosuppressive Tregs. Intestinal $CD103^+$DCs are important for Treg induction [487], and defects in this process can significantly impact systemic immune responses and inhibit tolerance induction [441,488,489]. This process requires $CD103^+$DC RA production [441], and RXR agonists can promote expression of the enzymes that produce RA [490]. Another lupus-suppressing process that could be enhanced by phytol and/or phytanic acid is the phagocytosis of apoptotic cells by macrophages, which is called efferocytosis [371,491,492]. Apoptotic cells are a source of nuclear self-antigens that can stimulate production of the anti-nuclear autoantibodies characteristic of lupus, and deficiencies in the ability of macrophages to clear apoptotic cells correlate with increased disease severity in SLE patients [380,493]. Due to its strong ability to activate RXR and PPARγ, phytanic acid could potentially enhance macrophage efferocytosis through these pathways [381,393-400]. Our subsequent experiments point towards a strong connection between efferocytosis and phytanic acid.

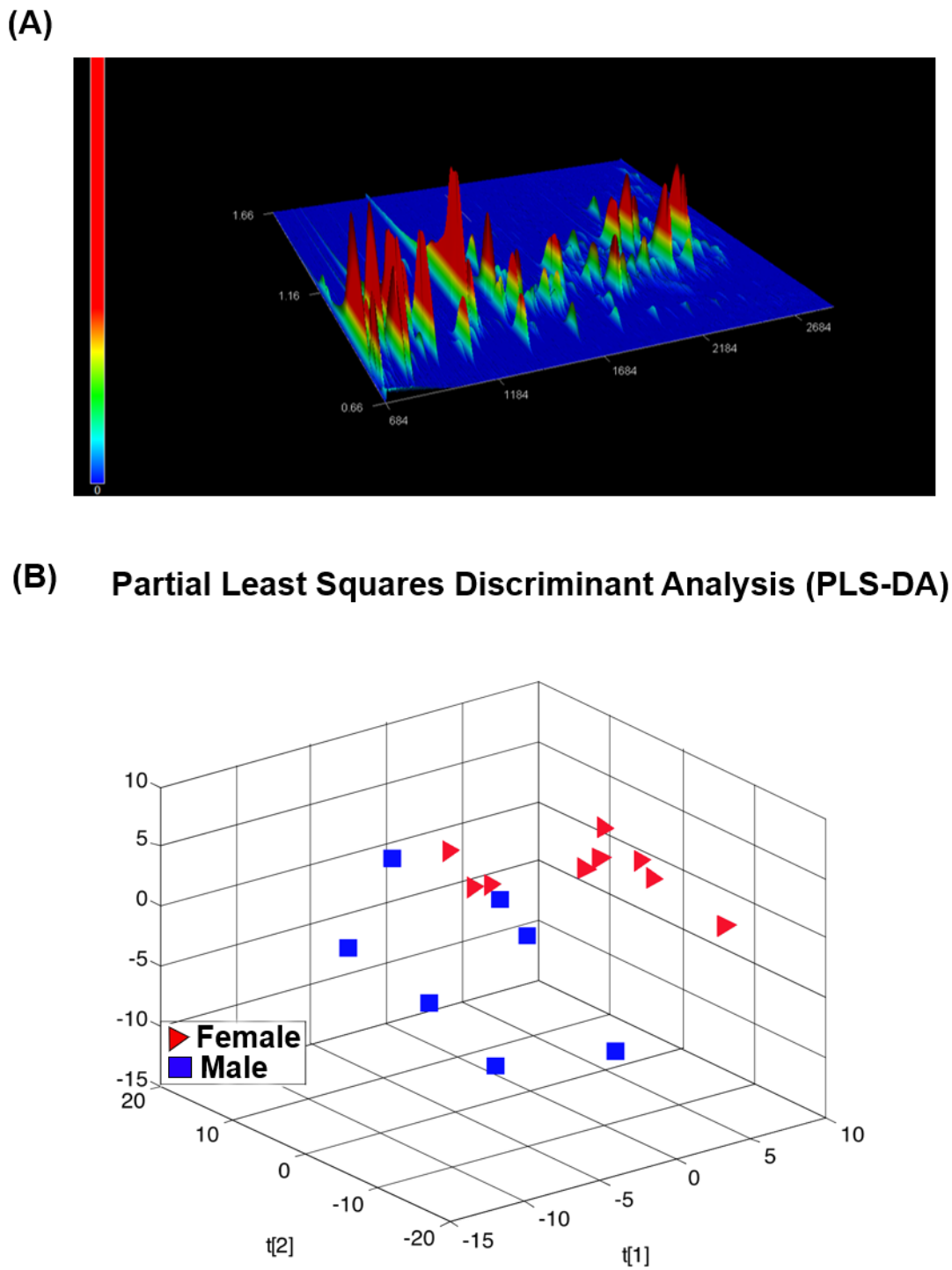


Figure 24. The gut metabolomic profile of adult female and male BWF1 mice differ. For Metabolite Experiment #1 feces were collected from 16-week-old female (n=9) and male (n=7) BWF1 mice and metabolites extracted using 80% methanol, derivatized with MSTFA, and analyzed by GCxGC-MS. **(A)** Representative GCxGC-MS chromatogram. **(B)** Partial least squares discriminant analysis (PLS-DA) of the metabolite profiles.

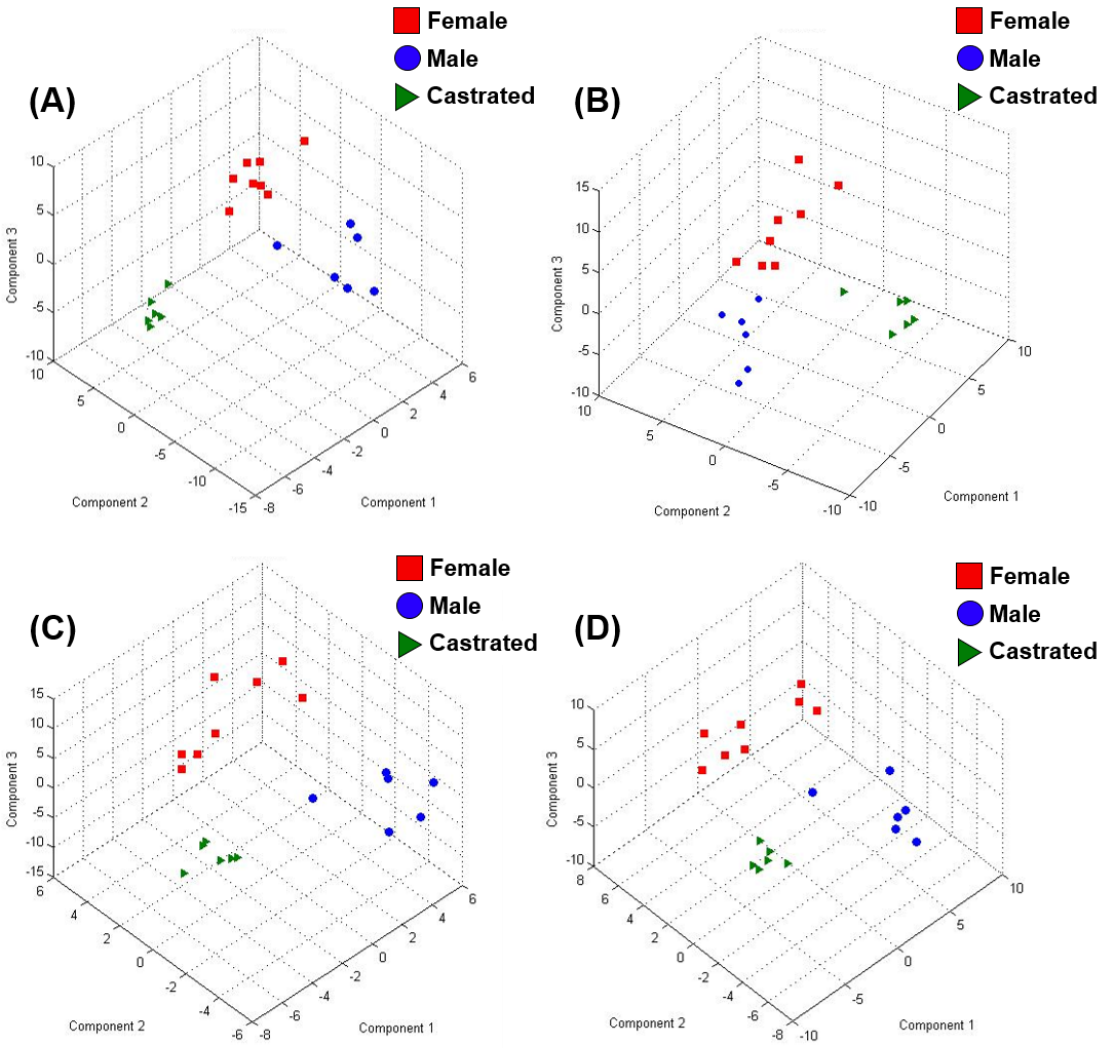


Figure 25. Multiple metabolomic analysis techniques show the BWF1 gut metabolomic profile differs with both sex and androgen depletion. For Metabolite Experiment #3, feces were collected from 16-week-old female (n=8), intact male (Male; n=6), and castrated male (Castrated; n=6) BWF1 mice (castrations were done at ~24 days of age). Metabolites were extracted using 80% methanol [420]. Partial least squares discriminant analysis (PLS-DA) was performed for the metabolite profiles analyzed via: **(A)** GCxGC-MS with MSTFA derivatization. **(B)** GCxGC-MS with MTBSTFA derivatization. **(C)** 2DLC-MS with negative ionization. **(D)** 2DLC-MS with positive ionization.

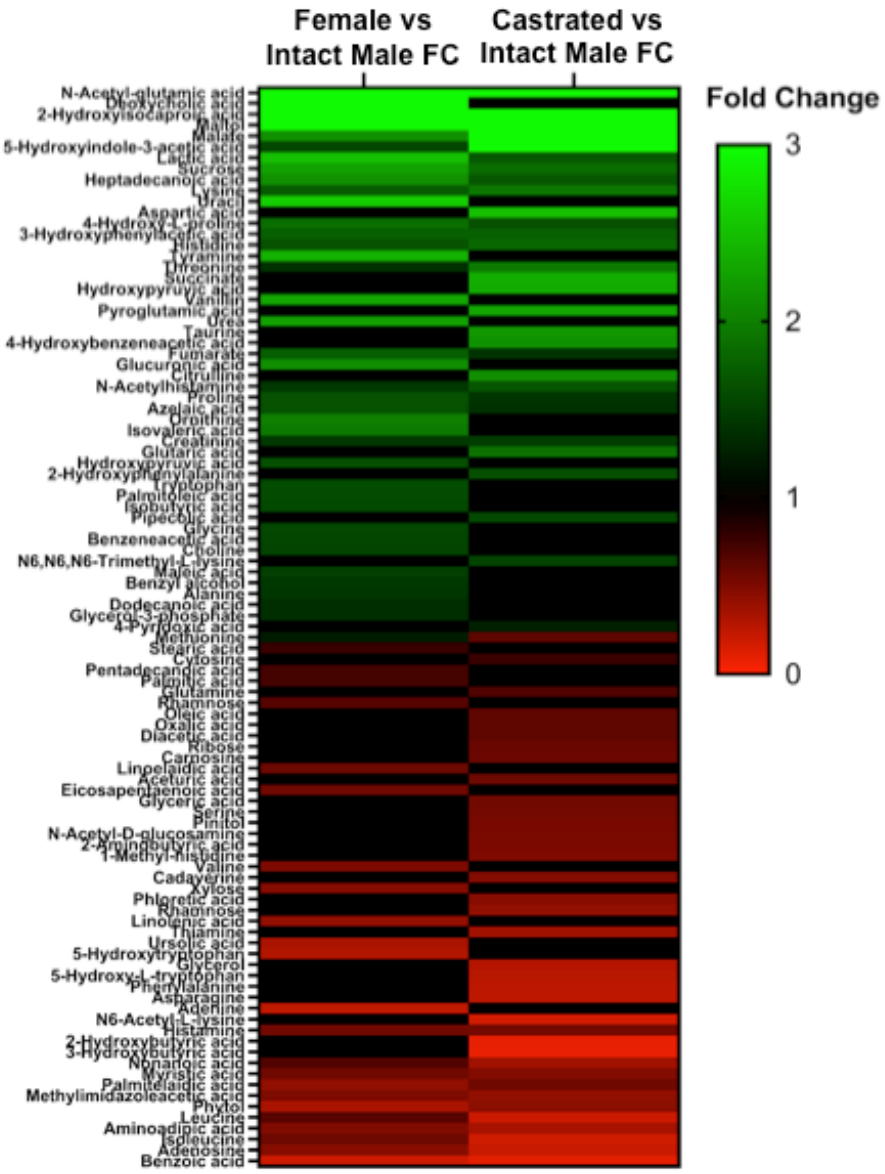
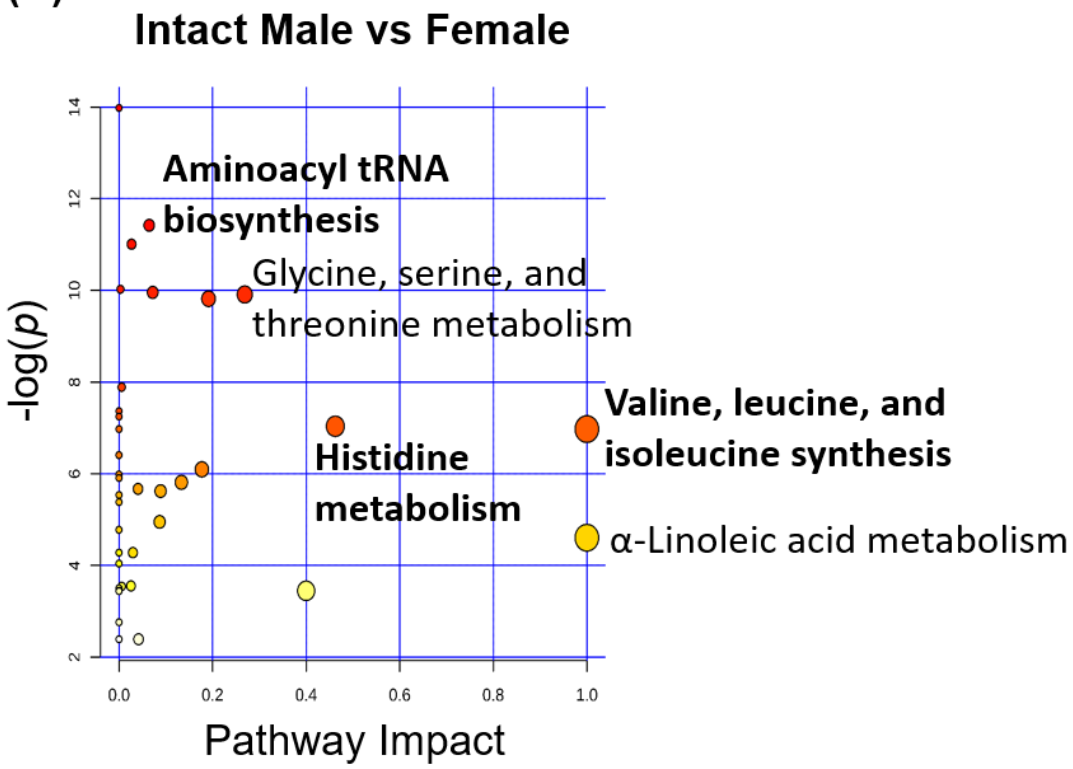


Figure 26. The metabolomic profile in castrated male mice is more similar to female than to intact male mice. For Metabolite Experiment #3, female (n=8), intact male (n=6), and castrated male (n=6) feces were collected and metabolites were analyzed as described in **Figure 25**. For each analysis method, pairwise Student's *t*-tests were used to identify metabolites that were significantly different between at least two of the groups **(Tables 3,4)**. The direction and fold change (FC) of metabolites significantly different between females vs intact males and/or between castrated males vs intact males are shown in a heatmap.

(A)


Intact Male vs Female
Aminoacyl tRNA biosynthesis
Glycine, serine, and threonine metabolism
Histidine metabolism
Valine, leucine, and isoleucine synthesis
α-Linoleic acid metabolism
-log(p)
Pathway Impact

(B)

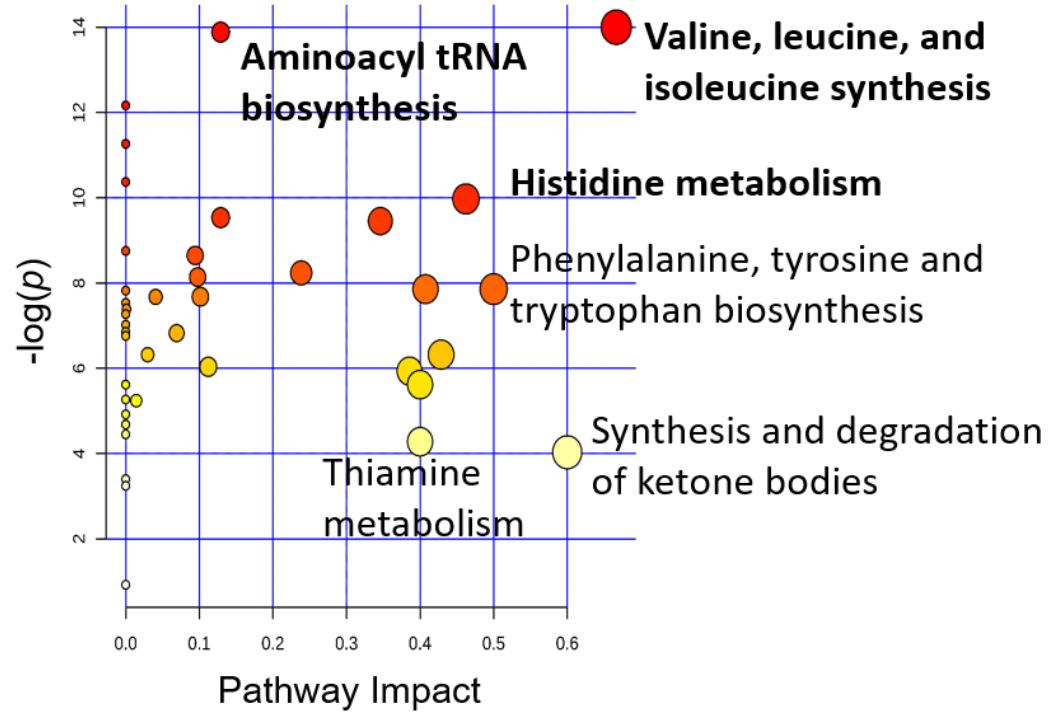

Intact Male vs Castrated
Aminoacyl tRNA biosynthesis
Valine, leucine, and isoleucine synthesis
Histidine metabolism
Phenylalanine, tyrosine and tryptophan biosynthesis
Synthesis and degradation of ketone bodies
Thiamine metabolism
-log(p)
Pathway Impact

Figure 27. Metabolic pathways that are different in female vs intact male mice are also often altered in castrated vs intact male BWF1 mice. For Metabolite Experiment #3, female (n=8), intact male (n=6), and castrated male (n=6) feces were collected and metabolites were analyzed as described in **Figure 25**. Metabolites that were significantly different between intact male and female or castrated male BWF1 mice were identified using pairwise Student's *t*-tests **(Tables 3,4)**. Using Metaboanalyst (v4.0), quantitative pathway enrichment analysis was performed on the metabolites that were significantly different between female and intact male BWF1 mice **(Table 3)** and on the metabolites that were significantly different between intact and castrated male BWF1 mice **(Table 4)**. The metabolites were matched against the KEGG database for *Mus musculus* and analyzed for pathway enrichment and topology [420]. **(A)** Pathway enrichment of metabolites that were significantly different between female and intact male BWF1 mice. **(B)** Pathway enrichment of metabolites that were significantly different between castrated vs intact male BWF1 mice.

