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**A ROLE FOR REGULATORY T CELLS IN
GENDER-BIASED DISEASE SUSCEPTIBILITY
TO MURINE LUPUS**

By

Colleen Tucker

**A Dissertation
Submitted to the Faculty of the
Graduate School of the University of Louisville
in Partial Fulfillment of the Requirements
for the Degree of**

Doctor of Philosophy

**Department of Microbiology and Immunology
University of Louisville
Louisville, Kentucky**

August 2010



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GENDER-BIASED DISEASE SUSCEPTIBILITY
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Colleen Tucker

A Dissertation Approved on

June 17, 2010

by the following Dissertation Committee

Dissertation Director

DEDICATION

This dissertation is dedicated to my parents

Cy and Marcy Tucker

who have shown me unending love and support.

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I would first like to thank my mentor, Dr. Michele Kosiewicz for her guidance on this project. She has taught me so much about being a scientist: how to design experiments, how to think critically, and how to interpret data. Her door has always been open throughout my time in her lab and I have truly enjoyed being her student.

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ABSTRACT

A ROLE FOR REGULATORY T CELLS IN GENDER-BIASED DISEASE SUSCEPTIBILITY TO MURINE LUPUS

Colleen Tucker

June 17, 2010

Females are more susceptible to autoimmune disease than males. In several mouse models of disease, castration of males exacerbates disease while androgen treatment ameliorates disease. These data suggest hormones can have an influence on disease susceptibility and progression. Regulatory T cells (Tregs), particularly the CD4⁺CD25⁺ Tregs, have been shown to be important in controlling autoimmune disease. Studies have shown that depleting regulatory T cells can cause severe autoimmune disease, and increasing regulatory T cell population size can protect from disease. We hypothesized that gender differences in regulatory T cell populations would correlate with differences in disease susceptibility. We used a spontaneous mouse model of systemic lupus erythematosus, (NZBxNZW)F1 (BWF1), in which only females develop full-blown kidney disease to investigate gender differences in regulatory T cell

percentages and function between females and males and their relationship to disease development.

First, we assessed differences in regulatory T cell function and number between young (before disease onset) female mice from four different strains, two autoimmune-prone strains, BWF1 and SJL, and two more autoimmune-resistant strains, C57BL/6 and BALB/c. We found no differences in *in vitro* suppressive function by CD4⁺CD25⁺ Tregs from any of the four strains when co-cultured with either syngeneic CD4⁺CD25⁻ responders and APCs or C57BL/6 CD4⁺CD25⁻ responders and APCs. We did, however, find lower percentages of CD4⁺ cells that expressed Foxp3 (CD4⁺Foxp3⁺ cells) in the periphery of BWF1 mice when compared to the other three strains of mice. The CD4⁺CD25⁺CD103⁺ cells are a potent memory/effector subset of regulatory T cells that are better suppressors than CD4⁺CD25⁺CD103⁻ Tregs both *in vitro* and *in vivo*. As found with the CD4⁺CD25⁺ Tregs, we also found no differences in the suppressive function of CD4⁺CD25⁺CD103⁺ cells from any of the four strains of mice. However, percentages of CD4⁺CD25⁺CD103⁺ cells were, again, decreased in the periphery of BWF1 mice compared to the other three strains. We found that reduced percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in the periphery of BWF1 mice were not due to defects in either thymic production or homeostatic proliferation of these cells. These data suggest that it may be the decreased Treg:Teffector cell ratio, and not a defect in inherent suppressive function, that render BWF1 mice more susceptible to autoimmune disease.

We next examined gender differences in regulatory T cell function and number between young female and male mice of the four strains. We found no differences in the

suppressive ability of either CD4⁺CD25⁺ or CD4⁺CD25⁺CD103⁺ Tregs between females and males of any strain. BWF1 mice were the only strain in which females had lower peripheral percentages of CD4⁺Foxp3⁺ cells than strain-matched males. Strikingly, females of all four strains had lower percentages of CD4⁺CD25⁺CD103⁺ cells in the periphery compared to strain-matched males. The lower percentages did not appear to be due to defects in either thymic production or homeostatic proliferation of the CD4⁺Foxp3⁺ or CD4⁺CD25⁺CD103⁺ cells in females of any strain studied. Taken together these data suggest that decreased percentages of Tregs in the periphery of female BWF1 mice compared to BWF1 males and other mouse strains, and not an inherent defect in Treg suppressive function, may contribute to their increased susceptibility to systemic lupus erythematosus.

Finally, we assessed regulatory T cell function and number in BWF1 mice with established disease and compared age-matched (~32-36 weeks of age) sick (with proteinuria \geq 300 mg/dl) females, non-sick females and males. We found no differences in suppressive function of either CD4⁺CD25⁺ or CD4⁺CD25⁺CD103⁺ cells from sick female, non-sick female or male mice when co-cultured with male CD4⁺CD25⁻ responders and male APCs *in vitro*. Surprisingly, we found significantly higher percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in the periphery of sick females that were nearly three times that seen in non-sick females and males. To evaluate the relationship between progression of disease and regulatory T cell function and number, we examined regulatory T cells at several time-points throughout disease development. The lower percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ regulatory cells found in young (9 week-old) pre-disease female BWF1 mice compared to

age-matched males disappeared as mice aged, i.e., by 20-24 weeks of age. We hypothesized that the 9 weeks time-point (before disease onset) may represent an important period during which having reduced proportions of the potent $CD4^+CD25^+CD103^+$ Treg population could render females more susceptible to disease. To determine whether decreasing the $CD4^+CD25^+CD103^+$ Tregs at this critical time-point could impact disease development later on, we depleted $CD103^+$ cells by administration of anti- $CD103$ antibody bi-weekly then bi-monthly beginning at 8 weeks of age in female and male BWF1 mice. We found that $CD103^+$ cell depletion early on accelerated disease onset and death in female BWF1 mice, and dramatically increased disease incidence and death in male BWF1 mice. These data suggest that 9 weeks may be a critical time-point in disease development and that reduced regulatory T cell populations in young pre-disease mice may render female BWF1 mice more susceptible to disease or alternatively, that higher regulatory T cell numbers in young pre-disease mice may help protect male BWF1 mice from disease.

The data presented in this dissertation suggest that reduced Treg:Teffector cell ratios and not an inherent defect in regulatory function may cause increased susceptibility to autoimmune disease. It also suggests that the potent $CD4^+CD25^+CD103^+$ regulatory T cell population may be more sensitive to hormonal influences, as females of all four strains studied had lower peripheral percentages of these cells than strain-matched males. Finally, these data suggest that the early pre-disease time-point in BWF1 disease progression represents a critical period of time where a reduction in regulatory T cells can accelerate disease and death.

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INTRODUCTION

Gender Differences in Autoimmune Disease

Women are more susceptible than men to most autoimmune diseases (1). This observation has prompted much research into sex differences and their effect on autoimmune disease susceptibility, particularly the effect of the differing hormonal environment on the immune system and disease incidence and progression (2-5). Females have stronger immune responses in general. In fact, female mice produce higher IgM and IgG antibody titers in response to antigens than males (6). Female T cells are also more reactive in a mixed leukocyte reaction than male T cells (7). This increased strength of the female immune response is also seen in humans. Pre-pubertal females given the rubella vaccine have higher antibody titers than pre-pubertal males (8). Females have also been shown to have a higher CD4 to CD8 T cell ratio than males (9). Differences in the amounts and types of cytokines produced between males and females have also been seen in both humans and mice. Female BCG-sensitized mice challenged with PPD produce more IFN- γ than males (10). CD4⁺ T cells from female multiple sclerosis patients can be induced to produce the anti-inflammatory cytokine IL-10 in response to high doses of estradiol, or the inflammatory cytokines IFN- γ and TNF- α in response to low doses of estradiol, demonstrating a dose-dependent estrogen effect (11). Treatment of activated mouse T cells with the androgen dihydrotestosterone (DHT) resulted in decreased

production of IL-4, IL-5, and IFN- γ (12). The highly reactive female immune system is useful for fighting off infections and other foreign antigens, but presents a problem in individuals genetically predisposed to autoimmune disease where the immune system is primed to respond to self-antigens.

Exposure to estrogens that are either produced naturally in females or present in estrogenic compounds such as oral contraceptives, fertilizers, insecticides and other man-made compounds can increase the risk of autoimmune disease (13, 14). Estrogen promotes higher CD4 to CD8 T cell ratios and increased antibody production by activated B cells (9, 15). Estrogen receptors (ER) are expressed on a variety of immune cells, indicating estrogens can directly influence the immune system. In fact, CD4⁺ T cells, CD8⁺ T cells, B cells, macrophages, and both splenic and bone marrow-derived dendritic cells (DCs) express ER (16, 17). Estrogen receptor expression, and thus, the ability of estrogen to influence immune cells, varies with cell type and developmental stage (16-18). In fact, the differentiation of bone marrow cells into DCs requires physiological amounts of estradiol *in vitro* (19). The hormonal environment can affect the differentiation of B cells. The B cells from mice treated with estradiol develop into marginal zone cells, while B cells from mice treated with prolactin develop into follicular cells (20). Estradiol treatment may protect mice from developing experimental autoimmune encephalomyelitis (EAE) by reducing TNF- α production by CD4⁺ T cells and preventing recruitment of inflammatory T cells and macrophages into the central nervous system (CNS) (21).

Androgens can also have an effect on immune cells. Androgens can indirectly promote thymocyte apoptosis (22). B cell populations increase after castration of male

C57BL/6 mice (23, 24). Castrated male mice also had decreased absolute numbers of T cells in their spleens, however, the few T cells they did have were highly activated, producing high levels of IL-2 and IFN- γ (23). Exposure of pregnant female mice to dehydroepiandrosterone (DHEA), a weak androgen produced in response to stress, causes decreased function of T cells in their male offspring (25). Alternatively, it may be the androgen/estrogen balance and not the presence of estrogen or absence of androgen that causes autoimmune disease susceptibility. Rheumatoid arthritis (RA) patients tend to have low serum levels of testosterone and high levels of estrogens (26). This may be due to increased aromatase enzyme activity in RA patients converting androgens to estrogens (27). In fact, inflammatory cytokines like TNF- α and IL-6, known to be increased in autoimmune diseases like RA and lupus, have been shown to stimulate aromatase activity, thereby increasing estrogen production (28, 29). However, estrogens have been shown to induce inflammatory cytokine production (11), so it is difficult to determine cause and effect in this system.

Gender is such a strong determining factor in autoimmune disease susceptibility that a significant amount of research has gone into hormonal treatments for several autoimmune diseases (30). Female SJL mice are more susceptible than males to adoptively transferred EAE, a mouse model of multiple sclerosis (MS) (31, 32). In fact, in an adoptive transfer model where myelin basic protein (MBP) specific T cells from females with EAE are transferred into non-diseased females and males, females develop more severe EAE (33). Female MBP-specific T cells, which have more of a T_H1 phenotype, are able to induce EAE with smaller numbers of adoptively transferred cells than male MBP-specific T cells, which have more of a T_H2 phenotype (34). This T_H2 bias

in SJL males may contribute to the fact that they do not have relapses in induced-EAE, while their female counterparts do (35). This indicates that it is something about the female environment that increases disease severity. Female SJL mice treated with DHT 90-day release pellets develop less severe and eventually remitting disease than placebo-treated females, that may be due in part to increased IL-10 production by MBP-specific T cells (31). Male SJL mice, which develop less severe disease in the adoptive transfer model of EAE, produce less IL-12 in draining lymph nodes than females with disease (36). Addition of IL-12 to male LN cells increases the MBP-specific response, indicating that IL-12 is an important cytokine in EAE disease progression (36). In a human study, 10 male MS patients were treated with a testosterone-containing gel. This testosterone treatment decreased CD4⁺ T cell numbers and IL-2 production while increasing TGF- β production (37).

While there is no known gender bias in human type 1 diabetes, females predominantly develop disease in the non-obese diabetic (NOD) mouse model of type 1 diabetes (38), and this model has been used extensively in hormonal studies. Androgen treatment can prevent islet destruction in adult female NOD mice (39). However, androgen treatment could not prevent NOD mice from developing diabetes through adoptive transfer of diabetic T cells (39). These data indicate that androgen treatment may prevent or reduce the induction of pathogenic T cell development in female NOD mice, resulting in decreased islet destruction (39). Castration increases the incidence of diabetes in male NOD mice, while oophorectomy decreases disease incidence in female NOD mice (40). Thus, androgens may have a protective effect in type I diabetes whereas estrogens may have a disease-enhancing effect. In fact, CD4⁺ T cells from NOD mice

treated with testosterone *in vitro* produce less of the inflammatory cytokine IFN- γ in response to IL-12 while CD4⁺ T cells treated with estrogen *in vitro* produce more IL-12-induced IFN- γ (41). These data support the hypothesis that the female bias toward developing type 1 diabetes in NOD mice may be due to increased estrogens and decreased androgens in diabetic females.

Rheumatoid arthritis (RA) is also more prevalent in females than males but is ameliorated during pregnancy, suggesting a role for sex hormones in disease susceptibility and progression (42, 43). Estrogens are present at high levels in the synovium of RA patients and are associated with increased inflammation at these sites (44). However, in rodent models of RA, the gender bias and hormonal influence on disease are more complicated. Different models of rheumatoid arthritis have different sensitivities to treatment with hormones. Castrated male rats develop accelerated and more severe adjuvant-induced arthritis than non-castrated males (45). However, in a type II collagen-induced model of rheumatoid arthritis in (B10QxDBA/1)F1 mice, in which males are more susceptible to disease, estrogen treatment suppresses disease in both castrated and non-castrated males while oophorectomy or testosterone treatment increases disease incidence in females (46). Physiologic levels of estrogen have also been used to treat castrated female DBA/1 mice with collagen-induced arthritis, ameliorating disease by suppressing T cell responses and stimulating B cell activity (47). Hormone replacement therapy, most commonly used after menopause, in human rheumatoid arthritis patients has not been shown, as it has in other autoimmune diseases, to be associated with disease flares, and may, in fact, reduce disease symptoms (48). However, human studies of RA are difficult to interpret because of small experimental population

size (48). Thus, sex hormones may play an important, though complicated, role in rheumatoid arthritis development and/or treatment.

Females develop systemic lupus erythematosus (SLE) far more often than males, and much research has focused on the influence of sex hormones on this disease (49, 50). In fact, SLE symptoms worsen during pregnancy, a time when estrogen and prolactin levels are high, and these hormones can have differing effects on B cell development into either marginal zone or follicular B cells, respectively (51). In a study of 94 non-pregnant female SLE patients, a high percentage of these women were found to have abnormal levels of many hormones, including estrogen and prolactin (52). In a study of 78 pregnancies in SLE patients, 65% had increased flares during pregnancy (53). Administration of external estrogens, whether through use of oral contraceptives or estrogen replacement therapy in post-menopausal women, has been associated with increased SLE disease severity (54-58). Similar to RA patients, SLE patients tend to have higher serum levels of estrogen and lower serum levels of androgen, which may be due to increased aromatase activity, also seen in these patients (59). Estradiol, and synthetic estrogens and their metabolites can cause DNA damage, including strand breakage, oxidized base formation, and adduct formation (60). DNA that has been modified by estradiol is bound more readily by dsDNA autoantibodies in human lupus patients (61). SLE patients also have abnormal estrogen metabolism. Urine samples from SLE patients have been found to contain 16 α -hydroxylated estradiol, a potent estrogen metabolite not normally found at high levels in urine (62, 63). Male patients suffering from SLE and Klinefelter's syndrome (XXY) have estrogen levels similar to female SLE patients (64). In one case study, a male Klinefelter's patient with SLE treated with physiologic levels of

testosterone had decreased lupus symptoms, including a lower titer of anti-DNA antibodies (65). While testosterone treatment causes many unwanted side effects, these data suggest that development of an androgenic compound that does not cause such severe side effects could be very useful for the treatment of lupus.

(NZBxNZW)F1 (BWF1) is a mouse model of SLE in which only females spontaneously develop disease (66, 67). Castration of male BWF1 lupus-prone mice causes them to develop disease at similar rates and severity as BWF1 females (68). In addition, both castrated male and ovariectomized female BWF1 mice treated with estrogen show increased disease severity and decreased survival, while mice treated with androgens are protected from disease (68). Male BWF1 fetuses have increased circulating estradiol compared to control C57BL/6 fetuses, and treatment with testosterone can decrease estradiol in BWF1, but not C57BL/6 fetuses (69). These data suggest that the BWF1 background predisposes mice to produce high levels of inflammation-promoting estrogens, as even males have increased levels compared to other strains of mice (69). Estrogen has been shown to interact with the estrogen receptor in female lupus patients to promote T cell activation and B cell autoantibody production (70). Estrogen receptor- α (ER α) deficiency can attenuate disease in female BWF1 mice (71). This attenuation may be due to decreased levels of the estrogen-regulated cytokine, IFN- γ , high levels of which are present in the serum of lupus patients and lupus-prone mice (71). In BWF1 mice, removal of estrogen ameliorates disease symptoms, as can be seen when these mice are treated with tamoxifen, an anti-oestrogen, which reduces autoantibody production (72). These data indicate an important role for sex hormones in susceptibility to SLE.

Differences in estrogen receptor signaling have also been found between strains of mice. Two lupus-prone mice, the BWF1 and MRL/MP-*lpr/lpr* strains, have decreased ER expression measured in the cytosol of cells of both the liver and the thymus compared to less autoimmune-prone BALB/c mice (73, 74). However, ERs from both lupus-prone strains had a higher binding affinity than ERs from BALB/c mice (73, 74). These differences in ER number and binding affinity may underlie some of the defects in estrogen signaling and metabolism seen in lupus patients and contribute to the susceptibility of patients and lupus-prone mice.

Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is an autoimmune disease that predominantly affects women. There is evidence for a genetic basis for SLE (75, 76). Ten percent of lupus patients have at least one first degree relative with lupus (77). In a study of monozygotic twins, if one twin had lupus there was a 24-58% chance the second twin also had disease, while in dizygotic twins, it was only a 2-5% chance (78, 79). However, these data not only indicate that there is a strong genetic predisposition in lupus, but also suggest that there are strong environmental factors.

In SLE, antibodies are produced against self-antigens such as double-stranded DNA, histones, chromatin, other nuclear proteins, etc (80). These antibodies combine with complement to form immune complexes, which deposit in the kidneys eventually causing glomerulonephritis and death (80). Many cytokines like IFN- α , TNF- α , and IL-10 are increased in the serum of both lupus patients and lupus-prone mice (81). SLE is a difficult disease to treat because there are such a wide variety of symptoms (82). Some

patients develop CNS pathology or skin rashes, while others develop severe glomerulonephritis (83). This makes it difficult to identify potential therapeutic targets.

Lupus patients and lupus-prone mice have a defect in their ability to clear apoptotic cells (84). In fact, in two lupus-prone strains of mice, the MRL/lpr and BWF1 strains, disease manifestation is associated with decreased phagocytosis by macrophages (85). Accumulation of apoptotic cells can impact disease. For example, injection of apoptotic B and T cells into young lupus-prone BWF1 mice results in increased IL-6 and IL-10 production by macrophages, increased lymphocyte recruitment to the site of injection, and acceleration of disease (86). Injection of apoptotic B cells, but not apoptotic T cells, induces a systemic T_H2 response, resulting in B cell autoantibody production and acceleration of disease (86).

Decreased apoptotic cell clearance may also affect DC function. DCs from lupus-prone mice have a low activation threshold. High mobility group box protein 1 (HMGB1) is a molecule associated with apoptotic cell chromatin that may participate in breaking tolerance to double stranded DNA (dsDNA) and nucleosomes (87). HMGB1-containing nucleosomes from apoptotic cells, which are found at high levels in the plasma of SLE patients, induce DC maturation and secretion of proinflammatory cytokines like IL-1 β , IL-6, and IL-10 (87). DC from lupus-prone mice produce more inflammatory cytokines in response to anti-CD40 or LPS stimulation than wildtype mice (88). Defects in DC can also be seen in human lupus patients. Myeloid DC from human lupus patients display an abnormal phenotype that is characterized by increased expression of maturation markers and inflammatory cytokines, and consequently an increase in T cell proliferation (89). Kidney-infiltrating myeloid DC from lupus patients have been associated with increased

levels of C1q, a component of the classical complement pathway (90). C1q production in the kidneys is associated with disease and may lead to increased presentation of autoantigens (90).

While defects in many cellular compartments are associated with disease in SLE, infiltration of T cells into the kidney is necessary for the development of end-stage kidney disease (91). In fact, male NZM2328 lupus-prone mice, which develop autoantibodies in their serum but not end-stage kidney disease, do not have detectable DC and T cell infiltration in their kidneys, whereas female NZM2328 mice, which develop full-blown disease, have detectable DC and T cell kidney infiltration (91). Furthermore, removal of T cells, either by thymectomy or antibody-mediated T cell depletion, prevents lupus development in the MRL-*lpr/lpr* mouse model of lupus (92, 93). Injecting kidney cells from sick mice into the thymi of MRL-*lpr/lpr* lupus-prone mice can delay glomerulonephritis development, but not autoantibody production, apparently by deleting kidney-specific, autoreactive T cells from the developing T cell pool (94).

There are many genetic factors that contribute to the development of disease in lupus patients and lupus-prone mice, including polymorphisms of molecules involved in antigen presentation, cytokine production, and immune complex clearance (75). In a study of 91 caucasian families, an association between the class II MHC molecule HLA-DR2 and lupus has been observed (95). HLA-DR2 has been linked with development of autoantibodies against dsDNA (96, 97). Variations in cytokine genes are also seen in lupus. Polymorphisms in the gene encoding TNF- α , a cytokine seen at reduced levels in the serum of both humans and mice with lupus compared to healthy controls, gene have also been found in both SLE patients and in lupus-prone mice (98-101). In another

example, expression of certain FcγRII and FcγRIII alleles has been associated with disease in humans (102-107). These disease-associated FcγR alleles may contribute to disease because these receptors bind less efficiently to IgG antibodies, preventing effective clearing of immune complexes (105, 108).

Mouse models have also been used to determine genetic factors contributing to SLE disease susceptibility. Morel and colleagues have used congenic mice to identify several susceptibility loci, including *Sle1a*, *Sle1b*, and *Sle1c* (109). These three loci break tolerance to chromatin and result in the production of anti-chromatin antibodies (110). *Sleb* is associated with polymorphisms in the signaling lymphocyte activation molecule (SLAM)/CD2 gene family, which plays a role in cell activation and signaling (111). *Sle1a* and *Sle1c* have been shown to be involved in autoreactive CD4⁺ T cell generation and a reduction in the size of the CD4⁺CD25⁺ regulatory T cell population (109, 112). *Sle1c* has been mapped to the NZM2410 complement receptor 2 (Cr2) allele (112). Polymorphisms in the Cr2 gene have also been found in human lupus patients (113, 114). The presence of the *Sle1a* locus alone causes a decrease in the number, but not function, of CD4⁺CD25⁺Foxp3⁺ Tregs, and causes DC to increase production of IL-6, rendering CD4⁺CD25⁻ T cells resistant to suppression (115, 116). *Sle1a* has been broken down into two intervals, *Sle1a.1* and *Sle1a.2* (117). Expression of both intervals is necessary for the *Sle1a* phenotypes (117). A strong candidate gene for *Sle1a.1* is *Pbx1*, which encodes a nuclear transcriptional regulator, is expressed by B cells and macrophages, and whose gene expression is seen in murine and human T cells (117). A strong candidate gene for *Sle1a.2* is *Sh2d1b*, which encodes Eat-2 (a SLAM associated protein involved in recruitment of Src kinases) and is expressed by natural killer cells, B cells, T cells, and

DC (117). As seen in human SLE patients, certain MHC class II haplotypes have been associated with disease in lupus-prone mice on specific backgrounds. For example, H-2^{d/z} is associated with disease susceptibility in BWF1 mice (118-120). A deficiency in Fas, which mediates activation induced cell death, has also been associated with B cell production of autoantibodies in a mouse model of lupus (121, 122).

Regulatory T Cells

Regulatory T cells (Tregs) have been shown to be important in controlling autoimmune disease (123, 124). Tregs are CD4⁺ cells that constitutively express IL-2 receptor α (CD25) (125). When CD4⁺CD25⁻ T cells are adoptively transferred into BALB/c athymic nude mice (*nu/nu*) mice, they cause severe autoimmune disease that can be prevented by cotransfer of CD4⁺CD25⁺ Tregs (125). CD25 is not an ideal marker for Tregs as it is also expressed on activated T cells and is not expressed on all Tregs (126). Foxp3 is a nuclear transcription factor with a forkhead-binding domain, encoded on the X-chromosome, and is the most reliable Treg marker, at least in mice (127, 128). Foxp3 is required for Treg function and development (127, 129-134). In fact, Foxp3 deficient (*scurfy*) mice do not develop Tregs and males die of severe autoimmune/inflammatory disease by 21 days of age (127). Disease in *scurfy* mice is due to the absence of Tregs as these mice can be rescued from disease by adoptive transfer of CD4⁺CD25⁺ cells from wildtype mice (127). Immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX), a severe disease affecting multiple organs, is also seen in humans with Foxp3 mutations (135). Foxp3 alone can confer regulatory ability. Retroviral gene transfer of Foxp3 into murine CD4⁺CD25⁻ effector T cells causes them to develop a

regulatory phenotype and function (127, 129). Foxp3's control of regulatory phenotype and function is dose-dependent. Ablation or attenuation of Foxp3 in Treg cells results in the loss of regulatory function and the development of severe autoimmune disease resembling that seen in scurfy mice (130, 132-134, 136).

In humans, TCR engagement induces Foxp3 expression (137), and it has been shown that effector T cells can express Foxp3 transiently when they are activated (138). Epigenetic Foxp3 DNA methylation is one way to determine whether a Foxp3⁺ cell is a Treg or an activated T cell transiently expressing the transcription factor, as Tregs but not activated effector T cells are demethylated at the Foxp3 locus (139). This Treg-specific Foxp3 DNA methylation is not unique to human cells and has also been observed in mice (140, 141).

It is unknown whether Tregs suppress effector T cell proliferation *in vivo* by directly suppressing the effector T cells or indirectly through interactions with DCs (124). Using 2-photon microscopy, Bluestone and colleagues were able to detect interactions of Tregs with DCs, but not with effector T cells (142). After interaction with Treg cells, DCs had shorter interactions with effector T cells (142). CTLA-4 is constitutively expressed on Tregs and on activated T cells, and interacts with DC through CD80 and CD86 (143-145). Addition of a blocking anti-CTLA-4 mAb *in vitro* can prevent Treg suppression of effector T cell proliferation (146). Mice in which CTLA-4 has been conditionally knocked out develop severe autoimmune disease similar to that found in scurfy mice, suggesting that CTLA-4 is critical for Treg suppressive function *in vivo* (147). Tregs influence DC function but DCs can influence Treg function as well. High

levels of IL-6 production by DCs can render effector T cells resistant to Treg-mediated suppression (148).

Another molecule expressed by CD4⁺CD25⁺ Tregs is the glucocorticoid-induced TNF receptor (GITR) (149, 150). It has been shown that treatment with blocking anti-GITR antibody can reduce Treg suppressive function both *in vitro* and *in vivo* (149, 150). However, GITR is also expressed on effector CD4⁺ and CD8⁺ T cells (149, 150). GITR acts as a costimulatory molecule for effector T cells, and treatment with anti-GITR antibody promotes proliferation and cytokine production (151). The decrease in Treg suppressive function seen with anti-GITR antibody treatment may in fact be due more to increased effector T cell proliferation.

Many factors control and impact Treg function (152). Naturally occurring CD4⁺CD25⁺ Tregs are activated by self-peptides presented by the MHC class II molecule *in vivo* (153). This activation allows Tregs to suppress *in vitro* without further TCR stimulation as long as IL-2 is present in culture (153). This may have relevance *in vivo*, as previously activated Tregs can suppress in a non-antigen specific manner in the presence of high enough levels of IL-2 or other cytokines (153). An interesting new finding reports that Tregs co-express Foxp3 along with transcription factors specific for T_H1 or T_H2 responses, such as interferon regulatory factor-4 (IRF-4) a molecule necessary for T_H2 responses, to help them more selectively control the type of immune response occurring in the tissue in which they are located (154). MicroRNA molecules such as miRNA155 that are small single-stranded RNA molecules found in the cytoplasm that degrade transcription products have also been found to be important in controlling Treg function both *in vivo* and *in vitro* (155, 156).

Tregs can also regulate B cells, a property that makes them an even more attractive target for SLE therapeutic treatment. Tregs have been shown to migrate to germinal centers and indirectly suppress B cells by suppressing the germinal center T-helper cells whose help is necessary for B cell function (157). Tregs can also directly suppress B cell Ig class switching and antibody production by inducing B cell apoptosis in both mice and humans (158, 159).

Regulatory T cells are a promising therapeutic target in SLE because they can interact with many different potentially autoreactive cell types and because reduced numbers of Tregs has been correlated with disease susceptibility in mice and disease progression in humans (160, 161). The correlation of Treg population size and function to disease in human lupus patients is controversial. Many groups have shown a reduced percentage of Tregs in the peripheral blood of active lupus patients as well as a reduction in regulatory function in suppression of cytokine production, *in vitro* suppression assays, and Foxp3 expression (162-164). Others have shown a reduction in Treg percentages in peripheral blood but no reduction in regulatory function or Foxp3 expression (165, 166). Still other groups have shown an increase in circulating CD4⁺Foxp3⁺ cells but a decrease in CD4⁺CD25^{hi} cells (167, 168). Another group found increased CD4⁺CD25⁺ cells in the peripheral blood of active lupus patients (169). Increasing Tregs with either plasmapheresis treatment or treatment with corticosteroids correlated with decreases in disease activity (168-170). Different groups may find such differences in Treg percentages and function because they are defining active phases of lupus differently or because they are defining CD4⁺Foxp3⁺ or CD4⁺CD25⁺ and not CD4⁺CD25^{hi} T cells as Tregs. The importance of distinguishing Tregs from activated T cells in these studies is

emphasized by the findings of Sakaguchi and colleagues. They have found a subset of human CD4⁺ T cells that co-express Foxp3 and RORC (an important transcription factor in T_H17 cell development) and produce IL-17 (171).

Tregs have been extensively studied in mouse models of lupus as well. (NZBxNZW)F1 (BWF1) and (NZBxSWR)F1 (SNF1) mice, mouse models of lupus, display lower percentages of CD4⁺CD25⁺ cells than non-autoimmune-prone BALB/c and DBA/1 mice (172) or (DBA/2xNZW)F1 mice (173). However, these studies used CD25 as a marker instead of the more specific Treg marker, Foxp3, and Foxp3 mRNA expression is shown in the CD4⁺CD25⁻ T cell subset of at least one of these studies (173). No difference in suppressive function was seen between BWF1 and (DBA/2xNZW)F1 CD4⁺CD25⁺ cells *in vitro* (173). The reduction in CD4⁺CD25⁺ cell percentages seen in BWF1 mice can be reversed by nasal tolerance induction with a histone peptide antigen H471 (172). Treatment of non-autoimmune (DBA/2xNZW)F1 mice with anti-CD25 monoclonal antibody increases serum levels of dsDNA autoantibodies (173), and treatment of female BWF1 mice starting at 3 days of age with anti-CD25 monoclonal antibody results in accelerated disease, including early development of high dsDNA autoantibody titers, increased serum levels of inflammatory cytokines, and glomerulonephritis (174). Neonatal thymectomy (i.e. interrupts Tregs development) of lupus-prone NZM2328 mice causes accelerated disease development, and adoptive transfer of CD25⁺ Tregs from asymptomatic mice ameliorates disease symptoms (175). In fact, several studies have shown that increasing the Treg population in lupus-prone mice can delay and/or ameliorate disease (176-179). Increasing the CD4⁺CD25⁺ Treg population by tolerizing lupus-prone SNF1 mice with a histone peptide antigen injected

subcutaneously every two weeks delays disease onset (176). Injection of a peptide based on the complementary-determining region 1 (hCDR1) every other day for a week starting at 8 weeks of age increases both CD4⁺CD25⁺ Treg percentages and TGF-β production and ameliorates disease in lupus-prone BWF1 mice (177). Adoptive transfer of ex vivo-expanded Tregs delays or prevents lupus development in BWF1 mice. (178, 179).

Interactions of regulatory T cells and APC in lupus can impact Treg function. In humans, APC from active lupus patients produce high levels of IFN-α, which may render Tregs non-functional (180). Treg function is restored when they are cultured with APC from healthy control patients (180). Mice expressing the NZM2410 lupus susceptibility locus *Sle1a* produce high levels of IL-6, rendering CD4⁺CD25⁻ effector T cells resistant to suppression by Tregs (115).

Tregs have been shown to be important in controlling many other autoimmune diseases. Decreased numbers of Tregs are also seen in the islets of a type 1 diabetes mouse model, the non-obese diabetic (NOD) mouse model (181). Reduced frequency of CD4⁺Foxp3⁺ Tregs and reduced Foxp3 expression per cell are seen in MS patients (182). Adoptive transfer of Tregs, which results in the doubling of the Treg population reduces disease severity in a model of EAE (183). Cotransfer of CD4⁺CD25⁺ Tregs with disease-causing CD4⁺CD45RB^{high} T cells can prevent colitis (184).

Natural Tregs develop in the thymus where they are positively selected on a high affinity TCR (185). TGF-β signaling is required for thymic development of Tregs (186). Thymically-derived Tregs have high TCR diversity, allowing them to regulate a wide variety of potentially autoreactive T cells in the periphery (187). Thymic development of Tregs requires MHC class II, but an activated subset of Tregs can develop in the

periphery of MHC class II^{-/-} mice (188, 189). Tregs can also develop independent of the thymus through peripheral conversion of CD4⁺CD25⁻ effector T cells to CD4⁺CD25⁺ Tregs (190, 191). This conversion requires TCR stimulation, TGF- β , and B7 costimulation (190, 191). In fact, these TGF- β converted cells, or inducible Tregs, function as well as natural Tregs to rescue scurfy mice from disease (192). It has been shown that, once activated, Tregs can suppress responder T cells in an antigen non-specific manner (193). However, new evidence suggests that to promote organ-specific tolerance, antigen specific Tregs are critical (194). In fact, several studies have shown that antigen specific Tregs are better at preventing diabetes in NOD mice than polyclonal Tregs (195-197).

CD103

The integrin $\alpha_E\beta_7$ (CD103) is a type I transmembrane protein expressed primarily on mucosal lymphocytes (198). The only known ligand for CD103 is E-cadherin, which is expressed primarily on epithelial cells in the gut, skin, and thymus (199-202). E-cadherin is expressed on medullary thymic epithelial cells, which are cells that have been shown to be necessary for Treg development in the thymus (202, 203). E-cadherin is upregulated during inflammation (204). There is also some evidence for E-cadherin-independent binding of CD103 to another unknown ligand expressed on human intestinal microvascular endothelial cells (205) and in oral epithelial and epidermal tissues (206).

CD103 expression identifies a very potent subset of regulatory T cells in both mice and humans (207-209). CD103⁺ Tregs have a memory/effector phenotype (210) and the subset represents a small fraction of the peripheral CD4⁺CD25⁺ Tregs. CD103⁺ Tregs

are better suppressors *in vitro* and *in vivo* than CD103⁻ Tregs (149, 207, 211). The increase in suppressive function does not appear to be due to increased IL-10 production by the CD103⁺ Treg subset, as CD103⁺ Tregs from IL-10 knockout mice are as suppressive as CD103⁺ Tregs from wildtype mice (211). The expression of CD103 on Tregs has been shown to function in retention of these cells at sites of inflammation (212). In fact, CD4⁺CD25⁺ Tregs purified from CD103-deficient mice were less effective than wildtype CD4⁺CD25⁺ Tregs at homing to and accumulating in sites of dermal inflammation in a mouse model of *Leishmania major* infection (212). It is thought that CD103 is induced and/or maintained at these sites of inflammation (207, 212) and CD103⁺ Tregs develop their increased suppressive function at sites of inflammation (213). CD103⁺ Tregs that lack E/P-selectin ligands (i.e., cannot migrate to sites of inflammation) were not as suppressive in a DTH model as wildtype CD103⁺ Tregs that were able to enter inflamed sites, though they expressed similar levels of Foxp3 and other markers such as CD62L (213). Effector/memory CD103⁺ Tregs, that have presumably been activated *in vivo*, can spontaneously (i.e., without additional activation) suppress CD4⁺CD25⁻ effector T cells in an *in vitro* suppression assay (189).

The homing and maintenance of lymphocytes to specific locations, whether to lymphoid organs or to non-lymphoid tissues, is very important for proper immune function (214). CD4⁺CD25⁺CD103⁺ Tregs have an “inflammation-seeking” phenotype, expressing adhesion molecules like ICOS, P-selectin ligand, and E-selectin ligand and chemokine receptors that promote migration towards inflammatory chemokines such as CXCL9, CCL17, and CCL20 (210). Expression of CD103 allows T cells to form filopodia and move towards E-cadherin (215). Migration to sites of inflammation is

necessary to suppress inflammatory reactions *in vivo*, but not to control the induction phase of an immune response in the lymph nodes (213). In fact, in bone marrow chimera studies where scurfy (Foxp3-deficient) bone marrow and CCR4^{-/-} bone marrow were transferred into Rag1^{-/-} recipients, the lack of CCR4 on CD103⁺ Tregs prevented migration to the skin and resulted in severe inflammatory disease in the skin (216).

The CD103⁺ subset of Tregs is important in disease settings. In an adoptive transfer model of wasting disease, CD4⁺CD25⁺CD103⁺ Tregs, but not CD4⁺CD25⁺CD103⁻ Tregs are able to prevent wasting disease (211). CD103-deficient ($\alpha_E^{-/-}$) mice have reduced T lymphocyte numbers in gut and vaginal mucosal tissue (217). CD103-deficient mice develop inflammatory skin lesions spontaneously, and transfer of splenocytes from CD103-deficient, but not wildtype, mice into *scid/scid* mice also induces skin inflammation (218). Donor CD103⁺ Tregs from DBA/2 mice transferred into recipient BALB/c mice with chronic graft-versus-host disease (GVHD) help reverse symptoms by trafficking to GVHD target tissues and suppressing donor CD4⁺ T cell proliferation (219).

There are other cells that express CD103. A small percentage of circulating CD8⁺ cells express CD103, produce IL-10, and have suppressive function in a cell-cell contact dependent manner *in vitro* (220). The CD8⁺CD103⁺ Tregs can be expanded by exposure to alloantigen *in vitro* (220). These CD8⁺CD103⁺ Tregs are also involved in a model of induced tolerance referred to as anterior chamber-associated immune deviation (ACAID) (221). Cytotoxic CD8⁺ T cells have also been shown to express CD103 (222-224). These CD8⁺CD103⁺ cytotoxic T cells bind target E-cadherin expressing cells and cause them to apoptose and have been associated with tissue destruction of exocrine glands in Sjogren's

syndrome, tissue destruction of lungs in a lung transplant model and tumor regression in a brain tumor model (222-224). CD103 has also been shown to be important in the development of CD4⁺CD8⁺ thymocytes (225). CD103 is a target of Runx3, a transcription factor responsible for the processes that downregulate CD4 on CD4⁺CD8⁺ thymocytes (225).

A subset of DCs also expresses CD103. They are found primarily in the gut, but a small subset can be found in the mesenteric lymph node (226). These CD103⁺ DCs from both gut and mesenteric lymph nodes can induce a gut-homing phenotype (CCR9⁺α₄β₇⁺) in T lymphocytes (226). CD103 expression on gut-associated DCs is necessary for protection against a T cell adoptive transfer model of colitis (227). CD103⁺ DCs are thought to mediate conversion of CD4⁺CD25⁻ effector cells to CD4⁺CD25⁺ Tregs via secretion of retinoic acid in the gut (228, 229). There is also a subset of CD103⁺ DCs in the skin that is able to effectively cross-present viral and self antigens (230). An invariant NKT cell subset, found in the peripheral lymph nodes and in skin, produces IL-17 and also expresses CD103 (231). So, while CD103 is expressed on a subset of CD4⁺CD25⁺ Tregs, it is also expressed on a variety of other cell types in the immune system. Thus, one can reason that CD103 itself does not confer regulatory function, but perhaps it is its role in retention and homing to specific tissues that gives CD103⁺ Tregs their increased suppressive function.

CD103 can be induced by TGF-β (232). For most integrins, a proximal promoter confers cytokine responsiveness, but for CD103, responsiveness is likely dependent on more distant control elements (233). One of these distant control elements may be the transcription factor Foxp3 because when CD4⁺CD25⁻ T cells are transduced with Foxp3,

they express CD103 (129). Other factors control the size of the CD4⁺CD25⁺CD103⁺ population of Tregs. CD47, whose ligands thrombospondin 1 (TSP-1) and SIRP- α are involved in inhibition of DC and T cell function, is important in controlling CD103⁺ Treg homeostatic proliferation (234), as seen in CD47 knockout mice, which have increased percentages of these Tregs due to unrestrained proliferation (235).

Strain Differences

Genetically different strains of mice can have dramatically different immune systems, and thus, different immune responses to infection and autoimmune disease. Non-autoimmune prone strains of mice have dsDNA-specific autoreactive B cells that remain inactive, while lupus-prone BWF1 mice have autoreactive B cells that produce high titers of anti-dsDNA antibodies (236). B cells from mice with a high affinity anti-DNA H chain transgene on a C57BL/6 background produce anti-dsDNA antibodies while B cells from mice with this same transgene on a BALB/c background do not produce anti-dsDNA antibodies (237). This transgene on the C57BL/6 background promotes repertoire shifting to L chains, which allow better DNA binding (237), illustrating the impact genetic background can have on disease promoting symptoms.

Some strains of mice are skewed to a more T_H1 or T_H2 response (238). These differences can cause some mouse strains to be more susceptible to different types of infections (239-241) and autoimmune diseases (242-245). BALB/c mice, for example, have an immune system skewed towards a T_H2 response (238), and are more susceptible to *Mycobacterium avium* infection, in which IL-10 has been shown to augment disease, than C57BL/6 mice, which have an immune system skewed more towards a T_H1 response

(241). Mice on a BALB/c background are less susceptible to *Pseudomonas aeruginosa* corneal infection than mice on a C57BL/6 background, indicating that a T_H1 skewed immune response renders mice more susceptible to this disease (239).

Strain differences impact autoimmune disease susceptibility. In a mouse model of experimental autoimmune uveitis (EAU) susceptible strains of mice had a T_H1 immune response, producing high levels of IFN- γ , IL-12, and IgG2a antibodies (242). Female SJL mice develop more severe EAE and pristane-induced lupus than age-matched males, hypothesized to be due to enhanced T_H2 cytokine production in females compared to males, however, C57BL/6 mice do not show this female bias for EAE (245). Another group found differences in disease severity and progression between SJL mice from 4 different commercial vendors (246). They found that the strains had variation in gene copy numbers of inflammation-associated *Naip1* (*Naip1* mRNA was detected in SJL brain tissue and in a neuronal cell line), and that these differences correlated with the development of either chronic or relapsing remitting disease patterns (246). Pre-diabetic NOD mice, a model of type 1 diabetes, had higher percentages of B cells, an important cell type in diabetes development, in their thymi than other non-diabetic strains (244). Strain differences in susceptibility to Grave's hyperthyroidism have been used to find candidate genes (associated with the immunoglobulin heavy chain variable region) responsible for the break in tolerance and the production of thyroid stimulating antibodies (247-251). Knockout of Fc γ RIIB on a C57BL/6 background results in lupus-like disease, while Fc γ RIIB knockout on a BALB/c background does not result in disease (252). This is due to the presence of a lupus-suppressor gene, *sbb2(a)*, which restricts the production of pathogenic IgG antibody isotypes in BALB/c mice (253).

Regulatory T cells play an important role in controlling autoimmune disease (123, 124). Variation in Treg populations of genetically different strains of mice may play a role in their susceptibility to autoimmune diseases. Howard and colleagues have reported that BALB/c mice have significantly higher percentages of CD4⁺CD25⁺ Tregs in the thymus and peripheral lymphoid organs than C57BL/6 mice, although both BALB/c and C57BL/6 CD4⁺CD25⁺ cells express similar levels of other regulatory markers like CD45RB, CD103, GITR, CTLA-4, and Foxp3 (254, 255). Interestingly, they also found that CD4⁺CD25⁻ responder cells from C57BL/6 mice are more resistant to suppression than BALB/c responder cells by CD4⁺CD25⁺ Tregs from both C57BL/6 and BALB/c mice in an *in vitro* suppression assay (254). Although Mathis et al. found no significant differences in CD4⁺CD25⁺ cell percentages in the thymus or periphery when comparing NOD mice to non-diabetic mice (244), a dramatically reduced TCR repertoire was seen in NOD Tregs when compared to C57BL/6 Tregs (256). Obviously, the size and quality of the immune response can vary dramatically between inbred strains of mice, and comparison between strains can be a very useful tool to discover susceptibility loci for a variety of autoimmune diseases.

Gender differences exist for many autoimmune diseases. Sex hormones impact the immune system and can be used to treat autoimmune disease. Androgens tend to be immunosuppressive, while estrogens tend to be immunoenhansive. Regulatory T cells are important components in controlling autoimmune disease. Regulatory Treg populations can vary between females and males and between different inbred strains of mice. With this in mind, we have investigated regulatory T cell populations in a mouse model of

lupus, comparing percentages between female and male lupus-prone BWF1 mice and comparing BWF1 Treg populations to other autoimmune-prone and less autoimmune-prone strains of mice.

MATERIALS AND METHODS

Mice.

Female C57BL/6, BALB/c, SJL, and (NZBxNZW)F1 (BWF1) mice were either purchased from The Jackson Laboratories (Bar Harbor, ME) or bred and maintained in our animal facility at the University of Louisville. Animals were maintained under the guidelines stipulated by the University of Louisville Institutional Animal Care and Use Committee.

Antibodies and reagents.

The following monoclonal antibodies were purchased from BD Pharmingen (CA): anti-CD4-PerCP, anti-CD8-APC, anti-CD25-APC, anti-CD25-PE, anti-CD25-PE-Cy7, anti-CD103-FITC, anti-Ki67-PE, anti-CD62L-APC, and anti-CD3 (2C11). Anti-Foxp3-PE and anti-Foxp3-APC were purchased from Ebioscience (CA).

Flow cytometric analysis.

Single cell suspensions of 2×10^6 lymph node, spleen, or thymus cells were incubated with Fc block for 15 minutes at 4°C and then labeled with appropriate monoclonal antibodies in staining buffer (DPBS, 1% FCS, 0.1% NaNO₃) for 15 minutes at 4°C in the dark, washed twice, and fixed in 2% formalin. For intracellular staining, cells were fixed and permeabilized following manufacturer's instructions. Stained cells

were collected and analyzed on a FACS caliber (BD Pharmingen) or an LSR (Beckon Dickinson).

Cell purification.

Spleen cells were purified using T cell enrichment columns (R&D Systems). These purified T cells were pooled with unfractionated LN cells and applied to CD4 enrichment columns (R&D Systems). For separation of CD25⁺ cells, enriched CD4⁺ T cells were then labeled with CD25-PE antibody. Following a 15 minute incubation at 4°C and wash with Miltenyi MACS buffer, cells were labeled with anti-PE beads (Miltenyi). Labeled CD4⁺ cells were then applied to an MS magnetic bead column and CD4⁺CD25⁻ and CD4⁺CD25⁺ cells were collected. For cell sorting, cells were labeled appropriately and sorted by a high-speed cell sorter (FACS Vantage or FACS Aria, BD Biosciences, CA) to >95% purity.

In vitro suppression assay.

The suppressive ability of magnetic bead purified CD4⁺CD25⁺ or sorted CD4⁺CD25⁺CD103⁺ cells was tested by culturing them with CD4⁺CD25⁻ responder T cells at varying ratios in the presence of 1x10⁵ irradiated spleen cells and 0.5ug/ml anti-CD3. Cells were cultured in super complete media (phenol red-free RPMI 1640, 10% heat-inactivated charcoal filtered FCS, 2mM glutamine, 10mM Hepes, 100U/ml penicillin G sodium, 100ug/ml streptomycin sulfate, and 10⁻⁵ M 2-mercaptoethanol) for 4 days at 37°C in 5% CO₂. On Day 3, the cells were pulsed with [³H] thymidine (0.5uCi)

for 18 hours. On Day 4, cells were harvested and radioactivity read on a scintillation counter.

Measurement of proteinuria.

Urine from female and male BWF1 mice was tested for the presence of protein bi-monthly starting at 20 weeks of age using Albustix[®] (Bayer). Urine was scored from 0 to 5, 0 indicating no protein detected in urine, 1 indicating 30mg/dL protein detected in urine, 2 indicating 100mg/dL protein detected in urine, 3 indicating 300mg/dL protein detected in urine, 4 indicating over 2000mg/dL detected in urine, and 5 indicating death. A mouse was considered sick when it scored 3 or above on two consecutive test dates.

Measurement of BrdU incorporation.

Mice were injected i.p. with 1mg BrdU every 12 hours for 3 days. After 72 hours, mice were sacrificed and their organs harvested. Single cell suspensions were surface labeled as described above, then intracellularly labeled with anti-BrdU fluorescent antibody following manufacturer's instructions (BD Pharmingen).

In vivo depletion of CD103.

8-week-old female and male BWF1 mice were injected i.p. with 1mg anti-CD103 monoclonal antibody (M290, BioXCell) or control IgG2a. These mice were treated twice a week with 0.5mg anti-CD103 antibody or control IgG2a from 8-12 weeks of age. Mice were then treated with a 0.5mg maintenance dose of anti-CD103 antibody or control

IgG2a once every 2 weeks from 12-20 weeks of age. Urine was tested every week for the presence of protein starting at 8 weeks of age.

Statistical analysis.

Data were subjected to analysis by student's t test or ANOVA and the Tukey-Dramer multiple comparisons test. All experiments were performed at least two times, and most performed at least 5 times with similar results. A p-value ≤ 0.05 is considered significant. * indicates $p < 0.05$, ** indicates $p < 0.005$, and *** indicates $p < 0.0005$.

RESULTS

SPECIFIC AIM I

Assessing regulatory T cell populations in young autoimmune-prone and less-autoimmune-prone strains of mice.

The CD4⁺CD25⁺Foxp3⁺ cells are important regulators of the immune response, and are particularly important for the control of autoreactive cells and the prevention of autoimmune diseases (123). A large number of studies that evaluate the number and function of CD4⁺CD25⁺Foxp3⁺ regulatory cells have been conducted in both humans and mice that develop autoimmune/inflammatory diseases, including systemic lupus erythematosus, multiple sclerosis and type 1 diabetes, but the results have been inconclusive, i.e., some have found defects in numbers and/or function whereas others have not (160, 162-169, 172, 173, 244). In the current study, we compared the numbers and function of regulatory T cells from four different strains of mice that have varying predispositions to developing autoimmune diseases, including the (NZBxNZW)F1 (BWF1) and SJL strains which tend to be autoimmune disease-prone and the BALB/c and C57BL/6 strains which are more autoimmune disease-resistant to determine whether differences in regulatory T cell number or function correlated with differences in susceptibility to disease.

CD4⁺CD25⁺ regulatory cells from young female C57BL/6, BALB/c, SJL and BWF1 mice function similarly in an *in vitro* suppression assay.

The CD4⁺CD25⁺ cells are the best studied of the regulatory T cells (123). Since their “discovery” in the mid-1990s, attention has focused on the relationship between CD4⁺CD25⁺ regulatory cell function/numbers and pre-disposition/susceptibility to autoimmune diseases (125, 172-174, 176, 178, 179). Female (NZBxNZW)F1 (BWF1) mice spontaneously develop systemic lupus erythematosus (SLE), and SJL mice are much more susceptible to induced autoimmune diseases than the C57BL/6 or BALB/c strains of mice (66, 67). For this reason, we compared the characteristics of Tregs in these four strains of mice. The first question we addressed was whether there were qualitative differences in Treg function, i.e., differences in the ability of these cells to suppress responder T cell proliferation *in vitro*, between the different strains of mice. Although the classic *in vitro* assay for Treg-mediated suppression of responder cell proliferation may not always reflect the ability of Treg populations to regulate autoreactive cells *in vivo*, it is a measure of inherent Treg function and a defect in this function, even *in vitro*, would very likely translate into a defect in function *in vivo*. In the following experiments, we tested Treg function using two variations of the classic *in vitro* assay for regulatory cell function. First, using a protocol that standardized all of the potential variables except for the Tregs themselves, we tested the ability of CD4⁺CD25⁺ cells from the various strains of mice to suppress the same CD4⁺CD25⁻ responder cell/APC combination. For these experiments, varying numbers of CD4⁺CD25⁺ cells from each of the four strains of mice were co-cultured with C57BL/6 CD4⁺CD25⁻ responder cells stimulated by C57BL/6 irradiated splenocytes as antigen presenting cells

(APC) and anti-CD3. We found that the Tregs from all four strains of mice suppressed proliferation of anti-CD3-activated C57BL/6 responder cells equally effectively (Figure 1A, 7A). Next, we tested the ability of CD4⁺CD25⁺ cells from the various strains of mice to suppress their own CD4⁺CD25⁻ responder cell/APC combination (i.e., CD4⁺CD25⁻ responders and APC that were syngeneic to the Tregs) in the presence of anti-CD3 antibody. As shown in Figure 1B, we found that CD4⁺CD25⁺ cells from all four strains of mice were equally effective at suppressing proliferation of anti-CD3-activated syngeneic responder cells *in vitro* (Figure 1B, 7B). In summary, we found no consistently significant differences in the ability of CD4⁺CD25⁺ Tregs from any of the four strains to suppress responder cell proliferation *in vitro* and we, therefore, concluded that there were no differences in inherent Treg function between the four strains of mice tested.

BWF1 mice have very low percentages of peripheral CD4⁺Foxp3⁺ cells by comparison to other strains of mice.

In addition to issues with regulatory function by Tregs, another contributing factor to the predisposition of humans and particular strains of mice to autoimmune disease could be an imbalance between the effector CD4 and regulatory T cell populations. In the following experiments, we performed a quantitative analysis of Tregs in the four different strains of mice. CD4⁺ cells collected from lymph node (LN) and spleen from the four different strains, BWF1, SJL, C57BL/6 and BALB/c, of young female mice were evaluated for expression of the transcription factor, Foxp3, a hallmark of CD4⁺CD25⁺ regulatory cells. In general, BWF1 mice had by far the lowest percentages of

CD4⁺Foxp3⁺ cells (i.e., percentage of CD4 cells expressing Foxp3) among the 4 strains evaluated, and in LN, had less than half the level found in C57BL/6 and BALB/c mice (Figure 2A). Although not quite as low, SJL mice also had low percentages of CD4⁺Foxp3⁺ cells in LN compared to BALB/c mice and C57BL/6, but fairly high percentages in spleen (Figure 2A). The patterns of percentages of CD4⁺CD25⁺ cells (i.e., percentages of CD4 cells expressing CD25) in both the LN and spleen were very similar to those found for CD4⁺Foxp3⁺ cells in all four strains (Figure 2C). The percentages of CD4⁺CD25⁺ cells that expressed Foxp3 were very similar in all four strains of mice (>90%, data not shown), and the level of Foxp3 expression (as indicated by the mean fluorescence intensity, MFI) in CD4⁺CD25⁺Foxp3⁺ cells was also similar for all four strains of mice (Figure 2D). Taken together, these data indicated that the Foxp3⁺ Treg to effector (Foxp3⁻) CD4 cell ratio (i.e., as reflected by the percentages of CD4⁺Foxp3⁺ cells) in the peripheral lymphoid organs differs between strains and tends to be lower in autoimmune-prone mice.

The analysis described above evaluated percentages, but not numbers, of cells in the various populations. Analysis of the numbers is important because it can reveal whether it is relative decreases in the numbers of CD4⁺ Foxp3⁺ cells or increases in the CD4⁺Foxp3⁻ cells that produce differences in the CD4⁺Foxp3⁺ to CD4⁺Foxp3⁻ ratios. We found that the SJL mice had the highest total numbers of all cells (Table 1) as well as CD4⁺ cells (Figure 6B) in both LN and spleen among the four strains evaluated. The SJL mice also had the highest numbers of both CD4⁺Foxp3⁺ cells (>4-fold more; Figure 2B) in LN and spleen of all four strains. C57BL/6 and BWF1 mice tended to have the lowest numbers of CD4⁺Foxp3⁺ cells (Figure 2B). Overall, the numbers of peripheral

CD4⁺Foxp3⁺, with the exception of SJL mice, did not vary dramatically between the strains, and we concluded that these lower numbers of CD4⁺Foxp3⁺ cells could not account for the differences in percentages.

BWF1 and SJL mice have low percentages of peripheral CD4⁺CD25⁺CD103⁺ cells.