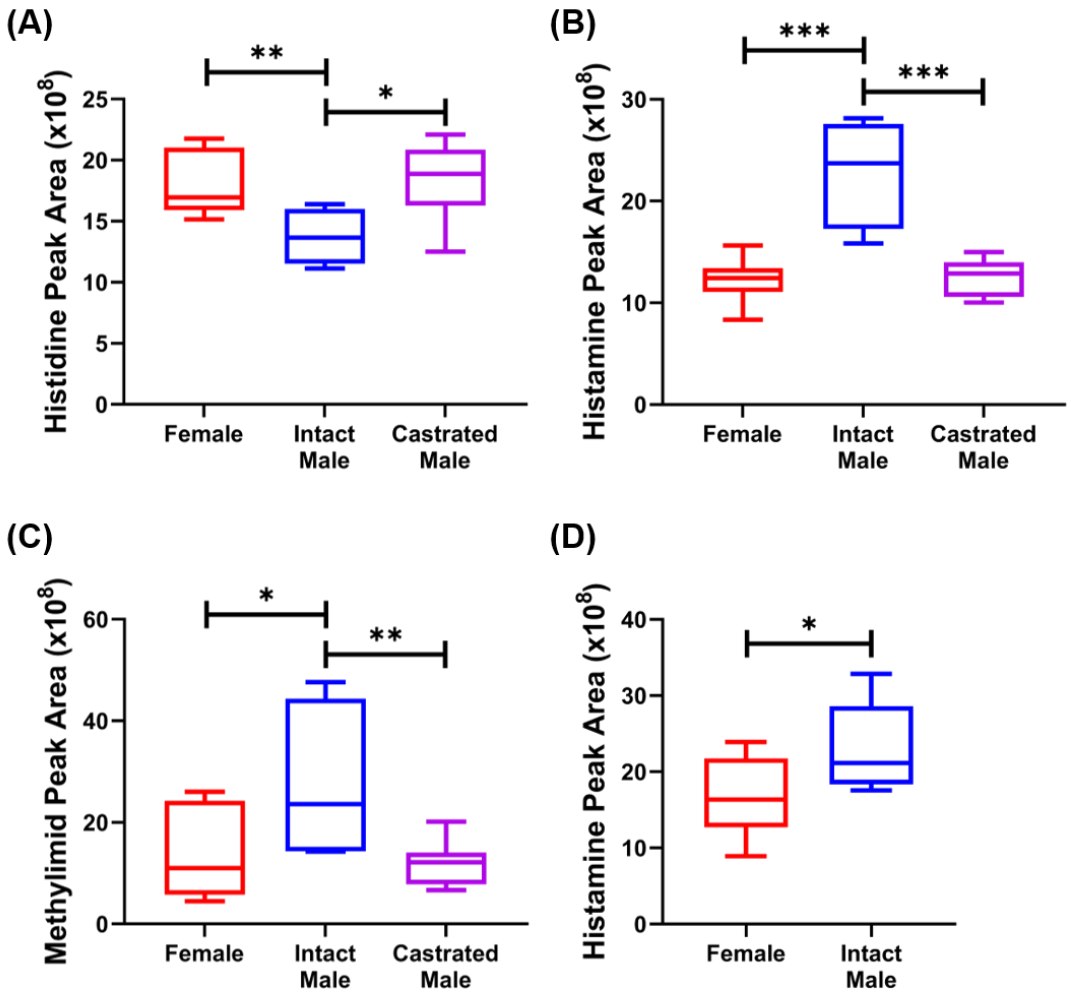


Figure 28. Histamine is more abundant in intact male than female or castrated male BWF1 mice. For Metabolite Experiment #3, feces were collected from 16-week-old female (n=8), and intact (Intact Male; n=6) and castrated (at ~24 days of age; n=6) male BWF1 mice. For Metabolite Experiment #2, feces were collected from 16-week-old female and male (Intact Male) BWF1 mice (n=9). Metabolites were extracted using 80% methanol, analyzed with 2DLC-MS/MS. Significantly different metabolites were identified using pairwise Student's *t*-tests **(Tables 2,3)**. **(A)** Metabolomic Experiment #3 - Histidine abundance. **(B)** Metabolomic Experiment #3 - Histamine abundance. **(C)** Metabolomic Experiment #3 - Methylimidazoleacetic acid (Methylimid) abundance. **(D)** Metabolomic Experiment #2 - Histamine abundance. *p<0.05, **p<0.01, ***p<0.001

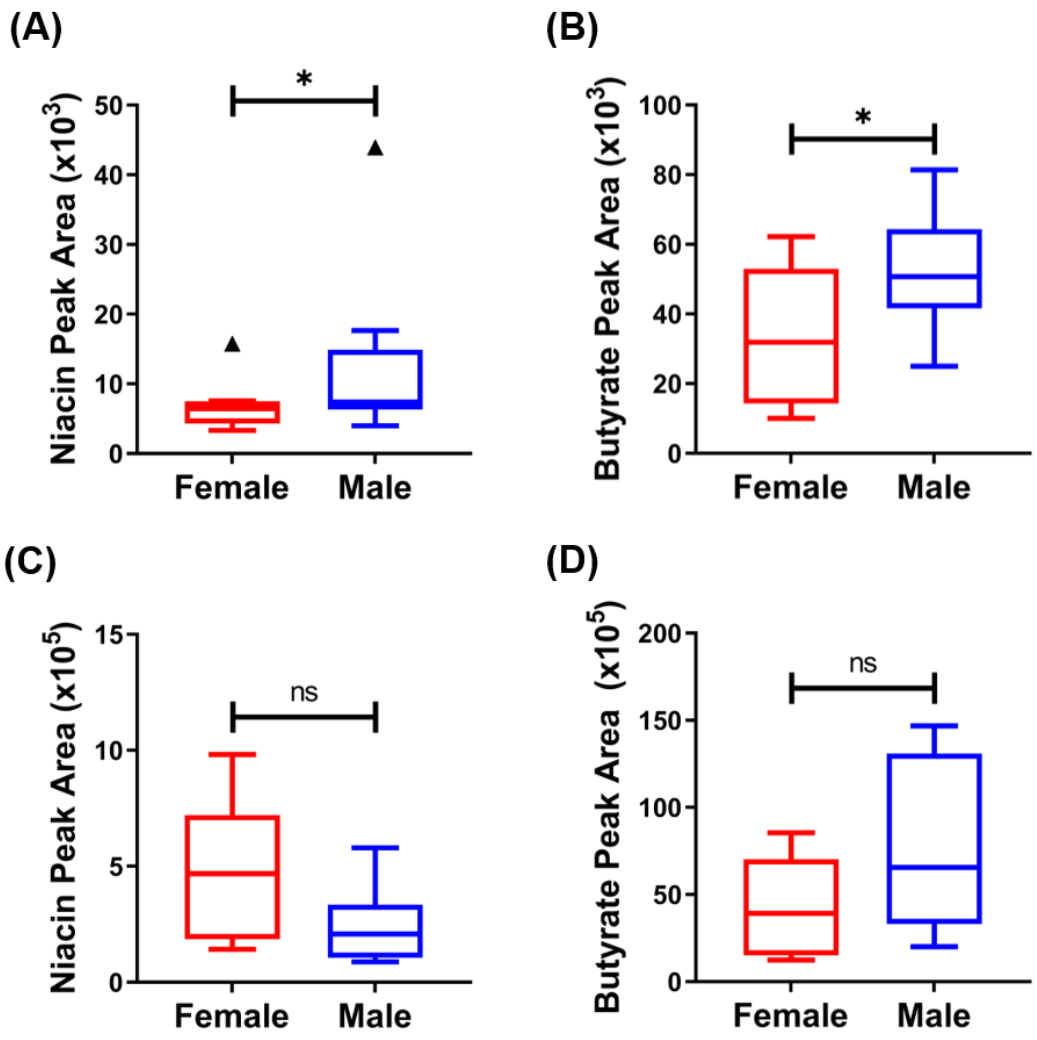


Figure 29. Niacin and butyrate were more abundant in male than female BWF1 mice in Metabolomic Experiment #2, but not Metabolomic Experiment #3. For Metabolite Experiments #2 & #3, feces were collected from 16-week-old female and male BWF1 mice. Metabolites were extracted from feces using 80% methanol, derivatized with MSTFA, analyzed by GCxGC-MS. Significantly different metabolites were identified using pairwise Student's *t*-tests **(Tables 2,3)**. **(A)** Metabolomic Experiment #2 - Niacin abundance (n=9). **(B)** Metabolomic Experiment #2 - Butyrate abundance (n=3). **(C)** Metabolomic Experiment #3 - Niacin abundance (female n=8, male n=6). **(D)** Metabolomic Experiment #3 - Butyrate abundance (female n=8, male n=6). Outliers are indicated as triangles. ns=not significant, *p<0.05

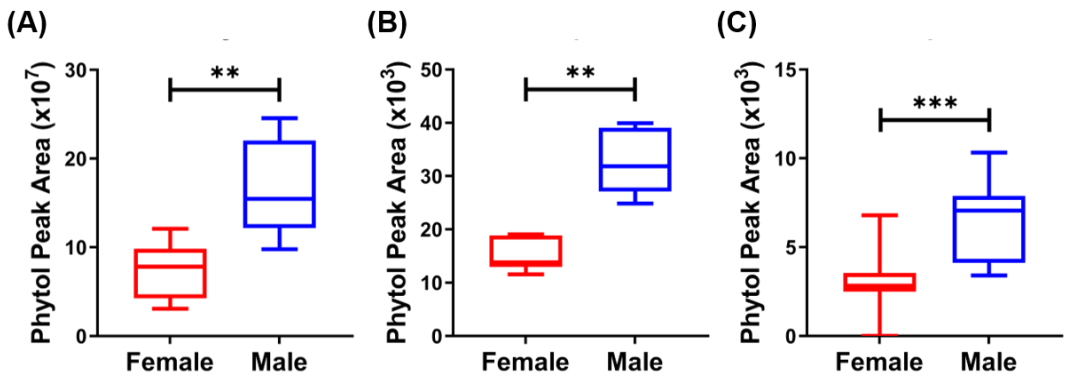


Figure 30. Three separate metabolomic analyses of female and male BWF1 feces have found phytol elevated in male BWF1 mice. For Metabolite Experiments #1, #2 & #3, feces were collected from 16-week-old female and male BWF1 mice. Metabolites were extracted from feces using 80% methanol, derivatized with MSTFA, analyzed by GCxGC-MS. Significantly different metabolites were identified using pairwise Student's *t*-tests **(Tables 1-3)**. **(A)** Metabolomic Experiment #1 - Phytol abundance (female n=9, male n=8). **(B)** Metabolomic Experiment #2 - Phytol abundance (n=9). **(C)** Metabolomic Experiment #3 - Phytol abundance (female n=8, male n=6). ** $p<0.01$, *** $p<0.001$

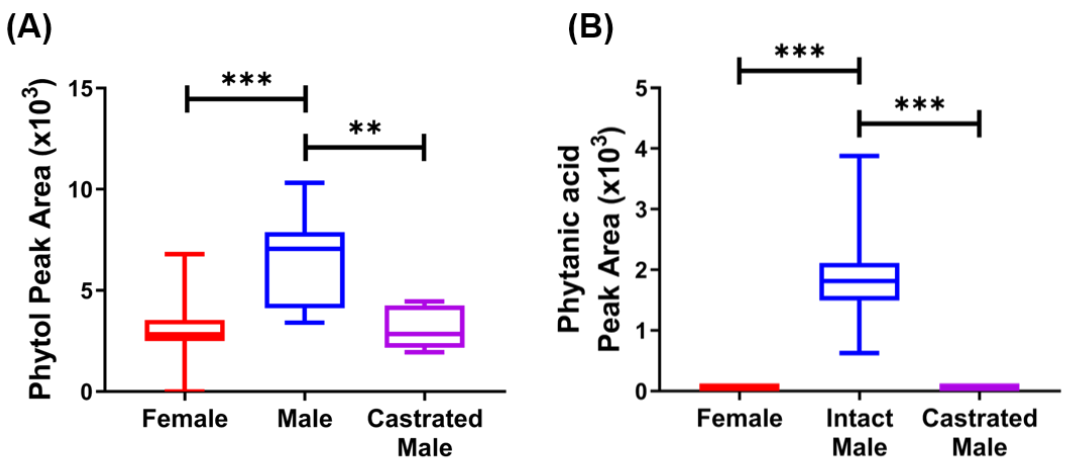


Figure 31. Phytol and the phytol derivative, phytanic acid, are both upregulated in intact male compared to both female and castrated male BWF1. For Metabolite Experiment #3, feces were collected from 16-week-old female (n=8), intact male (Male; n=6), and castrated (at ~24 days of age; n=6) male BWF1 mice. Metabolites were extracted from feces using 80% methanol, derivatized with MSTFA, analyzed by GCxGC-MS. Significantly different metabolites were identified using pairwise Student's *t*-tests **(Tables 3-5)**. **(A)** Metabolomic Experiment #3 - Phytol abundance [420]. **(B)** Metabolomic Experiment #3 – Phytanic Acid (PA) abundance. **p<0.01, ***p<0.001,

Table 1. Metabolomic Experiment #1. Metabolites with significantly different abundance between female and male BWF1 mice (female n=9, male n=8).

Name	*p*-value	Fold change (Male/Female)	Platform
Pentadecanoic acid	0.0027	3.23	GC×GC-MS_MSTFA
Methyl stearate	0.0008	3.03	GC×GC-MS_MSTFA
Phytol	0.0005	2.22	GC×GC-MS_MSTFA
1-Dichloromethyl(dimethyl) silyloxyhexadecane	0.0000	1.89	GC×GC-MS_MSTFA
Arachidonic acid; cis-5,8,11,14-Eicosatetraenoic acid	0.0346	1.69	GC×GC-MS_MSTFA
Paullinic acid	0.0395	1.61	GC×GC-MS_MSTFA
2-Hydroxysuccinic acid, Malic acid	0.0425	1.56	GC×GC-MS_MSTFA
Succinic acid	0.0264	1.52	GC×GC-MS_MSTFA
Ferulic acid	0.0230	1.49	GC×GC-MS_MSTFA
Kynurenic acid; 4-Hydroxyquinaldic acid	0.0361	1.39	GC×GC-MS_MSTFA
6-Hydroxynicotonic acid	0.0032	1.35	GC×GC-MS_MSTFA
3-Hydroxy-4-methoxybenzaldehyde	0.0451	1.32	GC×GC-MS_MSTFA
3-Methylglutaconic acid	0.0156	1.32	GC×GC-MS_MSTFA
5-Hexen-1-ol,hex-5-en-1-ol	0.0300	0.85	GC×GC-MS_MSTFA
(E)-2-Butenoic acid, trans-Crotonic acid	0.0149	0.48	GC×GC-MS_MSTFA
5-Aminovaleric acid; Pentanoic acid, 5-amino-	0.0193	0.47	GC×GC-MS_MSTFA

Table 2. Metabolomic Experiment #2. Metabolites with significantly different abundance between female and male BWF1 mice (n=8).

Name	*p*-value	Fold change (Male/Female)	Platform
Nicotinic Acid	2.50E-03	12.50	2DLC-MS/MS (+)
3-Hydroxy-3-methylglutaric Acid	2.63E-03	5.26	2DLC-MS/MS (-)
Nipecotic acid	1.10E-04	4.35	2DLC-MS/MS (+)
11-Octadecenoic acid, methyl ester	1.70E-04	4.00	GC×GC-MS_MSTFA
2-Pyrrolidone-5-carboxylic acid	1.20E-02	2.78	GC×GC-MS_MSTFA
4-Acetamidobenzaldehyde	2.82E-03	2.70	2DLC-MS/MS (+)
7-Hexadecenoic acid, methyl ester, (Z)-	5.53E-05	2.56	GC×GC-MS_MSTFA
Diethylene glycol	0.00E+00	2.56	GC×GC-MS_MSTFA
Glutamic Acid	2.83E-02	2.50	2DLC-MS/MS (+)
Hexadecanoic acid, 15-methyl-, methyl ester	6.80E-04	2.50	GC×GC-MS_MSTFA
6-Aminocaproic Acid	2.49E-03	2.38	2DLC-MS/MS (+)
9,12-Octadecadienoic acid (Z,Z)-, methyl ester	5.31E-05	2.38	GC×GC-MS_MSTFA
9,12,15-Octadecatrienoic acid (Z,Z,Z)-, methyl ester	1.10E-03	2.27	GC×GC-MS_MSTFA
Benzotriazole	1.04E-02	2.22	2DLC-MS/MS (+)
Hypoxanthine	5.10E-03	2.17	GC×GC-MS_MSTFA
N-Acetylputrescine	2.70E-03	2.13	GC×GC-MS_MSTFA
Phytol	0.00E+00	2.04	GC×GC-MS_MSTFA
Proline	9.64E-03	2.00	2DLC-MS/MS (-)
cis-7-Tetradecenol	4.50E-03	1.96	GC×GC-MS_MSTFA
DL-α-Methoxyphenylacetic Acid	7.72E-03	1.92	2DLC-MS/MS (-)
Hypoxanthine	1.39E-02	1.89	2DLC-MS/MS (-)
5-Aminovaleric acid	4.30E-03	1.72	GC×GC-MS_MSTFA
d-Mannose	3.90E-02	1.72	GC×GC-MS_MSTFA
Lysine	1.28E-02	1.69	2DLC-MS/MS (-)
Malic Acid	2.89E-02	1.69	2DLC-MS/MS (-)
Parabanic acid	6.80E-03	1.69	GC×GC-MS_MSTFA
Butyric Acid	2.60E-02	1.61	GC×GC-MS_MSTFA
L-Aspartic Acid	1.71E-02	1.61	2DLC-MS/MS (-)
Niacin	4.30E-02	1.61	GC×GC-MS_MSTFA
Cerulenin	2.70E-03	1.59	GC×GC-MS_MSTFA
Hydroxy-L-Proline	2.37E-02	1.59	2DLC-MS/MS (-)
Cadaverine	4.20E-04	1.54	GC×GC-MS_MSTFA
Serine	3.58E-05	1.54	2DLC-MS/MS (-)
2-Aminobutyric Acid	4.15E-02	1.52	2DLC-MS/MS (+)
Cyanuric acid	2.30E-02	1.52	GC×GC-MS_MSTFA

d-Galactose	5.90E-03	1.47	GC×GC-MS_MSTFA
D-Pinitol	3.30E-03	1.47	GC×GC-MS_MSTFA
3-tert-Butyladipic Acid	3.11E-02	1.43	2DLC-MS/MS (-)
Histamine	1.61E-02	1.41	2DLC-MS/MS (+)
Ribitol,	4.00E-02	1.39	GC×GC-MS_MSTFA
Serine	2.97E-02	1.37	2DLC-MS/MS (+)
1,3-Propanediol	1.20E-02	1.35	GC×GC-MS_MSTFA
Tartaric Acid	2.44E-02	1.33	2DLC-MS/MS (-)
Glycerol	2.00E-02	1.32	GC×GC-MS_MSTFA
1-Aminocyclohexanecarboxylic Acid	2.19E-02	1.30	2DLC-MS/MS (+)
D-Ribose	3.50E-02	1.28	GC×GC-MS_MSTFA
β-D-Talopyranose	4.30E-02	1.27	GC×GC-MS_MSTFA
Diethylamine	1.70E-02	1.16	GC×GC-MS_MSTFA
d-Glucose	4.00E-02	0.87	GC×GC-MS_MSTFA
Glycerol-3-phosphate	1.20E-02	0.80	GC×GC-MS_MSTFA
p-Coumaric acid	2.20E-02	0.71	GC×GC-MS_MSTFA
N,N-Diisopropylethylamine	2.93E-02	0.62	2DLC-MS/MS (+)
Adipic Acid	2.17E-02	0.60	2DLC-MS/MS (-)
4-Guanidinobutyric Acid	1.11E-02	0.58	2DLC-MS/MS (+)
6-Hydroxypicolinic Acid	2.97E-02	0.58	2DLC-MS/MS (-)
Pentadecanoic acid	1.50E-02	0.58	GC×GC-MS_MSTFA
Carnitine	1.53E-02	0.52	2DLC-MS/MS (+)
6-Chloro-5-methyl-1H-1,2,3-Benzotriazole	1.21E-02	0.51	2DLC-MS/MS (-)
6-Hydroxycaproic Acid	3.92E-02	0.50	2DLC-MS/MS (-)
Quinic Acid	4.03E-02	0.49	2DLC-MS/MS (-)
Creatinine	3.58E-02	0.47	2DLC-MS/MS (+)
Taurine	4.48E-02	0.47	2DLC-MS/MS (-)
Pyridoxine	2.04E-02	0.44	2DLC-MS/MS (+)
Linoleic Acid	4.87E-02	0.41	2DLC-MS/MS (-)
Deoxycholic Acid	1.02E-04	0.38	2DLC-MS/MS (-)
2-Aminonicotinic Acid	4.62E-02	0.37	2DLC-MS/MS (+)
L-Histidinol	2.47E-03	0.37	2DLC-MS/MS (+)
Pyridoxal	3.81E-02	0.35	2DLC-MS/MS (+)
Glycylproline	5.55E-03	0.34	2DLC-MS/MS (+)
8Z,11Z,14Z-Eicosatrienoic Acid	3.53E-02	0.31	2DLC-MS/MS (-)
4-Pyridoxic Acid	6.23E-04	0.30	2DLC-MS/MS (-)
O-Phosphoethanolamine,	1.50E-02	0.29	GC×GC-MS_MSTFA
Triethanolamine	8.77E-05	0.29	2DLC-MS/MS (+)
Taurine	1.05E-03	0.28	2DLC-MS/MS (+)
Oleic Acid	3.81E-02	0.27	2DLC-MS/MS (-)
β-Hydroxybutyric acid	1.80E-02	0.26	GC×GC-MS_MSTFA

Table 3. Metabolomic Experiment #3. Metabolites with significantly different abundance between female and male BWF1 mice. (female n=8, male n=6)

Name	*p*-value	Fold change (Male/Female)	Platform
Phytanic acid	7.05E-03	Male only	GC×GC-MS_MSTFA
Phytanic acid	1.51E-03	Male only	GC×GC-MS_MTBSTFA
Benzoic acid	0.00E+00	5.26	GC×GC-MS_MTBSTFA
Adenine	1.26E-02	4.00	2DLC-MS/MS (+)
5-Hydroxytryptophan	9.92E-03	3.33	GC×GC-MS_MSTFA
Ursolic acid	3.48E-03	3.03	2DLC-MS/MS (+)
Adenine	4.74E-02	2.50	2DLC-MS/MS (-)
Linolenic acid	1.73E-02	2.33	GC×GC-MS_MSTFA
Palmitelaidic acid	1.41E-03	2.33	GC×GC-MS_MSTFA
Phytol	3.58E-04	2.22	GC×GC-MS_MSTFA
Adenosine	3.05E-02	2.17	2DLC-MS/MS (+)
Phytol	2.95E-03	2.13	GC×GC-MS_MTBSTFA
Xylose	1.66E-02	2.08	GC×GC-MS_MSTFA
Aminoadipic acid	1.20E-02	2.00	2DLC-MS/MS (-)
Methylimidazoleacetic acid	4.42E-02	2.00	2DLC-MS/MS (+)
Valine	3.42E-04	2.00	GC×GC-MS_MTBSTFA
2-Hydroxycaproic acid	1.08E-02	1.96	2DLC-MS/MS (-)
Histamine	1.73E-04	1.82	2DLC-MS/MS (+)
Myristic acid	6.48E-04	1.82	GC×GC-MS_MSTFA
Eicosapentaenoic acid	3.13E-02	1.79	GC×GC-MS_MSTFA
Valine	4.10E-04	1.79	GC×GC-MS_MSTFA
Isoleucine	2.32E-03	1.75	GC×GC-MS_MTBSTFA
Linoelaidic acid	2.82E-02	1.72	GC×GC-MS_MSTFA
Myristic acid	3.30E-03	1.69	GC×GC-MS_MTBSTFA
Isoleucine	1.72E-02	1.64	GC×GC-MS_MSTFA
Leucine	3.19E-03	1.54	GC×GC-MS_MTBSTFA
Rhamnose	4.79E-02	1.52	GC×GC-MS_MSTFA
Nonanoic acid	3.50E-02	1.47	GC×GC-MS_MTBSTFA
Palmitic acid	1.98E-02	1.39	GC×GC-MS_MSTFA
Pentadecanoic acid	2.92E-02	1.37	GC×GC-MS_MSTFA
Stearic acid	1.48E-02	1.30	GC×GC-MS_MSTFA
Methionine	7.80E-03	0.81	GC×GC-MS_MTBSTFA
Histidine	8.52E-03	0.76	2DLC-MS/MS (+)
Glycerol-3-phosphate	3.47E-02	0.74	GC×GC-MS_MTBSTFA
Dodecanoic acid	4.15E-02	0.73	GC×GC-MS_MTBSTFA
Threonine	1.57E-02	0.72	2DLC-MS/MS (-)
Alanine	3.37E-02	0.71	2DLC-MS/MS (+)
Methionine	4.58E-02	0.71	2DLC-MS/MS (-)
Alanine	2.87E-03	0.70	GC×GC-MS_MTBSTFA
Benzyl alcohol	1.21E-02	0.69	GC×GC-MS_MTBSTFA
Creatinine	1.08E-02	0.69	2DLC-MS/MS (+)
N-Acetyl-histamine	1.37E-02	0.69	2DLC-MS/MS (+)

Alanine	2.62E-02	0.68	GC×GC-MS_MSTFA
Maleic acid	1.66E-02	0.67	GC×GC-MS_MTBSTFA
5-Hydroxyindole-3-acetic acid	3.30E-03	0.65	2DLC-MS/MS (+)
Benzeneacetic acid	4.53E-02	0.65	GC×GC-MS_MTBSTFA
Choline	1.96E-02	0.65	2DLC-MS/MS (+)
Glycine	7.13E-03	0.64	GC×GC-MS_MTBSTFA
Threonine	4.28E-02	0.64	2DLC-MS/MS (+)
Isobutyric acid	2.20E-02	0.63	GC×GC-MS_MTBSTFA
Palmitoleic acid	2.32E-02	0.63	GC×GC-MS_MTBSTFA
Tryptophan	1.07E-02	0.63	2DLC-MS/MS (+)
beta-Hydroxypyruvic acid	1.07E-03	0.62	GC×GC-MS_MSTFA
Azelaic acid	1.01E-02	0.61	GC×GC-MS_MTBSTFA
Histidine	2.67E-04	0.61	2DLC-MS/MS (-)
Proline	6.23E-03	0.61	GC×GC-MS_MSTFA
Tryptophan	1.83E-02	0.61	2DLC-MS/MS (-)
Lysine	1.47E-03	0.58	2DLC-MS/MS (+)
3-Hydroxyphenylacetic acid	2.22E-02	0.57	GC×GC-MS_MTBSTFA
Fumarate	1.80E-05	0.57	GC×GC-MS_MTBSTFA
Proline	1.01E-02	0.56	GC×GC-MS_MTBSTFA
4-Hydroxy-proline	5.45E-03	0.54	2DLC-MS/MS (-)
Methionine	2.55E-02	0.52	2DLC-MS/MS (+)
Isovaleric acid	3.17E-03	0.51	GC×GC-MS_MTBSTFA
Lysine	6.06E-03	0.50	2DLC-MS/MS (-)
Ornithine	4.94E-02	0.50	2DLC-MS/MS (+)
Glucuronic acid	2.78E-02	0.47	2DLC-MS/MS (-)
Heptadecanoic acid	2.55E-03	0.47	GC×GC-MS_MTBSTFA
Malate	2.54E-03	0.47	GC×GC-MS_MTBSTFA
Sucrose	1.34E-03	0.44	2DLC-MS/MS (-)
Urea	3.29E-03	0.44	GC×GC-MS_MTBSTFA
Vanillin	3.13E-02	0.43	GC×GC-MS_MTBSTFA
Tyramine	8.80E-03	0.42	GC×GC-MS_MSTFA
Lactic acid	2.93E-02	0.40	GC×GC-MS_MTBSTFA
Malate	4.19E-02	0.38	2DLC-MS/MS (-)
Uracil	3.26E-02	0.38	GC×GC-MS_MTBSTFA
2-Hydroxyisocaproic acid	2.01E-05	0.19	GC×GC-MS_MTBSTFA
Maltol	8.34E-04	0.18	GC×GC-MS_MTBSTFA
N-Acetyl-glutamic acid	3.44E-02	0.13	2DLC-MS/MS (-)
Deoxycholic acid	2.84E-06	0.09	2DLC-MS/MS (-)

Table 4. Metabolomic Experiment #3. Metabolites with significantly different abundance between intact male (Male) and castrated male (Castrated) BWF1 mice (n=6).

Name	*p*-value	Fold change (Male/Castrated)	Platform
Phytanic acid	9.30E-03	Male only	GC×GC-MS_MSTFA
Phytanic acid	6.99E-03	Male only	GC×GC-MS_MTBSTFA
3-Hydroxybutyric acid	3.05E-02	12.50	2DLC-MS/MS (-)
2-Hydroxybutyric acid	4.64E-02	11.11	2DLC-MS/MS (-)
Benzoic acid	5.08E-06	9.09	GC×GC-MS_MTBSTFA
Xylose	2.79E-06	9.09	GC×GC-MS_MSTFA
N6-Acetyl-lysine	5.83E-03	5.88	2DLC-MS/MS (+)
Phytol	6.35E-06	5.56	GC×GC-MS_MTBSTFA
Isoleucine	6.70E-05	5.26	GC×GC-MS_MSTFA
Leucine	3.93E-05	5.00	2DLC-MS/MS (+)
Adenosine	6.08E-05	4.76	2DLC-MS/MS (+)
Valine	5.22E-04	4.76	GC×GC-MS_MSTFA
Isoleucine	8.52E-05	4.35	GC×GC-MS_MTBSTFA
Valine	4.19E-05	4.35	GC×GC-MS_MTBSTFA
Leucine	4.45E-05	4.17	GC×GC-MS_MTBSTFA
Asparagine	2.21E-02	3.85	2DLC-MS/MS (+)
Phenylalanine	4.52E-04	3.85	GC×GC-MS_MTBSTFA
Leucine	3.10E-04	3.70	GC×GC-MS_MSTFA
5-Hydroxytryptophan	1.93E-02	3.57	GC×GC-MS_MSTFA
Glycerol	3.16E-04	3.45	GC×GC-MS_MTBSTFA
Phytol	7.47E-03	3.03	GC×GC-MS_MSTFA
Aminoadipic acid	9.58E-05	2.94	2DLC-MS/MS (-)
Valine	4.29E-05	2.94	2DLC-MS/MS (+)
Nonanoic acid	5.97E-05	2.78	GC×GC-MS_MTBSTFA
Thiamine	2.08E-02	2.70	2DLC-MS/MS (-)
3-Hydroxybutyric acid	4.81E-02	2.63	GC×GC-MS_MSTFA
Phenylalanine	1.57E-02	2.38	2DLC-MS/MS (-)
Methylimidazoleacetic acid	6.63E-03	2.33	2DLC-MS/MS (+)
Rhamnose	8.66E-05	2.33	GC×GC-MS_MSTFA
Tyrosine	1.02E-02	2.33	2DLC-MS/MS (-)
Carnosine	3.32E-04	2.27	2DLC-MS/MS (-)
Phenylalanine	2.41E-03	2.27	2DLC-MS/MS (+)
Methionine	9.40E-03	2.13	GC×GC-MS_MSTFA
Phloretic acid	3.44E-02	2.13	GC×GC-MS_MTBSTFA
Cadaverine	2.88E-02	2.08	GC×GC-MS_MSTFA
Myristic acid	8.52E-05	2.08	GC×GC-MS_MTBSTFA
Glutamine	1.43E-02	2.04	2DLC-MS/MS (+)
1-Methyl-histidine	7.82E-03	1.96	2DLC-MS/MS (+)
2-Aminobutyric acid	6.75E-03	1.96	GC×GC-MS_MTBSTFA
N-Acetyl-glucosamine	1.88E-03	1.92	2DLC-MS/MS (+)
Pinitol	2.82E-03	1.89	GC×GC-MS_MSTFA

Tyrosine	2.49E-02	1.89	GC×GC-MS_MSTFA
Histamine	2.25E-04	1.85	2DLC-MS/MS (+)
Serine	1.67E-03	1.85	GC×GC-MS_MTBSTFA
Glyceric acid	7.64E-03	1.82	GC×GC-MS_MTBSTFA
Palmitelaidic acid	2.15E-02	1.82	GC×GC-MS_MSTFA
Aceturic acid	5.80E-03	1.75	GC×GC-MS_MTBSTFA
Carnosine	4.00E-03	1.72	2DLC-MS/MS (+)
Ribose	1.20E-02	1.72	2DLC-MS/MS (-)
Methionine	2.17E-02	1.64	2DLC-MS/MS (-)
Diacetic acid	3.30E-03	1.61	GC×GC-MS_MTBSTFA
Methionine	3.17E-03	1.61	GC×GC-MS_MTBSTFA
Oleic acid	3.42E-02	1.61	GC×GC-MS_MSTFA
Oxalic acid	7.86E-03	1.61	GC×GC-MS_MTBSTFA
Methionine	3.32E-03	1.56	2DLC-MS/MS (+)
Glutamine	6.05E-03	1.45	2DLC-MS/MS (-)
Serine	1.47E-02	1.37	2DLC-MS/MS (-)
Cytosine	1.00E-02	1.30	GC×GC-MS_MTBSTFA
4-Pyridoxic acid	4.40E-02	0.80	2DLC-MS/MS (+)
Azelaic acid	4.02E-02	0.76	GC×GC-MS_MSTFA
Histidine	1.26E-02	0.75	2DLC-MS/MS (+)
Azelaic acid	4.04E-02	0.72	2DLC-MS/MS (-)
Fumarate	1.06E-02	0.72	GC×GC-MS_MTBSTFA
Proline	1.98E-02	0.71	2DLC-MS/MS (+)
Glutaric acid	4.34E-02	0.69	2DLC-MS/MS (-)
Creatinine	9.66E-05	0.68	2DLC-MS/MS (+)
Creatinine	2.00E-03	0.66	2DLC-MS/MS (-)
N6,N6,N6-Trimethyl-lysine	2.50E-02	0.66	2DLC-MS/MS (+)
Pipecolic acid	7.31E-04	0.64	GC×GC-MS_MTBSTFA
Uracil	4.26E-02	0.64	GC×GC-MS_MSTFA
Pipecolic acid	2.96E-03	0.63	GC×GC-MS_MSTFA
2-Hydroxyphenylalanine	8.32E-03	0.62	2DLC-MS/MS (+)
4-Hydroxy-Proline	2.56E-02	0.61	2DLC-MS/MS (+)
N-Acetyl-histamine	5.29E-04	0.60	2DLC-MS/MS (+)
Heptadecanoic acid	2.36E-03	0.59	GC×GC-MS_MTBSTFA
Lactic acid	1.63E-03	0.59	2DLC-MS/MS (-)
Urocanic acid	7.58E-04	0.59	2DLC-MS/MS (+)
3-Hydroxyphenylacetic acid	4.58E-03	0.57	GC×GC-MS_MSTFA
Histidine	1.53E-04	0.56	2DLC-MS/MS (-)
4-Hydroxy-proline	3.32E-03	0.54	2DLC-MS/MS (-)
Succinate	1.06E-02	0.54	GC×GC-MS_MSTFA
Sucrose	3.23E-03	0.54	2DLC-MS/MS (-)
Glutaric acid	9.92E-04	0.53	GC×GC-MS_MSTFA
Lysine	3.08E-04	0.52	2DLC-MS/MS (+)
Threonine	5.03E-03	0.51	2DLC-MS/MS (-)

Citrulline	3.41E-02	0.50	2DLC-MS/MS (-)
Lysine	5.43E-03	0.50	2DLC-MS/MS (-)
4-Hydroxybenzeneacetic acid	9.31E-03	0.47	GC×GC-MS_MSTFA
Citrulline	2.67E-02	0.47	2DLC-MS/MS (+)
Taurine	1.75E-03	0.46	GC×GC-MS_MTBSTFA
Pyroglutamic acid	4.52E-02	0.44	GC×GC-MS_MSTFA
Urocanic acid	4.07E-05	0.44	GC×GC-MS_MTBSTFA
beta-Hydroxypyruvic acid	7.63E-03	0.43	GC×GC-MS_MSTFA
Succinate	5.40E-04	0.43	2DLC-MS/MS (-)
Aspartic acid	2.78E-02	0.40	GC×GC-MS_MSTFA
5-Hydroxyindole-3-acetic acid	5.37E-05	0.28	2DLC-MS/MS (+)
Maltol	1.58E-03	0.26	GC×GC-MS_MTBSTFA
Malate	9.11E-04	0.23	GC×GC-MS_MTBSTFA
Malate	4.44E-04	0.20	GC×GC-MS_MSTFA
Malate	9.58E-06	0.19	2DLC-MS/MS (-)
2-Hydroxyisocaproic acid	3.62E-04	0.18	GC×GC-MS_MTBSTFA
N-Acetyl-glutamic acid	3.19E-06	0.06	2DLC-MS/MS (-)

Table 5. Metabolomic Experiment #3 (2018). Metabolites with significantly different abundance between female and castrated male BWF1 mice (female n=8, castrated n=6).

Name	*p*-value	Fold change (Female/Castrated)	Platform
Phenylalanine	4.74E-05	3.37	GC×GC-MS_MTBSTFA
Isoleucine	1.78E-04	3.21	GC×GC-MS_MSTFA
Methionine	2.19E-03	3.12	GC×GC-MS_MSTFA
Phenylalanine	5.66E-05	3.03	2DLC-MS/MS (-)
3-Aminobutyric acid	3.13E-03	2.98	GC×GC-MS_MTBSTFA
Tyrosine	9.92E-04	2.98	2DLC-MS/MS (-)
Phenylalanine	7.50E-06	2.86	2DLC-MS/MS (+)
Leucine	9.65E-06	2.75	GC×GC-MS_MSTFA
Leucine	2.43E-04	2.74	GC×GC-MS_MTBSTFA
3-Hydroxybutyric acid	3.65E-02	2.68	GC×GC-MS_MSTFA
Norvaline	6.79E-03	2.68	GC×GC-MS_MSTFA
3-Hydroxybutyric acid	1.17E-02	2.56	2DLC-MS/MS (-)
Isoleucine	1.09E-03	2.48	GC×GC-MS_MTBSTFA
Tyrosine	1.27E-02	2.37	2DLC-MS/MS (+)
Leucine	2.68E-03	2.36	2DLC-MS/MS (+)
Carnosine	3.06E-06	2.31	2DLC-MS/MS (-)
Methionine	1.68E-04	2.31	2DLC-MS/MS (-)
Hydroxyphenyllactic acid	4.54E-03	2.29	2DLC-MS/MS (-)
Valine	7.43E-04	2.25	2DLC-MS/MS (+)
Valine	2.97E-03	2.15	GC×GC-MS_MTBSTFA
Tryptophan	2.80E-04	2.02	2DLC-MS/MS (-)
Oxalic acid	1.48E-03	2.01	GC×GC-MS_MTBSTFA
Carnosine	1.50E-05	1.98	2DLC-MS/MS (+)
Methionine	9.48E-05	1.98	GC×GC-MS_MTBSTFA
4-Hydroxybenzoic acid	2.78E-02	1.92	GC×GC-MS_MTBSTFA
Methionine	2.96E-04	1.91	2DLC-MS/MS (+)
Nonanoic acid	6.16E-03	1.87	GC×GC-MS_MTBSTFA
Tryptophan	3.47E-04	1.87	2DLC-MS/MS (+)
Dodecanoic acid	3.35E-03	1.83	GC×GC-MS_MTBSTFA
Ornithine	2.82E-02	1.82	2DLC-MS/MS (+)
Serine	8.56E-05	1.81	GC×GC-MS_MTBSTFA
Glycine	5.82E-03	1.79	2DLC-MS/MS (-)
Urea	4.17E-02	1.77	GC×GC-MS_MTBSTFA
Citrate	2.84E-02	1.74	GC×GC-MS_MTBSTFA
Benzoic Acid	1.37E-02	1.73	GC×GC-MS_MTBSTFA
Benzeneacetic acid	1.79E-02	1.69	GC×GC-MS_MTBSTFA
Ribose	8.92E-04	1.68	GC×GC-MS_MSTFA
Diacetic acid	8.22E-03	1.66	GC×GC-MS_MTBSTFA
Tyrosine	4.36E-03	1.66	GC×GC-MS_MSTFA
N-Acetylneuraminic acid	4.22E-02	1.65	2DLC-MS/MS (+)
Glutamine	1.73E-03	1.60	2DLC-MS/MS (+)
Alanine	5.86E-04	1.54	GC×GC-MS_MTBSTFA

Glutamine	2.52E-03	1.53	2DLC-MS/MS (-)
Ribose	1.36E-02	1.50	2DLC-MS/MS (-)
Phloretic acid	4.24E-02	1.49	GC×GC-MS_MTBSTFA
Serine	9.09E-03	1.45	2DLC-MS/MS (+)
Glycine	1.16E-02	1.44	GC×GC-MS_MTBSTFA
5-Aminovaleric acid	1.86E-02	1.37	2DLC-MS/MS (+)
1-Methyl-L-histidine	1.91E-04	1.36	2DLC-MS/MS (+)
Alanine	4.18E-02	1.31	2DLC-MS/MS (+)
Serine	2.18E-03	1.30	2DLC-MS/MS (-)
2-Hydroxyphenylalanine	2.71E-02	0.79	2DLC-MS/MS (+)
Azelaic acid	2.39E-02	0.79	2DLC-MS/MS (-)
Creatinine	9.63E-05	0.74	2DLC-MS/MS (-)
Maleic acid	2.13E-02	0.74	2DLC-MS/MS (-)
2-Hydroxyvaleric acid	3.05E-03	0.63	2DLC-MS/MS (-)
N-Acetylhistamine	8.31E-04	0.62	2DLC-MS/MS (+)
Pentadecanoic acid	3.45E-03	0.55	GC×GC-MS_MSTFA
Succinate	1.61E-04	0.55	2DLC-MS/MS (-)
Succinate	3.49E-04	0.53	GC×GC-MS_MSTFA
4-Hydroxybenzeneacetic acid	7.24E-03	0.52	GC×GC-MS_MSTFA
Malate	1.88E-02	0.50	2DLC-MS/MS (-)
N-Acetyl-glutamic acid	1.73E-02	0.44	2DLC-MS/MS (-)
alpha-Ketoglutaric acid	1.11E-02	0.37	2DLC-MS/MS (-)
Malate	2.41E-03	0.26	GC×GC-MS_MSTFA

Table 6. Pathways affected by the metabolic differences between female and male groups (female n=8, male n=6).

Name	p-value	-LOG(p)	Impact
Aminoacyl-tRNA biosynthesis	8.48E-07	13.98	0.0000
Glyoxylate and dicarboxylate metabolism	1.10E-05	11.42	0.0645
Tyrosine metabolism	1.66E-05	11.01	0.0269
Alanine, aspartate and glutamate metabolism	4.42E-05	10.03	0.0032
Citrate cycle (TCA cycle)	4.74E-05	9.96	0.0721
Glycine, serine and threonine metabolism	4.96E-05	9.91	0.2688
Arginine and proline metabolism	5.44E-05	9.82	0.1914
Purine metabolism	3.75E-04	7.89	0.0057
Fatty acid biosynthesis	6.32E-04	7.37	0.0000
Pantothenate and CoA biosynthesis	7.11E-04	7.25	0.0000
Histidine metabolism	8.78E-04	7.04	0.4624
Valine, leucine and isoleucine biosynthesis	9.35E-04	6.98	1.0000
Valine, leucine and isoleucine degradation	9.35E-04	6.98	0.0000
Lysine biosynthesis	1.65E-03	6.41	0.0000
Lysine degradation	1.65E-03	6.41	0.0000
Tryptophan metabolism	2.25E-03	6.10	0.1772
Selenoamino acid metabolism	2.52E-03	5.98	0.0000
Nitrogen metabolism	2.72E-03	5.91	0.0000
Pentose and glucuronate interconversions	2.98E-03	5.81	0.1333
Galactose metabolism	3.44E-03	5.67	0.0407
Glycerophospholipid metabolism	3.61E-03	5.62	0.0889
Pyruvate metabolism	3.94E-03	5.54	0.0000
Starch and sucrose metabolism	4.59E-03	5.38	0.0000
Cysteine and methionine metabolism	7.08E-03	4.95	0.0869
Biosynthesis of unsaturated fatty acids	8.41E-03	4.78	0.0000
alpha-Linolenic acid metabolism	9.99E-03	4.61	1.0000
Primary bile acid biosynthesis	1.38E-02	4.28	0.0298
Cyanoamino acid metabolism	1.38E-02	4.28	0.0000
Methane metabolism	1.38E-02	4.28	0.0000
Porphyrin and chlorophyll metabolism	1.38E-02	4.28	0.0000
Fatty acid elongation in mitochondria	1.76E-02	4.04	0.0000
Fatty acid metabolism	1.76E-02	4.04	0.0000
Glycerolipid metabolism	2.86E-02	3.55	0.0256
Glutathione metabolism	2.89E-02	3.54	0.0057
Biotin metabolism	3.01E-02	3.50	0.0000
Ascorbate and aldarate metabolism	3.18E-02	3.45	0.4000
Inositol phosphate metabolism	3.18E-02	3.45	0.0000
Glycolysis or Gluconeogenesis	3.20E-02	3.44	0.0000

Table 7. Pathways affected by the metabolic differences between castrated and intact male groups (n=6).

Name	p-value	-LOG(p)	Impact
Valine, leucine and isoleucine biosynthesis	8.29E-07	14.00	0.6667
Aminoacyl-tRNA biosynthesis	9.30E-07	13.89	0.1290
Lysine degradation	5.19E-06	12.17	0.0000
Valine, leucine and isoleucine degradation	1.28E-05	11.27	0.0000
Lysine biosynthesis	3.13E-05	10.37	0.0000
Histidine metabolism	4.66E-05	9.97	0.4624
Glyoxylate and dicarboxylate metabolism	7.23E-05	9.54	0.1290
Alanine, aspartate and glutamate metabolism	7.84E-05	9.45	0.3460
Pyruvate metabolism	1.58E-04	8.75	0.0000
Arginine and proline metabolism	1.75E-04	8.65	0.0944
Glycine, serine and threonine metabolism	2.64E-04	8.24	0.2385
Citrate cycle (TCA cycle)	2.92E-04	8.14	0.0977
Phenylalanine, tyrosine and tryptophan biosynthesis	3.86E-04	7.86	0.5000
Phenylalanine metabolism	3.86E-04	7.86	0.4074
Nitrogen metabolism	4.00E-04	7.82	0.0000
Butanoate metabolism	4.62E-04	7.68	0.1015
Galactose metabolism	4.64E-04	7.67	0.0407
Tyrosine metabolism	5.33E-04	7.54	0.0000
Purine metabolism	6.17E-04	7.39	0.0010
Tryptophan metabolism	6.96E-04	7.27	0.0000
Propanoate metabolism	8.92E-04	7.02	0.0000
Biotin metabolism	1.05E-03	6.86	0.0000
Amino sugar and nucleotide sugar metabolism	1.09E-03	6.83	0.0692
Fatty acid biosynthesis	1.16E-03	6.76	0.0000
Taurine and hypotaurine metabolism	1.80E-03	6.32	0.4286
Primary bile acid biosynthesis	1.80E-03	6.32	0.0298
Cysteine and methionine metabolism	2.39E-03	6.04	0.1122
Glycerolipid metabolism	2.65E-03	5.93	0.3857
Methane metabolism	3.63E-03	5.62	0.4000
Cyanoamino acid metabolism	3.63E-03	5.62	0.0000
Sphingolipid metabolism	3.63E-03	5.62	0.0000
beta-Alanine metabolism	5.15E-03	5.27	0.0000
Glutathione metabolism	5.29E-03	5.24	0.0143
Starch and sucrose metabolism	7.30E-03	4.92	0.0000
Pyrimidine metabolism	9.26E-03	4.68	0.0000
D-Glutamine and D-glutamate metabolism	9.26E-03	4.68	0.0000
Pentose phosphate pathway	1.17E-02	4.45	0.0000
Thiamine metabolism	1.39E-02	4.28	0.4000
Synthesis and degradation of ketone bodies	1.78E-02	4.03	0.6000
Glycolysis or Gluconeogenesis	3.34E-02	3.40	0.0000
Vitamin B6 metabolism	3.91E-02	3.24	0.0000

Table 8. Pathways affected by the metabolic differences between female and castrated male groups (female n=8, castrated n=6).