A number of different markers have been associated with CD4⁺CD25⁺ regulatory cell phenotype and function (125, 127, 128, 143-147, 149, 150), and variations in their expression could affect regulatory T cell function *in vivo*. The integrin, CD103 ($\alpha_E\beta_7$), is expressed on the surface of cells and is involved in retaining cells at sites of inflammation via interaction with its ligand, E-cadherin (198, 199, 212). In the CD4⁺CD25⁺ regulatory cell population, CD103 also identifies a highly potent subset that is thought to represent a memory/effector phenotype (207, 210). Because of the unique and potentially important disease-preventing properties of this population of Tregs, we compared the function and quantity of CD4⁺CD25⁺CD103⁺ cells between the four strains of mice. In the following experiments, the regulatory function of sorted CD4⁺CD25⁺CD103⁺ cells from the four strains of mice was evaluated using the *in vitro* assay described above in which varying numbers of CD4⁺CD25⁺CD103⁺ cells from the different strains were co-cultured with their own CD4⁺CD25⁻ responder cell/APC combination (i.e., syngeneic CD4⁺CD25⁻ responders and APC) in the presence of anti-CD3 antibody. As shown in Figure 3D, the CD4⁺CD25⁺CD103⁺ cells from all four strains of mice were able to suppress syngeneic responders to the same extent (Figure 3D, 7C). We also performed a quantitative analysis of CD4⁺CD25⁺CD103⁺ cells in the lymphoid organs of the four strains of mice. The CD4⁺CD25⁺ cells from LN and spleen of the four strains of mice were evaluated for

expression of CD103. The percentages and numbers of CD4⁺CD25⁺CD103⁺ cells (i.e., percentage of CD4⁺CD25⁺ cells that express CD103) were dramatically lower in the LN and spleen of BWF1 mice compared to the other three strains of mice (Figure 3A, 6C). The percentages of CD4⁺CD25⁺CD103⁺ cells were also quite low in the LN of SJL mice, although they were actually fairly high in the spleen (Figure 3A). On the other hand, the percentages of CD4⁺CD25⁺CD103⁺ cells in the spleen, but not LN, of C57BL/6 mice were very low (Figure 3A). Interestingly, CD103 (MFI) expression in CD4⁺CD25⁺CD103⁺ cells from the LN was significantly and consistently lower in BWF1 mice than the other three strains and was also lower in cells from the spleen of BWF1 than either BALB/c or SJL mice (Figure 3B, 3C). There were no differences in the percentages of CD4⁺CD25⁺CD103⁺ cells that expressed Foxp3 (>90%) between any of the four strains or in the expression (MFI) of Foxp3 by this population (data not shown). Taken together, these data indicated that the autoimmune-prone strains of mice tended to have lower percentages of CD4⁺CD25⁺CD103⁺ cells at least in the LN, and in the case of BWF1 mice, lower expression of CD103.

Neither thymic production nor proliferation rate appear to account for the differences in either CD4⁺Foxp3⁺ cells or CD4⁺CD25⁺CD103⁺ cells percentages in the periphery between strains.

Several factors can influence the numbers/relative proportions of peripheral T cell populations, and these include thymic production of T cells and proliferation (non-antigen-induced) of peripheral T cell populations (185, 257). We first examined thymus glands from each of the four strains of mice. The BWF1 strain of mice had the highest

thymic weights and total cell numbers in the thymus, (Table 1), but not numbers of CD4⁺CD8⁻ cells (Figure 6B). The SJL mice had very high percentages and numbers of CD4⁺CD8⁻ cells in the thymus (Figure 6A, 6B). Importantly, the percentages of CD4⁺CD8⁻Foxp3⁺ cells in the thymus were comparable in the BALB/c, SJL and BWF1 strains whereas C57BL/6 strain had low percentages (Figure 4A). The SJL mice had dramatically higher numbers of CD4⁺CD8⁻Foxp3⁺ cells in the thymus (Figure 2B) than the other strains of mice which is not surprising since as mentioned above, this strain also had considerably higher numbers of thymic CD4 cells than the other strains of mice. BWF1 had comparable or even higher numbers of CD4⁺CD8⁻Foxp3⁺ cells in the thymus than either C57BL/6 or BALB/c mice.

Most evidence suggests that CD103 is upregulated in Tregs after activation in the periphery hence the hypothesis that CD4⁺CD25⁺ that express CD103 cells are effector and/or memory cells (207, 210, 212). However, CD4⁺CD25⁺CD103⁺ cells can be found in the thymus and some evidence suggests that at least some of these cells may also be produced in the thymus (258). For this reason, we analyzed the thymus of the four different strains of mice for CD4⁺CD25⁺CD103⁺ cells. The CD103⁺ cells, albeit in small numbers, could be detected in the thymus, and C57BL/6 mice had very low and BALB/c mice very high percentages (>5-fold higher than C57BL/6 mice) of CD4⁺CD25⁺CD103⁺ cells (Figure 4B). SJL mice had high numbers (Figure 6C) and moderately high percentages (Figure 4B; although still one-half of that found in BALB/c mice) of CD4⁺CD25⁺CD103⁺ cells in the thymus. From these data, we concluded that there appeared to be little relationship between the thymic production of CD4⁺Foxp3⁺ cells and CD4⁺CD25⁺CD103⁺ cells, at least in terms of their relative percentages in the periphery.

Furthermore, when we compared the proportion of CD4⁺Foxp3⁺ cells in the thymus to that in the periphery, we found that the thymic to peripheral CD4⁺Foxp3⁺ cell ratio in BWF1 was twice that of the other three strains (Figure 4C), suggesting that it is not likely to be the ability of the BWF1 thymus gland to produce CD4⁺Foxp3⁺ cells that is an issue here.

T cells cycle, albeit slowly, in the absence of their cognate antigen in a non-lymphopenic environment, and differences in proliferation rate of either the Tregs or effector cells could affect the Treg to effector T cell balance (i.e., the Treg percentages) in the periphery. To determine whether there were differences in the proliferation rate of T cell populations between the four strains of mice, LN and spleen cells from BWF1, C57BL/6, BALB/c, and SJL mice were labeled for CD4, CD25, Foxp3, CD103 and in order to identify actively proliferating cells, Ki67. The SJL mice tended to have greater percentages of both CD4⁺Foxp3⁺ cells and CD4⁺CD25⁺CD103⁺ cells that expressed Ki67 (i.e., cells that were in the active phases of the cell cycle) compared to the other three strains which all had comparable levels of proliferating cells (Figure 5A, 5C). The BALB/c, SJL and BWF1 mice had comparable percentages of CD4⁺Foxp3⁻ cells that were proliferating whereas C57BL/6 tended to have higher percentages of proliferating CD4⁺Foxp3⁻ cells (Figure 5B). To further examine the relationship between proliferating Tregs (CD4⁺Foxp3⁺ cells) and effector CD4 cells (CD4⁺Foxp3⁻ cells), we analyzed the ratio between these two populations. As shown in Figure 5D, the proliferating Treg to effector CD4 cell ratio tended to be somewhat higher in SJL mice compared to the other strains, and the differences in ratios did not correlate with differences in percentages of CD4⁺Foxp3⁺ cells in peripheral lymphoid organs. From these data, we concluded that

there did not appear to be any type of direct correlation or association between differences in effector or Treg CD4 cell proliferation and the percentages of CD4⁺Foxp3⁺ cells in the periphery.

Summary

In this study we compared regulatory T cell percentages and function between young females from four strains of mice. We found decreased percentages but not a decrease in inherent suppressive function of Tregs in BWF1 mice compared to the other strains. Differences in thymic production and homeostatic proliferation could not account for the differences in peripheral percentages seen between four strains of mice. These data suggest that reduced percentages of both CD4⁺Foxp3⁺ and memory/effector CD4⁺CD25⁺CD103⁺ subsets of Tregs in the periphery, and not decreased function, at least *in vitro*, appear to be an important difference between lupus-prone BWF1 mice and other autoimmune-prone SJL mice and less-autoimmune prone C57BL/6 and BALB/c mice.

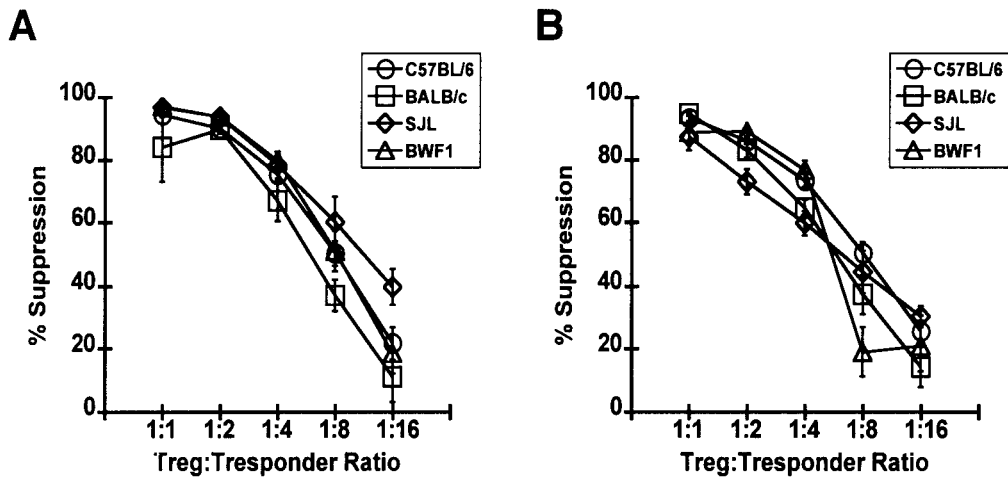


Figure 1. Regulatory function of CD4⁺CD25⁺ cells from four strains of mice.

CD4⁺CD25⁺ cells were purified from 9-week-old female C57BL/6, BALB/c, SJL, and BWF1 mice and varying numbers co-cultured with a constant number of (A) C57BL/6 CD4⁺CD25⁻ responder cells and APC (irradiated spleen cells) or (B) syngeneic CD4⁺CD25⁻ responder cells and APC and soluble anti-CD3 antibody. % suppression of positive control (responder cells alone) was calculated and the mean ± SEM presented for 4-5 separate experiments.

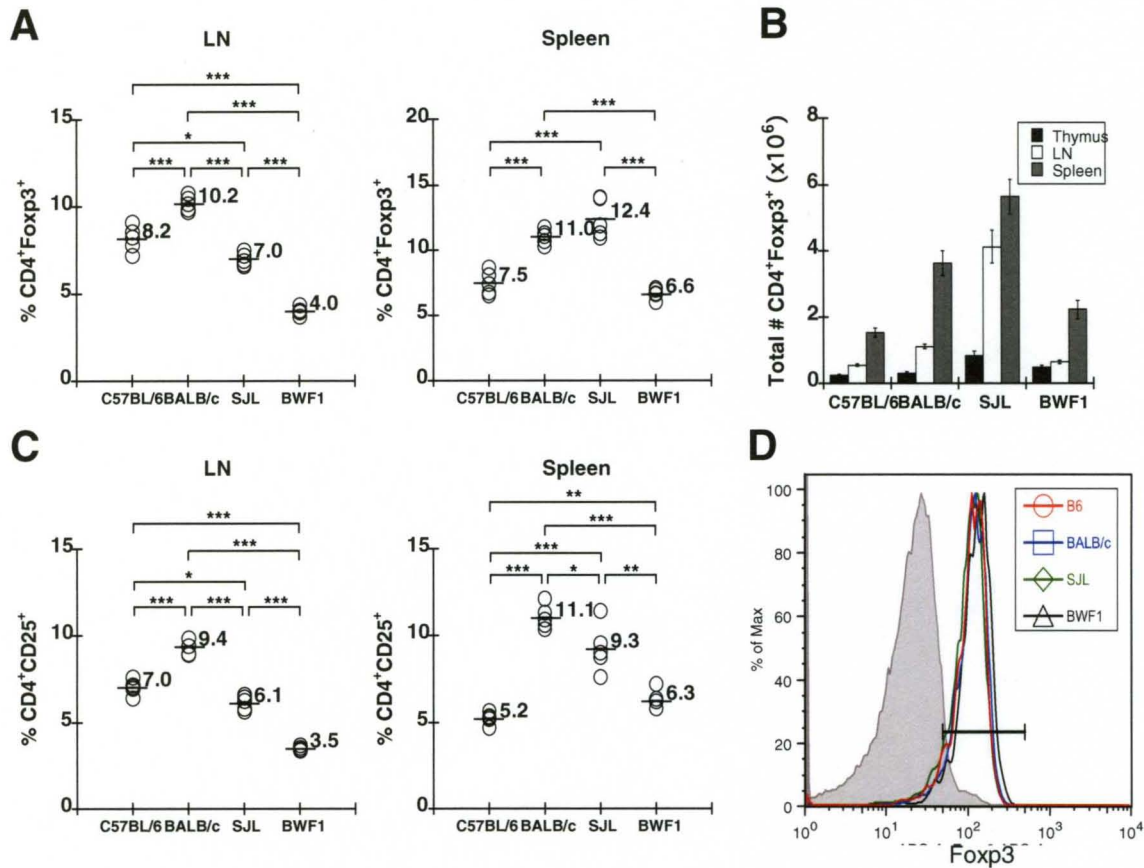
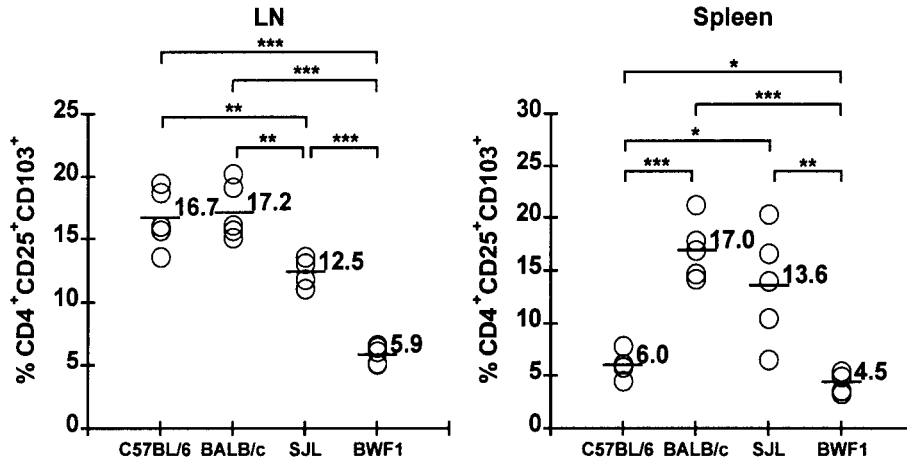
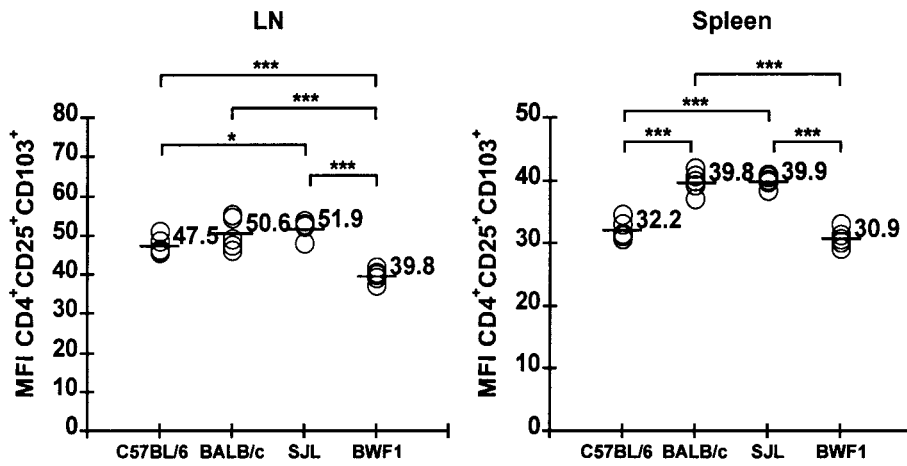


Figure 2. Percentages and numbers of CD4⁺Foxp3⁺ cells in four strains of mice. Cells from lymph nodes (LN), spleen and thymus of 9-week-old female C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, and anti-Foxp3 antibodies and analyzed by FACS[®]. (A) Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing Foxp3 in LN and spleen. (B) The total numbers of CD4⁺Foxp3⁺ cells in lymph nodes, spleen, and thymus (n=16) are shown. (C) Labeled CD4⁺ T cells were gated and analyzed for the percentages of cells expressing CD25 in LN and spleen. (D) Histogram of Foxp3 expression in CD4⁺CD25⁺

cells from a representative sample of LN cells from B6, BALB/c, SJL, and BWF1 mice. (A, C) A representative experiment of 5 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$.

A**B**

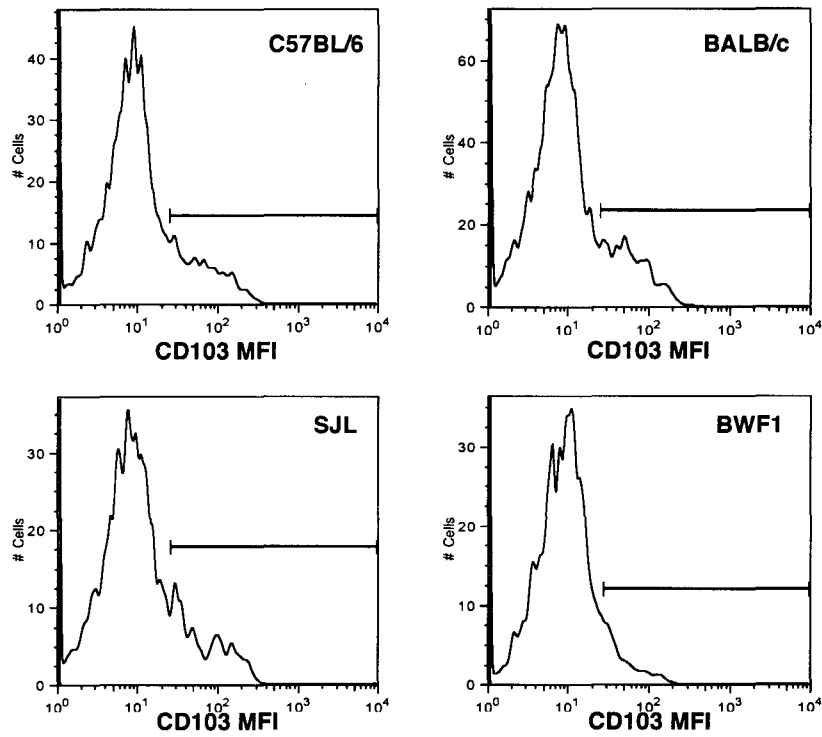
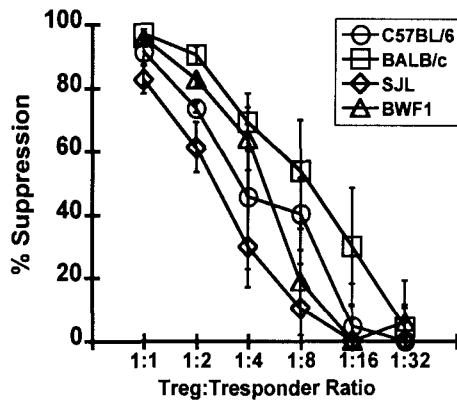
C**D**

Figure 3. Regulatory function and percentages of CD4⁺CD25⁺CD103⁺ cells in the periphery of four strains of mice. Cells from lymph nodes (LN) and spleen of 9-week-old female C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled

with anti-CD4, anti-CD25, and anti-CD103 antibodies and analyzed by FACS®. (A) Labeled CD4⁺CD25⁺ cells were gated and analyzed for the percentages of cells expressing CD103 in LN and spleen. (B) Labeled CD4⁺CD25⁺ cells were gated and analyzed for the per cell expression of CD103 in LN and spleen. (C) Histogram of CD103 expression in CD4⁺CD25⁺ cells from a representative sample of LN cells from B6, BALB/c, SJL and BWF1 mice. (A, B) A representative experiment of 5 performed is shown. Each symbol represents an individual animal. (D) CD4⁺CD25⁺CD103⁺ cells were purified from 9-week-old female C57BL/6, BALB/c, SJL, and BWF1 mice and sorted and varying numbers co-cultured with a constant number of syngeneic CD4⁺CD25⁻ responder cells and APC and soluble anti-CD3 antibody. % suppression of positive control (responder cells alone) was calculated and the mean ± SEM presented for 4-5 separate experiments. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

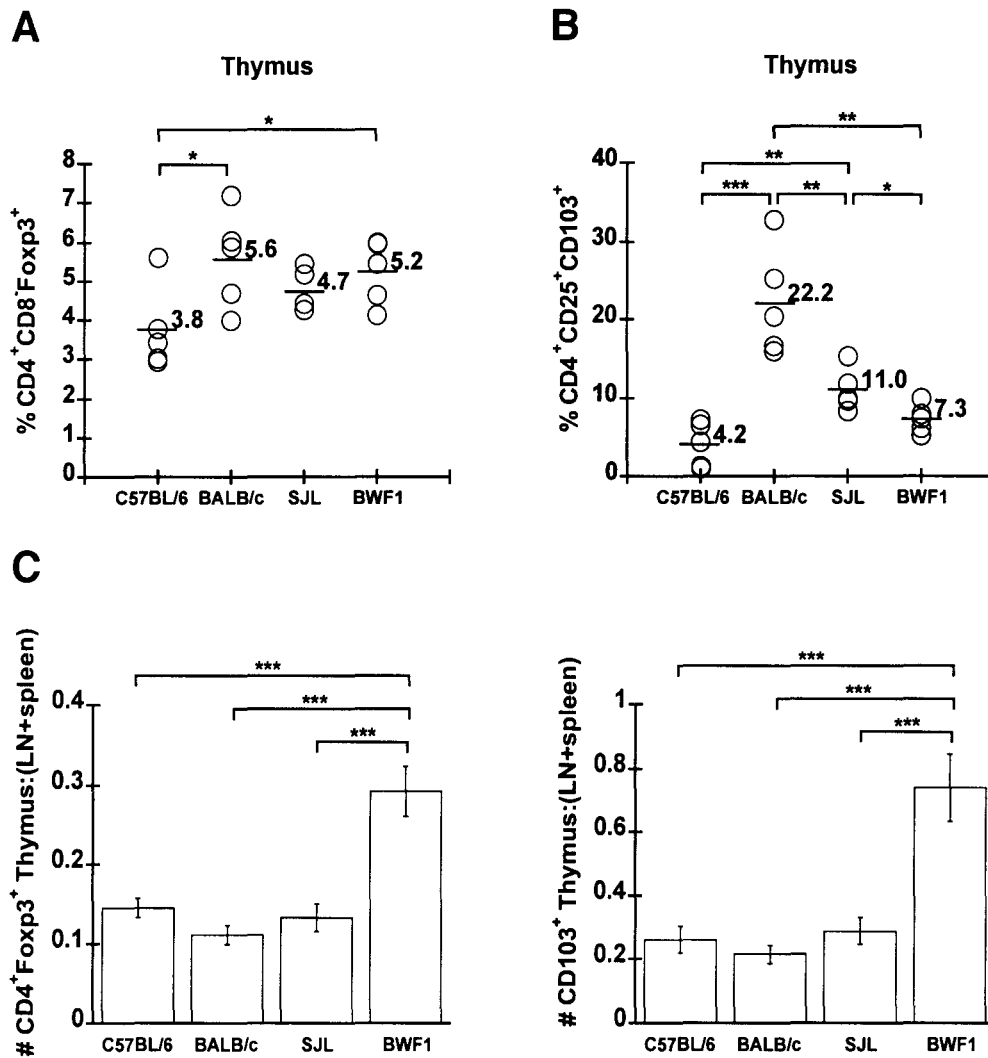
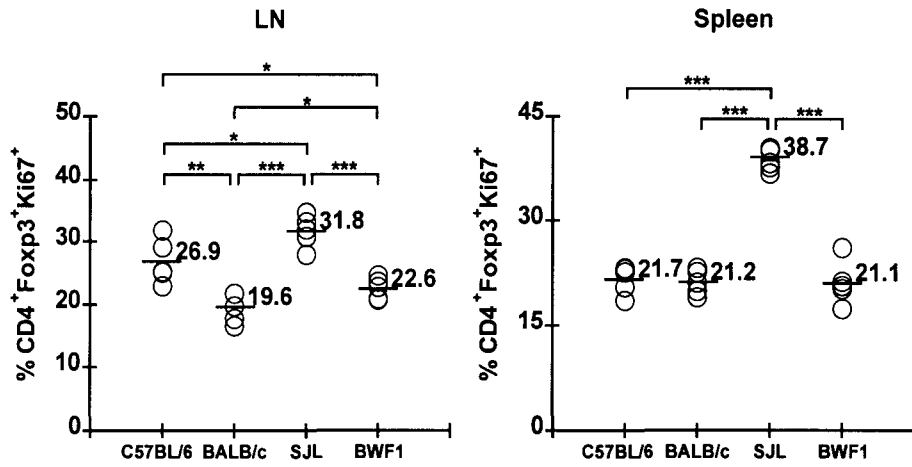
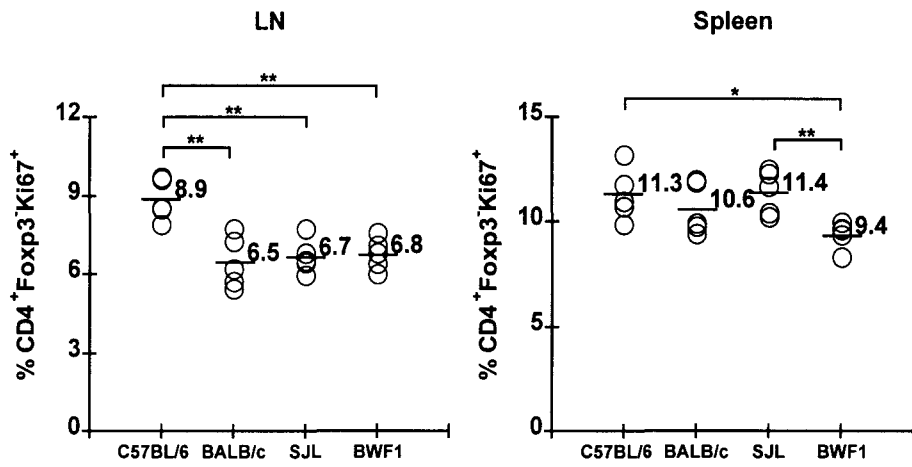
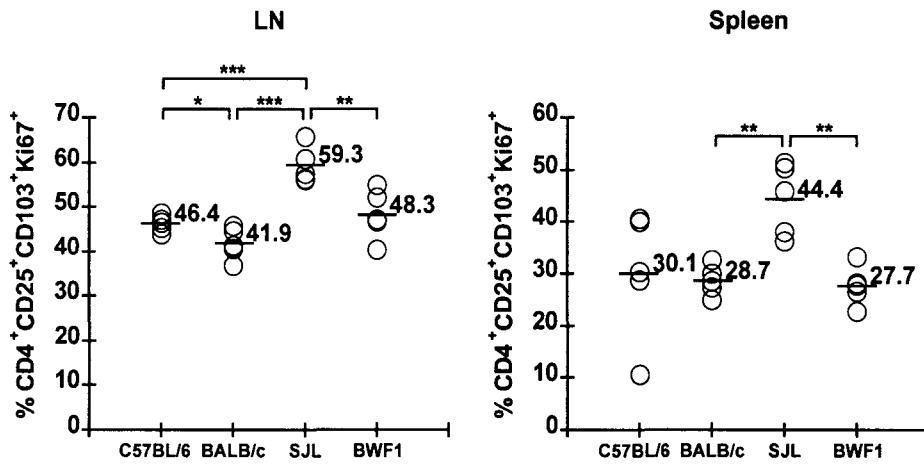


Figure 4. Percentages of CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in thymus of four strains of mice. Cells from thymus of 9-week-old female C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD8, anti-CD25, anti-CD103 and anti-Foxp3 antibodies and analyzed by FACS[®]. (A) Labeled CD4⁺CD8⁻ cells were gated and analyzed for the percentages of cells expressing Foxp3 in thymus. (B) Labeled CD4⁺CD8⁻CD25⁺ cells were gated and analyzed for the percentages of cells expressing CD103 in thymus. (C) Ratio of numbers of CD4⁺CD8⁻Foxp3⁺ cells in the

thymus to numbers of CD4⁺Foxp3⁺ cells in the periphery (i.e., LN and spleen). (A, B) A representative experiment of 5 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

A**B****C**

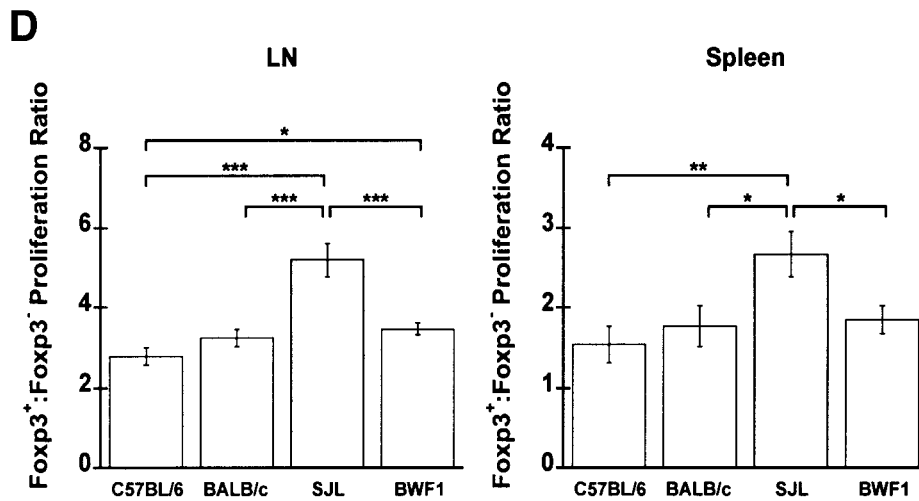


Figure 5. Percentages of proliferating $CD4^+Foxp3^+$, $CD4^+Foxp3^-$ and $CD4^+CD25^+CD103^+$ cells in the periphery of four strains of mice. Cells from lymph nodes (LN) and spleen of 9-week-old female C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, anti-Foxp3, anti-CD103 and anti-Ki67 antibodies and analyzed by FACS[®]. (A) Labeled $CD4^+Foxp3^+$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (B) Labeled $CD4^+Foxp3^-$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (C) Labeled $CD4^+CD25^+CD103^+$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (D) Proliferation ratios were calculated by dividing the percentage of proliferating ($Ki67^+$) $CD4^+Foxp3^+$ cells by the percentage of proliferating $CD4^+Foxp3^-$ cells from LN and spleen (n=15). (A, B, C) A representative experiment of 3 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

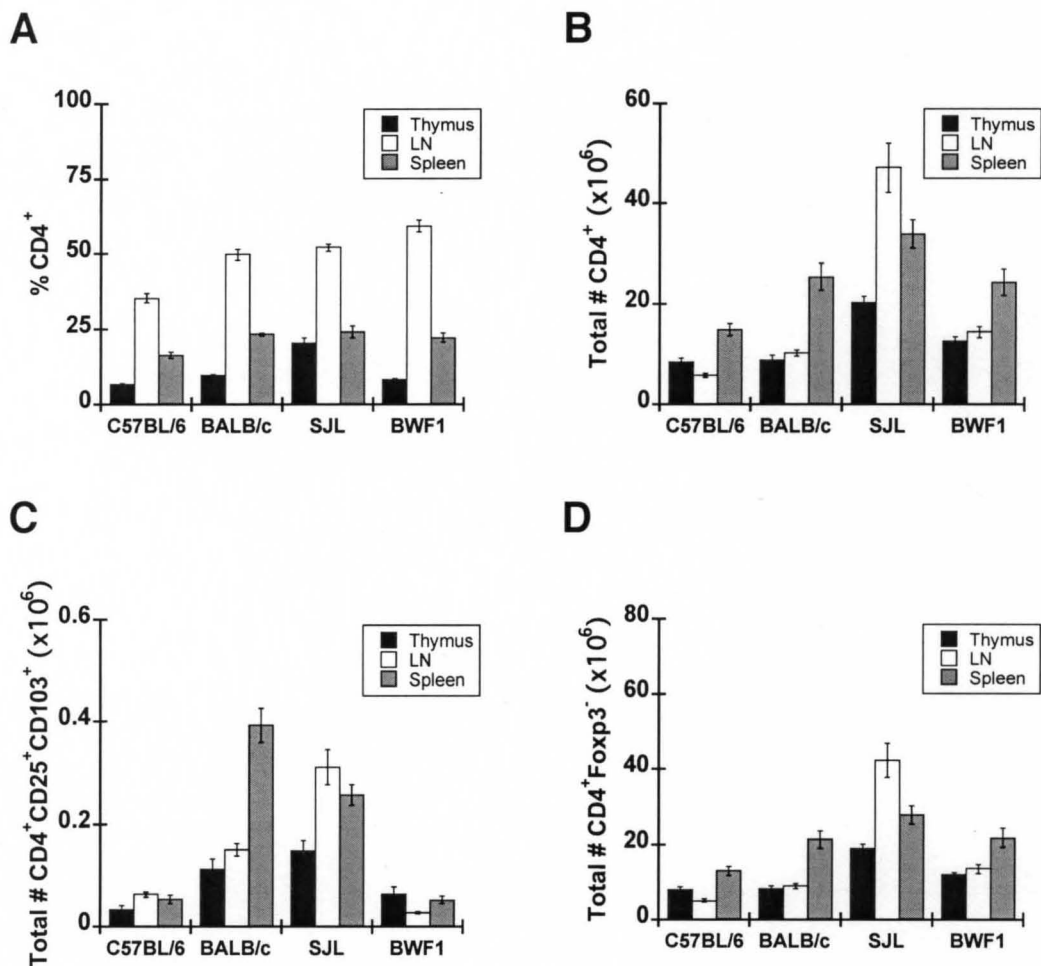
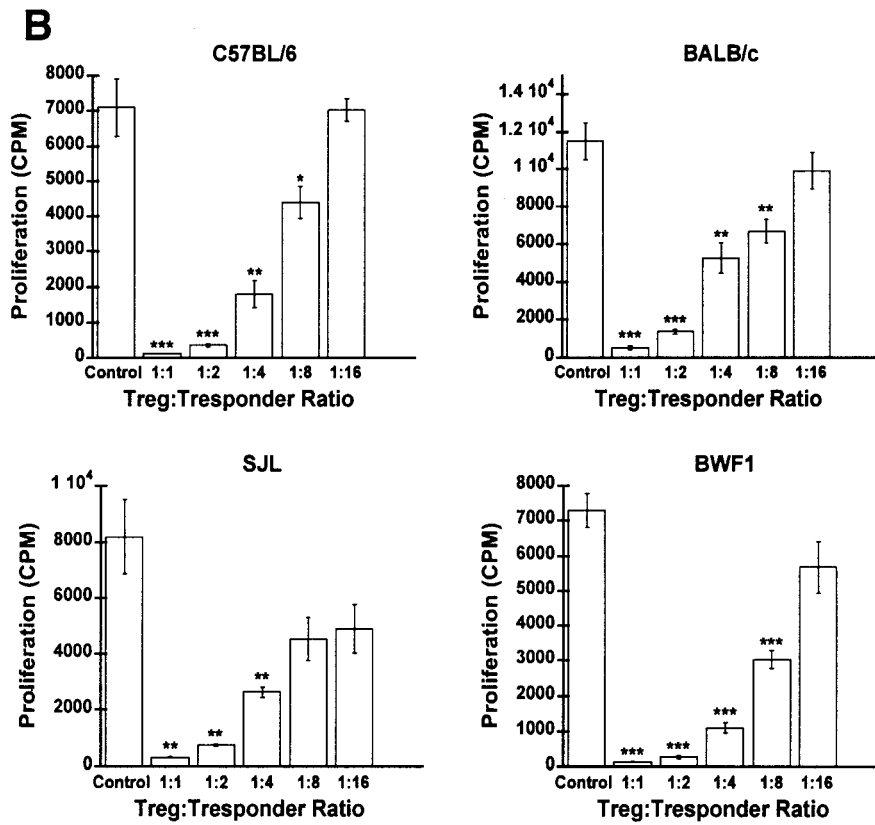
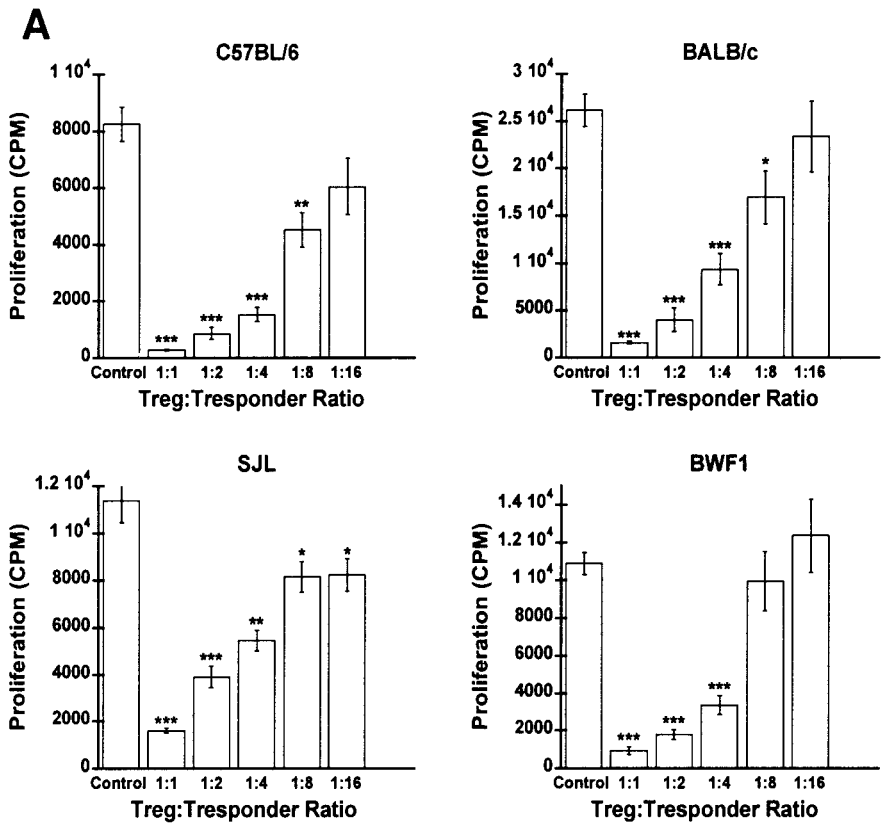


Figure 6. Percentages and numbers of CD4⁺ cells and numbers of CD4⁺CD25⁺CD103⁺ cells. Cells from thymus, lymph nodes (LN) and spleen of 9-week-old female C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, anti-CD103 and anti-Foxp3 antibodies and analyzed by FACS[®]. (A) Percentages and (B) numbers of CD4 single positive cells were determined. (C) Labeled CD4⁺CD25⁺ cells (CD4⁺CD8⁺CD25⁺ cells in the thymus) were gated and numbers of cells expressing CD103 were determined. (D) CD4⁺Foxp3⁻ cells (CD4⁺CD8⁺Foxp3⁻ cells in the thymus) were gated and numbers of cells were determined.



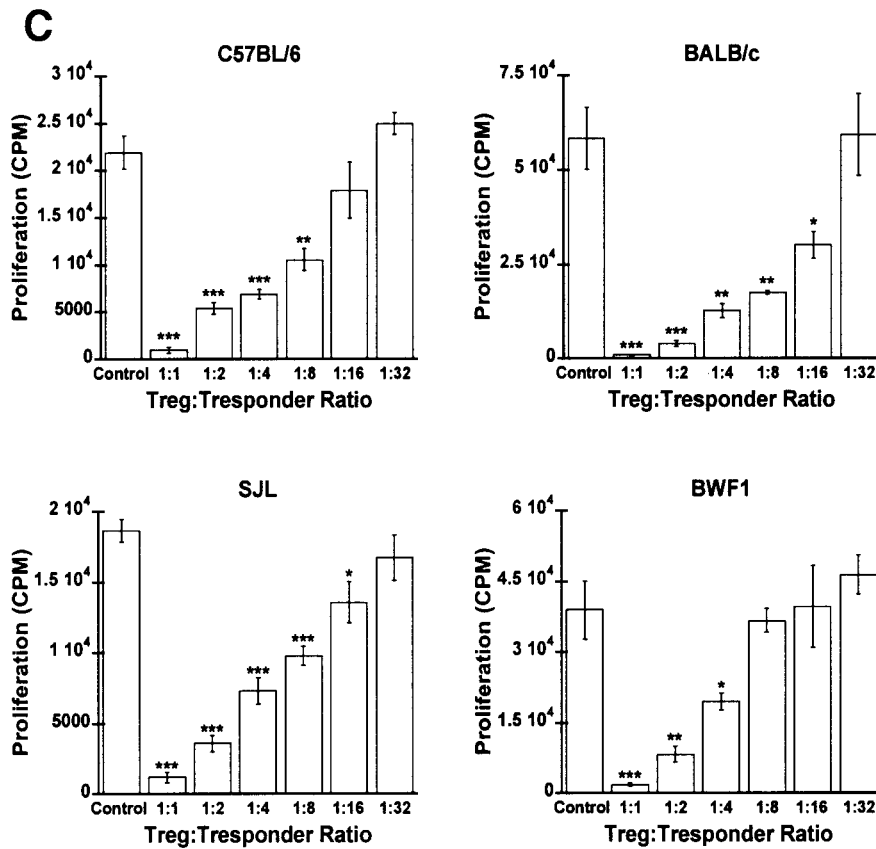


Figure 7. *In vitro* analysis of suppression by CD4⁺CD25⁺ and CD4⁺CD25⁺CD103⁺ cells from four strains of mice. CD4⁺CD25⁺ (A, B) or CD4⁺CD25⁺CD103⁺ (C) cells were purified from 9-week-old female C57BL/6, BALB/c, SJL, and BWF1 mice and varying numbers co-cultured with a constant number of (A, C) autologous or (B) C57BL/6 CD4⁺CD25⁻ responder cells and either (A, C) autologous or (B) C57BL/6 irradiated spleen cells and soluble anti-CD3 antibody. A representative experiment from among (A) 4-5 separate experiments or (B, C) 2-4 separate experiments is shown. Data are shown as mean counts per minute (CPM) ± SEM and statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, *** p<0.0005 compared to control.

Table 1: Thymic Weights (mg) and Total Cell #s (x10⁶)				
Strains	Thymic Weights (mg) n=20	Thymus Total Cell #s (x10⁶) n=16	LN Total Cell #s (x10⁶) n=16	Spleen Total Cell #s (x10⁶) n=16
C57BL/6	46.2 ± 2.3	149.3 ± 9.8	17.6 ± 1.0	106.5 ± 5.7
BALB/c	38.1 ± 0.9	109.0 ± 6.2	21.2 ± 1.2	124.1 ± 5.4
SJL	52.2 ± 2.4	121.7 ± 9.1	106.8 ± 10.3	163.0 ± 6.4
BWF1	78.9 ± 1.9	176.3 ± 10.6	26.4 ± 1.3	118.5 ± 6.9

Table 1. Thymic weights and cell numbers in thymus, lymph nodes and spleen in four strains of mice. Thymus, lymph nodes and spleen were collected from 9-week-old female C57BL/6, BALB/c, SJL and BWF1 mice and weights or cell numbers determined.

SPECIFIC AIM II

Decrease in regulatory T cell percentages, but not function, may contribute to female susceptibility to autoimmune disease.

Women are more susceptible to many autoimmune diseases than men (1). For example, women are nine times more likely to develop systemic lupus erythematosus (1). This increased susceptibility to disease can be seen in many mouse models of autoimmunity as well (66, 67, 109, 172, 173). It has been shown that regulatory T cells are an important cell type in the control of autoimmune disease (123). Mice that lack functional Foxp3, and thus lack regulatory T cells, live only to 21 days of age and develop severe autoimmune disease (127). These mice can be rescued from disease by adoptive transfer of regulatory T cells (127). To determine how gender differences in regulatory T cells could contribute to autoimmune disease susceptibility, we investigated regulatory T cell function and percentages in females and males from four different strains of mice, two autoimmune-prone strains, the (NZBxNZW)F1 (BWF1) and SJL strains, and two less autoimmune-prone strains, the C57BL/6 and BALB/c strains. Female BWF1 mice develop lupus-like disease spontaneously whereas males only develop disease after castration. SJL mice are susceptible to multiple induced autoimmune diseases, while C57BL/6 and BALB/c mice are both relatively resistant to induced disease.

CD4⁺CD25⁺ regulatory cells from young female C57BL/6, BALB/c, SJL, and BWF1 mice function similarly to strain-matched males in an *in vitro* suppression assay.

To compare inherent Treg function between females and males, we cultured varying numbers of bead-purified CD4⁺CD25⁺ cells from 8-9-week-old female and male mice of each strain in a standard *in vitro* suppression assay, using syngeneic male CD4⁺CD25⁻ T cells as responders, syngeneic male irradiated spleen cells as APC, and anti-CD3 antibody to stimulate. While *in vitro* suppression assays do not necessarily reflect exactly what is happening *in vivo*, they do allow for comparison of the suppressive function of regulatory T cells between groups in a controlled environment. We found no significant differences between female and male CD4⁺CD25⁺ Treg suppressive function *in vitro* in any of the four strains studied (Figure 8). This result was surprising for BWF1 mice in particular, as females are more susceptible to disease in this strain. These data suggest that there is no inherent defect in female Treg suppressive function in the autoimmune-prone and less autoimmune-prone strains in this study, although this does not rule out the possibility that differences in female and male Treg function exist *in vivo*.

Female lupus-prone BWF1 mice have significantly lower percentages of CD4⁺Foxp3⁺ Tregs than BWF1 males.

The CD4⁺CD25⁺ regulatory T cells from female autoimmune-prone mice did not show decreased suppressive function *in vitro* compared to males (Figure 8). This led us to hypothesize that perhaps it was a decreased ratio or absolute number of Tregs in female mice and not a defect in their function that contributed to their disease susceptibility. Although there were no differences in the percentages of CD4⁺ T cells between female

and male mice of any of the four strains studied in either LN or spleen (Table 2), female BWF1 mice did have significantly lower CD4⁺Foxp3⁺ cell percentages in the LN, but not spleen, than males (Figure 9A). No consistent gender differences were seen in the percentages of CD4⁺Foxp3⁺ cells in either LN or spleen of C57BL/6, BALB/c, or SJL mice, however C57BL/6 females trended toward having lower percentages in experiments even when they were not actually significantly lower (Figure 9A, Table 3).

Although the percentage of CD4⁺Foxp3⁺ cells was significantly lower in the LN of female BWF1 mice, there were no differences between female and male mice in the total cell number of CD4⁺Foxp3⁺ cells in LN or spleen (Figure 9B). Interestingly, in the LN of both SJL and BALB/c mice, females tended to have statistically higher total CD4⁺Foxp3⁺ cell numbers than males, although not always significant (Figure 9B, Table 4A). This increase in CD4⁺Foxp3⁺ cell numbers in LN of female SJL and BALB/c mice compared to male mice is no doubt due to the greater number of CD4⁺ cells found in LN, in general, in female compared to male SJL mice (Table 5A). C57BL/6 mice showed no gender difference in CD4⁺Foxp3⁺ total cell number (Figure 9B, Table 4). Similar to CD4⁺Foxp3⁺ cell percentages, BWF1 females had significantly lower percentages of CD4⁺CD25⁺ cells in LN, but not spleen, while none of the other strains showed a gender difference in percentages of these cells (Figure 9C). There were no gender differences in per cell expression of Foxp3 in CD4⁺CD25⁺ T cells in any of the four strains studied (Figure 9D, Table 6). Together, these data suggest that in BWF1 mice, in which females are more susceptible to disease, a lower Treg:effector cell ratio in female mice could contribute to increased disease susceptibility.

Female mice from all four strains have significantly lower percentages of the potent CD4⁺CD25⁺CD103⁺ regulatory T cell subset.

CD103 ($\alpha_E\beta_7$) is an integrin expressed on the surface of a more potent subset of Tregs that are thought to represent a memory/effector population (207-209). CD4⁺CD25⁺CD103⁺ Tregs have been shown to be more potent suppressors than CD4⁺CD25⁺CD103⁻ Tregs both *in vitro* and *in vivo* in mouse models of autoimmune disease (149, 207, 211). CD103 helps retain Tregs at sites of inflammation where they are thought to receive activation signals contributing to their memory/effector Treg phenotype (212). To evaluate CD103⁺ Treg function *in vitro*, we cultured varying numbers of sorted CD4⁺CD25⁺CD103⁺ cells from female and male mice of each strain in a standard *in vitro* suppression assay, using syngeneic male CD4⁺CD25⁻ T cells as responders, syngeneic male irradiated spleen cells as APC, and stimulated by anti-CD3 antibody. Again, as with the CD4⁺CD25⁺ Tregs, we found no significant differences between female and male CD4⁺CD25⁺CD103⁺ Treg suppressive function *in vitro* in any of the four strains studied (Figure 10D). Thus, an inherent defect in female Treg suppressive function is not responsible for increased female susceptibility to disease, although this does not rule out the possibility that differences in female and male Treg function exist *in vivo*.

Females of all four strains of mice studied, which included both autoimmune-prone and less autoimmune-prone strains of mice, had significantly lower percentages of CD4⁺CD25⁺CD103⁺ cells in their LN compared to their male counterparts (Figure 10A). Interestingly, only lupus-prone female BWF1 mice had significantly lower percentages of CD4⁺CD25⁺CD103⁺ cells in spleens than male BWF1 mice (Figure 10A). Females from

both C57BL/6 and BWF1 mouse strains had significantly lower total CD4⁺CD25⁺CD103⁺ cell numbers than strain-matched males in LN, but not spleen (Table 7). Interestingly, not only did females of each strain have lower percentages of CD4⁺CD25⁺CD103⁺ cells in LN compared to males, but female BWF1 and C57BL/6 mice also expressed lower levels of CD103 than males in LN (Figure 10B, 10C, Table 8). These data together suggest that, particularly in the LN, gender may strongly influence CD4⁺CD25⁺CD103⁺ Treg populations. Gender differences, presumably hormonal differences, appear to impact this CD103⁺ Treg population more than the CD4⁺Foxp3⁺ Tregs in general.

Thymic production of CD4⁺Foxp3⁺ or CD4⁺CD25⁺CD103⁺ Tregs does not play a role in the lower percentages of these cells in the periphery.

To determine whether a defect in thymic CD4⁺Foxp3⁺ and/or CD4⁺CD25⁺CD103⁺ cell production was the cause of decreased Treg percentages in the periphery in females, we examined Treg populations in the thymus. We found no differences between female and male thymic percentages of CD4⁺Foxp3⁺ cells in the thymus of any of the four strains studied (Figure 11A, Table 3C). Interestingly, we found that both BALB/c and SJL mice had higher total CD4⁺Foxp3⁺ cell numbers in females than males, and while not always significant, both C57BL/6 and BWF1 females tended to have higher total CD4⁺Foxp3⁺ cell numbers as well (Figure 9B, Table 4C). Although females had higher thymic total cell numbers in all four strains, only female SJL and female BWF1 mice had higher thymic weights than strain-matched males (Table 9A).

There were no differences in percentages of thymic CD4⁺CD25⁺CD103⁺ cells between females and males in any of the four strains studied (Figure 11B). It is unclear whether the CD4⁺CD25⁺CD103⁺ cells found in the thymus develop there or have simply re-entered from the periphery, so we cannot completely rule out gender differences in thymic production (259). However, these data together suggest that it is unlikely that a defect in thymic production of Tregs is responsible for the decreased percentages of CD4⁺Foxp3⁺ cells seen in BWF1 females and the decreased percentages of CD4⁺CD25⁺CD103⁺ cells found in females of all four strains in the periphery.

Differences in non-Treg proliferation *in vivo* may account for decreased CD4⁺Foxp3⁺ peripheral percentages in female BWF1 mice, but proliferation does not appear to play a role in decreased female CD4⁺CD25⁺CD103⁺ peripheral percentages.

T cells cycle in the periphery, and it is, in part, through this mechanism that peripheral T cell populations, including Tregs, are maintained (257). A higher proliferation rate of male CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells *in vivo* compared to females could account for lower peripheral Treg percentages in females. Alternatively, if the CD4⁺Foxp3⁻ (non-Treg) proliferation rate is higher in females than males, this could also account for decreased percentages of Tregs in the periphery of females. To detect proliferating cells, we used the Ki67 marker, which identifies cells in active cell cycle. It was only the BALB/c strain that exhibited differences in percentages of proliferating CD4⁺Foxp3⁺ cells in either the LN or spleen (Figure 12A). However, the BALB/c strain does not have a gender difference in CD4⁺Foxp3⁺ percentages in the periphery, so the

significance of the differences in the proliferation rate is unclear. Interestingly, in the LN, female BWF1 mice had a higher percentage of proliferating CD4⁺Foxp3⁻ cells (non-Treg) than males, which, as discussed above, may contribute to the decreased ratio of CD4⁺Foxp3⁺:CD4⁺Foxp3⁻ cells found in the periphery in these mice (Figure 12B). None of the other three strains of mice showed gender differences in CD4⁺Foxp3⁻ proliferation in the LN (Figure 12B). SJL mice were the only strain with gender differences in the percentages of proliferating CD4⁺Foxp3⁻ cells in the spleen, with more proliferating CD4⁺Foxp3⁻ cells in females than males (Figure 12B). However, there were no gender differences in the percentage of CD4⁺Foxp3⁺ cells in the periphery (either LN or spleen) of SJL mice. Interestingly, there were no gender differences in the percentages of proliferating CD4⁺CD25⁺CD103⁺ cells in any of the four strains studied in the LN or spleen (Figure 12C). This indicates that differences in proliferation of these cells *in vivo* do not account for gender differences in peripheral percentages of CD4⁺CD25⁺CD103⁺ cells.

BWF1 mice were the only strain of the four studied where females had decreased percentages of CD4⁺Foxp3⁺ cells in the LN when compared to age- and strain-matched males (Figure 9A). Interestingly, BWF1 mice were also the only strain in which the ratio of proliferating Foxp3⁺:Foxp3⁻ cells was significantly lower than the proliferation ratio in BWF1 males (Figure 12D). These data together suggest that increased proliferation of CD4⁺Foxp3⁻ non-Tregs, which decreases the Foxp3⁺:Foxp3⁻ proliferation ratio, in female BWF1 mice compared to males may contribute to the decreased CD4⁺Foxp3⁺ percentages seen in the LN. Furthermore, although one of the characteristics of CD4⁺CD25⁺CD103⁺ cells is that they proliferate at a much greater rate than

CD4⁺CD25⁺CD103⁻ cells, the lower percentages of these cells in the periphery of females was not due to a lower proliferation rate among these cells.

Summary

In this study, we have found that regulatory:effector T cell ratio and not inherent suppressive function of Tregs, are reduced in the periphery of autoimmune-prone mice. The potent CD4⁺CD25⁺CD103⁺ regulatory T cell subset may be most sensitive to hormonal influence, as these cells are found at lower percentages in the periphery of female mice of all four strains studied in comparison to strain-matched males. Neither a defect in thymic production or differences in homeostatic proliferation could account for the differences in peripheral percentages seen between females and males. Other mechanisms will need to be explored to explain the lower CD4⁺CD25⁺CD103⁺ Treg percentages seen in female mice and will be discussed further in the general discussion.