Name	p-value	-LOG(p)	Impact
Aminoacyl-tRNA biosynthesis	2.15E-08	17.66	0.1290
Histidine metabolism	3.08E-06	12.69	0.0000
Alanine, aspartate and glutamate metabolism	3.22E-06	12.65	0.2131
Valine, leucine and isoleucine degradation	3.68E-06	12.51	0.0000
Cysteine and methionine metabolism	5.71E-06	12.07	0.1122
Arginine and proline metabolism	8.61E-06	11.66	0.1274
Valine, leucine and isoleucine biosynthesis	1.17E-05	11.36	1.0000
Phenylalanine metabolism	1.98E-05	10.83	0.4074
D-Glutamine and D-glutamate metabolism	3.78E-05	10.18	0.0000
Sphingolipid metabolism	4.12E-05	10.10	0.0000
Phenylalanine, tyrosine and tryptophan biosynthesis	4.19E-05	10.08	1.0000
Butanoate metabolism	4.80E-05	9.94	0.1015
Tyrosine metabolism	5.04E-05	9.90	0.1405
Glycine, serine and threonine metabolism	5.27E-05	9.85	0.5073
Methane metabolism	5.27E-05	9.85	0.4000
Cyanoamino acid metabolism	5.27E-05	9.85	0.0000
Citrate cycle (TCA cycle)	1.21E-04	9.02	0.1924
Synthesis and degradation of ketone bodies	1.44E-04	8.84	0.6000
Selenoamino acid metabolism	1.45E-04	8.84	0.0000
Purine metabolism	1.82E-04	8.61	0.0000
Propanoate metabolism	4.87E-04	7.63	0.0000
Pentose phosphate pathway	6.36E-04	7.36	0.0000
Nitrogen metabolism	1.03E-03	6.87	0.0000
Tryptophan metabolism	1.20E-03	6.73	0.1772
Pyruvate metabolism	1.20E-03	6.73	0.0000
Pyrimidine metabolism	1.85E-03	6.29	0.0000
Fatty acid biosynthesis	1.97E-03	6.23	0.0000
Glyoxylate and dicarboxylate metabolism	2.46E-03	6.01	0.2581
Glutathione metabolism	2.81E-03	5.88	0.0057
Pantothenate and CoA biosynthesis	3.73E-03	5.59	0.0000
Primary bile acid biosynthesis	7.80E-03	4.85	0.0298
Porphyrin and chlorophyll metabolism	7.80E-03	4.85	0.0000
Ubiquinone and other terpenoid-quinone biosynthesis	8.80E-03	4.73	0.0000

SPECIFIC AIM 3

Sex-based differences in splenic macrophage efferocytosis may be mediated by a male microbiota associated metabolite

Introduction

Like most autoimmune diseases, the multi-organ autoimmune disease systemic lupus erythematosus (SLE) has a pronounced sex bias, as about 9 in every 10 people affected by SLE are women [1,2,4-8]. There are complex genetic and environmental factors that predispose individuals to SLE, and the mechanisms that play a role in its development are still not completely understood. Cells and molecules of both the innate and adaptive immune responses are clearly involved in the pathogenesis [494]. Lupus is characterized by the activation of autoreactive T and B cells resulting in the production of anti-nuclear autoantibodies that form immune complexes that are deposited in the skin, kidneys, and other organs. The resulting inflammation is multiorgan can manifest as lupus nephritis, vasculitis, pulmonary hypertension, or stroke, and over 50% of lupus patients eventually develop one or more of these complications, and although there are treatments that mitigate symptoms, there is no cure [3,5,9-13]. The NZBxNZW F1 (BWF1) mouse strain is an excellent spontaneous model of lupus, and exhibits many of the features of the human disease including a definite sex bias. Female BWF1 mice spontaneously develop anti-dsDNA autoantibodies, glomerulonephritis,

lymphadenopathy, splenomegaly in a manner that resembles the progression of disease in humans whereas male BWF1 have much lower disease incidence and severity [16].

Many innate immune activities play a major role in the initiation and progression of lupus. Dendritic cells (DCs) present self-antigens to T cells, which then promote B cell maturation [495]. Deficiencies in complement proteins result in impaired clearance of the immune complexes that drive kidney inflammation [496], and plasmacytoid DCs produce IFNα in response to these immune complexes, which promotes autoantibody production [497]. However, all of these activities depend on the deficient macrophage phagocytosis of apoptotic cells [371]. This process, known as efferocytosis, sequesters nuclear antigen containing apoptotic debris away from other immune cells, suppressing the stimulation of anti-nuclear autoantibody production [370]. Macrophages isolated from SLE patients or lupus-prone female BWF1 mice are defective at phagocytosing apoptotic cells (i.e., efferocytosis) compared to healthy controls [386,391,392,498], and disease severity in SLE patients is strongly correlated with deficient efferocytosis [380,493].

Several factors are thought to play roles in the higher incidence of SLE in females. One is the incomplete inactivation of the X chromosome that can result in overexpression of X-linked genes that are involved in the immune response and promote autoimmunity [90-92]. Sex hormones are also thought to play a major role in both human and BWF1 mouse sex bias. Female sex hormones, estrogens, are generally immunostimulatory, and there is evidence that they promote lupus progression [58,102,103]. On the other hand, male sex hormones, androgens, are strongly immunosuppressive and anti-inflammatory and are believed to protect against lupus [103,122-125]. Both women and men with SLE have decreased levels of androgens

[115,138-144]. Additionally, SLE is more prevalent in men with Klinefelter syndrome, who naturally have lower androgen levels and higher estrogen levels than XY males [146,147]. Many innate and adaptive immune cells express androgen receptor, including macrophages, monocytes, mast cells, neutrophils, B cells, and T cells [499]. The effects of androgen signaling on immune cells are many and diverse. They include promoting neutrophil differentiation, suppressing macrophage inflammatory responses to pathogens, limiting T cell proliferation, promoting Treg expansion, and decreasing the numbers of circulating B cells [499,500].

Accumulating evidence indicates that the gut microbiota can have a major impact on immune functions systemically as well as locally. Consequently, the gut microbiota can affect the progression of a large variety of diseases including autoimmune diseases. [218,436]. Most autoimmune diseases, including SLE, are associated with dysbiosis, an imbalance between beneficial microbes and potential pathobionts [184,256,257,276-280,282,283], as well as altered microbial metabolite production [333-336,348,360-362]. An increasing number of studies have found that the intestinal microbiota of SLE patients differs significantly from those of healthy controls [276-283,437]. Clearly, there is a strong connection between lupus and the microbiota. Furthermore, sex also significantly affects the microbiota, suggesting that sex-based differences in microbiota function could potentially contribute to the sex bias of lupus [182,300-303]. We and others have found sex differences in the gut microbiota in mouse models of autoimmune disease, including lupus **(Fig. 11)** [323-325].

One of the major ways the microbiota can affect autoimmune diseases is via the production of immunoregulatory metabolites [327], e.g., short chain fatty acids (SCFAs).

Among many other anti-inflammatory effects, SCFAs can promote regulatory T-cell induction and suppress inflammasome activation [328-332]. Multiple autoimmune diseases including multiple sclerosis, IBD, and spondyloarthritis have been found to be associated with decreased levels of SCFAs and SCFA-producing bacteria and reconstitution of these metabolites can alleviate disease in mouse models of these diseases [333-339]. Also, production of SCFAs and other metabolites can be affected by androgens [218,324,420,436,501,502]. Considering the significant effect alterations in immunomodulatory metabolites can have on autoimmune diseases, this suggests that sex-based differences in metabolites could contribute to the sex bias in lupus-prone BWF1 mice [327]. For this reason, we previously compared the fecal metabolomic profiles of disease-prone female and castrated male BWF1 mice and disease-resistant intact male BWF1 mice. The metabolomic profiles of these mice differed significantly, and we identified several immunomodulatory metabolites that were elevated in the intact male BWF1 mice compared to the female and castrated male BWF1 mice [420]. One of these was phytol, which is produced from the breakdown of chlorophyll and has RXR and PPARγ agonist activities [449,450]. Phytol is converted by host enzymes to phytanic acid, which was completely absent from the female and castrated male BWF1 feces but present in the BWF1 feces (manuscript in preparation). Phytanic acid has even more potent agonist activity compared to its parent compound, phytol, and it strongly activates the PPARγ/RXR, LXR/RXR, and PPARδ/RXR signaling complexes [449-453,463]. Phytanic acid could potentially affect disease progression in a number of ways, as these pathways are involved in a wide range of immune processes central to lupus progression [383,400,457,459,486].

In this paper, we further investigate the functional differences in immune activities between female and male BWF1 mice. The splenocyte transcriptomes of disease-susceptible female BWF1 mice, and disease-resistant male BWF1 and female BALB/c mice were compared using RNA sequencing (RNA-seq), and we found disease resistance was associated with enrichment of phagocytosis-promoting genes. Based on these data, we compared efferocytic activity in splenic macrophages from female and male BWF1 mice and found that male macrophages were significantly more effective at phagocytosing apoptotic cells than macrophages from female BWF1 mice. We found similar differences in macrophage efferocytosis between intact and castrated male BWF1 mice, where macrophages from intact male BWF1 mice were significantly more effective at phagocytosing apoptotic cells than macrophages from castrated male BWF1 mice. Additional analysis of the RNA-seq data suggested that increased PPARγ and LXR signaling might play a role in higher male efferocytic activity [393-399,503]. Our previous metabolomic study had found that male BWF1 mice had higher than either female or castrated male mice of a metabolite, phytol, which can activate those pathways **(Fig. 31A)** [420,456]. Phytol's derivative, phytanic acid, was also elevated in intact male BWF1 mice and is an even more potent activator of the PPARγ and LXR pathways **(Fig. 31B)** [449-453,463]. *In vitro* and *in vivo* treatments with phytanic acid increased macrophage efferocytosis and expression of the efferocytosis-promoting gene CD36. We show that this effect is dependent on both LXR/RXR and PPARγ/RXR signaling. These findings shed new light on how the microbiota can influence disease progression in the BWF1 mouse model and could have implications on the development of novel lupus therapies.

Results

Splenic transcriptome and macrophage gene expression analysis suggests that male BWF1 macrophages mice may have greater efferocytic activity

To investigate the sex differences in immune functions in BWF1 mice, we first compared the splenocyte gene expression profile of adult disease-prone female BWF1 mice to disease-resistant male BWF1 and female BALB/c mice by RNA sequencing (RNA-seq) [16,420]. Hierarchical clustering analysis of differentially expressed genes revealed three prominent clusters of genes that varied between the three groups based on strain, sex, or disease susceptibility **(Fig. 32A)**. The heatmap of relative gene expression shows that despite differing in sex and genetics, there is a significant cluster of genes differentially regulated in both the disease-resistant male BWF1 and female BALB/c mice compared to the disease-prone female BWF1 mice. This suggests that the lower susceptibility to lupus in male BWF1 and female BALB/c mice is associated with differential expression of a set of splenic genes. To investigate what specific biological processes differed in the disease-prone and non-disease-prone mice, we performed Gene ontology: Biological Process (GO:BP) analysis of the genes significantly upregulated in male BWF1 or female BALB/c mice compared to the female BWF1 mice **(Fig. 32B)**. Functional analysis revealed similarities in some upregulated pathways between the disease resistant male BWF1 and BALB/c female mice relative to female BWF1 mice. Interestingly, both male BWF1 and female BALB/c spleens had increased levels of genes that positively regulate phagocytosis. This matches previous studies which have shown that apoptotic cell clearance by phagocytosis is deficient in SLE patients and mouse models of lupus. There is significant evidence that this deficiency promotes lupus onset

and progression [371,491,492]. Macrophages are the cells primarily responsible for apoptotic cell phagocytosis, referred to as efferocytosis, and macrophages isolated from SLE patients and lupus-prone mice have been shown to be intrinsically defective at efferocytosis compared to healthy controls [379,387,504,505]. We examined the phagocytosis-related genes that were enriched in male BWF1 mice **(Table 9)**. One of them, LRP1, is known to be expressed by macrophages and directly involved in efferocytosis. It is a surface receptor that is specifically involved in binding apoptotic cells and promoting their phagocytosis [506]. The RNA-seq analysis showing that the expression of LRP1 is doubled in male macrophages **(Fig. 32C)** was confirmed using qPCR and flow cytometry; male BWF1 $CD11b^{+}F4/80^{+}$ splenic macrophages express higher levels of LRP1 than female BWF1 splenic macrophages **(Fig. 32D,E; 33A; 40A)**.

Androgens have been associated with protection from lupus [115,140,143], and we and others have found that castration dramatically accelerates and increases severity of disease and decreases survival of male BWF1 mice [17,133,420]. To determine if androgens affect LRP1 expression, male BWF1 mice were castrated and LPR1 mRNA and protein expression were evaluated. Androgen depletion resulted in lower levels of LRP1 mRNA and protein expression in castrated compared to intact male BWF1 splenic macrophages **(Fig. 32F,G; 40B)**. This lower expression of LRP1 correlates with the greater susceptibility to lupus in castrated male BWF1 [420]. Taken together, these data suggest that disease resistant intact male BWF1 mice appear to have more efficient macrophage efferocytosis than disease-prone female and castrated male BWF1 mice.

Splenic macrophages from intact male BWF1 mice have higher efferocytic activity than macrophages from either female or androgen-depleted male BWF1 mice

Both SLE patients and lupus-prone mice exhibit deficient macrophage clearance of apoptotic cells (efferocytosis), which results in accumulation of nuclear antigen-containing debris that promotes disease progression [371,391,491,492]. The spleen is a major site of apoptotic cell clearance [379,387,504,505], and our analyses of splenocyte and splenic macrophage gene expression suggested that macrophages from lupus-prone female BWF1 mice might have deficient efferocytic activity compared to males **(Fig. 32)**. To investigate this, we compared the ability of splenic macrophages from female and male BWF1 mice to phagocytose apoptotic cells. Adherent cells from digested spleens were incubated with CTV-labeled apoptotic thymocytes and were analyzed by flow cytometry to determine the percentage of $F4/80^{+}CD11b^{+}$ macrophages that had phagocytosed the labeled apoptotic cells. Representative figures for this flow cytometry assay are shown in **Figure 34**. As shown in **Figure 35A**, male BWF1 splenic macrophages were significantly more efficient than female macrophages as shown by the fold change of CTV^{+} macrophages. To control for the possibility of a bystander effect of another adherent cell type, we repeated the assay using sorted $F4/80^{+}CD11b^{+}$ cells, and confirmed that the male splenic macrophages had higher efferocytic activity compared to female splenic macrophages **(Fig. 35B)**.

Since we had found that androgen depletion via castration resulted in decreased expression of the efferocytic receptor LRP1, we tested whether castration also reduced macrophage efferocytosis using either adherent or sorted splenic macrophages from adult castrated or intact male mice. We found that androgen depletion decreases splenic

macrophage phagocytosis of apoptotic cells **(Fig. 35C,D)**. Additionally, when mice were treated with the androgen receptor blocker, flutamide, splenic macrophage efferocytosis was decreased by comparison to vehicle-treated male controls **(Fig. 35E)**. Since multiple studies have found that androgen signaling either has no effect or even decreases phagocytic activity in macrophages [507-511], the positive effect of androgen (as indicated by our findings that both androgen depletion and androgen receptor signaling blockade impair efferocytosis) on macrophage efferocytosis that we find in our studies may not be due to a direct effect of androgen on macrophages. One way that androgen is known to indirectly affect immune responses is by altering the production of immunomodulatory metabolites produced by gut bacteria [218,436,501,502].

Treatment with a male microbiota-associated metabolite increases the ability of female splenic macrophages to phagocytose apoptotic cells

Having established that male BWF1 macrophages are more efficient at efferocytosis, and efferocytosis is impaired by androgen depletion, we next investigated the underlying mechanisms. Transcription factor enrichment analysis of our RNA-seq data showed the set of genes upregulated in male BWF1 vs female BWF1 spleens was enriched for genes stimulated by LXR and PPARγ signaling **(Fig. 36A; Table 10,11)**. PPARγ and LXR stimulation are known to upregulate many efferocytosis-promoting genes, but this raised the question of what was stimulating the LXR and PPARγ genes in the male splenocytes. In a previous study, we found that feces from female, and intact and castrated male BWF1 mice exhibit different metabolite profiles. In that study, we found differences in the levels of a potentially immunomodulatory metabolite that could affect PPARγ and

LXR signaling. The acyclic diterpene alcohol, phytol can activate both the PPARγ and the LXR pathways [449,450,456], and was elevated in intact male BWF1 mice compared to female and castrated male BWF1 mice [420]. Phytol is converted by host enzymes to phytanic acid, which has similar but even more potent agonist activity compared to its parent compound phytol, and strongly activates PPARγ/RXR, LXR/RXR, and PPARδ/RXR signaling [449-453,463]. We have found that phytanic acid is completely absent from female and castrated male BWF1 feces but present in intact male BWF1 feces (Unpublished data). Since signaling via the PPARγ/RXR, LXR/RXR or PPARδ/RXR pathways is known to promote efferocytosis [381,393-400], we hypothesized that phytanic acid could potentially increase the ability of splenic macrophages to phagocytose apoptotic cells. To test this, female BWF1 adherent or sorted splenic macrophages were pre-treated with phytanic acid then cultured with CTV-labeled apoptotic cells. Phytanic acid (PA) significantly increased the ability of female BWF1 splenic macrophages to phagocytose apoptotic cells **(Fig. 36B,C)**. Phytanic acid also increased efferocytosis in macrophages from castrated male BWF1 mice **(Fig. 36D)**. These findings suggested that higher levels of phytanic acid and its precursor, phytol, found in the male gut microbiota could contribute to the higher levels of splenic macrophage efferocytosis found in males. If this is the case, then treating female BWF1 mice with phytanic acid *in vivo* should increase splenic macrophage efferocytosis. To test this, female BWF1 mice were fed phytanic acid-containing microspheres that are taken up by the gut and slowly release their payload [422,423]. After 4 weeks of treatment, spleens were harvested and macrophage efferocytosis was evaluated *in vitro*. Macrophages from female BWF1 mice fed phytanic acid-containing microspheres were

significantly more efficient at efferocytosis than macrophages from female control mice fed empty microspheres **(Fig. 36E)**.

The intestinal production of phytanic acid's precursor, phytol, is completely dependent on gut microbiota metabolism [460-462]. Therefore sex-based differences in the BWF1 microbiota are the most likely source of the elevated fecal levels of both phytol and phytanic acid in male BWF1 mice compared to female BWF1 mice [420]. If the male microbiota is the source of these metabolites, then transfer of male microbiota into female mice should also enhance macrophage-mediated efferocytosis after transfer into recipient female mice. To determine whether male microbiota could affect macrophage function in female BWF1 mice, we transferred adult male BWF1 cecal contents (containing the microbiota) into female BWF1 mice at regular intervals starting at weaning and continuing until 16 weeks of age. At that time, we harvested the spleens and assayed the efferocytic activity of the macrophages. We found that transfer of adult male, but not female (negative control), cecal contents into female BWF1 mice increased the ability of female splenic macrophages to phagocytose apoptotic cells to levels found in positive control male mice that received male cecal contents **(Fig. 36F)**. Altogether, these data suggest that the male-upregulated microbiota metabolite, phytol and its derivative phytanic acid could play a role in the sex differences in BWF1 macrophage efferocytosis.

Phytanic acid affects macrophage efferocytosis via LXR/RXR and PPARγ/RXR pathways

Phytanic acid can stimulate PPARδ/RXR, LXR/RXR, and PPARγ/RXR signaling [449-453,463], and each of these pathways can potentially stimulate macrophage phagocytosis of apoptotic cells [381,393-400]. To investigate which pathways phytanic acid stimulates to enhance macrophage efferocytosis, splenic macrophages from female BWF1 mice were treated with phytanic acid along with inhibitors that block one or all of those signaling pathways. We found that when RXR-mediated pathways were blocked with the RXR antagonist HX531, phytanic acid treatment could no longer enhance macrophage efferocytosis, suggesting that RXR pathways are required for phytanic acid action on macrophages **(Fig. 37A)**. Similarly, blocking the PPARγ/RXR heterodimer-mediated pathway with the PPARγ antagonist, GW9662 or the LXR/RXR heterodimer-mediated pathway with LXR antagonist, GSK2033 also prevented phytanic acid treatment from increasing macrophage efferocytosis **(Fig. 37B,C)**. These data indicate that activation of both the PPARγ/RXR and LXR/RXR pathways is required for phytanic acid to increase macrophage ability to phagocytose apoptotic cells, and their activities are not redundant. However, inhibiting the PPARδ/RXR-mediated pathway with the PPARδ antagonist, GSK0660 did not affect the enhancement of efferocytosis by phytanic acid, indicating that phytanic acid does not act via the PPARδ pathway **(Fig. 37D)**. In summary, phytanic acid increases macrophage efferocytosis via a PPARγ and LXR pathway dependent mechanism.