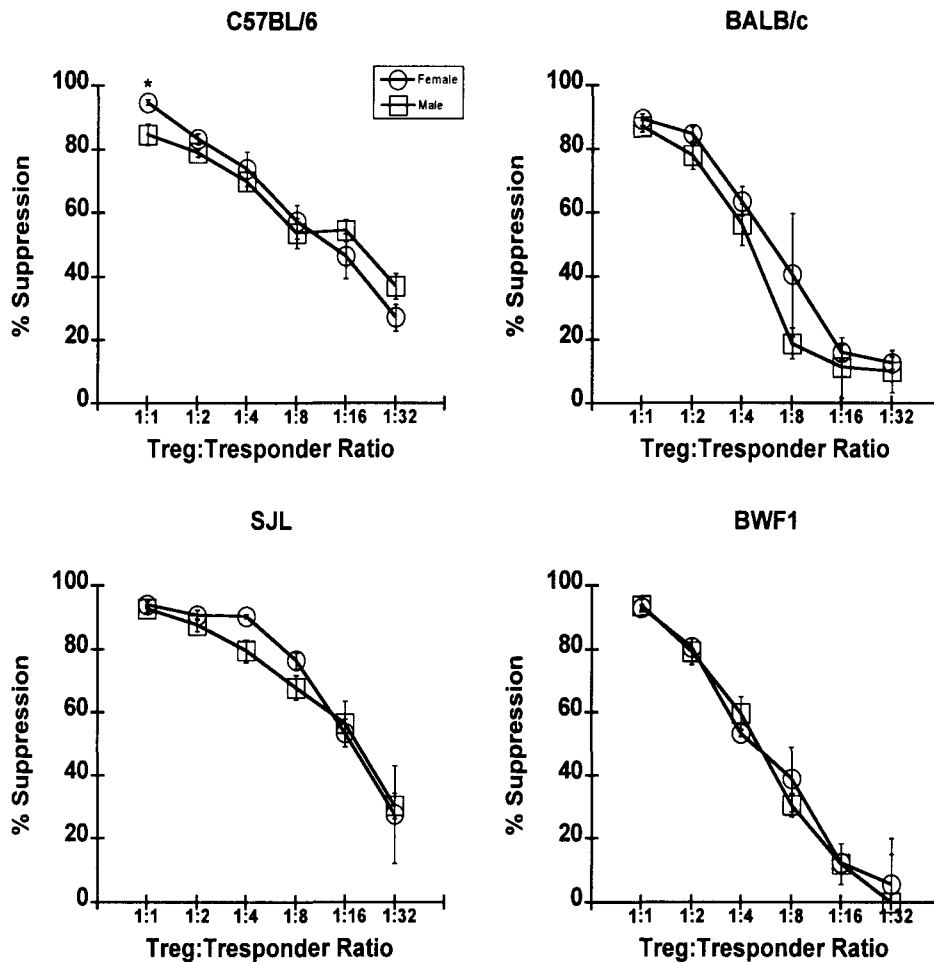
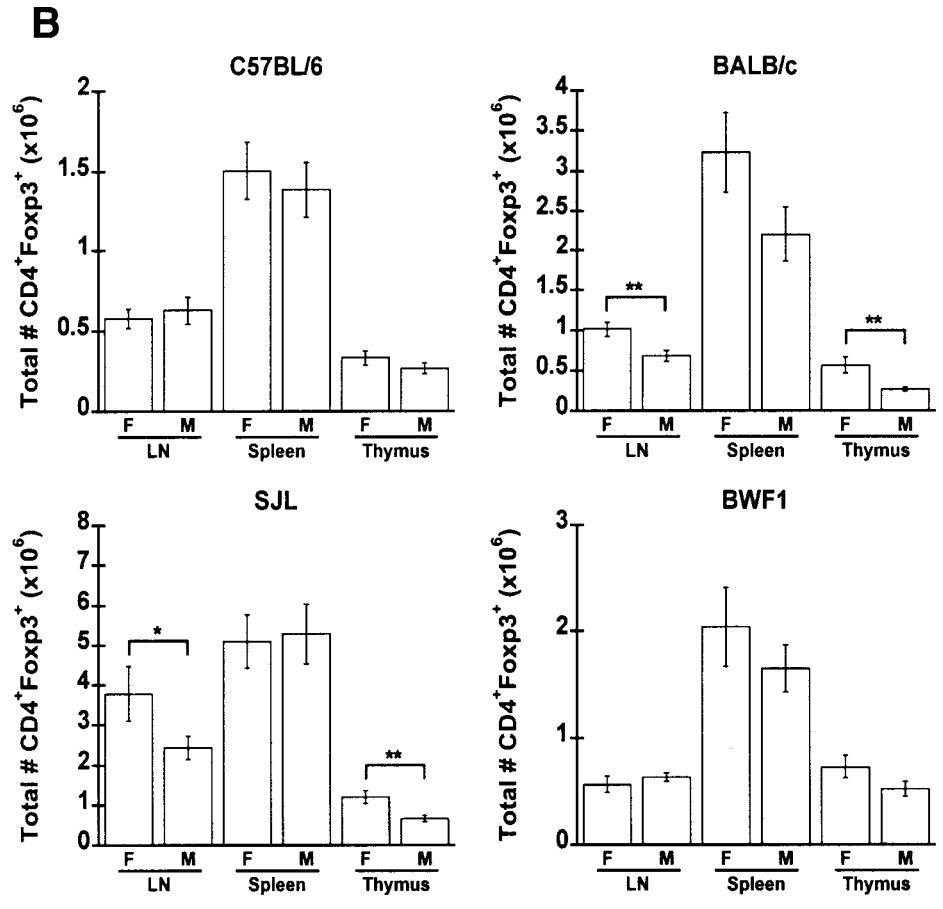
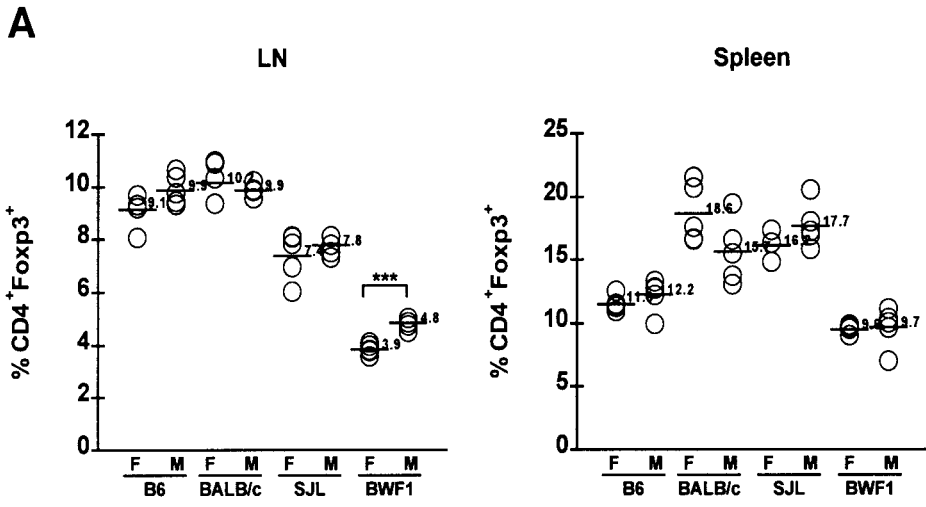


Figure 8. Regulatory function of female vs. male $CD4^+CD25^+$ cells from four strains of mice. $CD4^+CD25^+$ cells were purified from 8-9-week-old female and male C57BL/6, BALB/c, SJL, and BWF1 mice and varying numbers co-cultured with a constant number of syngeneic male $CD4^+CD25^-$ responder cells and male APC and soluble anti-CD3 antibody. % suppression of positive control (responder cells alone) was calculated and the mean \pm SEM presented. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$.



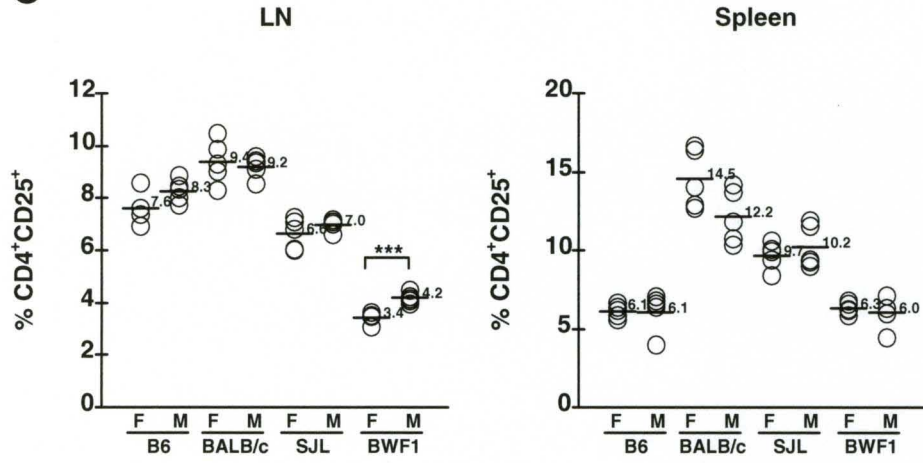
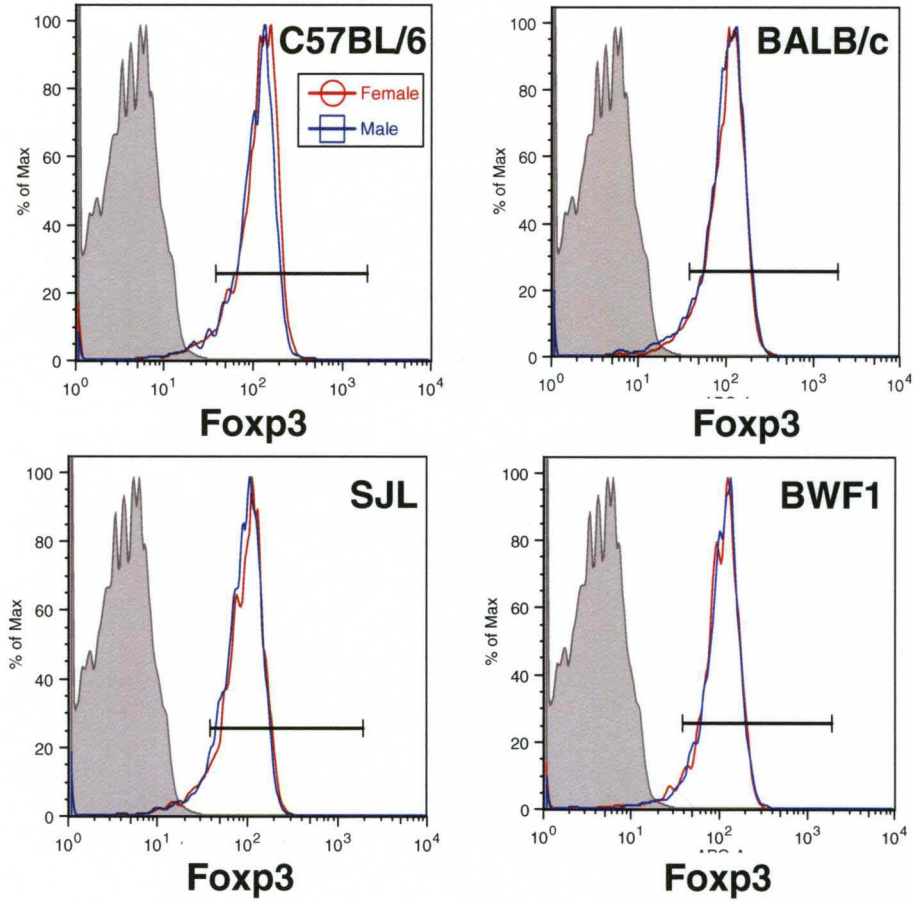
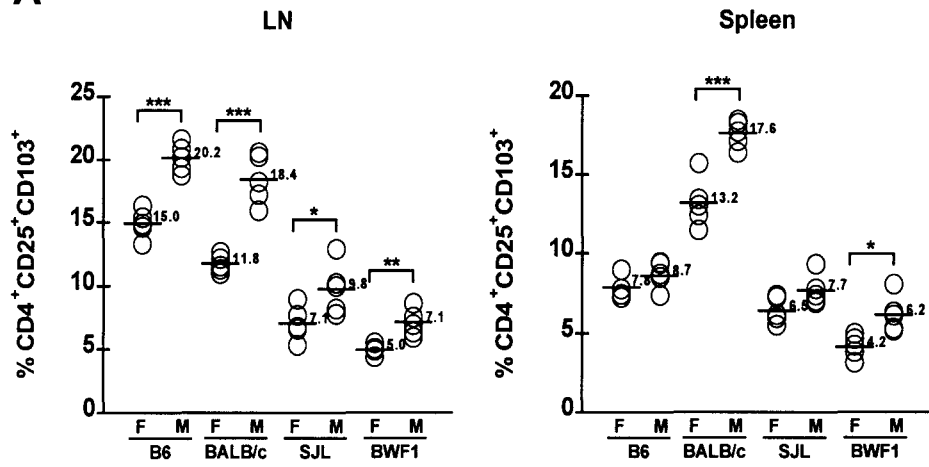
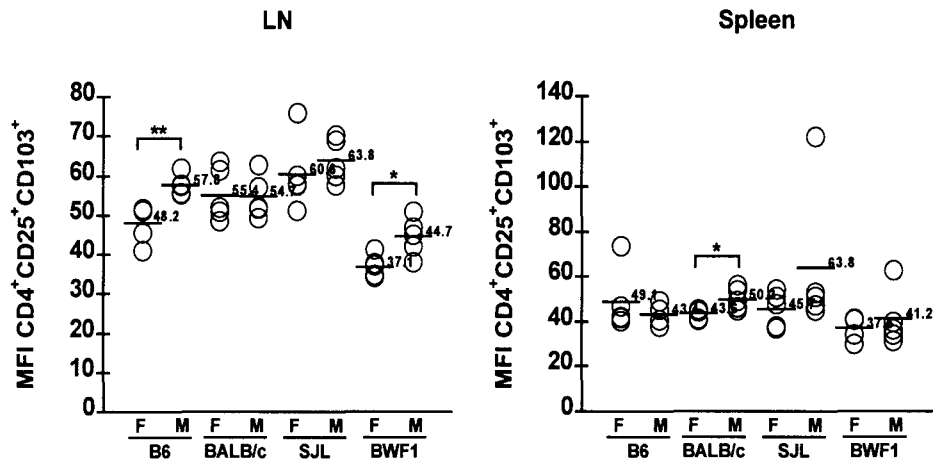
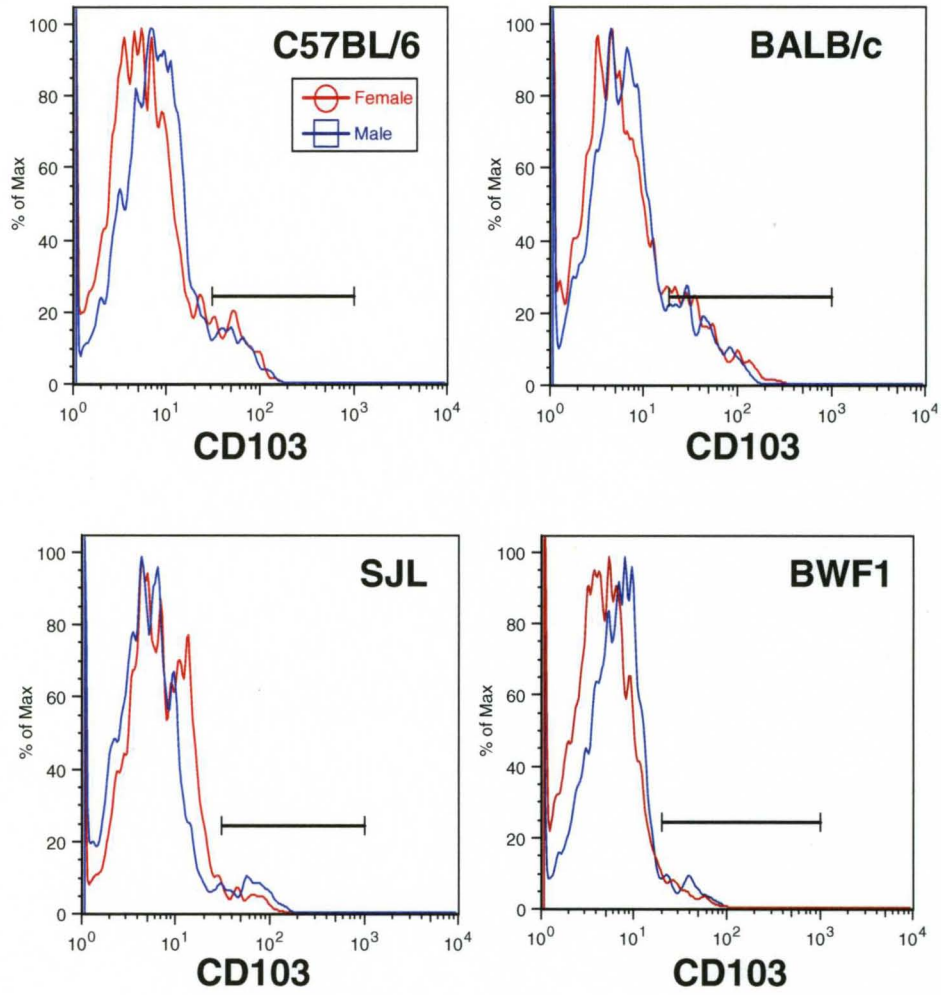
C**D**

Figure 9. Percentages and numbers of CD4⁺Foxp3⁺ cells in four strains of mice. Cells from lymph nodes (LN) and spleen of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, and anti-Foxp3 antibodies and analyzed by FACS[®]. (A) Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing Foxp3 in LN and spleen. (B) The total numbers of CD4⁺Foxp3⁺ cells in lymph nodes, spleen, and thymus are shown. (C) Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing CD25 in LN and spleen. (D) A sample histogram showing Foxp3 expression on CD4⁺CD25⁺ cells in LN is shown. (A, C) A representative experiment of 4-9 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

A**B**

C



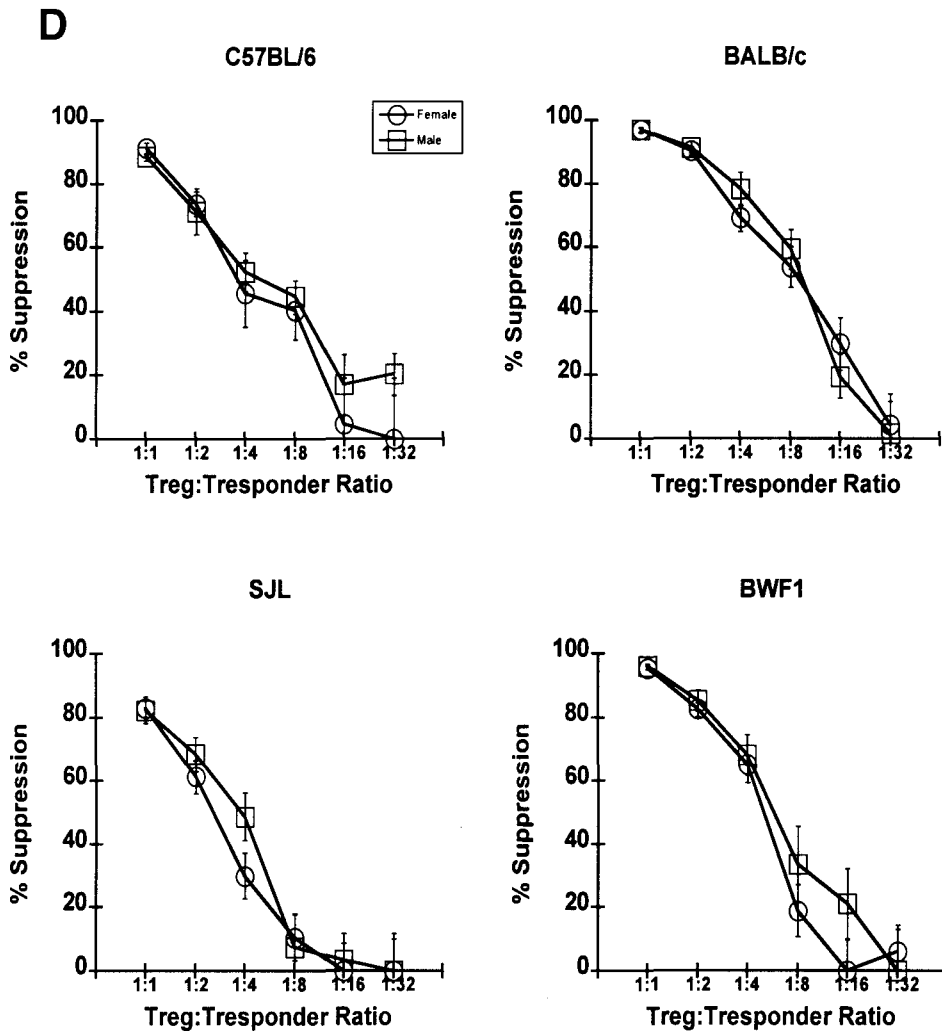


Figure 10. Percentages and suppressive function *in vitro* of CD4⁺CD25⁺CD103⁺ cells in four strains of mice. Cells from lymph nodes (LN) and spleen of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, and anti-CD103 antibodies and analyzed by FACS[®]. (A) Labeled CD4⁺CD25⁺ cells were gated and analyzed for the percentages of cells expressing CD103 in LN and spleen. (B) Labeled CD4⁺CD25⁺ cells were gated and analyzed for the per cell expression of CD103 in LN and spleen. (C) A sample histogram

showing CD103 expression on CD4⁺CD25⁺ cells in LN is shown. (D)

CD4⁺CD25⁺CD103⁺ cells were purified from female and male C57BL/6, BALB/c, SJL, and BWF1 mice and varying numbers co-cultured with a constant number of syngeneic male CD4⁺CD25⁻ responder cells and APC and soluble anti-CD3 antibody. % suppression of positive control (responder cells alone) was calculated and the mean \pm SEM presented. (A,B) A representative experiment of 4-9 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

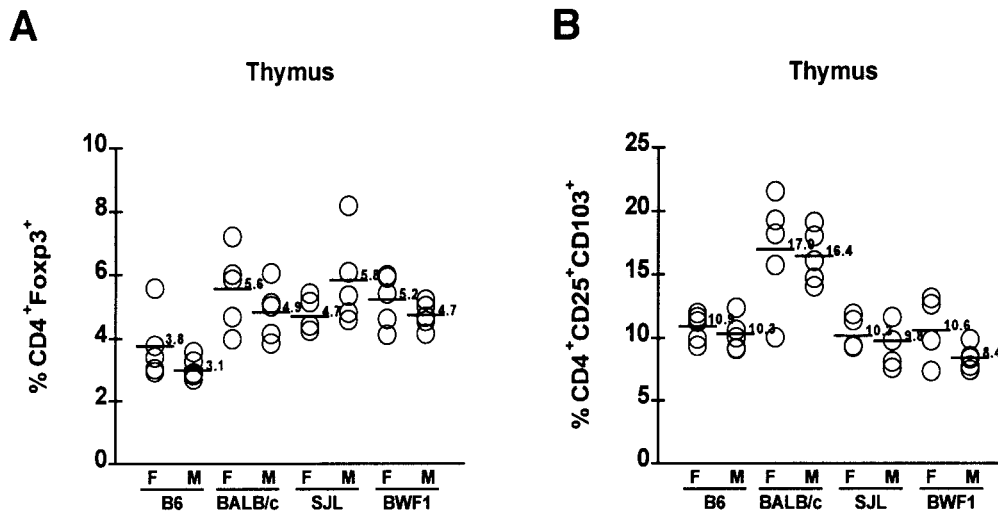
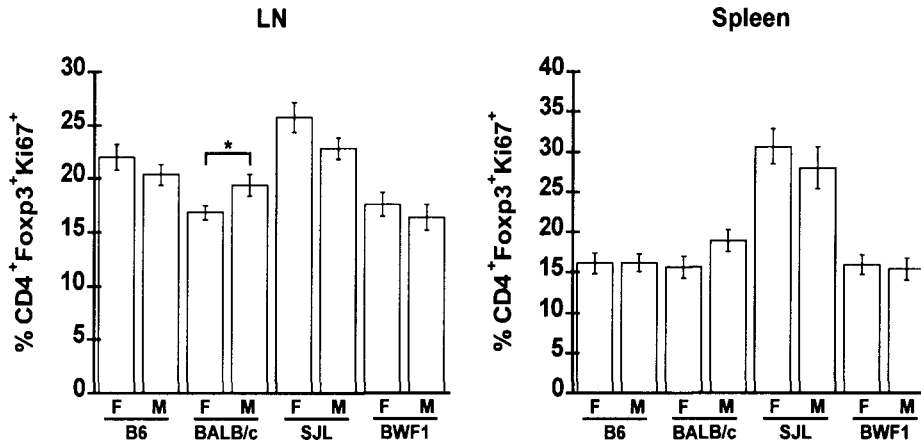
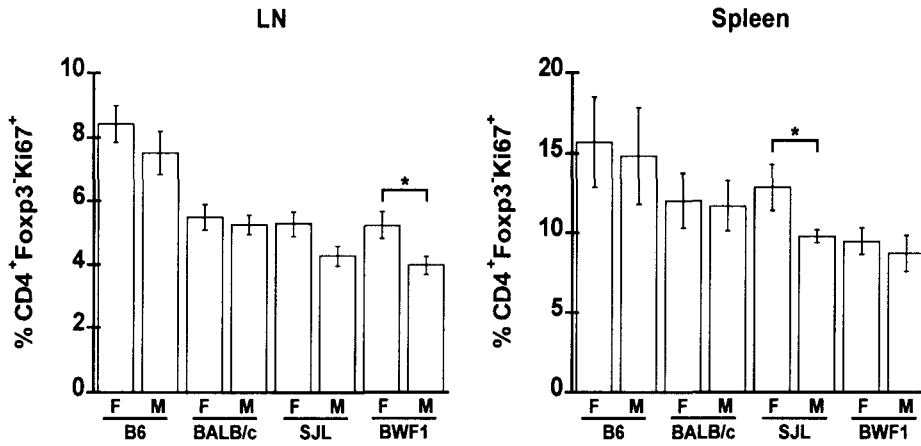
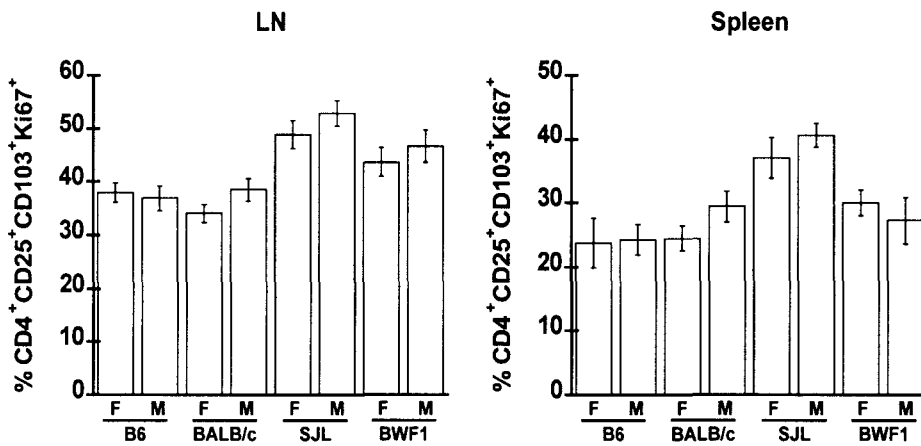


Figure 11. Percentages of CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in thymus in four strains of mice. Cells from lymph nodes (LN) and spleen of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, anti-CD103, and anti-Foxp3 antibodies and analyzed by FACS[®]. Labeled cells were gated and analyzed for (A) percentages of CD4⁺ cells expressing Foxp3 and (B) percentages of CD4⁺CD25⁺ cells expressing CD103. A representative experiment of 4 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

A**B****C**

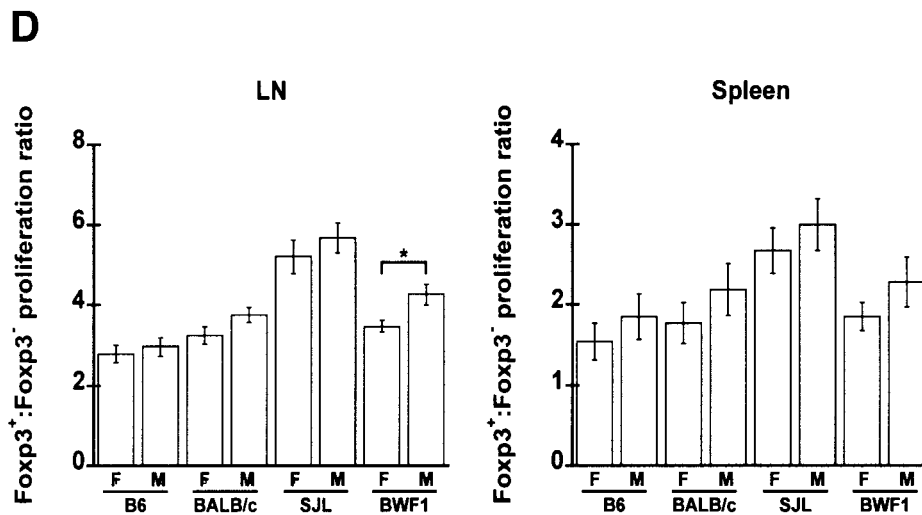


Figure 12. Percentages of proliferating $CD4^+Foxp3^+$, $CD4^+Foxp3^-$ and $CD4^+CD25^+CD103^+$ cells in the periphery of four strains of mice. Cells from lymph nodes (LN) and spleen of 9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, anti-Foxp3, anti-CD103 and anti-Ki67 antibodies and analyzed by FACS[®]. (A) Labeled $CD4^+Foxp3^+$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (B) Labeled $CD4^+Foxp3^-$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (C) Labeled $CD4^+CD25^+CD103^+$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (D) Labeled $CD4^+Foxp3^+$ and $CD4^+Foxp3^-$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. Proliferation ratios were calculated by dividing the percentage of proliferating ($Ki67^+$) $CD4^+Foxp3^+$ cells by the percentage of proliferating $CD4^+Foxp3^-$ cells from LN and spleen. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test (n=15). * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	33.0 ± 2.3	35.4 ± 1.5	33.4 ± 1.8	**38.4 ± 0.4	37.1 ± 3.1	35.1 ± 1.4	31.5 ± 2.6	37.8 ± 0.8	28.8 ± 2.3
	Male	25.5 ± 3.1	32.9 ± 1.6	27.7 ± 2.9	34.5 ± 0.8	34.1 ± 2.8	31.9 ± 1.0	31.4 ± 1.0	37.9 ± 0.8	30.5 ± 1.1
BALB/c	Female	**50.6 ± 1.2	49.9 ± 1.9	51.4 ± 3.8	56.5 ± 0.6	55.9 ± 1.0	53.4 ± 1.5	60.4 ± 1.0		
	Male	44.1 ± 1.4	47.9 ± 2.1	41.0 ± 3.1	55.5 ± 1.0	55.2 ± 0.9	52.7 ± 3.1	58.5 ± 1.0		
SJL	Female	44.2 ± 2.1	52.2 ± 1.2	36.0 ± 5.1	50.6 ± 0.8	25.7 ± 3.2	24.2 ± 3.6	49.3 ± 1.3	51.9 ± 1.6	53.5 ± 0.6
	Male	49.0 ± 3.7	47.6 ± 2.5	47.2 ± 3.1	50.3 ± 0.6	31.6 ± 1.0	28.4 ± 1.6	49.2 ± 1.3	51.9 ± 0.9	53.1 ± 0.7
BWF1	Female	46.1 ± 6.5	59.4 ± 1.9	55.3 ± 4.8	63.6 ± 0.3	65.0 ± 0.7	*62.2 ± 0.9	65.5 ± 0.6	65.1 ± 0.8	
	Male	58.5 ± 3.2	57.3 ± 3.6	55.7 ± 1.0	64.2 ± 2.0	62.0 ± 2.0	58.7 ± 0.3	64.0 ± 0.8	64.5 ± 0.6	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	7.9 ± 0.6	16.5 ± 1.0	11.6 ± 1.0	17.4 ± 0.4	17.3 ± 1.1	23.7 ± 4.4	*19.2 ± 0.5	*19.8 ± 0.4	13.4 ± 1.6
	Male	7.0 ± 0.7	15.3 ± 0.6	12.3 ± 0.5	16.6 ± 1.1	18.0 ± 1.3	18.3 ± 0.5	17.1 ± 0.7	21.4 ± 0.5	16.1 ± 0.6
BALB/c	Female	12.7 ± 1.6	**23.4 ± 0.4	17.0 ± 2.1	**30.8 ± 1.0	29.0 ± 1.0	26.1 ± 1.0	29.8 ± 1.3		
	Male	11.1 ± 0.7	19.4 ± 0.9	13.1 ± 1.0	25.9 ± 0.8	29.7 ± 1.7	26.9 ± 1.2	27.0 ± 0.8		
SJL	Female	13.6 ± 0.7	24.2 ± 2.1	19.2 ± 1.8	31.6 ± 0.9	19.5 ± 1.3	19.7 ± 1.4	28.5 ± 1.0	29.7 ± 1.3	28.6 ± 1.5
	Male	13.3 ± 0.7	24.5 ± 1.2	19.5 ± 1.8	29.1 ± 1.1	20.3 ± 0.7	18.2 ± 0.8	27.5 ± 1.0	27.3 ± 0.6	27.4 ± 0.7
BWF1	Female	12.3 ± 1.0	22.3 ± 1.5	17.0 ± 2.5	31.4 ± 0.8	33.0 ± 0.3	26.0 ± 2.2	*32.2 ± 0.6	31.2 ± 1.8	
	Male	10.1 ± 2.0	21.0 ± 1.7	18.3 ± 2.4	28.6 ± 1.6	33.0 ± 1.4	29.1 ± 1.2	29.8 ± 0.7	31.9 ± 1.3	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	5.9 ± 0.2	6.8 ± 0.4	5.0 ± 0.3	7.5 ± 0.4	*8.7 ± 0.3	*40.2 ± 0.7	**8.2 ± 0.5		*6.8 ± 0.9
	Male	5.5 ± 0.1	7.6 ± 0.2	5.4 ± 0.3	7.9 ± 0.2	7.9 ± 0.2	35.7 ± 1.6	6.5 ± 0.4		9.7 ± 0.7
BALB/c	Female	9.2 ± 0.3	9.7 ± 0.3	**6.0 ± 0.2	12.7 ± 0.6					
	Male	8.2 ± 0.4	10.6 ± 0.2	10.2 ± 1.2	12.0 ± 0.7					
SJL	Female	*20.0 ± 1.5	**20.5 ± 1.7	17.6 ± 0.6	***26.3 ± 0.9	6.1 ± 0.4	35.0 ± 4.2	20.5 ± 1.7	22.5 ± 1.4	
	Male	15.8 ± 1.0	17.6 ± 0.6	18.2 ± 0.6	20.2 ± 0.5	8.5 ± 1.7	24.7 ± 1.5	17.5 ± 0.9	21.9 ± 0.9	
BWF1	Female	8.2 ± 0.7	8.5 ± 0.4	8.1 ± 0.6	**10.4 ± 0.2			10.4 ± 0.2	12.7 ± 0.4	
	Male	9.0 ± 0.4	10.4 ± 0.4	7.9 ± 0.4	12.2 ± 0.4			11.4 ± 0.6	11.9 ± 0.8	

Table 2. Percentages of CD4⁺ cells in four strains of mice. Cells from lymph nodes (LN), spleen, and thymus of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4 antibody and analyzed by FACS[®]. Lymphocytes were gated and analyzed for the percentage of cells expressing CD4 in (A) LN, (B) spleen, and (C) thymus. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	**8.0 ± 0.2	8.2 ± 0.3	9.1 ± 0.3	**10.0 ± 0.3	*10.1 ± 0.4	22.2 ± 3.1	*10.1 ± 0.4	12.5 ± 0.5	8.1 ± 0.8
	Male	9.9 ± 0.5	8.5 ± 0.5	9.9 ± 0.3	12.6 ± 0.5	11.7 ± 0.5	22.1 ± 4.6	12.1 ± 0.7	14.0 ± 1.0	8.0 ± 1.0
BALB/c	Female	8.4 ± 0.4	10.2 ± 0.2	10.2 ± 0.4	11.7 ± 0.1	12.3 ± 0.6	*7.3 ± 0.7	12.6 ± 0.7		
	Male	9.5 ± 0.5	10.2 ± 0.4	9.9 ± 0.1	11.6 ± 0.5	12.9 ± 0.4	9.7 ± 0.7	13.3 ± 0.7		
SJL	Female	6.7 ± 0.4	7.0 ± 0.2	7.4 ± 0.4	9.4 ± 0.3	8.3 ± 0.4	13.1 ± 0.8	12.2 ± 1.0	11.0 ± 1.3	7.7 ± 0.2
	Male	7.1 ± 0.2	7.1 ± 0.4	7.8 ± 0.2	9.9 ± 0.3	8.6 ± 0.6	10.1 ± 1.2	12.0 ± 0.5	12.2 ± 0.4	7.5 ± 0.4
BWF1	Female	4.8 ± 0.8	*4.0 ± 0.1	***3.9 ± 0.1	**4.9 ± 0.1	4.5 ± 0.4	*4.6 ± 0.1	**6.8 ± 0.4	**4.7 ± 0.2	
	Male	4.1 ± 0.1	4.5 ± 0.2	4.8 ± 0.1	5.5 ± 0.1	4.9 ± 0.3	5.3 ± 0.2	8.8 ± 0.3	6.1 ± 0.3	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	10.7 ± 0.6	7.5 ± 0.4	11.6 ± 0.3	10.9 ± 0.3	11.1 ± 0.7	28.5 ± 6.5	**11.4 ± 0.4	13.6 ± 0.4	9.9 ± 0.9
	Male	12.5 ± 0.9	6.8 ± 0.4	12.2 ± 0.6	10.9 ± 0.2	11.1 ± 0.8	18.2 ± 1.9	14.8 ± 0.9	13.6 ± 0.5	9.6 ± 0.8
BALB/c	Female	15.9 ± 0.7	11.0 ± 0.2	18.6 ± 1.0	14.0 ± 0.2	14.2 ± 0.3	*11.8 ± 0.7	15.3 ± 1.5		
	Male	15.5 ± 1.7	9.9 ± 0.5	15.7 ± 1.1	14.6 ± 0.6	13.4 ± 0.5	8.9 ± 0.7	15.9 ± 0.2		
SJL	Female	22.2 ± 1.5	12.4 ± 0.7	16.2 ± 0.6	**17.0 ± 0.4	19.3 ± 0.8	20.9 ± 0.8	22.8 ± 0.9	21.4 ± 0.7	14.9 ± 0.6
	Male	18.3 ± 1.4	12.7 ± 0.8	17.7 ± 0.8	18.7 ± 0.3	18.2 ± 0.6	19.3 ± 0.6	21.6 ± 0.4	22.4 ± 1.5	15.3 ± 0.4
BWF1	Female	11.2 ± 1.0	*6.6 ± 0.2	9.6 ± 0.2	9.8 ± 0.4	8.4 ± 0.2	12.3 ± 0.7	7.8 ± 0.3	9.0 ± 0.2	
	Male	10.4 ± 0.9	7.4 ± 0.3	9.7 ± 0.7	10.4 ± 0.4	8.7 ± 0.5	11.4 ± 0.5	8.7 ± 0.5	8.9 ± 0.4	

Table 3C: Thymus % CD4 ⁺ Foxp3 ⁺										
Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	1.5 ± 0.2	3.8 ± 0.5	2.3 ± 0.2	3.8 ± 0.2	2.8 ± 0.1	9.2 ± 0.7	**6.7 ± 0.2		0.5 ± 0.1
	Male	1.1 ± 0.1	3.1 ± 0.2	2.4 ± 0.2	4.0 ± 0.1	2.7 ± 0.2	11.1 ± 1.4	5.3 ± 0.2		0.4 ± 0.1
BALB/c	Female	1.8 ± 0.4	5.6 ± 0.6	4.0 ± 0.2	***6.8 ± 0.3					
	Male	2.0 ± 0.1	4.9 ± 0.4	3.1 ± 0.3	4.5 ± 0.2					
SJL	Female	2.5 ± 0.3	4.7 ± 0.2	*3.7 ± 0.2	***7.5 ± 0.3	12.8 ± 0.4	5.5 ± 0.2	7.2 ± 0.3	7.3 ± 0.5	
	Male	2.6 ± 0.1	5.8 ± 0.6	4.7 ± 0.3	5.6 ± 0.2	12.9 ± 0.6	6.0 ± 0.2	7.5 ± 0.3	6.3 ± 0.4	
BWF1	Female	3.3 ± 0.2	5.2 ± 0.4	4.8 ± 0.2	6.7 ± 0.3			5.6 ± 0.2	7.9 ± 0.3	
	Male	3.2 ± 0.4	4.7 ± 0.2	5.0 ± 0.3	6.3 ± 0.3			5.7 ± 0.2	7.0 ± 0.3	

Table 3. Percentages of CD4⁺ cells that are Foxp3⁺ in four strains of mice. Cells from lymph nodes (LN), spleen, and thymus of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4 and anti-Foxp3 antibodies and analyzed by FACS[®]. Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing Foxp3 in (A) LN, (B) spleen, and (C) thymus. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		0.400 ± 0.037	0.462 ± 0.052	0.751 ± 0.060	0.714 ± 0.050				
	Male		0.467 ± 0.086	0.432 ± 0.035	0.845 ± 0.126	0.611 ± 0.049				
BALB/c	Female		0.741 ± 0.097	*1.123 ± 0.107	1.113 ± 0.123			1.212 ± 0.128		
	Male		0.482 ± 0.055	0.578 ± 0.073	0.877 ± 0.089			1.177 ± 0.149		
SJL	Female		2.811 ± 0.290	1.461 ± 0.200	*5.783 ± 0.745	2.683 ± 0.248				
	Male		1.824 ± 0.292	1.821 ± 0.099	3.191 ± 0.455	1.841 ± 0.267				
BWF1	Female		0.380 ± 0.030	*0.399 ± 0.061	0.785 ± 0.096	0.704 ± 0.083		1.240 ± 0.151	0.902 ± 0.067	
	Male		0.473 ± 0.100	0.697 ± 0.035	0.696 ± 0.029	0.966 ± 0.232		1.516 ± 0.176	0.955 ± 0.132	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		1.033 ± 0.144	0.994 ± 0.150	2.094 ± 0.055					
	Male		0.778 ± 0.100	1.124 ± 0.120	1.914 ± 0.136					
BALB/c	Female		**1.806 ± ± 0.188	1.922 ± 0.535	***4.882 ± ± 0.201			3.740 ± 0.695		
	Male		0.887 ± 0.064	1.647 ± 0.161	3.336 ± 0.131			3.639 ± 0.294		
SJL	Female		3.238 ± 0.837	3.580 ± 0.800	7.116 ± 0.435					
	Male		3.158 ± 0.567	3.442 ± 0.582	7.683 ± 0.445					
BWF1	Female		0.937 ± 0.211	1.140 ± 0.218	*3.244 ± 0.258	2.335 ± 0.179		2.213 ± 0.115	*2.177 ± 0.120	
	Male		1.054 ± 0.175	1.017 ± 0.068	2.391 ± 0.104	2.424 ± 0.080		2.391 ± 0.212	1.808 ± 0.084	

Table 4C: Thymus CD4 ⁺ Foxp3 ⁺ Cell #s (x10 ⁶)										
Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		0.286 ± 0.047	0.177 ± 0.032	0.456 ± 0.030	0.352 ± 0.015				
	Male		0.197 ± 0.025	0.186 ± 0.043	0.362 ± 0.029	0.265 ± 0.044				
BALB/c	Female		0.364 ± 0.076	0.233 ± 0.020	***0.892 ± 0.045					
	Male		0.260 ± 0.029	0.173 ± 0.042	0.342 ± 0.030					
SJL	Female		0.900 ± 0.271	0.784 ± 0.086	**1.632 ± 0.152	*1.286 ± 0.141				
	Male		0.398 ± 0.062	0.555 ± 0.161	0.897 ± 0.088	0.756 ± 0.089				
BWF1	Female		0.527 ± 0.091	0.467 ± 0.064	**1.010 ± 0.136			1.039 ± 0.062	**1.465 ± 0.101	
	Male		0.442 ± 0.093	0.671 ± 0.208	0.487 ± 0.066			1.030 ± 0.076	0.878 ± 0.107	

Table 4. Numbers of CD4⁺ cells that are Foxp3⁺ in four strains of mice. Cells from lymph nodes (LN), spleen, and thymus of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4 and anti-Foxp3 antibodies and analyzed by FACS[®]. The total numbers of CD4⁺ cells that express Foxp3 in (A) lymph nodes, (B) spleen, and (C) thymus are shown. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8
C57BL/6	Female	4.8 ± 0.3	5.1 ± 0.4	7.6 ± 0.7	7.1 ± 0.7				
	Male	5.0 ± 0.8	4.3 ± 0.4	6.7 ± 0.9	5.3 ± 0.5				
BALB/c	Female	7.3 ± 0.8	**10.7 ± 0.5	9.5 ± 1.0			9.7 ± 0.8		
	Male	4.8 ± 0.5	5.8 ± 0.7	7.5 ± 0.6			8.8 ± 0.8		
SJL	Female	*40.4 ± 3.4	19.7 ± 1.3	**62.2 ± 8.1	**32.3 ± 1.8				
	Male	23.4 ± 3.7	23.0 ± 1.9	31.8 ± 3.5	21.0 ± 1.7				
BWF1	Female	9.5 ± 0.6	10.4 ± 1.6	16.0 ± 1.8	15.6 ± 1.2		18.1 ± 1.6	19.2 ± 1.5	
	Male	10.2 ± 2.1	14.6 ± 1.2	12.6 ± 0.5	19.4 ± 4.1		17.1 ± 1.7	15.4 ± 1.4	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8
C57BL/6	Female	12.7 ± 1.4	8.4 ± 1.3	19.3 ± 0.7					
	Male	11.6 ± 1.5	8.8 ± 1.1	18.0 ± 1.2					
BALB/c	Female	*16.6 ± 1.4	9.5 ± 2.4	***35.0 ± 1.6			23.9 ± 3.1		
	Male	9.5 ± 0.8	9.5 ± 0.5	22.9 ± 1.2			22.8 ± 1.7		
SJL	Female	24.1 ± 5.1	21.2 ± 5.2	42.1 ± 3.0					
	Male	23.1 ± 3.4	19.8 ± 4.1	41.2 ± 2.7					
BWF1	Female	13.9 ± 3.2	11.8 ± 2.0	***33.0 ± 1.5	27.9 ± 2.2		29.0 ± 0.9	24.3 ± 1.7	
	Male	13.8 ± 1.6	10.0 ± 1.0	23.0 ± 0.7	28.2 ± 2.1		27.3 ± 1.8	20.6 ± 1.6	

Table 5C: Thymus CD4 ⁺ CD8 ⁻ Cell #s (x10 ⁶)									
Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8
C57BL/6	Female	8.2 ± 0.8	7.1 ± 1.4	**12.1 ± 0.6	*12.5 ± 0.5				
	Male	6.1 ± 0.5	7.5 ± 0.5	9.0 ± 0.6	9.6 ± 0.9				
BALB/c	Female	6.8 ± 0.7	5.7 ± 0.7	***13.2 ± 0.9					
	Male	5.6 ± 0.6	5.7 ± 1.1	7.6 ± 0.6					
SJL	Female	*18.7 ± 3.9	20.8 ± 1.5	*21.9 ± 2.1	*10.0 ± 1.0				
	Male	6.2 ± 0.2	12.1 ± 4.0	16.0 ± 1.2	5.9 ± 0.7				
BWF1	Female	10.6 ± 0.6	9.4 ± 0.8	**14.9 ± 1.5			18.7 ± 0.5	*18.5 ± 1.3	
	Male	8.8 ± 1.7	13.0 ± 3.2	7.7 ± 1.0			18.0 ± 1.2	12.6 ± 1.4	

Table 5. Numbers of CD4⁺ cells in four strains of mice. Cells from lymph nodes (LN), spleen, and thymus of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4 antibody and analyzed by FACS[®]. The total numbers of CD4⁺ in (A) lymph nodes, (B) spleen, and (C) thymus are shown. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	126.2 ± 2.9	120.6 ± 2.2	169.4 ± 3.9	*340.4 ± 7.0	535.8 ± 6.3	497.1 ± 7.5	*247.5 ± 4.8	312.5 ± 9.0	549.0 ± 7.2
	Male	123.2 ± 1.2	121.8 ± 3.6	162.2 ± 6.2	366.8 ± 7.8	520.1 ± 10.7	541.0 ± 36.1	227.4 ± 7.1	429.6 ± 97.6	676.3 ± 66.1
BALB/c	Female	112.0 ± 2.7	122.4 ± 1.8	153.4 ± 1.8	*347.0 ± 4.1	433.2 ± 14.9	407.8 ± 21.8	65.0 ± 1.9		
	Male	117.0 ± 2.3	123.2 ± 1.9	150.0 ± 3.8	299.6 ± 14.3	471.8 ± 13.2	421.6 ± 32.9	66.8 ± 2.9		
SJL	Female	109.8 ± 1.8	125.8 ± 2.6	147.8 ± 4.8	314.7 ± 14.0	462.0 ± 14.8	747.9 ± 37.0	304.0 ± 3.7	159.8 ± 5.4	528.7 ± 8.1
	Male	112.6 ± 2.2	118.6 ± 5.2	148.6 ± 3.2	346.2 ± 6.8	450.5 ± 3.7	697.4 ± 37.4	323.6 ± 7.9	147.2 ± 6.1	558.9 ± 14.4
BWF1	Female	119.2 ± 2.5	123.8 ± 2.8	**152.4 ± 2.8	342.2 ± 2.0	289.0 ± 2.7	348.9 ± 15.8	160.5 ± 3.8	**250.7 ± 9.5	
	Male	119.0 ± 1.1	125.6 ± 3.7	164.0 ± 1.4	333.0 ± 14.1	287.7 ± 9.1	357.9 ± 19.3	265.3 ± 73.6	386.9 ± 39.3	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	98.9 ± 2.1	93.5 ± 3.7	139.4 ± 3.9	282.2 ± 11.0	*567.2 ± 11.9	885.7 ± 346.1	271.4 ± 6.8	324.5 ± 8.0	547.3 ± 22.8
	Male	98.6 ± 1.1	89.9 ± 1.2	126.4 ± 4.3	279.8 ± 7.7	506.3 ± 22.8	538.8 ± 9.3	298.1 ± 20.8	300.3 ± 8.2	536.9 ± 7.7
BALB/c	Female	99.9 ± 1.5	92.3 ± 2.0	131.0 ± 3.7	296.1 ± 3.7	403.4 ± 13.2	378.6 ± 4.1	64.6 ± 3.5		
	Male	100.4 ± 1.5	87.0 ± 3.8	119.6 ± 4.8	267.0 ± 13.0	381.2 ± 16.5	356.9 ± 19.6	62.7 ± 1.0		
SJL	Female	***104.2 ± 1.0	91.4 ± 2.2	126.2 ± 3.6	*265.2 ± 9.7	456.8 ± 16.1	694.6 ± 24.9	301.4 ± 8.2	158.0 ± 5.3	560.2 ± 14.7
	Male	98.3 ± 0.4	84.7 ± 3.2	125.8 ± 2.8	290.6 ± 3.1	440.1 ± 22.8	667.7 ± 17.9	286.6 ± 6.0	153.6 ± 3.0	545.1 ± 23.7
BWF1	Female	99.6 ± 1.4	86.9 ± 1.8	126.4 ± 3.3	253.6 ± 4.4	268.5 ± 9.4	349.2 ± 11.6	209.2 ± 13.0	*227.4 ± 4.6	
	Male	97.6 ± 1.2	94.0 ± 3.1	127.0 ± 3.0	265.1 ± 4.4	286.6 ± 5.0	321.5 ± 9.0	209.0 ± 9.0	349.3 ± 38.7	

Table 6. Per cell expression of Foxp3 on CD4⁺ cells in four strains of mice. Cells from lymph nodes (LN) and spleen of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4 and anti-Foxp3 antibodies and analyzed by FACS[®]. Labeled CD4⁺ cells were gated and analyzed for the per cell expression (MFI) of Foxp3 in (A) LN and (B) spleen. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Table 7A: LN CD4 ⁺ CD25 ⁺ CD103 ⁺ Cell #s (x10 ⁶)										
Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		0.052 ± 0.010	0.059 ± 0.008	0.085 ± 0.007	0.120 ± 0.018				
	Male		0.076 ± 0.008	0.077 ± 0.005	0.136 ± 0.023	0.122 ± 0.007				
BALB/c	Female		0.151 ± 0.016	0.194 ± 0.030	0.107 ± 0.014			0.105 ± 0.013		
	Male		0.108 ± 0.013	0.131 ± 0.023	0.140 ± 0.017			0.117 ± 0.015		
SJL	Female		0.398 ± 0.096	0.164 ± 0.014	0.322 ± 0.064	0.187 ± 0.012				
	Male		0.227 ± 0.035	0.159 ± 0.005	0.255 ± 0.058	0.172 ± 0.038				
BWF1	Female		0.024 ± 0.002	*0.019 ± 0.003	0.031 ± 0.004	0.016 ± 0.002		0.042 ± 0.005	0.059 ± 0.007	
	Male		0.033 ± 0.008	0.052 ± 0.007	0.039 ± 0.004	0.024 ± 0.005		0.058 ± 0.008	0.066 ± 0.008	

Table 7B: Spleen CD4 ⁺ CD25 ⁺ CD103 ⁺ Cell #s (x10 ⁶)										
Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		0.026 ± 0.004	0.032 ± 0.006	0.095 ± 0.007					
	Male		0.013 ± 0.004	0.047 ± 0.009	0.077 ± 0.007					
BALB/c	Female		*0.234 ± 0.030	0.282 ± 0.077	0.470 ± 0.038			0.224 ± 0.030		
	Male		0.120 ± 0.027	0.203 ± 0.015	0.437 ± 0.023			0.197 ± 0.016		
SJL	Female		**0.268 ± 0.015	0.240 ± 0.035	*0.248 ± 0.019					
	Male		0.133 ± 0.025	0.153 ± 0.009	0.317 ± 0.013					
BWF1	Female		0.036 ± 0.007	0.030 ± 0.008	0.086 ± 0.009	0.031 ± 0.010		0.144 ± 0.010	**0.120 ± 0.009	
	Male		0.016 ± 0.002	0.032 ± 0.008	0.084 ± 0.011	0.040 ± 0.002		0.123 ± 0.007	0.087 ± 0.002	

Table 7C: Thymus CD4 ⁺ CD25 ⁺ CD103 ⁺ Cell #s (x10 ⁶)										
Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		*0.022 ± 0.001	0.010 ± 0.003	0.057 ± 0.006					
	Male		0.011 ± 0.003	0.017 ± 0.002	0.041 ± 0.004					
BALB/c	Female		0.069 ± 0.014	*0.065 ± 0.010	**0.166 ± 0.028					
	Male		0.041 ± 0.009	0.034 ± 0.003	0.063 ± 0.005					
SJL	Female		0.147 ± 0.060	0.107 ± 0.018	*0.176 ± 0.023					
	Male		0.068 ± 0.009	0.076 ± 0.025	0.101 ± 0.015					
BWF1	Female		0.032 ± 0.008	0.028 ± 0.003	*0.105 ± 0.021					
	Male		0.040 ± 0.008	0.029 ± 0.003	0.040 ± 0.007					

Table 7. Numbers of CD4⁺CD25⁺CD103⁺ cells in four strains of mice. Cells from lymph nodes (LN), spleen, and thymus of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, and anti-CD103 antibodies and analyzed by FACS[®]. Labeled CD4⁺CD25⁺ cells (CD4⁺CD8⁺CD25⁺ cells in the thymus) were gated and numbers of cells expressing CD103 were determined in (A) LN, (B) spleen, and (C) thymus. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	**48.2 ± 2.1	82.1 ± 5.1	91.1 ± 3.3	*54.3 ± 1.1	*55.8 ± 1.8	78.9 ± 2.0	49.4 ± 0.9	**43.3 ± 1.3	65.8 ± 1.5
	Male	57.8 ± 1.1	90.5 ± 6.6	93.8 ± 6.7	57.7 ± 0.9	61.6 ± 1.3	85.2 ± 3.7	54.4 ± 4.3	48.6 ± 0.8	62.7 ± 2.3
BALB/c	Female	55.4 ± 3.0	**69.9 ± 2.4	94.5 ± 4.9	51.6 ± 0.5	48.9 ± 1.0	*63.9 ± 3.7	273.1 ± 21.0		
	Male	54.7 ± 2.4	79.5 ± 1.2	111.0 ± 7.1	55.1 ± 2.4	46.8 ± 3.9	52.0 ± 2.5	304.3 ± 15.7		
SJL	Female	60.6 ± 4.1	**78.2 ± 4.0	*146.0 ± 8.5	56.4 ± 1.8	70.2 ± 2.7	66.9 ± 2.8	**80.5 ± 0.9	**58.7 ± 2.7	*49.7 ± 0.9
	Male	63.8 ± 2.5	98.5 ± 1.2	119.0 ± 4.5	61.2 ± 1.6	79.6 ± 5.7	76.3 ± 3.3	95.7 ± 3.2	70.6 ± 1.0	53.7 ± 1.5
BWF1	Female	*37.1 ± 1.2	49.4 ± 2.5	70.4 ± 6.3	45.0 ± 0.8	29.9 ± 2.7	***44.3 ± 0.9	422.4 ± 20.1	**34.4 ± 1.2	
	Male	44.7 ± 2.2	55.7 ± 3.9	81.7 ± 3.1	44.8 ± 0.5	40.1 ± 3.9	52.8 ± 1.2	457.9 ± 14.8	38.5 ± 0.4	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	49.1 ± 6.3	**60.1 ± 2.5	43.4 ± 1.1	31.8 ± 1.0	**36.1 ± 1.0	77.6 ± 20.9	31.6 ± 0.7	28.1 ± 0.6	46.8 ± 10.1
	Male	43.4 ± 2.5	74.5 ± 2.9	47.7 ± 4.0	33.6 ± 1.4	41.0 ± 1.0	56.9 ± 1.6	30.3 ± 1.6	28.5 ± 0.4	50.3 ± 3.5
BALB/c	Female	*43.6 ± 1.0	*69.4 ± 1.6	73.1 ± 7.1	*35.6 ± 0.2	37.4 ± 0.6	34.8 ± 1.1	145.0 ± 6.1		
	Male	50.3 ± 2.2	89.7 ± 8.1	100.6 ± 18.5	36.7 ± 0.3	38.1 ± 0.4	33.5 ± 0.4	155.6 ± 13.2		
SJL	Female	45.7 ± 3.5	74.2 ± 6.4	61.4 ± 3.4	*36.2 ± 0.6	46.2 ± 0.7	59.5 ± 4.9	57.2 ± 1.2	42.5 ± 0.4	35.1 ± 0.6
	Male	63.8 ± 14.6	70.0 ± 7.2	52.6 ± 2.3	38.0 ± 0.4	44.7 ± 2.7	50.0 ± 1.1	60.9 ± 3.6	44.1 ± 0.9	34.5 ± 0.5
BWF1	Female	37.6 ± 2.3	*55.2 ± 4.0	46.6 ± 2.3	**30.0 ± 0.7	21.7 ± 0.9	*33.4 ± 1.1	350.5 ± 3.4	22.5 ± 0.6	
	Male	41.2 ± 5.6	81.0 ± 7.4	100.1 ± 49.6	32.6 ± 0.3	22.3 ± 1.6	38.5 ± 1.0	348.4 ± 11.1	23.1 ± 0.6	

Table 8. Per cell expression of CD103 of CD4⁺CD25⁺ cells in four strains of mice. Cells from lymph nodes (LN) and spleen of 8-9-week-old female and male C57BL/6 (B6), BALB/c, SJL and (NZBxNZW)F1 (BWF1) mice were labeled with anti-CD4, anti-CD25, and anti-CD103 antibodies and analyzed by FACS[®]. Labeled CD4⁺CD25⁺ cells were gated and analyzed for the per cell expression (MFI) of CD103 in (A) LN and (B) spleen. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Table 9A: Thymic Weights (mg)

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female	51.0 ± 4.1	51.6 ± 3.8	47.8 ± 1.4		*52.4 ± 2.0				
	Male	54.4 ± 1.8	40.6 ± 4.8	44.8 ± 3.0		41.4 ± 3.0				
BALB/c	Female	34.4 ± 2.0	37.2 ± 1.6	**40.0 ± 1.7						
	Male	33.4 ± 3.3	35.4 ± 3.2	26.8 ± 3.3						
SJL	Female	*53.2 ± 4.7	**49.4 ± 4.0	47.2 ± 3.0		***69.2 ± 2.0				
	Male	36.0 ± 3.0	28.8 ± 4.0	37.2 ± 4.9		35.3 ± 2.8				
BWF1	Female	*78.4 ± 4.3	**80.4 ± 3.1	*81.6 ± 4.6					***69.2 ± 2.5	
	Male	59.4 ± 4.0	66.4 ± 2.0	66.6 ± 3.3					47.8 ± 2.6	

Table 9B: LN Cell #s (x10⁶)

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		17.8 ± 1.9	*16.9 ± 0.9	20.8 ± 1.9	21.0 ± 1.3				
	Male		19.9 ± 3.4	21.5 ± 1.2	21.1 ± 3.1	17.8 ± 0.4				
BALB/c	Female		*19.7 ± 1.8	22.8 ± 2.3	17.8 ± 1.9			17.2 ± 1.6		
	Male		13.5 ± 1.1	19.3 ± 2.7	14.4 ± 1.2			15.8 ± 1.5		
SJL	Female		*99.7 ± 11.2	56.5 ± 4.0	**133.9 ± 17.7	*156.6 ± 24.1				
	Male		65.8 ± 3.9	51.0 ± 2.6	68.2 ± 7.5	77.6 ± 4.9				
BWF1	Female		20.7 ± 1.8	26.4 ± 1.2	26.4 ± 2.9	29.0 ± 1.1		31.3 ± 2.9	30.9 ± 2.4	
	Male		22.7 ± 1.8	29.7 ± 2.7	21.6 ± 0.7	34.6 ± 8.5		31.8 ± 3.2	25.3 ± 2.5	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		117.7 ± 9.2	107.0 ± 4.2	120.7 ± 4.1					
	Male		120.3 ± 13.1	118.3 ± 8.4	116.1 ± 4.1					
BALB/c	Female		117.7 ± 15.4	**100.3 ± 5.6	***125.0 ± 1.6			97.6 ± 12.7		
	Male		93.3 ± 8.0	134.7 ± 4.3	97.4 ± 3.1			89.0 ± 6.9		
SJL	Female		192.3 ± 11.9	*161.7 ± 9.8	152.3 ± 8.5					
	Male		199.0 ± 26.2	252.3 ± 20.1	161.8 ± 3.5					
BWF1	Female		145.3 ± 14.7	91.3 ± 7.7	*116.5 ± 5.9	90.2 ± 6.9		105.6 ± 3.9	*82.1 ± 3.3	
	Male		104.0 ± 16.1	95.7 ± 4.9	92.5 ± 7.1	92.4 ± 4.4		107.0 ± 8.0	67.6 ± 4.0	

Strains	Gender	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7	Expt. #8	Expt. #9
C57BL/6	Female		**146.3 ± 6.2	160.0 ± 12.9	**180.9 ± 8.9	153.2 ± 10.0				
	Male		93.3 ± 8.3	157.2 ± 14.1	128.7 ± 8.5	129.4 ± 10.7				
BALB/c	Female		*89.2 ± 4.0	115.2 ± 13.3	**119.5 ± 11.7					
	Male		66.7 ± 5.8	66.8 ± 21.2	72.6 ± 6.0					
SJL	Female		122.7 ± 33.1	129.6 ± 13.8	93.9 ± 10.6	**182.4 ± 13.6				
	Male		45.0 ± 1.6	77.6 ± 23.6	89.3 ± 8.9	86.0 ± 18.7				
BWF1	Female		158.0 ± 10.8	152.0 ± 22.7	**158.4 ± 14.3			188.2 ± 6.2	*148.2 ± 9.6	
	Male		108.8 ± 22.7	180.0 ± 35.6	75.0 ± 10.4			166.3 ± 13.4	108.4 ± 9.3	

Table 9. Thymic weights and cell numbers in thymus, lymph nodes and spleen in four strains of mice. Thymus, lymph nodes and spleen were collected from 8-9-week-old female and male C57BL/6, BALB/c, SJL and BWF1 mice and weights or cell numbers determined. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

SPECIFIC AIM III

Assessing regulatory T cell changes throughout disease progression in lupus-prone (NZBxNZW)F1 mice.