Phytanic acid may increase macrophage phagocytosis by upregulating the expression of the pro-efferocytic receptor CD36

As described earlier **(Fig. 32C-G)**, we have found that the efferocytosis-associated receptor, LRP1, was upregulated in intact male compared to female and castrated male BWF1 mice. Interestingly, LRP1 has been shown to potentiate the effects of LXR and/or PPARγ signaling, including the upregulation of other molecules associated with efferocytosis in macrophages [415,512]. These data suggest that LXR and/or PPARγ signaling could act, possibly in concert with LRP1 that is expressed at higher levels in males, by upregulating surface receptors involved in efferocytosis. CD36, MERTK, and TIMD4 are all surface receptors that are known to be directly involved in efferocytosis in macrophages, and have been shown to be upregulated by LXR and/or PPARγ signaling [372,384,393,396,404,408-410,412,413,513-521]. To determine whether expression of any of these surface markers differs between female and intact and castrated male macrophages and, could, therefore, potentially contribute to the differences in efferocytosis between splenic macrophages from female and intact and castrated male BWF1 mice, we analyzed their expression via flow cytometry. We found that while MERTK and TIMD4 expression did not differ between female and male BWF1 macrophages, CD36 was expressed at higher levels in male BWF1 splenic macrophages compared to female mice **(Fig. 38A; 33B; 40C-E)**. AXL, MFGE8, and C1QA are also involved in efferocytosis and upregulated by LXR and/or PPARγ, and defects in them are associated with lupus [375,382,393,400,405,522-533]. To evaluate AXL, MFGE8, and C1QA expression, we compared mRNA levels of these molecules by qPCR in sorted splenic macrophages from female and male BWF1 mice, but found no

differences in expression **(Fig. 39)**. Additionally, CD36 was also expressed at higher levels in intact male compared to castrated male BWF1 mice **(Fig. 38B; 40F)**. CD36 can be regulated by LXR and/or PPARγ signaling [393,409,410], and we have found that phytanic acid can affect efferocytosis via signaling through one or both of these pathways **(Fig. 37A-C)**. For these reasons, we determined whether *in vitro* treatment of female or castrated male macrophages with phytanic acid could upregulate CD36. Treatment with phytanic acid *in vitro* induced increased CD36 expression in both female and castrated male BWF1 macrophages. **(Fig. 38C,D; 40G,H)**, and feeding microspheres containing phytanic acid to female BWF1 mice also increased CD36 expression in splenic macrophages **(Fig. 38E, 40I)**. Since CD36 is involved in phagocytosis of apoptotic cells and phytanic acid increases both CD36 and phagocytosis of apoptotic cells, we wondered whether phytanic acid could be enhancing phagocytosis of apoptotic cells by upregulating CD36 expression. To determine whether CD36 is required for phytanic acid mediated enhanced phagocytosis of apoptotic cells, we cultured female macrophages with phytanic acid and a CD36-blocking antibody. When CD36 was blocked, there was no difference in the ability to phagocytose apoptotic cells between phytanic acid and vehicle treated-macrophages **(Fig. 38F),** suggesting that CD36 is, at least, required for phytanic acid's effects on macrophage phagocytic activity. Taken together, these data suggest that a male microbiota-associated metabolite, phytanic acid, can enhance efferocytosis in female and castrated male BWF1 macrophages. This effect is dependent on LXR and PPARγ signaling and is abrogated by blocking the pro-efferocytic receptor CD36 **(Fig. 41)**.

Discussion

The incidence of severe glomerulonephritis and kidney failure in SLE patients has changed little since the 1990s as progress in the development of new treatments for SLE has plateaued [177-179]. A more complete understanding of the factors contributing to lupus incidence and progression could potentially allow the development of new paradigms of SLE management. One area that is not fully understood is what factors make women and males with Klinefelter's much more susceptible to SLE [1,4,6-8]. Higher dosage of X-linked immune genes due to incomplete X-inactivation has been proposed as a cause of this sex bias [90-92]. The higher levels of immune-promoting estrogen hormones and lower levels of immunosuppressive androgen hormones have also been shown to contribute to the higher incidence in women and men with Klinefelter's syndrome [146,147,149-152]. The BWF1 mouse model of lupus as the course of disease closely mimics the characteristics of SLE humans. Incidence of autoantibodies and glomerulonephritis is much higher in female BWF1 mice, and androgens protect against disease [16,17,133]. However, while the sex differences in disease progression are well characterized, the immunological differences that make BWF1 female mice susceptible and male BWF1 mice resistant are largely unexplored. Here we compared the splenic transcriptomes of disease-prone female BWF1 mice to disease resistant male BWF1 mice and healthy control BALB/c mice. We found that a significant cluster of genes was similarly regulated in the male BWF1 and female BALB/c mice in comparison to the disease-prone female BWF1 mice. The fact that a subset of genes varied not by sex or stain, but by disease susceptibility suggested that the lower lupus susceptibility of male BWF1 mice could be related to differences in splenic immune processes. Subsequent

functional analysis of genes upregulated in male BWF1 and female BALB/c compared to female BWF1 found similarities in what functional processes were enriched in the two disease-resistant groups compared to the disease-prone group. Of particular note was an enrichment in the positive regulation of phagocytosis in both the male BWF1 and female BALB/c. This was significant because a deficiency in the clearance of apoptotic cells by phagocytosis is associated with increased lupus susceptibility [371,491,492].

The process by which phagocytes engulf apoptotic and necrotic cells is called efferocytosis [370]. This process allows the removal apoptotic debris in a manner that promotes anti-inflammatory responses. This is crucial to the maintenance of self-tolerance, as stimulation of immune responses against apoptotic cells and the self-antigens they contain could result in autoimmunity [371]. Rapid ingestion of apoptotic cells prevents the potentially autoimmune stimulating antigens from being exposed to other immune cells and potentially driving immune responses. At the same time, the anti-inflammatory cytokines produced by the phagocytes suppress nearby immune cells that may have been exposed to the apoptotic debris [370-372]. Macrophages are the cells primarily responsible for efferocytosis, and defects in macrophage efferocytosis are strongly associated with the development of SLE. Macrophages from SLE patients exhibit reduced ability to phagocytose apoptotic cells, and accumulation of apoptotic cells is seen in SLE patient tissues [370,371,380,493,523]. This defect is also present in murine models of lupus, as peritoneal macrophages from female BWF1 mice are deficient at efferocytosis compared to healthy control female BALB/c mice [391]. Our findings from the RNA-seq suggested that the sex bias of lupus in BWF1 mice might be partially due to deficient efferocytic activity in female BWF1 mice. If male BWF1

splenic macrophages are like those of healthy control mice and are intrinsically more efficient at efferocytosis, we would expect them to express higher levels of genes directly involved in the efferocytic process. We examined the pro-phagocytosis genes upregulated in male BWF1 compared to the disease-prone female BWF1 mice and we identified one gene, LRP1, which is expressed in macrophages and is responsible for binding apoptotic cells and promoting their ingestion [405,506]. The other phagocytosis promoting genes are either not expressed in macrophages (SFTPA1) or not directly involved in phagocytosis (IL2RB, RAC2, and IL1B) [534-538]. Measuring expression in sorted male and female BWF1 splenic macrophages showed that male splenic macrophages do express higher levels of LRP1. Also, macrophages from androgen depleted lupus-prone male BWF1 mice expressed lower levels of LRP1 compared to intact male BWF1 mice. Together, these findings support the theory that there could be a sex-based difference in splenic macrophage efferocytic efficiency.

Based on these findings, we compared the ability of female and male BWF1 splenic macrophages to phagocytose apoptotic cells, and found male BWF1 splenic macrophages are more efficient at efferocytosis. Furthermore, this difference was androgen dependent, as male BWF1 mice that had undergone androgen depletion by castration had splenic macrophage with deficient efferocytosis compared to macrophages from intact male BWF1 mice. This finding that male BWF1 mice exhibit increased splenic macrophage efferocytosis in an androgen dependent manner sheds new light on the causes of sex bias in the lupus BWF1 mouse model. An early study on lupus in the BWF1 mouse strain found that while removal of the spleen had no effect on the progression of disease in female mice, in the male mice splenectomy resulted in

accelerated anti-nuclear antibody development and decreased survival [539]. Our findings suggest a possible explanation for this sex-based difference in the effect of spleen removal. The spleen is a major site of apoptotic cell clearance [379,387,504,505], and reductions in the body's ability to clear apoptotic cells promote lupus progression [371,391,491,492]. The increased disease in splenectomized male mice could, therefore, be driven by an increase in the accumulation of apoptotic debris. Since, as our findings show, splenic macrophages in male BWF1 mice are more efficient at efferocytosis than those from female BWF1 mice, the removal of the spleen would have a bigger impact on the overall apoptotic cell clearing ability of the male mice.

While we had identified a defect in splenic macrophage efferocytosis in female and androgen-depleted male BWF1 mice, the source of the defect was not clear. The defect was linked to lower levels of androgen signaling (i.e., due to depletion of androgens by castration), which raised the question of whether macrophages, which are known to express androgen receptors, could be directly affected by androgens to increase phagocytosis of apoptotic cells. However, an examination of the literature shows that this is unlikely. Multiple studies have found either that treatment with androgens actually suppressed macrophage phagocytic activity [508,540-543], or that androgen treatment had no effect on macrophage phagocytosis [544,545]. Estrogen, conversely, generally has a pro-phagocytic effect [546,547]. These opposite effects of sex hormones may be why macrophages from healthy female rats and mice have been shown to have higher phagocytic activity than those from male rats and mice [74,75]. However, it has also been shown that castration of male mice decreases peritoneal macrophage phagocytic activity [548]. Taken together, this suggests that androgens are important for macrophage

phagocytosis, but that this effect is not mediated by a direct androgen-macrophage interaction.

One way that androgens can indirectly influence the immune system is by altering intestinal metabolite profiles. Androgens can affect both host and microbial metabolic activities, and alterations in metabolite production can have a major on the immune system [218,324,436,501,502]. In our studies described in the Metabolite section of this dissertation, we explored the functional differences between intact male and female BWF1 microbiota by comparing their fecal metabolite levels. We found that in all three experiments, male mice had higher levels of phytol **(Fig. 30)**, a metabolite which is derived from chlorophyll and exhibits RXR and PPARγ agonist activity [420]. We also found phytol abundance was greater in intact than castrated male BWF1 mice **(Fig. 31A)**. Once phytol is absorbed from the intestine it is converted to phytanic acid, which readily travels in the bloodstream and is an even more potent agonist activity than phytol and activates the PPARγ/RXR, PPARδ/RXR, and LXR/RXR heterodimeric signaling complexes [393-399,503]. When BWF1 fecal phytanic acid levels were tested, only the male feces had detectable levels; it was completely absent from the female and castrated male feces **(Fig. 31B)**.

LXR/RXR and PPARγ/RXR signaling are known to promote efferocytosis via many different mechanisms [381,393,397,404,409,410,414,549-560]. Also, our RNA-seq data showed that male BWF1 splenic macrophages expressed higher levels of LXR and PPARγ-stimulated genes. This made the phytanic acid an obvious candidate for investigation as a source of the sex and sex-hormone-based differences in macrophage efferocytosis. We investigated the effect of phytanic acid treatment on female BWF1

splenic macrophages, and found that it increased their efferocytic activity. We also performed these experiments with splenic macrophages from androgen-depleted male BWF1 mice, and found similar results. This suggests that treatment with phytanic acid might be able to correct the deficiency in efferocytosis that correlates with lower androgen signaling. The naturally higher levels of phytanic acid and its precursor, phytol, in male BWF1 mice may contribute to the higher level of efferocytosis in male BWF1 splenic macrophages. This hypothesis was strengthened by the fact that female BWF1 mice treated with phytanic acid had macrophages with higher efferocytic activity.

While mammals can convert phytol into phytanic acid, they lack the genes to produce phytol [460-462]. Therefore, the intestinal microbiota is the most likely source of the increased levels of phytol and phytanic acid seen in intact male BWF1 mice. This fits with the findings of recent studies in the literature, which have suggested that the gut microbiota could be a source of the sex bias seen in lupus. Studies have found that patients with systemic lupus erythematosus and other autoimmune diseases have been found to have dysbiotic (disrupted) microbiomes [184,256,257,276-280,282,283]. Multiple mechanisms by which the microbiota can influence the immune system have been identified, including molecular mimicry of self-antigens, direct interactions with intestinal immune cells that induce differentiation, and the production of immunomodulatory metabolites [218,436]. Furthermore, sex hormones can significantly affect the microbiota, suggesting that BWF1 mouse androgens levels could affect lupus progression by influencing the composition and functions of the microbiota [276-280,282,283,300-302]. This possibility was supported by the findings of two recent studies, which showed that the sex bias in the NOD model of Type 1 diabetes was linked

to differences in microbiota composition between male and female mice. They found that male mice contained a higher abundance of microbes that could protect them against developing diabetes, and that in the absence of the microbiota, the sex bias was absent [323,324]. One of the studies found that disease was suppressed in female NOD mice that received a microbiota transfer from male NOD mice. However, this protective microbiota phenotype was dependent on androgen, showing that the immune system, sex hormones, and the microbiota are closely interconnected [324]. Also, a recently published paper on the SWRxNZB F1 (SNF1) mouse model found differences in microbiota composition between intact male, androgen-depleted male, and female mice, but failed to find that these alterations significantly affected disease progression. However, the fact that they also found androgen depletion didn't significantly alter disease progression suggests that their model may not have been well suited to studying the relationship between sex hormones, the microbiota, and lupus [26]. We have also found differences in microbiota composition in feces between female and male, and intact and castrated male BWF1 mice. To investigate whether these differences were connected to the differences in efferocytosis, we transferred male microbiota into female BWF1 mice and found it improved splenic macrophage efferocytosis. This suggested that the sex differences in BWF1 splenic macrophage efferocytosis may be mediated in part by the microbiota.

These findings have obvious implications for potential SLE therapeutics. Increasing macrophage efferocytosis could potentially suppress lupus progression. In fact, there is some evidence that glucocorticoid therapy acts in part by increasing efferocytosis, but long-term use of glucocorticoid-based therapy is associated with irreversible side effects and in some cases can lead to a Th17/Th1 imbalance that

promotes inflammation [178,561,562]. However, the agonist activity of phytanic acid is too nonspecific for it to be a viable therapy, since it can activate any permissive RXR-containing heterodimer. For our findings to inform future research, it was crucial to understand what pathways are involved in the pro-efferocytic effect of phytanic acid. Phytanic acid can stimulate signaling through the PPARδ/RXR, LXR/RXR, and PPARγ/RXR complexes [449-453,463], and all of these pathways can stimulate macrophage phagocytosis [381,393-400]. However, we conclusively showed that the pro-efferocytosis effect of phytanic acid is dependent on LXR/RXR and PPARγ/RXR signaling but not on PPARδ/RXR signaling.

This leaves open the question of what LXR and/or PPARγ stimulated genes is phytanic acid upregulating to promote phagocytosis. We compared female and male BWF1 splenic macrophage expression of canonical efferocytic mediators that are stimulated by PPARγ and/or LXR agonists. AXL, MFGE8, and C1QA fit these criteria, and defects in their expression are associated with lupus [375,382,393,400,405,522-533], but we found no differences in splenic macrophage transcript levels between male and female BWF1 mice. We also tested CD36, MERTK, and TIMD4 protein levels, since they are well established important mediators of efferocytosis [372,384,393,396,404,408-410,412,413,513-521]. Of these three, only the pro-efferocytic receptor CD36 exhibited a sex-based difference in expression. CD36, a cell surface receptor that binds apoptotic cells and facilitates their phagocytosis [408], was expressed at higher levels in male BWF1 than female BWF1 splenic macrophages. Macrophages from castrated male BWF1 mice also had decreased CD36 expression compared to intact male BWF1 mice. CD36 expression can be enhanced by LXR and/or PPARγ signaling, and its expression

was highest in male BWF1 mice, which have been found to have higher levels of intestinal metabolites, phytol and phytanic acid, which can activate these receptors [420]. We found treating splenic macrophages with phytanic acid increased their levels of CD36 expression, and feeding female BWF1 mice phytanic acid-containing microspheres increased splenic macrophage CD36 expression. This suggests that the higher levels of CD36 expression in male BWF1 splenic macrophages could be due to higher production of phytanic acid and its precursor phytol in the intestine. Furthermore, functional CD36 is required for phytanic acid's enhancement of macrophage efferocytosis, which suggests there could be a link between phytanic acid's ability to upregulate CD36 and its ability to improve macrophage efferocytosis.

In conclusion, we have shown that disease-resistant male BWF1 mice have splenic macrophages that are more efficient at efferocytosis than macrophages from disease-prone female or androgen depleted castrated male BWF1 mice. This deficiency can be ameliorated by treatment with the metabolite phytanic acid, which is more abundant in males and activates the efferocytosis-promoting LXR and PPARγ pathways. This effect of phytanic acid may be mediated in part by upregulating CD36 expression. Transferring microbiota from male BWF1 to female BWF1 mice also increases macrophage phagocytosis, which supports the hypothesis that differences in microbiota metabolite production between male and female BWF1 mice result in differences in macrophage phagocytosis of apoptotic cells. This sex-based difference in efferocytosis could contribute to lupus progression, which sheds new light on the source of the sex bias in the BWF1 mouse model of lupus and suggests possible avenues of research into new treatments for lupus.

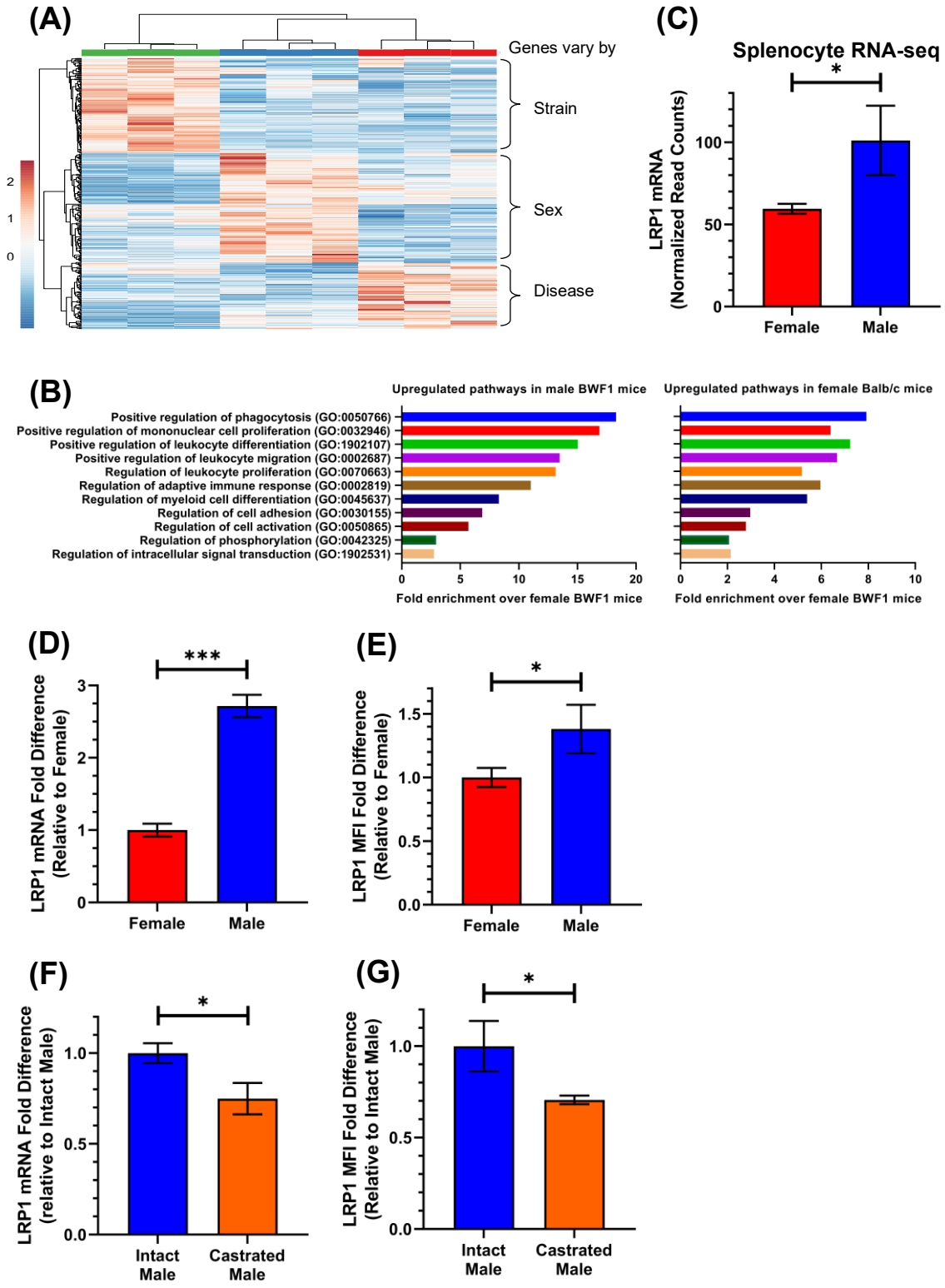

(A)
Genes vary by
Strain
Sex
Disease
2
1
0
(C)
Splenocyte RNA-seq
*
LRP1 mRNA
(Normalized Read Counts)
100
50
0
Female
Male
(B)
Upregulated pathways in male BWF1 mice
Upregulated pathways in female Balb/c mice
Positive regulation of phagocytosis (GO:0050766)
Positive regulation of mononuclear cell proliferation (GO:0032946)
Positive regulation of leukocyte differentiation (GO:1902107)
Positive regulation of leukocyte migration (GO:0002687)
Regulation of leukocyte proliferation (GO:0070663)
Regulation of adaptive immune response (GO:0002819)
Regulation of myeloid cell differentiation (GO:0045637)
Regulation of cell adhesion (GO:0030155)
Regulation of cell activation (GO:0050865)
Regulation of phosphorylation (GO:0042325)
Regulation of intracellular signal transduction (GO:1902531)
0 5 10 15 20
Fold enrichment over female BWF1 mice
0 2 4 6 8 10
Fold enrichment over female BWF1 mice
(D)
***
LRP1 mRNA Fold Difference
(Relative to Female)
3
2
1
0
Female
Male
(E)
*
LRP1 MFI Fold Difference
(Relative to Female)
1.5
1.0
0.5
0.0
Female
Male
(F)
*
LRP1 mRNA Fold Difference
(relative to Intact Male)
1.0
0.5
0.0
Intact Male
Castrated Male
(G)
*
LRP1 MFI Fold Difference
(Relative to Intact Male)
1.0
0.5
0.0
Intact Male
Castrated Male

Figure 32. Splenocyte transcriptome and macrophage gene expression analyses indicate that male BWF1 macrophages may have greater efferocytic activity. Splenocytes from 16-week-old female and male BWF1, and female BALB/c mice were collected and gene expression was analyzed by RNA-seq. **(A)** Heat map of hierarchically clustered differentially expressed genes in splenocytes from female BALB/c, and female and male BWF1 mice (n=3). **(B)** Selected GO:BP pathways enriched in male BWF1 and female BALB/c compared to female BWF1 splenocytes. **(C)** Lrp1 normalized read levels in female and male BWF1 splenocytes. **(D)** $F4/80^{+}CD11b^{+}$ splenic macrophages from 16-week-old female and male BWF1 mice were sorted and Lrp1 transcript levels were measured by qPCR (n=6). **(E)** Expression of LRP1 16-week-old female and male BWF1 $CD11b^{+}F4/80^{+}$ $^{+}$splenic macrophages measured by flow cytometry as mean fluorescence intensity (MFI) fold difference (n=5). **(F)** $CD11b^{+}F4/80^{+}$splenic macrophages from 16-week-old intact and castrated male BWF1 mice were sorted and Lrp1 transcript levels were measured by qPCR (n=6). **(G)** Expression of LRP1 16-week-old intact and castrated male BWF1 $CD11b^{+}F4/80^{+}$ splenic macrophages measured by flow cytometry as mean fluorescence intensity (MFI) fold difference (n=5). *p<0.05, **p<0.01, ***p<0.001

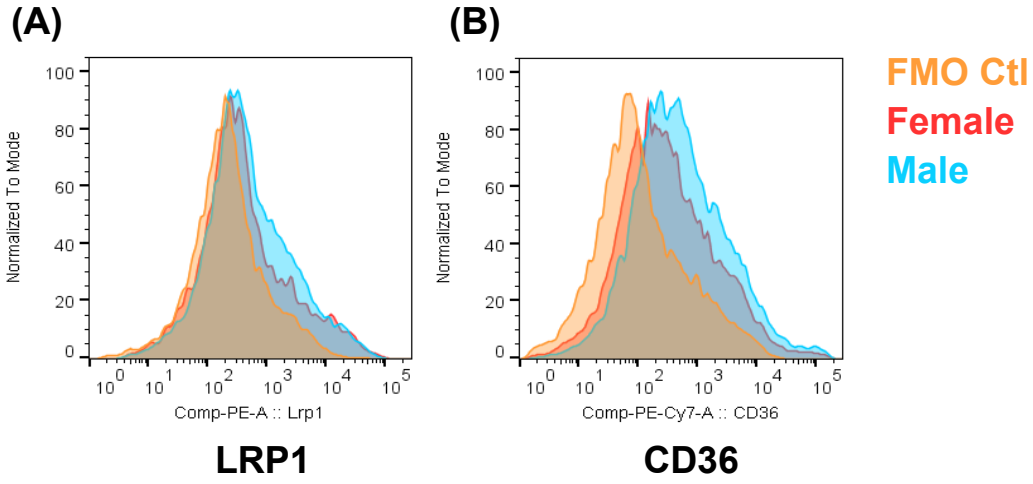


Figure 33. Representative histograms of LRP1 and CD36 labeling of female vs male CD11b$^+$F4/80$^+$ cells. Splenocytes from 16-week-old female or male BWF1 mice were collected and labeled with CD11b, F4/80 and LRP1 or CD36 antibodies and analyzed by flow cytometry. Expression of LRP1 or CD36 by CD11b$^+$F4/80$^+$ cells was evaluated. Fluorescence-minus-one controls (FMO Ctl) were used.

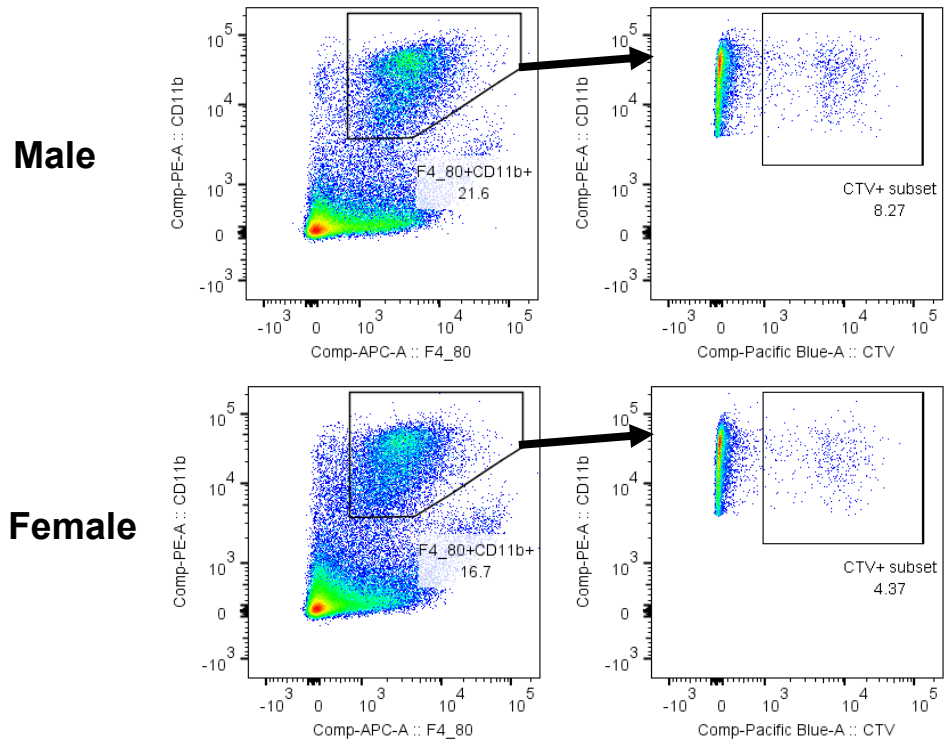


Figure 34. Representative gating of CD11b^{+}F4/80^{+} cells among adherent cells that were positive for the apoptotic thymocyte marker CellTraceTM Violet (CTV). Splenocytes from 16-week-old female and male BWF1 mice were collected and macrophages enriched by selecting for adherent cells. Phagocytosis of apoptotic cells by macrophages was assayed by culture with CellTrace^{TM} Violet (CTV)-labeled apoptotic thymocytes for one hour followed by analysis of percentages of CD11b^{+}F4/80^{+} cells that were CTV^{+}.

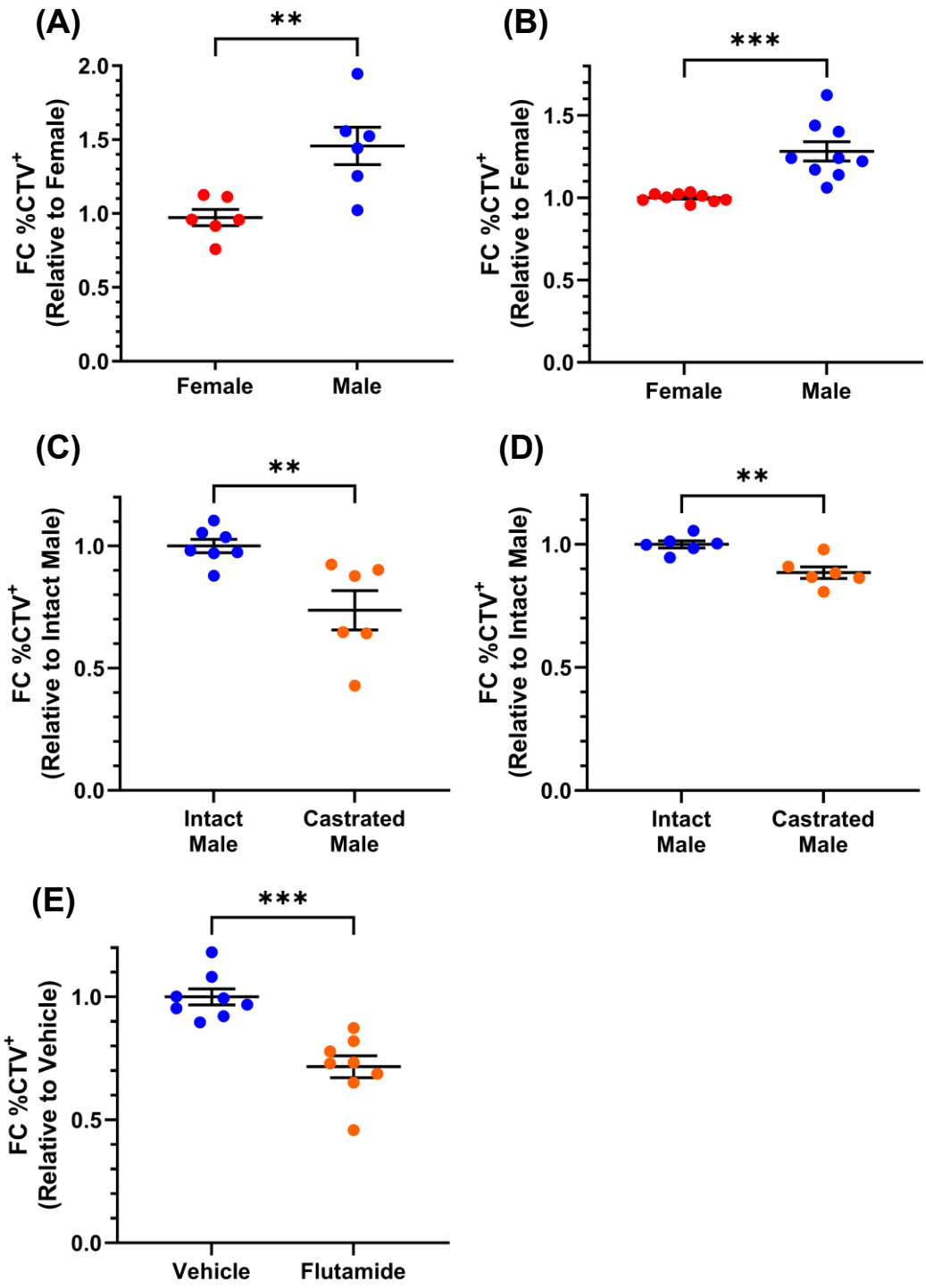

(A)
**
FC %CTV+ (Relative to Female)
2.0
1.5
1.0
0.5
0.0
Female
Male
(B)
***
FC %CTV+ (Relative to Female)
1.5
1.0
0.5
0.0
Female
Male
(C)
**
FC %CTV+ (Relative to Intact Male)
1.0
0.5
0.0
Intact Male
Castrated Male
(D)
**
FC %CTV+ (Relative to Intact Male)
1.0
0.5
0.0
Intact Male
Castrated Male
(E)
***
FC %CTV+ (Relative to Vehicle)
1.0
0.5
0.0
Vehicle
Flutamide

Figure 35. Splenic macrophages from disease-prone female or castrated male BWF1 mice are deficient in their ability to phagocytose apoptotic cells compared to disease-resistant intact male BWF1 mice. Splenocytes from 16-week-old BWF1 mice were collected and macrophages either enriched by selecting for adherent cells or sorted for $CD11b^+F4/80^+$ cells by FACS. Phagocytosis of apoptotic cells by macrophages was assayed by culture with CellTrace™ Violet (CTV)-labeled apoptotic thymocytes for one hour followed by analysis of percentages of $CD11b^+F4/80^+$ cells that were CTV^+. **(A)** Adherent splenocytes from female or male BWF1 mice were labeled with CD11b and F4/80 antibodies, and $CD11b^+F4/80^+$ cells evaluated for $\%CTV^+$ n=6). **(B)** Sorted $CD11b^+F4/80^+$macrophages from female or male BWF1 mice were evaluated for $\%CTV^+$ cells (n=9). **(C)** Adherent splenocytes from intact male or castrated male BWF1 mice were labeled with CD11b and F4/80 antibodies, and $CD11b^+F4/80^+$ cells evaluated for $\%CTV^+$ (n=6-7). **(D)** Sorted $CD11b^+F4/80^+$macrophages from intact male or castrated male BWF1 mice were evaluated for $\%CTV^+$ cells (n=6). **(E)** Adherent splenocytes from male BWF1 mice implanted with time-release pellets containing vehicle or androgen receptor inhibitor, flutamide, were labeled with CD11b and F4/80 antibodies, and $CD11b^+F4/80^+$ macrophages were evaluated for $\%CTV^+$ cells (n=8). Data are reported as fold change (FC) in $\%CTV^+$ cells. *$p<0.05$, **$p<0.01$, ***$p<0.001$

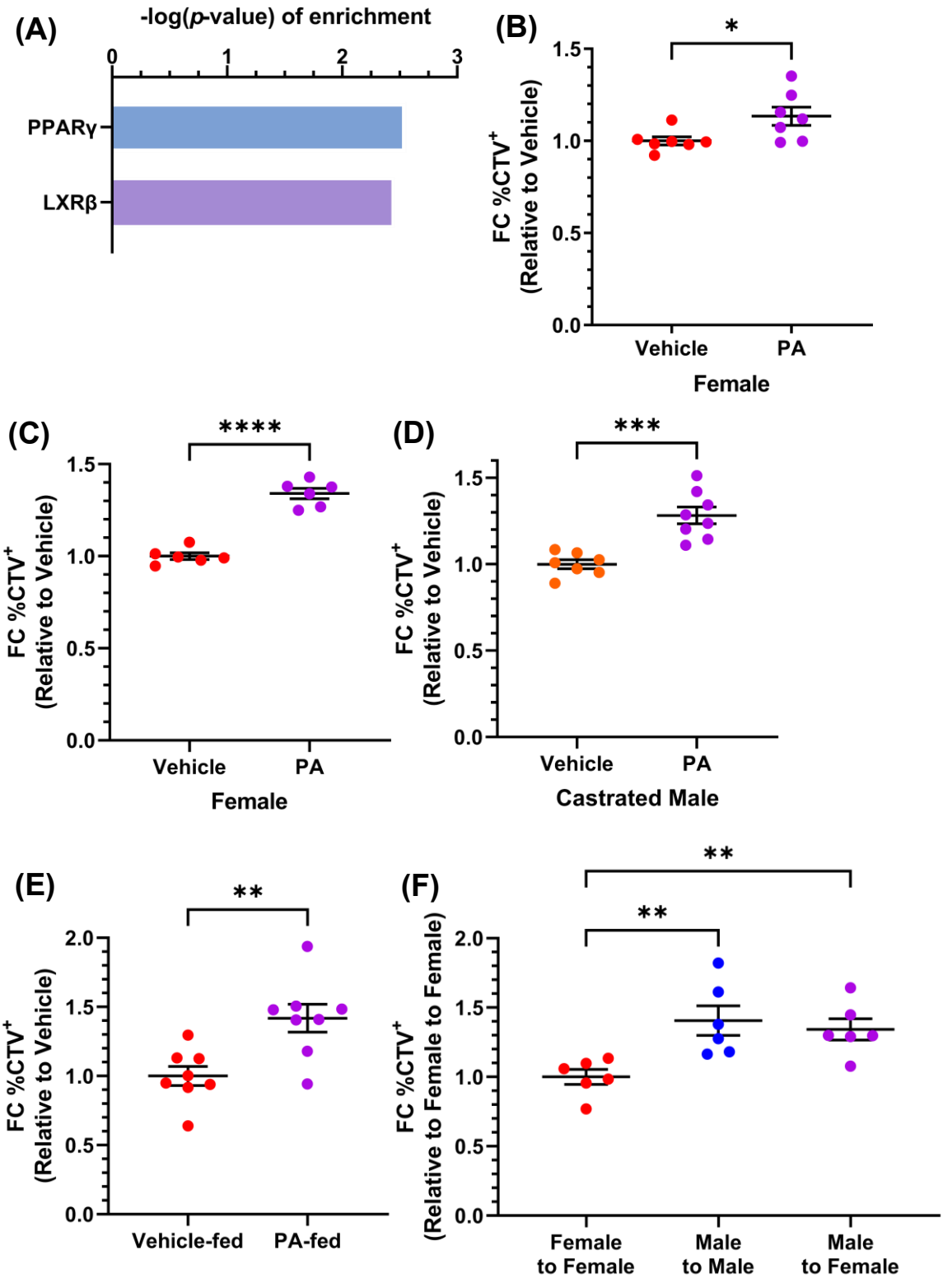

(A)
-log(p-value) of enrichment
0
1
2
3
PPARγ
LXRβ
(B)
*
FC %CTV+ (Relative to Vehicle)
1.5
1.0
0.5
0.0
Vehicle
PA
Female
(C)
****
FC %CTV+ (Relative to Vehicle)
Vehicle
PA
Female
(D)
***
FC %CTV+ (Relative to Vehicle)
Vehicle
PA
Castrated Male
(E)
**
FC %CTV+ (Relative to Vehicle)
2.0
1.5
1.0
0.5
0.0
Vehicle-fed
PA-fed
(F)
**
**
FC %CTV+ (Relative to Female to Female)
Female to Female
Male to Male
Male to Female

Figure 36. A male microbiota associated metabolite, phytanic acid, increases the ability of adult BWF1 splenic macrophages to phagocytose apoptotic cells. (A) Gene expression in female and male splenocytes from 16-week-old BWF1 mice was determined by RNA-seq. *Metacore* Transcription Factor Enrichment analysis shows the fold-enrichment of upregulated genes in male vs female splenocytes that are controlled by the transcriptional regulators PPARγ and LXRβ (n=3). Splenocytes from 16-week-old female **(B, C)** or castrated male **(D)** BWF1 mice were collected and macrophages either enriched by selecting for adherent cells and labeling with CD11b and F4/80 antibodies **(B)** or sorted for $CD11b^+F4/80^+$ cells by FACS **(C, D)**. Cells were pre-treated with 50 μM phytanic acid (PA) or ethanol (Vehicle) for 48 hours then phagocytosis of apoptotic cells by macrophages was assayed by culture with CellTrace™ Violet (CTV)-labeled apoptotic thymocytes for one hour followed by analysis of percentages of $CD11b^+F4/80^+$ cells that were CTV^+ (B, C, D; n=6-7). **(E)** Female BWF1 mice were treated with PBS (Vehicle) or phytanic acid (PA) encapsulated in microspheres 3x per week for 4 weeks beginning at 12 weeks of age then splenocytes were collected and sorted for $CD11b^+F4/80^+$ cells by FACS. Phagocytosis of apoptotic cells by macrophages was assayed by culture with CTV-labeled apoptotic thymocytes for one hour followed by analysis of percentages of $CD11b^+F4/80^+$ cells that were CTV^+ (n=8). **(F)** Twenty-four-day-old (shortly after weaning) female BWF1 mice were fed cecal contents from adult female (negative control) or male (experimental) BWF1 mice, or 24-day-old male BWF1 mice were fed cecal contents from adult male BWF1 mice (positive control). Mice were then fed cecal contents monthly until 16 weeks of age when splenocytes were collected and sorted for $CD11b^+F4/80^+$ cells by FACS. Phagocytosis of apoptotic cells by macrophages was assayed by culture with CTV-labeled apoptotic thymocytes for one hour followed by analysis of percentages of $CD11b^+F4/80^+$ cells that were CTV^+ (n=6). Data are reported as fold change (FC) in $\%CTV^+$ cells (B-F). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$

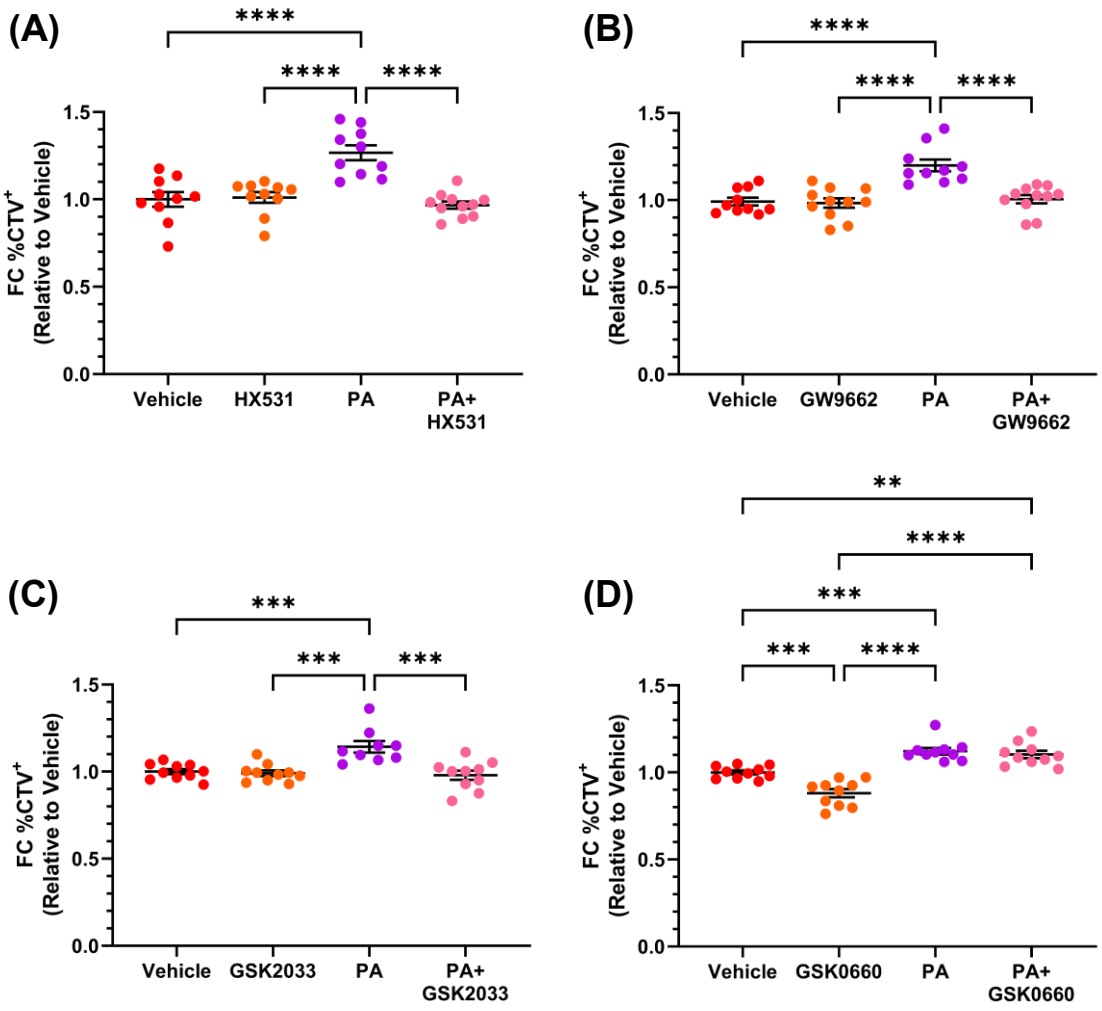
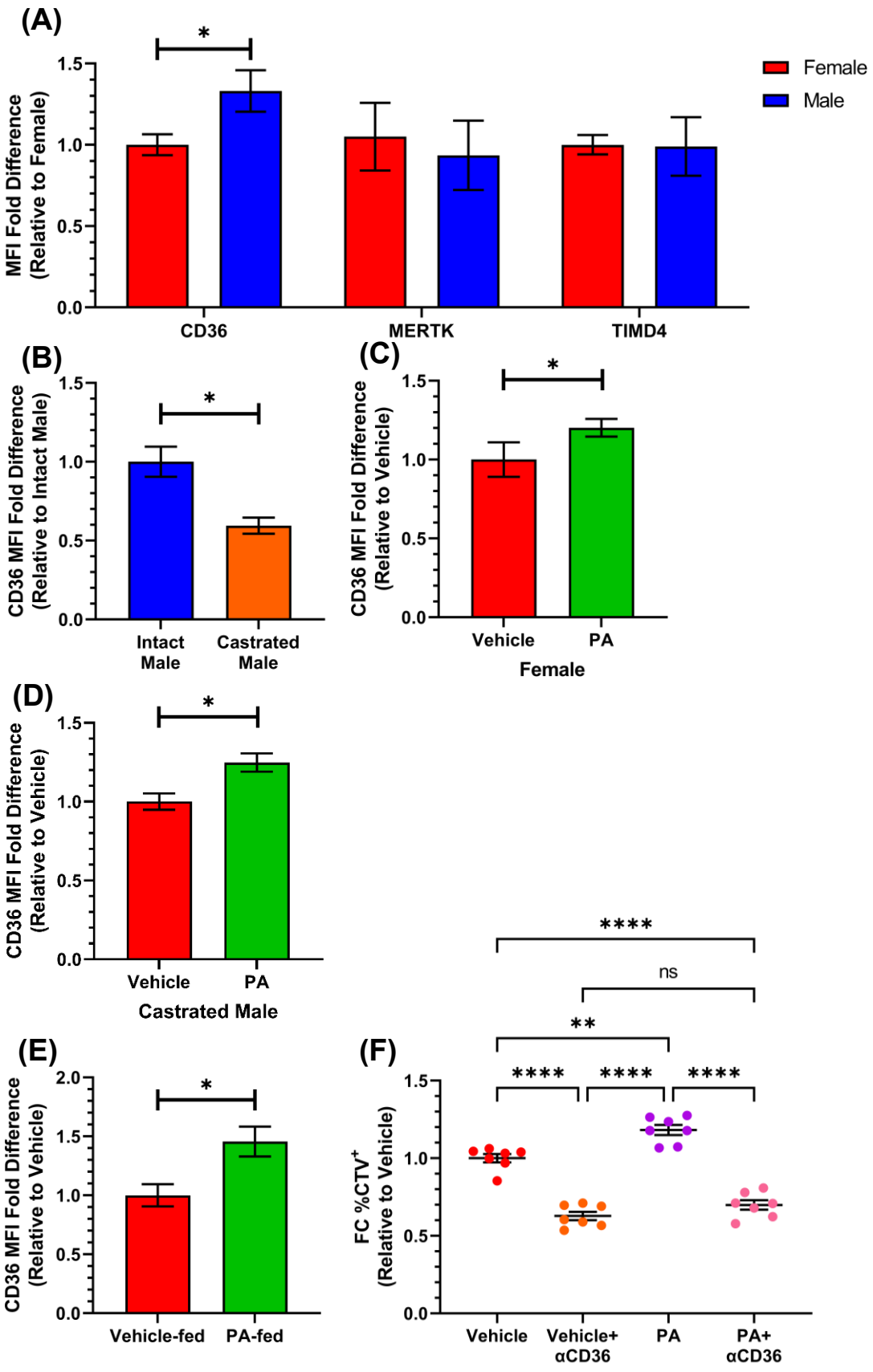