The (NZBxNZW)F1 (BWF1) strain of mice is used as a model of systemic lupus erythematosus in which females spontaneously develop disease (66 , 67). Antibodies against self-antigens, such as dsDNA and histones, are produced and associate with complement to form immune complexes (80). These antibodies can be detected in the serum at approximately 16 weeks of age in female BWF1 mice in our facility. Immune complexes deposit in the kidney glomeruli and cause kidney failure (80). Infiltration of T cells into the kidney has been shown to be necessary for end-stage kidney disease development (91). Glomerulonephritis is detected by the presence of protein in urine. We begin analyzing for protein in the urine when BWF1 mice are approximately 28 weeks of age in our facility. Male BWF1 mice also develop antibodies, but they do so later than females, and rarely develop full-blown SLE/glomerulonephritis. In our facility, approximately 50% of female BWF1 mice have developed disease by 32 weeks of age, while 0% of males have disease at this timepoint (Figure 13). Accumulating evidence indicates that regulatory T cells (Tregs) play an important role in controlling autoimmune disease (123). Here we have investigated the differences in Treg populations between females and males throughout disease progression to determine whether differences in these cells were associated with susceptibility to disease.

Sick female BWF1 mice had no defect in suppressive function *in vitro*, and had higher percentages of CD4⁺Foxp3⁺ cells in the periphery than age-matched non-sick females and males.

BWF1 mice were considered “sick” when protein levels in urine reached ≥ 300 mg/dL and were maintained for two consecutive weeks. Regulatory T cells have been shown to be important in controlling autoimmune disease (123), so we hypothesized that perhaps there was some inherent defect in sick female Treg suppressive function allowing for disease development and progression. We compared suppressive function of Tregs from sick females, non-sick females, and males in a standard *in vitro* suppression assay. We co-cultured bead-purified CD4⁺CD25⁺ cells from age-matched sick females, non-sick females, and males at varying ratios with male CD4⁺CD25⁻ responder T cells, irradiated male spleen cells for APC, and anti-CD3. We found no differences in the suppressive function of CD4⁺CD25⁺ cells *in vitro* between sick females, non-sick females, and males (Figure 14A).

In another mouse model of systemic lupus erythematosus derived from the BWF1 strain, Morel and colleagues also found no defect in the suppressive function of sick female Tregs, but did find that sick female CD4⁺CD25⁻ responder cells were resistant to suppression (115, 116). This resistance to suppression was mediated by increased IL-6 production by bone marrow-derived DC (115, 116). With this in mind, we compared CD4⁺CD25⁻ responder cell susceptibility to suppression as well as the ability of APC to support suppression between age-matched sick females, non-sick females, and males. We found that sick female CD4⁺CD25⁻ responder cells were suppressed at similar levels to

non-sick female and male CD4⁺CD25⁻ responder cells (Figure 14B). Morel and colleagues found excess IL-6 production by DC caused CD4⁺CD25⁻ responder cells to become resistant to suppression *in vitro* (115, 116). However, we found no defect in the ability of sick female APC to support suppression compared to age-matched non-sick female and male APC, i.e., APC from sick females did not confer resistance to suppression on responder cells (Figure 14C). These data together indicate that there are no inherent defects in the ability of sick CD4⁺CD25⁻ responder cells to be suppressed or in the ability of APC from sick female mice to support suppression of sick female Tregs to suppress disease.

Foxp3, the best Treg marker to date, is a transcription factor necessary for Treg function (127). Interestingly, 32-36-week-old sick females had significantly higher percentages of CD4⁺ T cells that were Foxp3⁺ in lymph nodes (LN) and spleen than age-matched non-sick females and males, whose percentages in LN and spleen were similar to one another (Figure 15A). Sick females also had higher total CD4⁺Foxp3⁺ cell numbers than age-matched non-sick females and males in LN (Figure 15B). This is consistent with higher total cell numbers in general in LN and spleen in sick female mice compared to age-matched non-sick female and male total cell numbers (Table 10). We found similar differences in the percentages of CD4⁺ cells that express CD25 as we did in percentages of CD4⁺ cells that express Foxp3, i.e. sick females had the highest percentages (Figure 15C). However, we found no difference in per cell Foxp3 expression by CD4⁺CD25⁺ cells between sick females and age-matched non-sick females and males in LN or spleen (Figure 15D, Table 11). Thus, sick female BWF1 mice have higher

percentages of Tregs than age-matched non-sick females and males, and these cells are functional suppressors *in vitro*.

Sick females had a higher percentage of CD4⁺CD25⁺CD103⁺ cells than age-matched non-sick females and males with no defect in suppressive function *in vitro*.

A subset of very potent Tregs expresses the integrin, $\alpha_E\beta_7$ (CD103) (207). CD103 is thought to be involved in retention of this subset of Tregs at sites of inflammation (212). CD103⁺ Tregs are more potent suppressors than CD103⁻ Tregs, both *in vitro* and *in vivo*, and are considered an effector/memory subset of Tregs (149, 207, 210, 211). This subset of CD103⁺ Tregs has been shown to be effective at controlling autoimmune/inflammatory diseases such as wasting disease and colitis (211). As we did with the CD4⁺CD25⁺ cells, we compared the suppressive function of CD4⁺CD25⁺CD103⁺ cells between age-matched sick females, non-sick females, and males using a standard *in vitro* suppression assay. We co-cultured CD4⁺CD25⁺CD103⁺ cells sorted to >95% purity from sick females, non-sick females, and males at varying ratios with male CD4⁺CD25⁻ responder cells, male irradiated spleen cells as APCs, and anti-CD3. We found no significant difference in suppressive ability of CD4⁺CD25⁺CD103⁺ cells between age-matched sick females, non-sick females, and males (Figure 16D).

Consistent with data found for CD4⁺Foxp3⁺ cells as a whole, we found that 32-36 week-old sick female mice had significantly higher percentages and total cell numbers of the potent CD4⁺CD25⁺CD103⁺ cells than age-matched non-sick females and males in LN and spleen (Figure 16A, Table 12). Sick females had similar per cell CD103 expression

on CD4⁺CD25⁺CD103⁺ cells as non-sick females and males in LN and spleen (Figs. 16B & C). Thus, there were no inherent defects in CD4⁺CD25⁺CD103⁺ suppressive function in sick females, and the CD4⁺CD25⁺CD103⁺ cell population was actually increased in sick BWF1 mice.

Although it is not clear whether the thymus is actually a major source of CD4⁺CD25⁺CD103⁺ cells, we next investigated whether sick females had defects in thymic production of these cells. We found that, as in the periphery, sick females had higher percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ subsets in the thymus (Figure 17). All types of T cells can traffic into the thymus from the periphery (259), so while our data did not suggest a defect in thymic CD4⁺CD25⁺CD103⁺ cell production in sick females, it does not rule out the possibility that CD4⁺CD25⁺CD103⁺ cells from the periphery, in which sick females have a higher percentages of CD4⁺CD25⁺CD103⁺ cells, are trafficking back into the thymus.

Sick female Tregs were unable to control effector T cell proliferation *in vivo*.

To determine whether differences in homeostatic proliferation were responsible for differences in peripheral Treg percentages between 32-36-week-old sick female, non-sick female, and male BWF1 mice, we stained LN and spleen cells with an antibody against Ki67, a marker for proliferating cells. Although sick females had higher percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in LN and spleen (Figure 15A, 16A), their CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells did not proliferate more *in vivo* than in age-matched non-sick females and males (Figure 18A, 18C). However, CD4⁺Foxp3⁻ effector T cells did proliferate significantly more in sick female LN and

tended to proliferate more in spleen than in age-matched non-sick females and males (Figure 18B). The Ki67 proliferation data were confirmed with BrdU staining. Further analysis showed that the $\text{Foxp3}^+:\text{Foxp3}^-$ proliferation ratio was significantly lower in both the LN and spleen of sick female mice by comparison to both non-sick female and male BWF1 mice (Figure 18D). These data suggest two things. First, the high percentages of $\text{CD4}^+\text{Foxp3}^+$ and $\text{CD4}^+\text{CD25}^+\text{CD103}^+$ cells found in the periphery of sick female BWF1 mice are not due to either increased proliferation of these cells or a decreased proliferation of the $\text{CD4}^+\text{Foxp3}^-$ cells. Secondly, the increased proliferation of the $\text{CD4}^+\text{Foxp3}^-$ cells in sick female mice suggest that sick female Tregs, while able to suppress proliferation of $\text{CD4}^+\text{CD25}^-$ cells *in vitro* (Figure 14A), appear unable to suppress proliferation of the same population *in vivo*.

Sick female Tregs were able to traffic to kidney LN

We next examined the ability of sick female Tregs to traffic to sites where their suppressive function would likely be needed in late stage disease. T cell infiltration into the kidney is necessary for end stage kidney disease (91), so the lymphoid organs draining the kidney may be an important site for Tregs to carry out their suppressive function. For this purpose, we analyzed kidney LN for the presence of $\text{CD4}^+\text{Foxp3}^+$ and $\text{CD4}^+\text{CD25}^+\text{CD103}^+$ cells. We found that sick females had higher percentages of both $\text{CD4}^+\text{Foxp3}^+$ and $\text{CD4}^+\text{CD25}^+\text{CD103}^+$ cells in the kidney LN compared to age-matched non-sick females and males (Figure 19), suggesting that the Tregs were getting to the site where the Treg targets, the kidney-specific effector T cells, would likely be induced.

However, these data do not rule out the possibility that the Tregs are not infiltrating the kidney itself where they may also be required to suppress pathogenic T cell function.

Downregulation of CD62L is required for T cell egress from LN (260).

CD4⁺CD25⁺CD103⁺ cells are known as a memory/effector subset of Tregs that can traffic to and are retained at sites of inflammation (210, 212). If these CD103⁺ Tregs are unable to downregulate CD62L and leave the LN, they may be unable to traffic to inflammatory sites, which may prevent them from performing important regulatory functions and thus, protect from disease. We evaluated CD62L expression on CD4⁺CD25⁺CD103⁺ Tregs, and found that sick females actually had lower percentages of CD4⁺CD25⁺CD103⁺CD62L^{hi} cells and, therefore, more CD62L^{lo} cells than age-matched non-sick females and males in both LN and spleen (Figure 20), suggesting that this population can and does downregulate CD62L and is, therefore, not “stuck” in the LN because of its inability to properly regulate CD62L expression.

Regulatory T cell percentages change with disease progression in BWF1 mice.

As discussed above, we found that sick females had much higher percentages and numbers of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ Tregs in the periphery than age-matched non-sick females and males (Figure 15A & B, 16A, Table 12). To determine how these populations change over time, we compared CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cell populations in female and male BWF1 mice at different time-points during disease development, i.e., at 9, 20, 24-28, and 32-36 weeks of age. As reported in specific aim 2, at 9 weeks, females had significantly lower percentages of CD4⁺ cells that are Foxp3⁺ (CD4⁺Foxp3⁺) than males in LN (21). At 20 weeks of age,

female and male percentages of CD4⁺Foxp3⁺ cells in LN were not significantly different, but the percentages still tended to be lower in females than males (Figure 21). By 24-28 weeks, there were no differences in percentages of CD4⁺Foxp3⁺ cells in LN between females and males (Figure 21). At 32-36 weeks of age, sick females had significantly higher percentages of CD4⁺Foxp3⁺ cells in the LN than age-matched non-sick females and males (Figure 21). In the spleen, there were no differences between female and male percentages of CD4⁺Foxp3⁺ cells at 9 weeks, 20 weeks, or 24-28 weeks (data not shown). It was not until 32-36 weeks that differences were seen in the spleen, with sick females having significantly higher percentages of CD4⁺Foxp3⁺ cells (Figure 15A). Percentages of CD4⁺CD25⁺CD103⁺ cells in the LN followed the same pattern as percentages of CD4⁺Foxp3⁺ cells; at 9 weeks, females were significantly lower; there was no difference at 20 weeks but females trended lower; and at 24-28 weeks there was no difference between females and males (Figure 22). Again, at 32-36 weeks, sick females had significantly higher percentages of CD4⁺CD25⁺CD103⁺ cells than age-matched non-sick females and males (Figure 22). In spleen, 9 week old BWF1 females had significantly lower CD4⁺CD25⁺CD103⁺ percentages than males, there was no significant difference at 20 weeks or 24-28 weeks of age, and at 32-36 weeks, sick females had significantly higher percentages of CD4⁺CD25⁺CD103⁺ cells than age-matched non-sick females and males (data not shown). In addition, we found no difference in suppressive function of CD4⁺CD25⁺CD103⁺ Tregs *in vitro* between females and males at any age tested (Figure 23). These data suggest that 9 weeks of age may be an important time-point during disease development and the appropriate Treg to effector T cell balance may be critical (Fig. 24).

At nine weeks of age, pre-disease female BWF1 mice had no defect in Treg suppressive function *in vitro* but may be unable to control effector T cell proliferation *in vivo*.

We found that 9 weeks of age (pre-disease) may represent an interesting and important time-point during disease development when gender differences in Treg populations may set the scene for gender differences in disease susceptibility later in life. As shown in specific aim 2 and discussed above, both CD4⁺CD25⁺ and CD4⁺CD25⁺CD103⁺ cells from 9-week-old female BWF1 mice were as effective as male cells in suppressing CD4⁺CD25⁻ responders (Figure 8, 10D, 23A). To normalize the experimental conditions so that results between females and males were comparable, regulatory function by female and male Tregs in those experiments were tested using male responders and APCs. As mentioned previously, Morel and colleagues found CD4⁺CD25⁻ responder cells from sick females were resistant to suppression in another mouse model of lupus, which appeared to be due to increased IL-6 production by DC (115, 116). For this reason, we first compared sensitivity of female and male CD4⁺CD25⁻ responder T cells to suppression by Tregs *in vitro*. We co-cultured varying numbers of bead-purified CD4⁺CD25⁺ cells from 9-week-old BWF1 male mice with a fixed number of female or male CD4⁺CD25⁻ responder cells, male irradiated spleen cells as APCs, and anti-CD3. Interestingly, we found that female CD4⁺CD25⁻ responder cells appeared to be more susceptible to suppression than male cells (Figure 25A). To determine whether sick female DC could render CD4⁺CD25⁻ responder cells resistant to suppression, we co-cultured varying numbers of bead-purified CD4⁺CD25⁺ cells from males with male

CD4⁺CD25⁻ responder cells, female or male irradiated spleen cells as APCs, and anti-CD3. We found no significant differences between the ability of 9-week-old female and male APCs to support suppression *in vitro* (Figure 25B). Finally, to determine whether suppressive function of female Tregs in an environment where all cell components of the assay were either from females or males, we co-cultured varying numbers of bead-purified CD4⁺CD25⁺ cells from females or males with gender-matched CD4⁺CD25⁻ responder cells and irradiated spleen cells as APCs, and anti-CD3. We found no differences in Treg suppressive function in the all female group compared to the all male environment *in vitro* (Figure 25C). These data together suggest that females do not have a defect in inherent suppressive function or in the ability of their responder T cells to be suppressed, but do have reduced Treg percentages and numbers when compared to males that could influence their susceptibility to disease. However, CD4⁺Foxp3⁻ effector T cells proliferated more *in vivo* in 9-week-old females than in males (Figure 12B). These data may suggest that while the female Tregs are functional, the responder T cells sensitive to suppression and the APC capable of supporting suppression *in vitro*, there may be some other factor(s) that may interfere with the ability of Tregs to function properly *in vivo*. And this appears to be true at both the early time-point before disease onset at 9 weeks of age as well as after disease is established by 32-36 weeks of age, and may be indicative of early uncontrolled activation of T cells.

Pre-disease CD103-depletion *in vivo* accelerated and enhanced disease in female and male lupus-prone BWF1 mice.

We found reduced proportions of Tregs in the peripheral lymphoid organs of 9-week-old female BWF1 mice compared to males (Figure 9A, 10A, 21, 22), and we propose that this is a critical early timepoint during disease development. Previous studies have shown that depletion of CD25⁺ cells in female BWF1 with an anti-CD25 monoclonal antibody at 3 days of age resulted in increased dsDNA production and urine protein levels ($\geq 300\text{mg/dL}$) by 4.5 months of age (174), suggesting that early depletion of CD25⁺ cells could accelerate disease in this strain of mice. We were interested in determining whether depleting the CD103⁺ Treg population had an impact on disease progression in both females and males during the period of development when females had lower percentages of this population. For this experiment, we depleted CD103⁺ cells using a depleting monoclonal antibody. Anti-CD103 antibody or an isotype control antibody was administered to female and male mice twice a week from 8-12 weeks of age and twice a month from 12-20 weeks of age (Figure 24). This treatment regimen almost completely depleted all CD103⁺ cells during this time-period in the blood (Figure 26). We found that CD103 depletion accelerated disease in both females and males when compared to isotype-treated animals (Figure 27A, 27C). In females, both disease onset and death were accelerated in anti-CD103-treated compared to isotype-treated mice (Figure 27). In fact, 100% of anti-CD103 treated females in experiment #1 were dead by 42 weeks of age, whereas the same percent mortality was not seen in isotype antibody-treated females until 53 weeks of age (Figure 27B, 27D). However, the most striking data

were seen in the males. Although anti-nucleic antibodies can be detected in males fairly early on, we rarely see full-blown disease, and if we do it is after 60 weeks of age. After treatment with anti-CD103 antibody, we saw a dramatic increase in both disease incidence (50-70% by 50 weeks) and mortality (30-70% by 60 weeks; Figure 27). These data indicate that loss of CD103⁺ Tregs early on can impact disease development and severity and give support to the hypothesis that reduced female Treg numbers and percentages at 9 weeks of age compared to males may set the stage for their increased disease susceptibility later in life.

Summary

In this study, we found that 32-36-week-old sick female mice have higher percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in the periphery when compared to age-matched non-sick females and males. Sick female Tregs do not show a defect in suppressive function. We found 9-week-old females had lower percentages of CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in the periphery compared to age-matched males. Nine weeks appears to be an important time period in disease progression. Depletion of CD103⁺ cells in females and males starting at 8 weeks decreased time to disease and increased death in both female and male BWF1 mice. Thus, decreased Treg numbers and percentages in female BWF1 mice early in development may increase their susceptibility to disease.

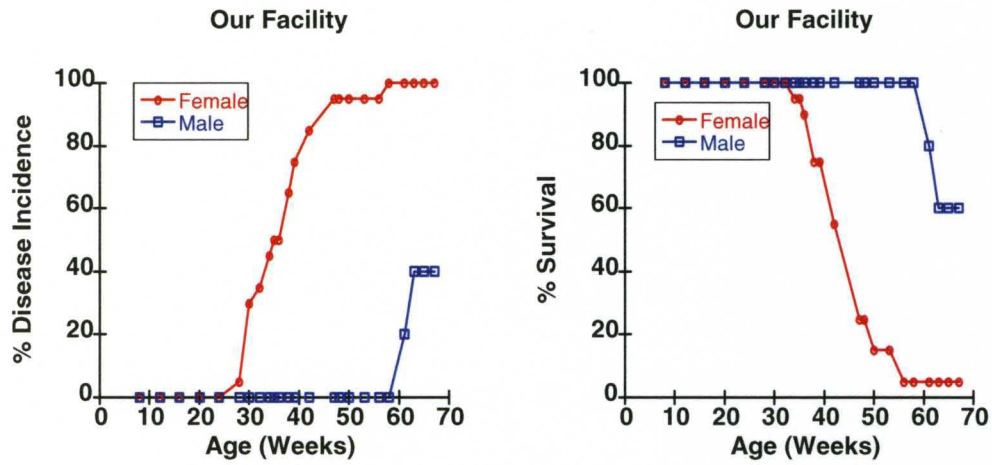


Figure 13. Disease progression in our facility. Urine from female and male BWF1 mice was tested for protein starting at 20 weeks of age. Mice were considered “sick” when the protein in their urine reached a score of 3 ($\geq 300\text{mg/dL}$) for two consecutive weeks. (A) Disease incidence. (B) Survival. (females $n=20$, males $n=5$).

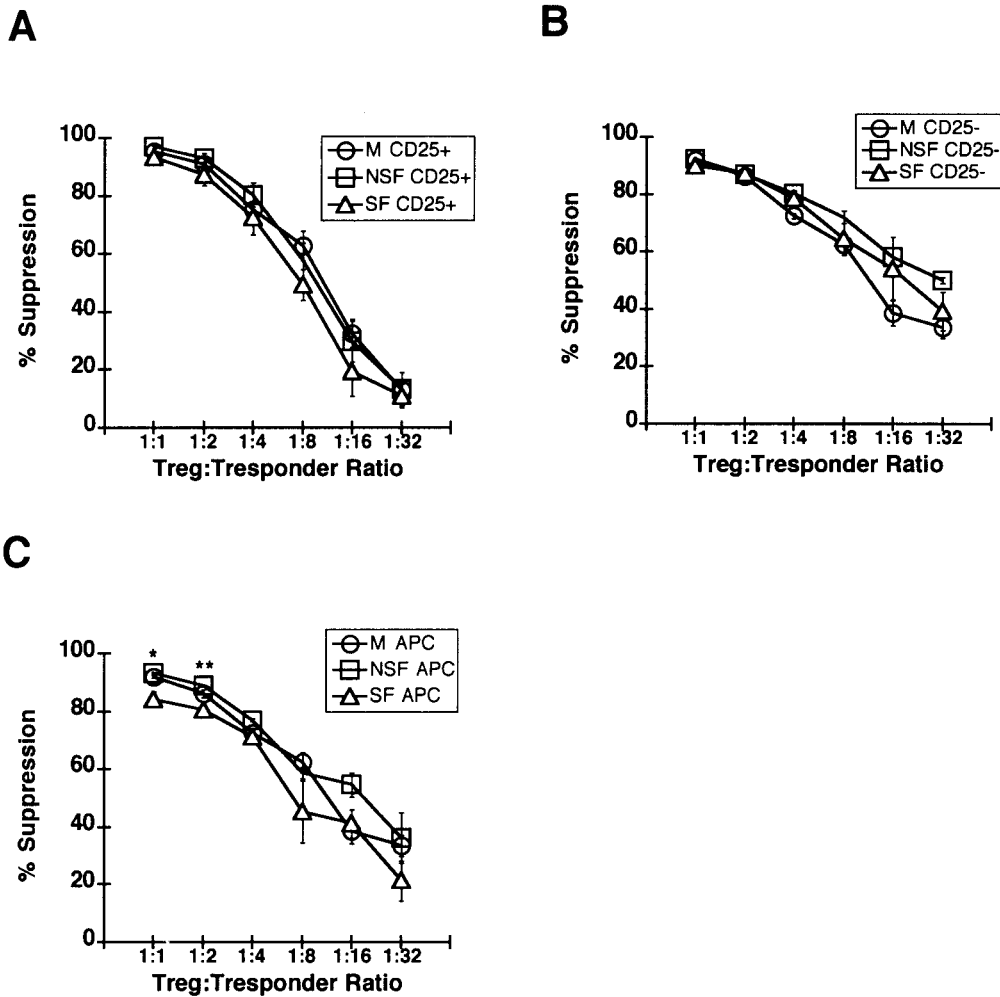
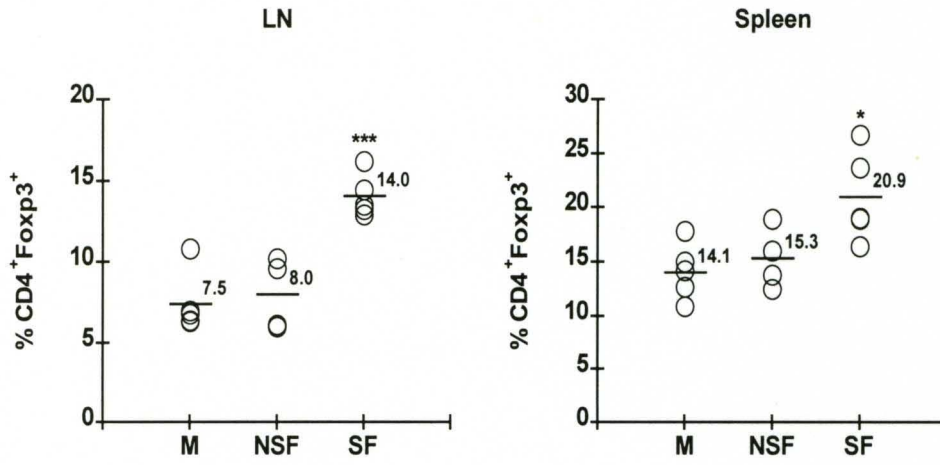
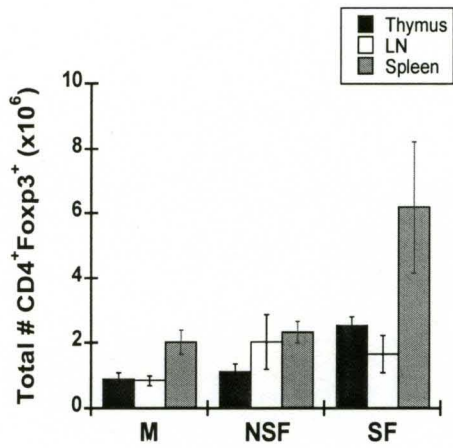
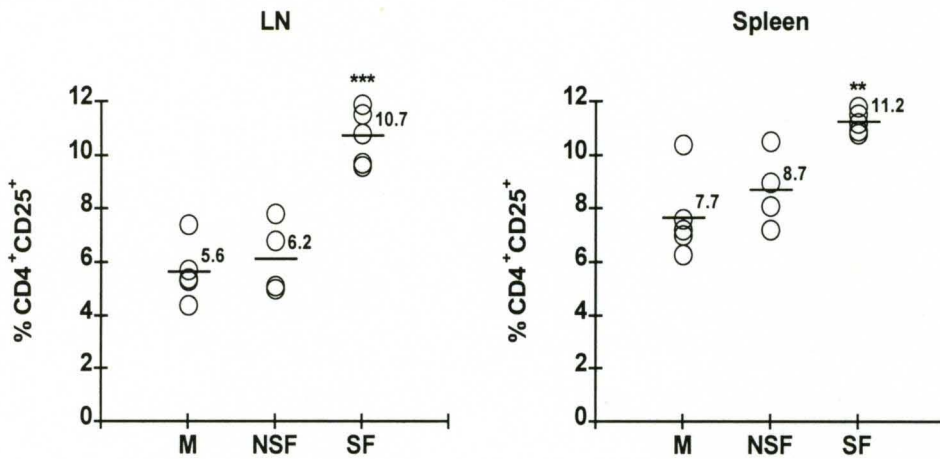