Figure 37. Phytanic acid increases macrophage phagocytosis in an LXR/RXR and PPARγ/RXR dependent, and PPARδ/RXR independent manner. Splenocytes from adult female BWF1 mice were collected and sorted for $CD11b^{+}F4/80^{+}$cells by FACS. Cells were pre-treated with 50 µM phytanic acid (PA), ethanol (Vehicle) and/or nuclear receptor (RXR, PPARγ, LXR, PPARδ) inhibitors for 48 hours then phagocytosis of apoptotic cells by macrophages was assayed by culture with CellTrace™ Violet (CTV)-labeled apoptotic thymocytes for one hour followed by analysis of percentages of $CD11b^{+}F4/80^{+}$ cells that were CTV^{+}. **(A)** 10 µM of RXR inhibitor HX531 (n=10). **(B)** 10 µM of PPARγ inhibitor GW9662 (n=10). **(C)** 5 µM of LXR inhibitor GSK2033 (n=10). **(D)** 10 µM of PPARδ inhibitor GSK0660 (n=10). Data are reported as fold change (FC) in $\%CTV^{+}$ cells. $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$


(A)
MFI Fold Difference (Relative to Female)
Female
Male
CD36
MERTK
TIMD4
(B)
CD36 MFI Fold Difference (Relative to Intact Male)
Intact Male
Castrated Male
(C)
CD36 MFI Fold Difference (Relative to Vehicle)
Vehicle
PA
Female
(D)
CD36 MFI Fold Difference (Relative to Vehicle)
Vehicle
PA
Castrated Male
(E)
CD36 MFI Fold Difference (Relative to Vehicle)
Vehicle-fed
PA-fed
(F)
FC %CTV+ (Relative to Vehicle)
Vehicle
Vehicle+ αCD36
PA
PA+ αCD36
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ns
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Figure 38. Male splenic macrophages express higher levels of CD36, and phytanic acid increases CD36 expression. Splenocytes from 16-week-old female, male, or castrated male BWF1 mice were collected and labeled with CD11b, F4/80, and pro-efferocytic receptor (CD36, MERTK, or TIMD4) antibodies and analyzed by flow cytometry. Expression is shown as mean fluorescence intensity (MFI) fold difference and comparisons were done with Student's *t*-tests. **(A)** CD36, MERTK, and TIMD4 expression in female and male $CD11b^+F4/80^+$ cells (n=5). **(B)** CD36 expression in intact and castrated male $CD11b^+F4/80^+$ cells (n=5). **(C)** CD36 expression in female $CD11b^+F4/80^+$ cells treated with 50 μM phytanic acid (PA) or ethanol (Vehicle), for 24 hours (n=5). **(D)** CD36 expression in castrated male $CD11b^+F4/80^+$ cells treated with 50 μM phytanic acid (PA) or ethanol (Vehicle), for 24 hours (n=5). **(E)** Female BWF1 mice were treated with PBS (Vehicle) or phytanic acid (PA) encapsulated in microspheres 3x per week for 2 weeks then splenocytes were collected. CD36 expression in $CD11b^+F4/80^+$ cells was analyzed (n=3). **(F)** Splenocytes from 16-week-old female BWF1 mice were collected and sorted for $CD11b^+F4/80^+$ cells by FACS. Sorted cells were pre-treated for 48 hours with 50 μM phytanic acid (PA) or ethanol (Vehicle). Then they were treated with or without blocking anti-CD36 antibody for 30 mins and phagocytosis of apoptotic cells was assayed by culture with CellTrace™ Violet (CTV)-labeled apoptotic thymocytes for one hour followed by analysis of percentages of $CD11b^+F4/80^+$ cells that were CTV^+. Data are reported as fold change (FC) in $\%CTV^+$ cells (n=7). *p<0.05, **p<0.01, ****p<0.0001

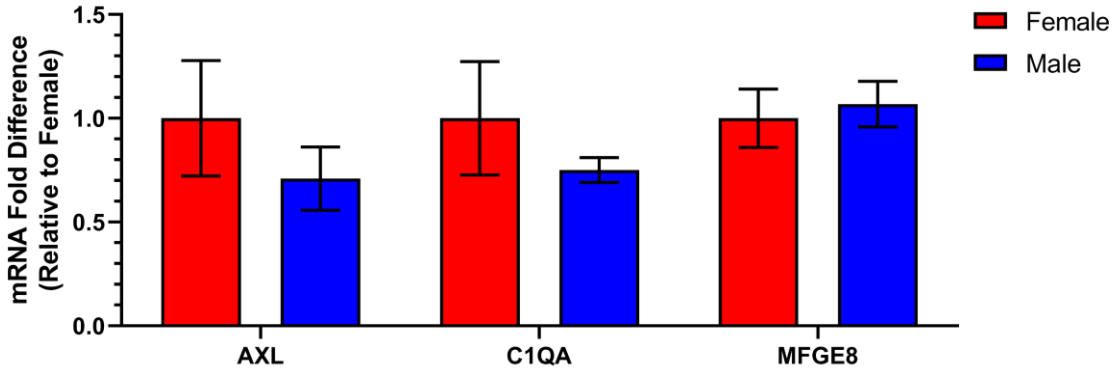

Figure 39. Expression of pro-efferocytic genes in female and male BWF1 splenic macrophages. Splenocytes from 16-week-old female and male BWF1 mice were collected and sorted for $CD11b^{+}F4/80^{+}$cells by FACS. AXL, C1QA, and MFGE8 transcript levels were measured by qPCR (n=6).

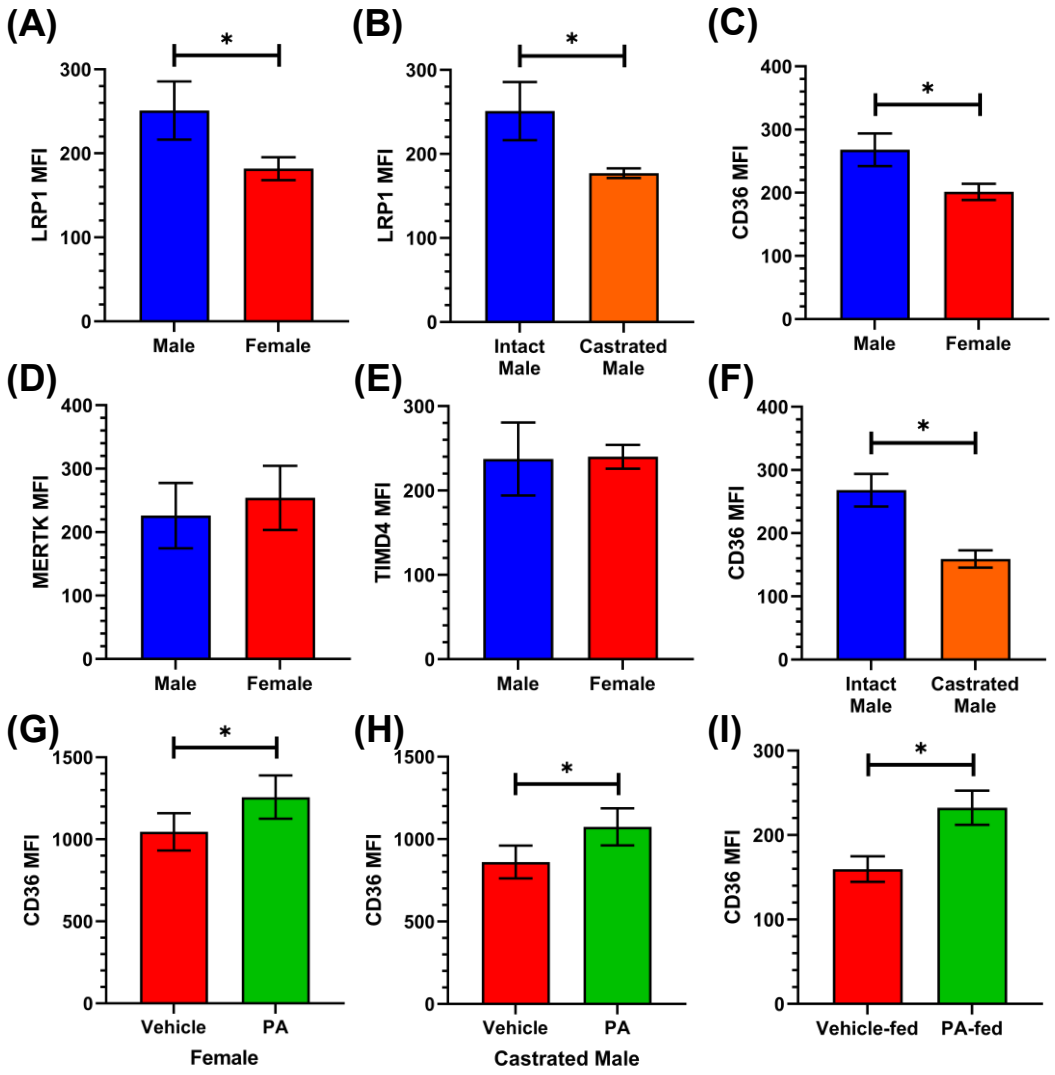

Figure 40. Raw Mean Fluorescence Intensity (MFI) data for pro-efferocytic expression analysis by flow cytometry. Splenocytes from 16-week-old female, male, or castrated male BWF1 mice were collected and labeled with CD11b, F4/80, and pro-efferocytic receptor (LRP1, CD36, MERTK, or TIMD4) antibodies and analyzed by flow cytometry. Comparisons were done using Student's t-tests. **(A)** Expression (MFI) of LRP1 in female and male $CD11b^{+}F4/80^{+}$ cells (n=5). **(B)** Expression of LRP1 in intact and castrated male $CD11b^{+}F4/80^{+}$ cells (n=5). **(C)** Expression of CD36 in female and male $CD11b^{+}F4/80^{+}$ cells (n=5). **(D)** Expression of MERTK in female and male $CD11b^{+}F4/80^{+}$ cells (n=5). **(E)** Expression of TIMD4 in female and male $CD11b^{+}F4/80^{+}$ cells (n=5). **(F)** Expression of CD36 in intact and castrated male $CD11b^{+}F4/80^{+}$ cells (n=5). **(G)** Expression of CD36 in female $CD11b^{+}F4/80^{+}$ cells treated with 50 µM phytanic acid (PA) or ethanol (Vehicle) for 24 hours (n=5). **(H)** Expression of CD36 in castrated male $CD11b^{+}F4/80^{+}$ cells treated with 50 µM phytanic acid (PA) or ethanol (Vehicle) for 24 hours (n=5). **(I)** Female BWF1 mice were treated with vehicle (control) or phytanic acid encapsulated in microspheres 3x per week for 2 weeks then splenocytes were collected. Expression of CD36 in $CD11b^{+}F4/80^{+}$ cells was analyzed (n=3). *p<0.05, **p<0.01, ***p<0.001

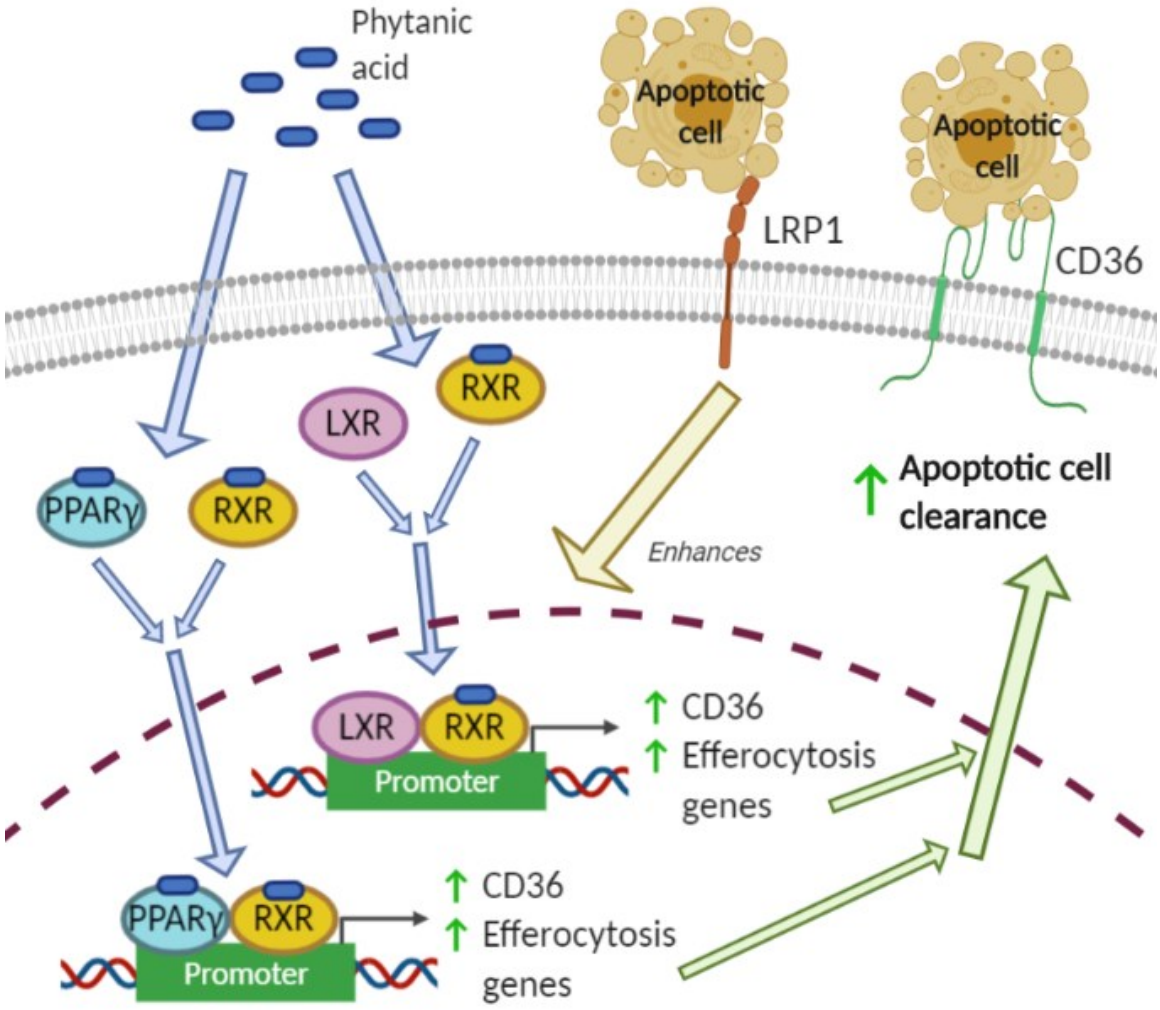

Figure 41. Proposed mechanism of phytanic acid enhancement of macrophage efferocytosis. Phytanic acid binds PPARγ and RXR and activates signaling through the LXR/RXR and PPARγ/RXR heterodimers. This leads to increased expression of CD36 and other pro-efferocytic genes, which in turn leads to enhanced clearance of apoptotic cells. Created with Biorender.com.

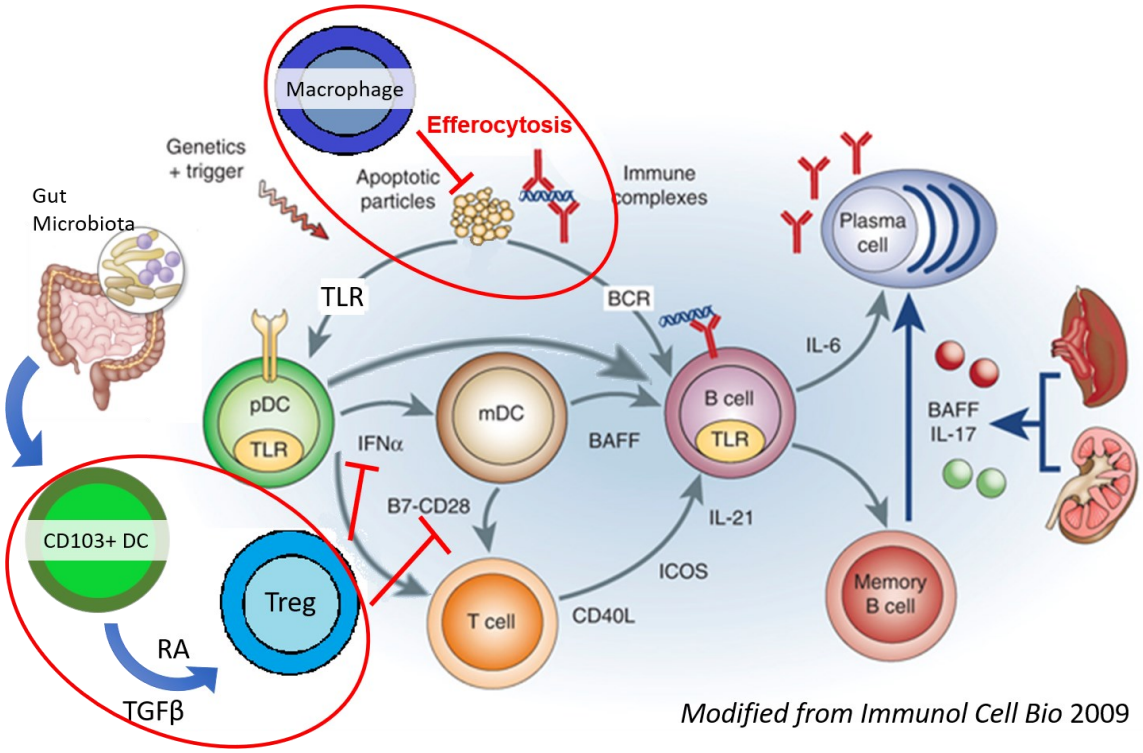

Figure 42. There are many immune cells involved in lupus progression. Defective macrophage clearance of apoptotic cells leads to a buildup of inflammatory nuclear antigen-containing debris. Myeloid dendritic cells (mDC) activate T cells and B cells, and T cells activate B cells. The apoptotic debris further stimulates anti-nuclear antigen responses CD103$^+$ dendritic cells (CD103$^+$ DC) induce regulatory T cells (Treg) that produce anti-inflammatory cytokines and suppress the activation/function of T cells. Plasmacytoid dendritic cells produce IFNα that promote B cell maturation and antibody production.

Table 9. Pro-phagocytosis genes upregulated in splenocytes from male compared to female BWF1 mice.

Gene Symbol	Gene Name
SFTPA1	Pulmonary Surfactant Protein A1
LRP1	LDL Receptor-Related Protein 1
IL1B	Interleukin 1 Beta
IL2RB	Interleukin 2 Receptor, Beta Chain
RAC2	Rac Family Small GTPase 2
HSPA8	Heat Shock Protein 8

Table 10. PPARγ-regulated genes upregulated in male compared to female BWF1 splenocytes

Gene Symbol	Gene Name	Gene Function
RPLP1	Ribosomal Protein Lateral Stalk Subunit P1	Ribosomal phosphoprotein 60S subunit component
SLC3A2	Solute Carrier Family 3 Member 2	Activator of dibasic and neutral amino acid transport, regulates intracellular calcium levels
ELOVL5	ELOVL Fatty Acid Elongase 5	Fatty acid elongase 1, involved in the elongation of long-chain polyunsaturated fatty acids

Table 11. LXR-regulated genes upregulated in male compared to female BWF1 splenocytes

Gene Symbol	Gene Name	Gene Function
CTSB	Cathepsin B	Lysosomal cysteine protease with both endopeptidase and exopeptidase activity that may play a role in protein turnover
TCF7	Transcription Factor 7	T-cell/lymphocyte high mobility group box transcription activator, plays role in NK cell and ILC development
CD3E	CD3-Epsilon	Critical part of the TCR-CD3 complex involved in antigen recognition
S1PR1	Sphingosine-1-Phosphate Receptor 1	Binds sphingosine-1 phosphate with high affinity and induces cell-cell adhesion
FOS	FBJ Osteosarcoma Oncogene	Regulator of cell proliferation, differentiation, and transformation
ACP5	Acid Phosphatase 5, Tartrate Resistant	Catalyzes the conversion of orthophosphoric monoester to alcohol and orthophosphate
LRP1	LDL Receptor Related Protein 1	Apoptotic cell clearance, amyloid precursor protein clearance, lipid homeostasis, intracellular signaling

DISCUSSION

A change in animal facilities coincided with a loss of the previously discovered ability of male microbiota to protect against lupus when transferred into female BWF1 mice, but the protective ability has since been restored **(Fig. 23)**. We compared the microbiota from periods when the male microbiota was protective originally (A Tower), when it was not protective (CTRB Early), and when the protective ability was restored (CTRB Recent). We found that while the female and male microbiota profiles were significantly different for all three periods, the degree of significance was higher during the A Tower and CTRB Recent periods compared to the CTRB Early period. Analysis of specific taxa abundance showed that in the A Tower, *Bacteroides* was higher in male than female BWF1, *Clostridium* abundance was ~10-20%, and *Alistipes* was detectable. In contrast in the CTRB Early experiments, *Bacteroides* was almost undetectable or was higher in female than male BWF1 mice, *Clostridium* was considerably higher at 30-40% abundance, and *Alistipes* was completely absent. For experiments in the CTRB Recent period, the taxa had returned to abundances more similar to that seen in the A Tower. *Bacteroides* was present and higher in male than female BWF1 mice, *Clostridium* abundance was ~5-15%, and *Alistipes* was present. Additionally, the same species in those genera were predominant as in the A Tower and CTRB Early: *B. acidifaciens*, *C. leptum*, *A. putredinis*, and *A. timonensis*. *Bacteroides* was the taxon most obviously associated with the periods when male microbiota was protective, but for CTRB Early

Experiment 4, female BWF1 mice had high *Bacteroides* but were not protected against disease. This could potentially be due to the fact that *Clostridium* was also high and *Alistipes* were absent in CTRB Early Experiment 4. High *Clostridium* could potentially promote lupus susceptibility or block the actions of beneficial microbiota. It is also possible that *Alistipes* and *Bacteroides* act cooperatively to suppress lupus. These hypotheses were supported by the comparison of cecal transfer experiments from the CTRB Early and CTRB recent periods. While high *Bacteroides* did not correlate with protection in the CTRB Early cecal transfer experiment, a high ratio of *Bacteroides* to *Clostridium* abundances did **(Fig. 22B)**; recipients of male microbiota with a high *Bacteroides*/*Clostridium* ratio had significantly suppressed disease whereas recipients of male microbiota with a low *Bacteroides*/*Clostridium* ratio did not, suggesting a possible explanation for why high *Bacteroides* in female mice in CTRB Early Experiment 4 did not correlate with protection against disease. *C. leptum* has been found to be enriched in SLE patient gut microbiota compared to healthy controls, and reduction of disease activity via standard treatments correlated with reduction of *C. leptum* abundance [439]. Another study found that intestinal retinoic acid synthesis was suppressed by bacteria from the class Clostridia, which includes *Clostridium* [440]. Retinoic acid is important for the induction of tolerance-promoting T regulatory cells (Tregs) [441], and SLE is associated with lower levels of Tregs [442]. Our lab has also found defects in Treg induction due to deficient retinoic acid production (manuscript in preparation). This suggests that an overabundance of *Clostridia* could potentially increase susceptibility to lupus by suppressing retinoic acid-dependent Treg induction, resulting in an environment that is too pro-inflammatory for the beneficial actions of *Bacteroides* to have any effect

on disease progression. In other words, the beneficial actions of the male microbiota may depend on a certain level of Treg induction. This could explain why in the CTRB Early cecal transfer experiment, the female recipients of female microbiota were not protected from disease despite having high *Bacteroides*. Further study of the effect of *Clostridium* on retinoic acid production in BWF1 mice will be required. The fact that males during the CTRB Early period did not develop disease despite having almost no *Bacteroides* and high *Clostridium* seems to argue against *Clostridium* promoting disease progression. However, despite having suboptimal microbiota compositions, the CTRB Early male mice still were naturally producing androgens. Treating lupus-prone female BWF1 mice with testosterone suppresses disease [133], and this effect is likely the reason that the CTRB Early male mice did not develop lupus.

We also found that not only the microbiota compositions but also the metabolomic profiles of female, intact male, and castrated male BWF1 mice differed. Together with our findings that male microbiota could suppress disease in female BWF1 mice, this suggested that the taxa that were higher in male than female BWF1 mice might produce metabolites that could suppress lupus. We subsequently identified metabolites that were consistently higher in male than female BWF1 feces and investigated them for whether they had the potential to influence lupus progression. Our results suggested that histamine production by the gut microbiota is upregulated in intact male BWF1 mice compared to female and castrated male mice. Histamine can have anti-inflammatory effects on the intestine, which could prevent the "leaky gut" phenomenon that has been linked to increased lupus susceptibility [297,357]. Determining what bacterial species are responsible for the increased histamine production could be informative. *Lactobacillus*

reuteri and *Lactobacillus gasseri* have been shown to produce histamine, but both of those were elevated in female mice and so are unlikely to be the cause of elevated histamine in male mice [563]. It is unknown whether *Bacteroides* can produce histamine, but a recent study offered some evidence that hints it might. Higher microbiota production of histamine has been found to promote other allergic diseases [564], and a recent study of children with Immunoglobulin E-mediated cow's milk allergy (IgE CMA) found that *Bacteroides* was highly elevated in the gut microbiota of children with IgE CMA compared to healthy controls [565]. This could potentially indicate that *Bacteroides* is promoting IgE CMA disease by producing histamine, though additional studies would be needed to confirm it. If *Bacteroides* did produce histamine, it would fit with our findings that *Bacteroides* abundance and histamine levels both are higher in male than female BWF1 mice. Unfortunately, we do not have metabolite data for the CTRB Early cecal transfer experiment where *Bacteroides* was higher in female recipients of female than male microbiota. Analysis of the metabolite profiles from this experiment and the CTRB recent experiment could shed additional light on what metabolites are associated with disease suppression, higher abundances of *Bacteroides*, and lower levels of *Clostridium*. There is another possible interpretation of both our data and the IgE CMA study, though. It is possible that higher levels of histamine favor higher *Bacteroides* abundance, so the higher levels of histamine in our mice affect lupus indirectly by promoting growth of beneficial *Bacteroides*.

Another interesting finding was that in every experiment where high *Bacteroides* abundance correlated with disease protection, *Alistipes* was present. This suggests that the successful suppression of lupus in female recipients might require both species to be

present in the male microbiota. However, this does not necessarily imply that both *Alistipes* and *Bacteroides* directly influence lupus. In fact, since *Alistipes* abundance is not higher in CTRB Recent male to female recipients than female to female recipients, it is unlikely that it directly suppresses lupus. It could potentially produce a precursor to a beneficial metabolite produced by *Bacteroides*. For example, *Alistipes* could produce histidine, and *Bacteroides* could convert it into histamine. Alternatively, *Alistipes* could enhance engraftment of *Bacteroides* in female recipients of male microbiota. It is still largely unknown what factors determine whether a microbiota transplant results in a temporary change followed by a return to the norm or in a stable engraftment of the donor bacteria. However, it is known that the compositions of both the donor and the recipient matter, though the effects of specific taxa are still being studied. Several studies have found that *Alistipes* species are among the most likely to stably engraft in the donor; these included *Alistipes putredinis*, which was one of the predominant species of *Alistipes* in our mice [566-568]. It is not clear, though, whether *Alistipes* higher engraftment ability also benefits other taxa in the donor microbiota. To determine whether *Bacteroides* is more successfully engrafted when *Alistipes* is present or absent as well as the effects of *Bacteroides* and *Alistipes* on lupus progression, we could transfer monocultures of *Alistipes* and/or *Bacteroides* into female BWF1 mice and compare both the effect on disease and the abundance of the donated strains at different time points. There is also some evidence in the literature that higher *Bacteroides* abundance promotes engraftment of donor microbiota. A recent study of the long-term microbiota of patients who had received fecal microbiota transfers to treat *Clostridium difficile* found that the higher the abundance of *Bacteroides* in the donor, the more the microbiota composition of the

recipient resembled the original donor composition at one year post transfer [569]. This suggests that *Bacteroides* in BWF1 mice might not be affecting microbiota directly but instead promoting the engraftment of other beneficial microbes. However, no other taxa besides *Bacteroides* correlated with suppression of lupus in recipients of male microbiota, so this is less likely than *Alistipes* promoting *Bacteroides* engraftment.

We also found that the metabolite, phytol, was consistently higher in male than female BWF1 mice for all three metabolomic experiments. This metabolite and its product, phytanic acid, can activate the RXR and PPARγ receptors, which means that they could potentially suppress lupus progression via several mechanisms. RXR agonists are known to increase retinoic acid production [490], so phytol and phytanic acid might increase intestinal $CD103^+$ dendritic cell (DC) retinoic acid-dependent induction of immunosuppressive Tregs by enhancing $CD103^+$ DC retinoic acid production **(Fig. 42)**. This is supported by our lab's findings that gut $CD103^+$ DCs from female BWF1 mice are less effective at inducing Tregs than male-derived gut $CD103^+$ DCs, and have a defect in retinoic acid synthesis that can be restored by treatment with phytanic acid (manuscript in preparation). RXR and PPARγ agonists are also known to upregulate genes promoting macrophage efferocytosis [393,394]. This could improve clearance of the apoptotic cell debris that is known to promote lupus progression. We focused our efforts on investigating macrophage efferocytosis and found, through transcriptomic and protein expression analysis, that the expression of the pro-efferocytosis receptor LRP1 is higher in male than female BWF1 splenic macrophages. We then tested the ability of splenic macrophages to phagocytose apoptotic cells *in vitro* and found more efficient efferocytic activity in intact male than either female or castrated male BWF1 splenic

macrophages. The fact that the intact male BWF1 mice had higher splenic macrophage efferocytic activity as well as higher abundance of phytol and phytanic acid suggested that there could be a connection between the two. To test this, we treated female BWF1 splenic macrophages with phytanic acid, and found that this improved their ability to phagocytose apoptotic cells in a PPARγ and LXR-dependent manner **(Fig. 41)**. Further investigations showed that feeding female BWF1 mice either phytanic acid or male BWF1 microbiota improved splenic macrophage efferocytosis, which strengthens the connection between the differing male and female microbiota profiles, the higher levels of phytol/phytanic acid male compared to female BWF1 mice, and the greater efferocytic activity of male splenic macrophages. We also determined that phytanic acid treatment upregulated the pro-efferocytic receptor CD36, and that CD36 activity was required for the enhancement of efferocytosis by phytanic acid, suggesting a possible mechanism **(Fig. 41)**. It is worth noting that when LRP1 binds apoptotic cells, it can enhance PPARγ signaling. Since male BWF1 splenic macrophages express higher levels of LRP1, this suggests that phytanic acid might be more effective at promoting macrophage efferocytosis in male than female macrophages.

As described in **Figure 41**, our data indicate that phytanic acid can increase the ability of macrophages to phagocytose apoptotic cells by activating PPARγ. However, in addition to increasing macrophage ability to sequester self-antigen-containing debris away from other immune cells, PPARγ stimulation also promotes macrophage production of anti-inflammatory cytokines, including TGFβ and IL-10 [394], and causes the macrophages to adopt a more M2-like phenotype [570]. These cytokines can suppress the inflammatory responses that drive lupus [394], providing another mechanism by which

phytanic acid-activated macrophages can suppress disease. This is not limited to macrophages in the spleen, either. Because phytanic acid is readily transported through circulation, it may also affect kidney-infiltrating macrophages. M2 macrophages have been found to suppress inflammation in chronic kidney disease and promote wound healing and tissue remodeling, which can help prevent kidney damage [571]. Phytanic acid, therefore, could potentially suppress disease not only by improving clearance of apoptotic cells, but also by influencing kidney infiltrating macrophages into an anti-inflammatory phenotype. The ability of phytanic acid to induce an anti-inflammatory phenotype in macrophages could be tested by treating macrophages with phytanic acid or vehicle, and then challenging them with LPS and measuring their cytokine production. The relevance of these effects on macrophages to lupus progression could be tested by feeding female BWF1 mice phytanic acid or vehicle and then isolating kidney infiltrating macrophages and comparing production of pro- and anti-inflammatory cytokines.

Additionally, macrophages are far from the only type of immune cell that is affected by PPARγ signaling, and there are many immune cells involved in lupus progression **(Fig. 42)**. In dendritic cells, PPARγ stimulation downregulates the costimulatory molecules CD80, CD83, and CD40 and upregulates the coinhibitory molecule B7H1. This makes the dendritic cells less effective at activating T-cells which in turn reduces the activation of B cells by activated T-cells [572]. T cells can also be directly affected by PPARγ agonists, as PPARγ stimulation suppresses T follicular helper responses, which are important for the maturation of antibody-producing B cells [573]. Phytanic acid is unlikely to suppress lupus via interaction with B cells, though, as PPARγ

activation has been shown to increase B cell antibody production [574]. Clearly, there are many possible mechanisms by which phytanic acid could be affecting lupus progression.

CONCLUSION

Our microbiota composition, metabolomic, and macrophage data strongly suggest that the microbiota from male BWF1 mice is distinctly different both in composition and metabolic activity from the female BWF1 mice. These differences likely play a role in the ability of the male microbiota to suppress lupus when transferred into female BWF1 mice. However, as we found when we compared data from the A Tower, CTRB Early, and CTRB Recent periods, when the *Bacteroides*/*Clostridium* ratio is low and *Alistipes* is absent like they were in the CTRB Early period, female recipients of male microbiota are not protected **(Fig. 23)**. These data illustrate how environmental factors have a major impact on microbiota composition. Microbiota function was altered as well, resulting in a loss of the ability of the male BWF1 microbiota to suppress disease. These experiments, therefore, simultaneously demonstrate the tremendous potential of therapeutic microbiota transfers and the inherent challenges of developing a large-scale stable and effective treatment based on the entire microbiota profile of a healthy person. Even if a donor whose microbiota has the desired effect can be identified, any number of things can cause the microbiota profile of the donor to change, and the environment of a human donor is much less controlled than that of a laboratory mouse. Ideally, specific beneficial bacterial strains can be identified, but here too our results highlight potential pitfalls. Our data suggest that high *Clostridium* may block the therapeutic effect of other bacterial strains,

meaning that even a therapy that works in some patients may not work in others if they have high levels of a strain that counters the effect of the probiotic bacteria.

If the therapeutic effect is mediated via the production of an immunomodulatory metabolite, it may be possible to bypass the bacteria entirely and design a treatment based on that metabolite. In our model we showed the disease suppressing effect of male BWF1 microbiota may be partially due to higher abundance of the microbial metabolite phytol and its derivative, phytanic acid, in male BWF1 mice. By activating PPARγ and LXR signaling, phytanic acid can upregulate the pro-efferocytic receptor CD36 and promote splenic macrophage efferocytosis. Defective efferocytosis contributes to lupus progression, so enhancement of efferocytic activity in macrophages could potentially suppress disease. This higher production of an efferocytosis-enhancing metabolite could potentially contribute to the lower incidence of lupus in male BWF1 mice. The BWF1 mouse model mimics the sex-bias seen in SLE patients, raising the possibility that sex-based differences in the microbiota might also indicate that the microbiota could also play a part in women being more susceptible to SLE.

Looking at the bigger picture, these findings show that treatments that alter the microbiota could be useful for managing SLE. This will require greater understanding of the complex and numerous mechanisms connecting the immune system and the microbiota, and our findings represent a significant step in that direction. Probiotics, prebiotics, or other therapeutic modalities designed to encourage the growth of beneficial bacteria and/or reduce the prevalence of harmful bacteria may become a standard part of the treatment a lupus patient receives.

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Dissertation Completion Award, University of Louisville	2020
American Association of Immunologists Travel Award (Hawaii)	2020
American Association of Immunologists Travel Award (Washington, DC)	2017
Integrated Programs in Biomedical Sciences Fellowship, U of L	2014-2016
Wisconsin Academic Excellence Scholarship	2008-2012
The Chairs Award Scholarship	2011
Alpha Lambda Delta/Phi Eta Sigma Honor Society member	2010-Present
Phi Kappa Phi Honor Society member	2010-Present
Wisconsin Academic Excellence Scholarship	2008-2012
Robert C. Byrd Honors Scholarship	2008-2012
Blugold Fellowship	2008-2010

Skills

Immune cell (T-cell, macrophage, cDC, pDC) culture and functional assays; primary immune cell isolation; flow cytometry; ELISA; Western blots; qPCR; fluorescence microscopy; plasmid cloning and assembly; mouse handling & surgery; tissue collection & processing

Software programs

QIIME2, PanFP, STAMP, PICRUSt, Metaboanalyst, MetScape

Research Experience

University of Louisville | Department of Microbiology & Immunology
PhD Researcher, *(Dr. Michele Kosiewicz)* 2014-2021
Determining the microbiota-immune interactions underlying the disease-suppressing effects of male gut microbiota in a mouse model of systemic lupus erythematosus.

- Applied a variety of bioinformatic analysis techniques to microbiota and metabolite profile data to investigate the lupus-suppressing effects of microbiota transfers
- Developed plans for new projects based on microbiota and metabolite findings
- Designed and executed experiments that demonstrated the therapeutic effect of a small-molecule microbiota metabolite in a mouse model of lupus
- Planned and carried out a project that identified enhancement of macrophage phagocytosis as a mechanism of the microbiota metabolite's therapeutic effect
  - ❖ Optimized and performed a broad array of immune cell functional assays to identify what immune cell phenotypes were altered by treatment
  - ❖ Used genomic and transcriptomic analyses to measure treatment effects
  - ❖ Tracked disease via quantitative and functional analyses of serum autoantibodies
  - ❖ Characterized the signaling pathways involved in the therapeutic effect

University of Wisconsin - Eau Claire | Department of Chemistry 2010-2012
Undergraduate Research Assistant, *(Dr. David Lewis)*
Synthesizing a more effective and safer derivative of the anticoagulant Warfarin.

- Synthesized vitamin K analogues for testing as potential adjuvants to Warfarin
- Analyzed structure of candidate molecules using spectroscopy techniques
- This project eventually led to a patent (US8765982B2)

University of Wisconsin - Eau Claire | Department of Biochemistry 2008-2010
Undergraduate Research Assistant, *(Dr. Warren Gallagher)*
Investigating the metal binding and reducing abilities of the bacterial peptide methanobactin.

Publications

F. Yuan*, **J. Harder***, J. Ma, X. Yin, X. Zhang[#] and M.M. Kosiewicz[#]. Using multiple analytical platforms to investigate the androgen depletion effects on fecal metabolites in a mouse model of systemic lupus erythematosus. (*co-first authors; [#]co-corresponding authors) *Journal of Proteome Research*. 2020 Jan 10.1021/acs.jproteome.9b00558

Harder, J., Ma, J., Alard, P., Zhang, X., Yuan, F. and Kosiewicz, M. Female lupus-prone mouse macrophages have efferocytic ability restored by male microbiota-associated metabolites. *Autoimmunity*. (In preparation)

A. Y. Chhabra*, **J. Harder***, J. Ma*, P. Alard, X. Zhang, V. Jala, H. Bodduluri and M. M. Kosiewicz. A role for hormones, gut microbiota and tolerogenic CD103DC in protection of male (NZBxNZW)F1 (BWF1) mice from lupus. (*co-first authors) (In preparation)

Presentations (National/International)

James Harder, Jing Ma, Pascale Alard, Xiang Zhang, Fang Yuan, and Michele M. Kosiewicz. Male microbiota-associated metabolites restore macrophage apoptotic cell clearance function in female lupus-prone mice. American Association of Immunologists 105rth Meeting, Honolulu, HI, May 2020. (Oral presentation)

Ma, J., **Harder, J.**, Alard, P., Kosiewicz, M. Androgens may mediate protection through an effect on immunoregulation in lupus-prone mice. American Association of Immunologists 105rth Meeting, Honolulu, HI, May 2020.

Fang Yuan*, **James Harder***, Xinmin Yin, Xiang Zhang, and Michele Kosiewicz. Using Multiple Analytical Platforms to Investigate the Androgen Depletion Effects on Fecal Metabolites of Systemic Lupus Erythematosus Mouse Model. ASMS 2020, Houston, TX (*co-first authors)

James Harder*, Jing Ma*, Anita Y. Chhabra, Pascale Alard, Xiang Zhang, Fang Yuan, Rachel Ferrill, Yuan Hua, and Michele M. Kosiewicz. Androgens may influence lupus development via an effect on the composition and metabolic activities of intestinal microbiota in BWF1 mice. American Association of Immunologists 104rd Meeting, San Diego, CA, May 2019. (*co-first authors)

James Harder*, Jing Ma*, Anita Y. Chhabra, Pascale Alard, Xiang Zhang, Fang Yuan, Rachel Ferrill, Yuan Hua, and Michele M. Kosiewicz. Androgens may influence lupus development via an effect on the composition and metabolic activities of intestinal microbiota in BWF1 mice. Autumn Immunology Conference, Chicago, IL, November 2019.

James Harder, Anita Y. Chhabra, Jing Ma, Pascale Alard, Xiang Zhang, Fang Yuan, Yuan Hua, Rachel Ferrill, and Michele M. Kosiewicz. Androgens regulate microbiota composition, function and protective properties in lupus-prone mice. Lupus 21st Century 2018 Conference, Armonk, New York, September 2018.

James Harder, A.Y. Chhabra, Pascale Alard, X. Zhang, Y. Hua, R. Ferrill, and Michele M. Kosiewicz. Male microbiota-associated metabolite protects female BWF1 mice from lupus possibly by restoring tolerogenic $CD103^+$ DC function. American Association of Immunologists 104rd Meeting, Washington, DC, May 2017. (Oral presentation)

Pascale Alard, **James Harder**, Thomas Fausnaught, Anuj Chhabra, and Michele M. Kosiewicz. Diferuloylmethane prevents type 1 diabetes development via inhibition of IFNγ-producing T cells. American Association of Immunologists 103rd Meeting, Seattle, Washington, May 2016.

Presentations (Local)

James Harder*, Jing Ma*, Anita Y. Chhabra, Pascale Alard, Xiang Zhang, Fang Yuan, Rachel Ferrill, Yuan Hua, and Michele M. Kosiewicz. Androgens may influence lupus development via an effect on the composition and metabolic activities of intestinal microbiota in BWF1 mice. Research! Louisville, September 10-13, 2019, Louisville, Kentucky.

Tsogtbaatar, E.; Anderson, K.M.; Doyle, P.M.; **Harder, J.W**.; Lewis, D.E. "Synthesis and reactions of vitamin K analogues." Wisconsin Science and Technology Symposium 2011, University of Wisconsin-Whitewater, Whitewater, Wisconsin.

Tsogtbaatar, E.; Doyle, P.M.; Meulemans, D.R.; McKenney, R.K.; **Harder, J.W**.; Klemm, B.J.; Lewis, D.E. "Synthesis and Reactions of Bridged Vitamin K Analogues: An Exercise in Frustration." Wisconsin Science and Technology Symposium 2010, University of Wisconsin-Green Bay, Green Bay, Wisconsin.

Harder, J.W.; Mulheron, H.; Gallagher, W. “Unraveling the Mysteries of how Methanobactin Binds and Reduces Copper Ions.” UW-Eau Claire Student Research Day 2010, University of Wisconsin-Eau Claire, Eau Claire, Wisconsin.