Figure 14. Regulatory function *in vitro* in 32-36-week-old BWF1 mice. (A) To test for $CD4^+CD25^+$ regulatory function, $CD4^+CD25^+$ cells were purified from 32-36-week-old sick female, non-sick female, and male BWF1 mice and varying numbers co-cultured with a constant number of male $CD4^+CD25^-$ responder cells, male APC, and soluble anti-CD3 antibody. % suppression of positive control (responder cells without Tregs) was calculated and the mean \pm SEM presented. (B) To test for $CD4^+CD25^-$ responder cell sensitivity to suppression, $CD4^+CD25^+$ cells were purified from male 32-36-week-old

BWF1 mice and varying numbers co-cultured with a constant number of 32-36-week-old sick female, non-sick female, and male CD4⁺CD25⁻ responder cells, male APCs, and soluble anti-CD3 antibody. % suppression of positive control (responder cells without Tregs) was calculated and the mean \pm SEM presented. (C) To test for the ability of APCs to support suppression, CD4⁺CD25⁺ cells were purified from male 32-36-week-old BWF1 mice and varying numbers co cultured with a constant number of male CD4⁺CD25⁻ responder cells, sick female, non-sick female, or male APCs, and soluble anti-CD3 antibody. % suppression of positive control (responder cells without Tregs) was calculated and the mean \pm SEM presented. A representative experiment of 3 performed is shown. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

A**B****C**

D

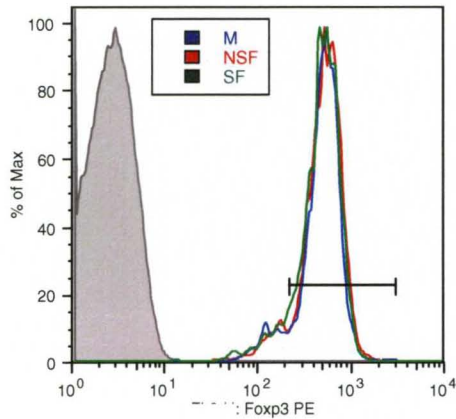
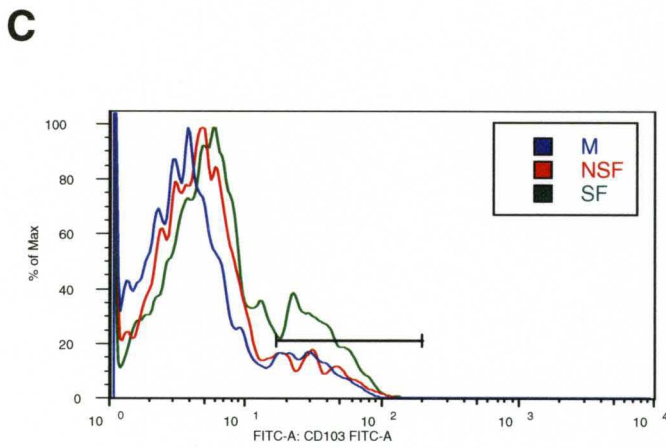
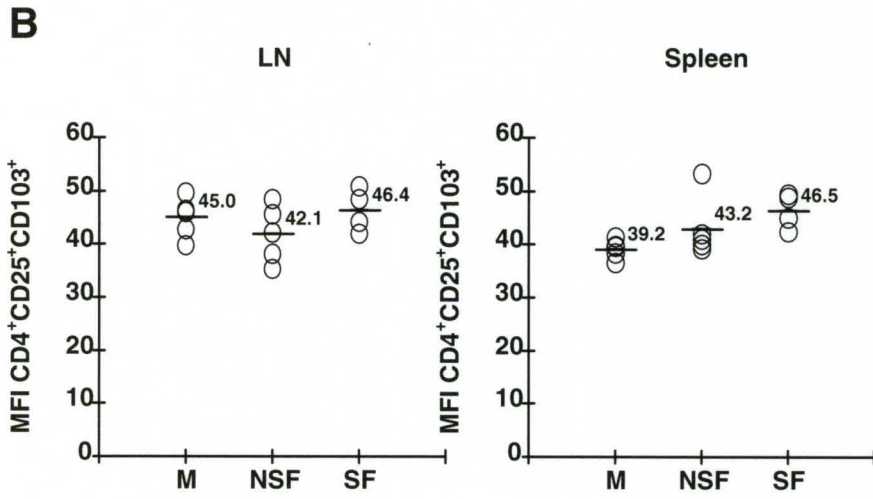
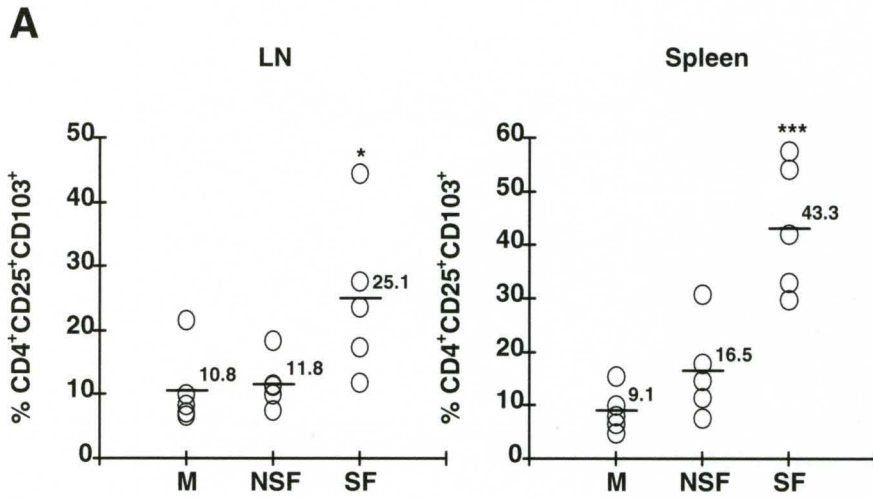


Figure 15. Percentages and numbers of CD4⁺Foxp3⁺ cells in 32-36-week-old BWF1 mice. Cells from lymph nodes (LN), spleen, and thymus of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4, anti-CD25, and anti-Foxp3 antibodies and analyzed by FACS[®]. (A) Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing Foxp3 in LN and spleen. (B) The total numbers of CD4⁺Foxp3⁺ cells are shown in LN and spleen. (C) Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing CD25 in LN and spleen. (D) A sample histogram showing Foxp3 expression on CD4⁺CD25⁺ cells in LN is shown. (A,C) A representative experiment of 4-6 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.



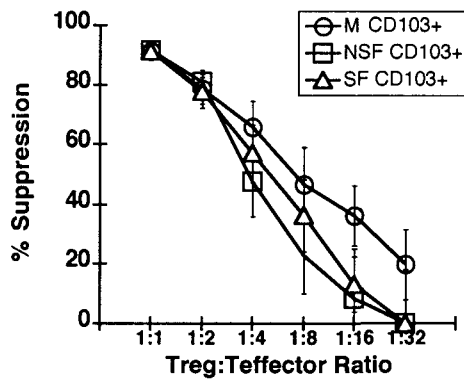
D

Figure 16. Percentages and *in vitro* suppressive function of CD4⁺CD25⁺CD103⁺ cells in 32-36-week-old BWF1 mice. Cells from lymph nodes (LN) and spleen of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4, anti-CD25, and anti-CD103 antibodies and analyzed by FACS[®]. (A) Labeled CD4⁺CD25⁺ cells were gated and analyzed for the percentages of cells expressing CD103 in LN and spleen. (B) Labeled CD4⁺CD25⁺ cells were gated and analyzed for the per cell expression of CD103 in LN and spleen. (C) A sample histogram showing CD103 expression on CD4⁺CD25⁺ cells in LN is shown. (D) CD4⁺CD25⁺CD103⁺ cells were purified from 32-36-week-old sick female, non-sick female, and male BWF1 mice and varying numbers co-cultured with a constant number of male CD4⁺CD25⁻ responder cells, male APC, and soluble anti-CD3 antibody. % suppression of positive control (responder cells alone) was calculated and the mean \pm SEM presented. (A,B) A representative experiment of 3-7 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$.

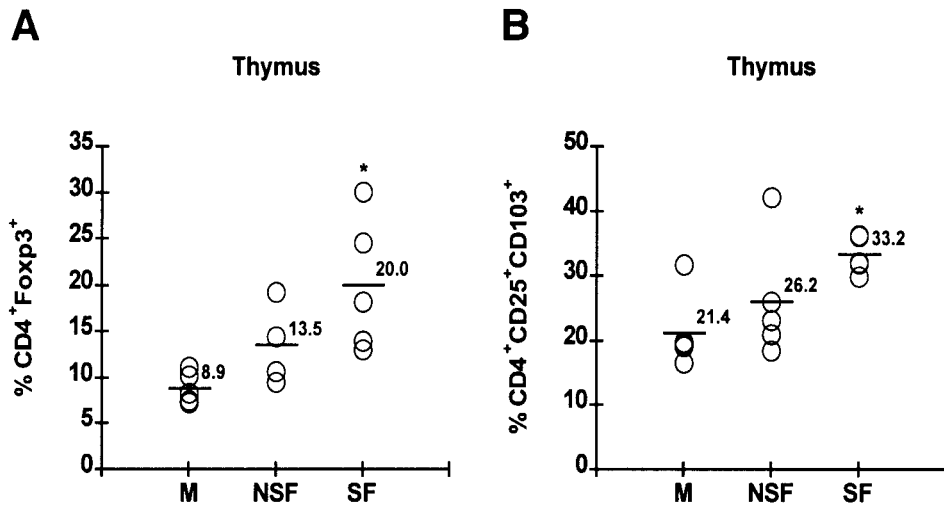
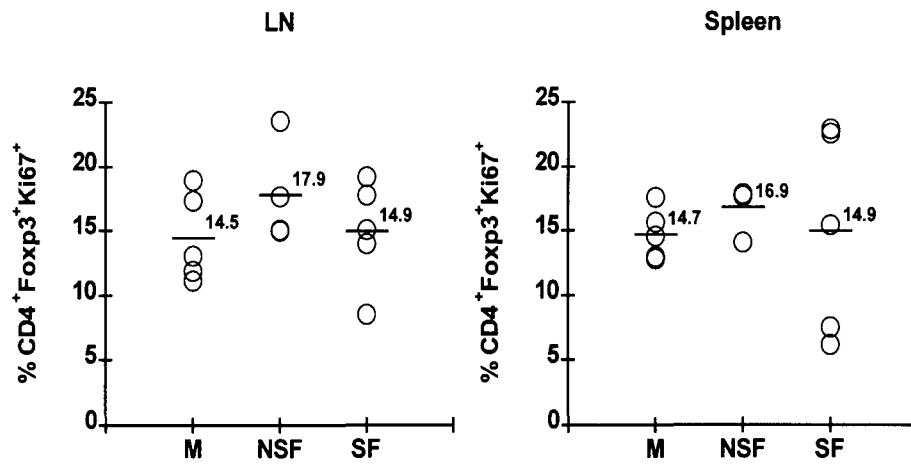
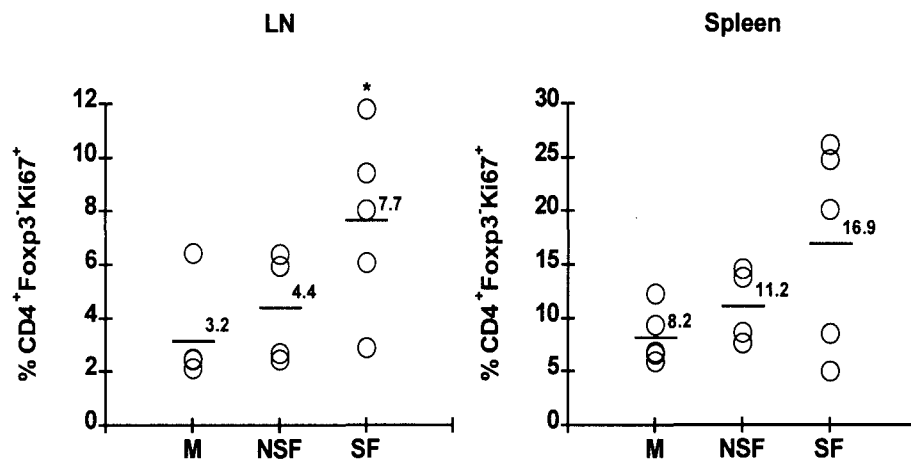


Figure 17. Percentages of CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ cells in thymus of 32-36-week-old BWF1 mice. Cells from the thymus of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4, anti-CD25, anti-CD103, and anti-Foxp3 antibodies and analyzed by FACS[®]. Labeled cells were gated and analyzed for (A) percentages of CD4⁺ cells expressing Foxp3 and (B) percentages of CD4⁺CD25⁺ cells expressing CD103. A representative experiment of 3-7 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

A**B**

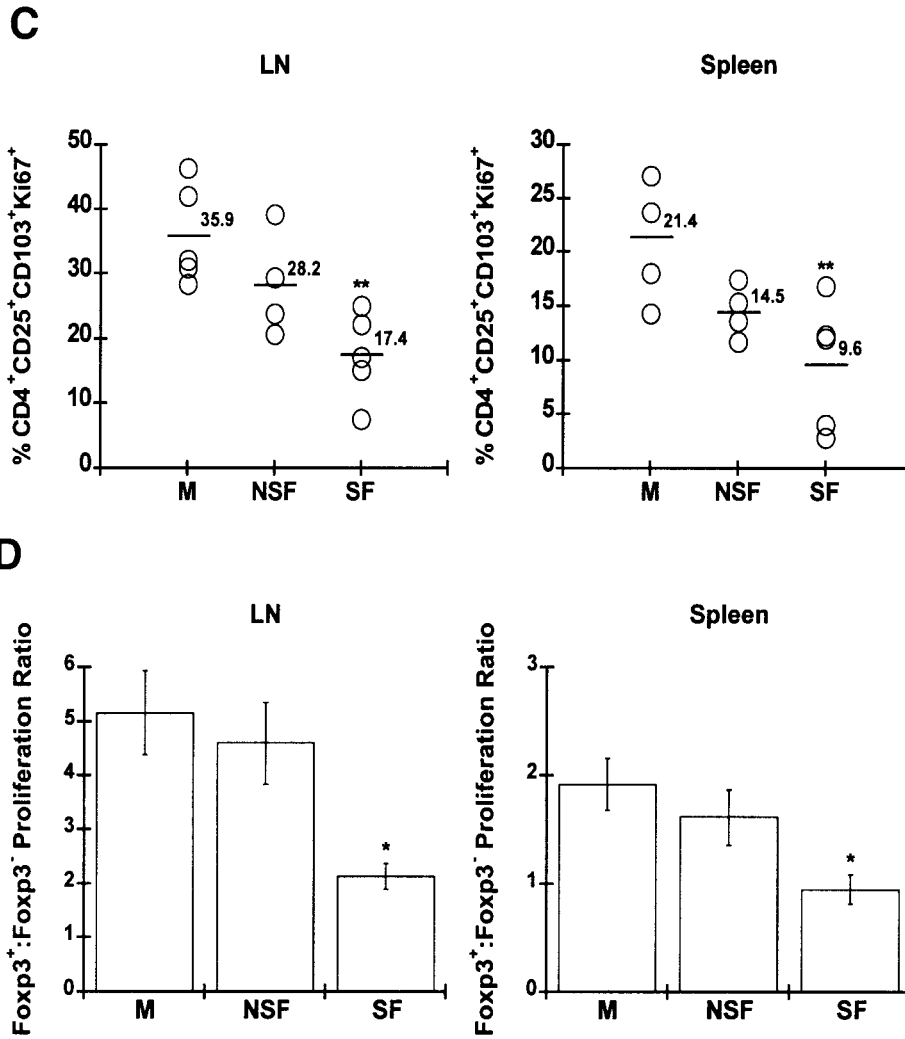


Figure 18. Percentages of proliferating $CD4^+Foxp3^+$, $CD4^+Foxp3^-$, and $CD4^+CD25^+CD103^+$ cells in the periphery of 32-36-week-old sick female, non-sick female, and male BWF1 mice. Cells from LN and spleen of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4, anti-CD25, anti-CD103, anti-Foxp3, and anti-Ki67 antibodies and analyzed by FACS[®]. (A) Labeled $CD4^+Foxp3^+$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (B) Labeled $CD4^+Foxp3^-$ cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (C) Labeled $CD4^+CD25^+CD103^+$

cells were gated and analyzed for the percentages of cells expressing Ki67 in LN and spleen. (D) Proliferation ratios were calculated by dividing the percentage of proliferating (Ki67⁺) CD4⁺Foxp3⁺ cells by the percentage of proliferating CD4⁺Foxp3⁻ cells from LN and spleen. A representative experiment of 3 performed is shown. (A-C) Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

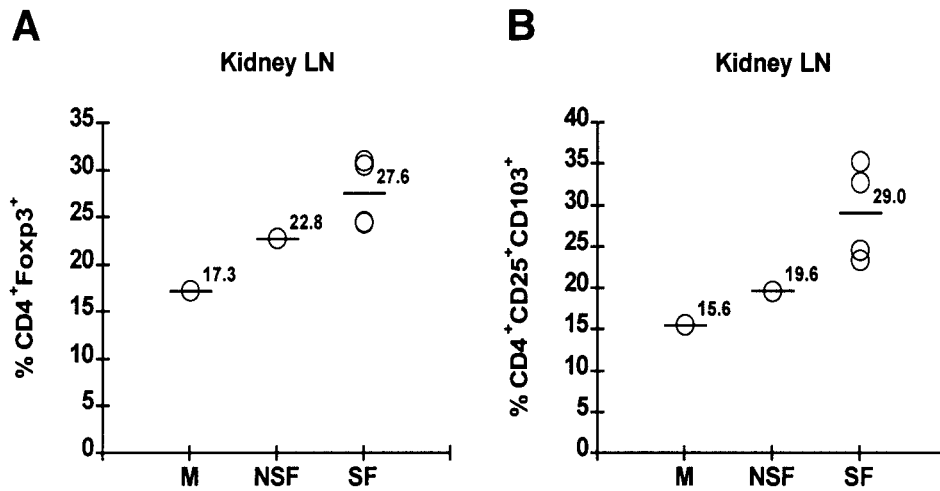


Figure 19. Regulatory T cell percentages in kidney draining LN from 32-36-week-old BWF1 mice. Cells from kidney draining LN of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4, anti-CD25, anti-CD103, and anti-Foxp3 antibodies and analyzed by FACS[®]. (A) Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing Foxp3 and (B) Labeled CD4⁺CD25⁺ cells were gated and analyzed for the percentages of cells expressing CD103. A representative experiment of 2 performed is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

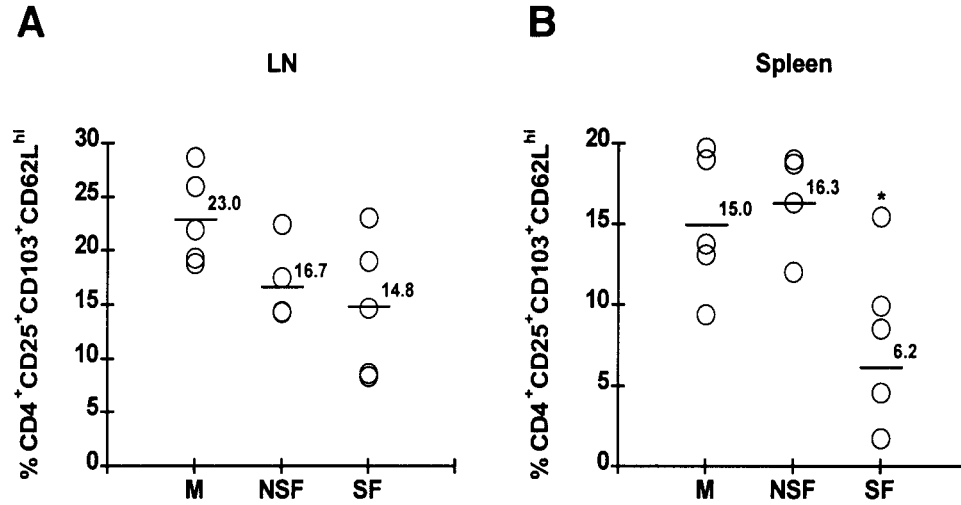


Figure 20. Percentages of CD4⁺CD25⁺CD103⁺CD62L^{hi} cells in 32-36-week-old BWF1 mice. Cells from lymph nodes (LN) and spleen of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4, anti-CD25, anti-CD103, and anti-CD62L antibodies and analyzed by FACS[®]. Labeled CD4⁺CD25⁺CD103⁺ cells were gated and analyzed for the percentages of cells expressing CD62L^{hi} in (A) LN and (B) spleen. A representative experiment of 4 is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

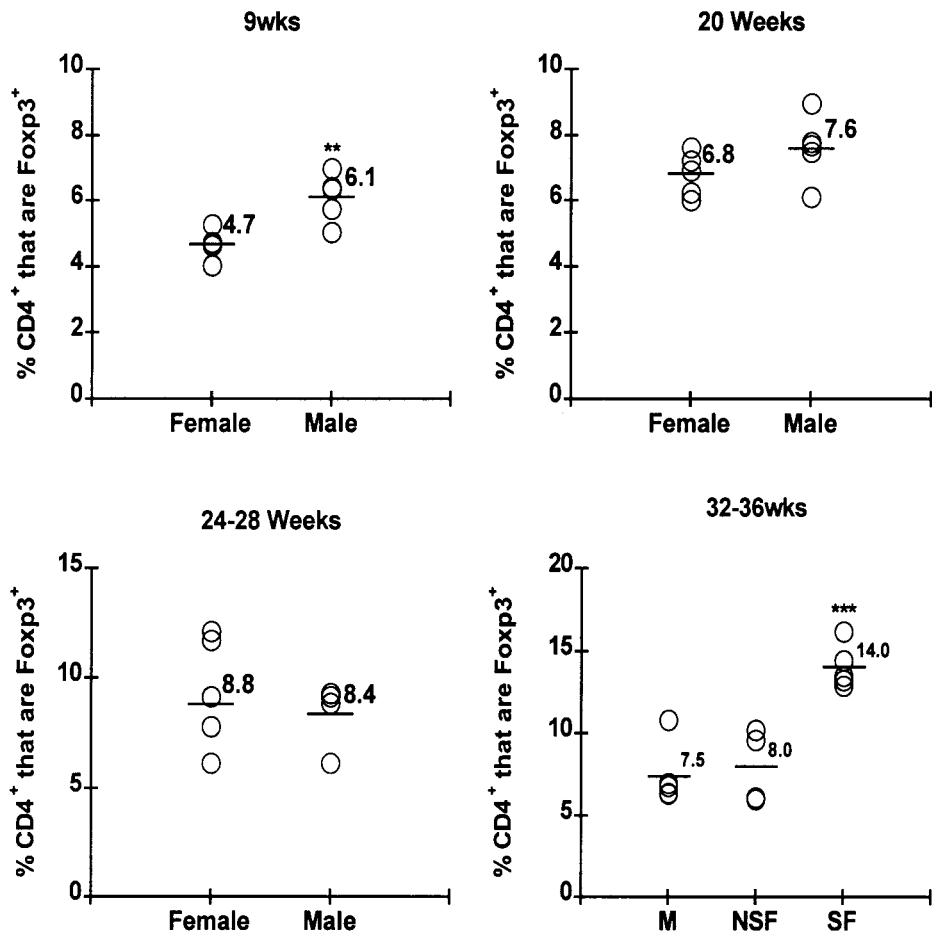


Figure 21. Percentages of CD4⁺Foxp3⁺ cells in BWF1 mice. Cells from lymph nodes (LN) of female and male 9-week-old, 20-week-old, 24-28-week-old, and sick female, non-sick female, and male 32-36-week-old mice were labeled with anti-CD4 and anti-Foxp3 antibodies and analyzed by FACS[®]. Labeled CD4⁺ cells were gated and analyzed for the percentages of cells expressing Foxp3. A representative experiment is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

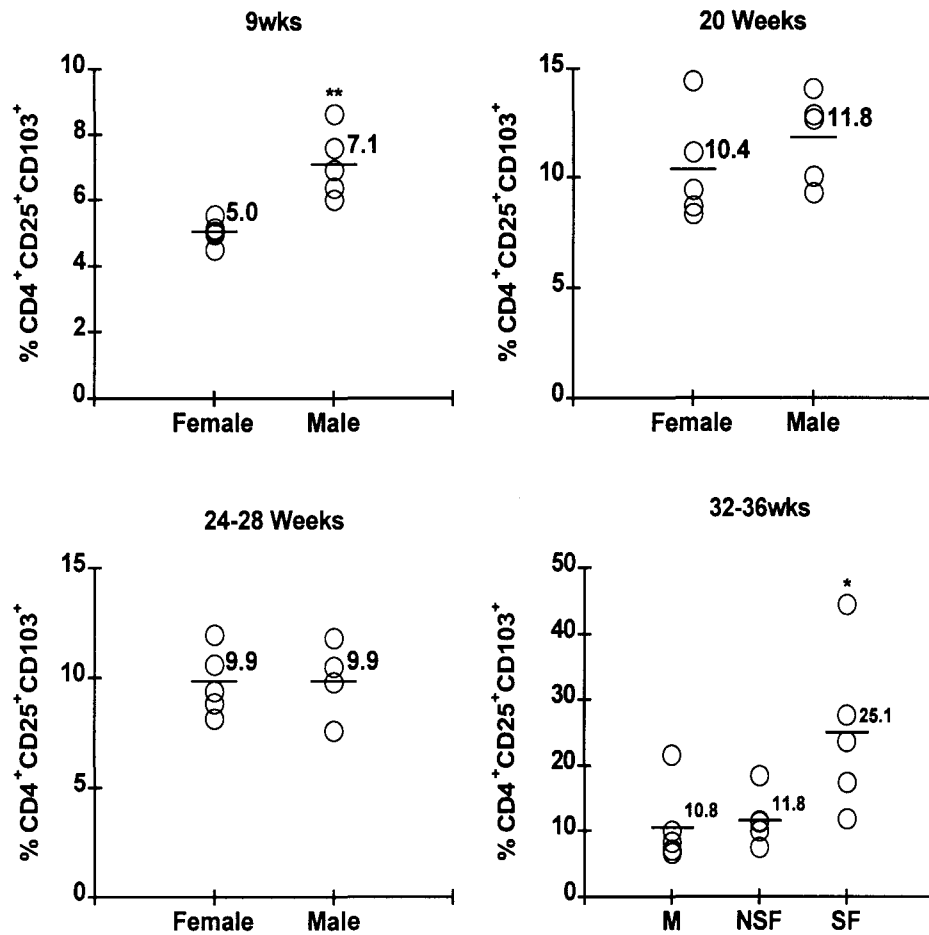


Figure 22. Percentages of CD4⁺CD25⁺CD103⁺ cells in BWF1 mice. Cells from lymph nodes (LN) of female and male 9-week-old, 20-week-old, 24-28-week-old, and sick female, non-sick female, and male 32-36-week-old mice were labeled with anti-CD4, anti-CD25, and anti-CD103 antibodies and analyzed by FACS[®]. Labeled CD4⁺CD25⁺ cells were gated and analyzed for the percentages of cells expressing CD103. A representative experiment is shown. Each symbol represents an individual animal. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

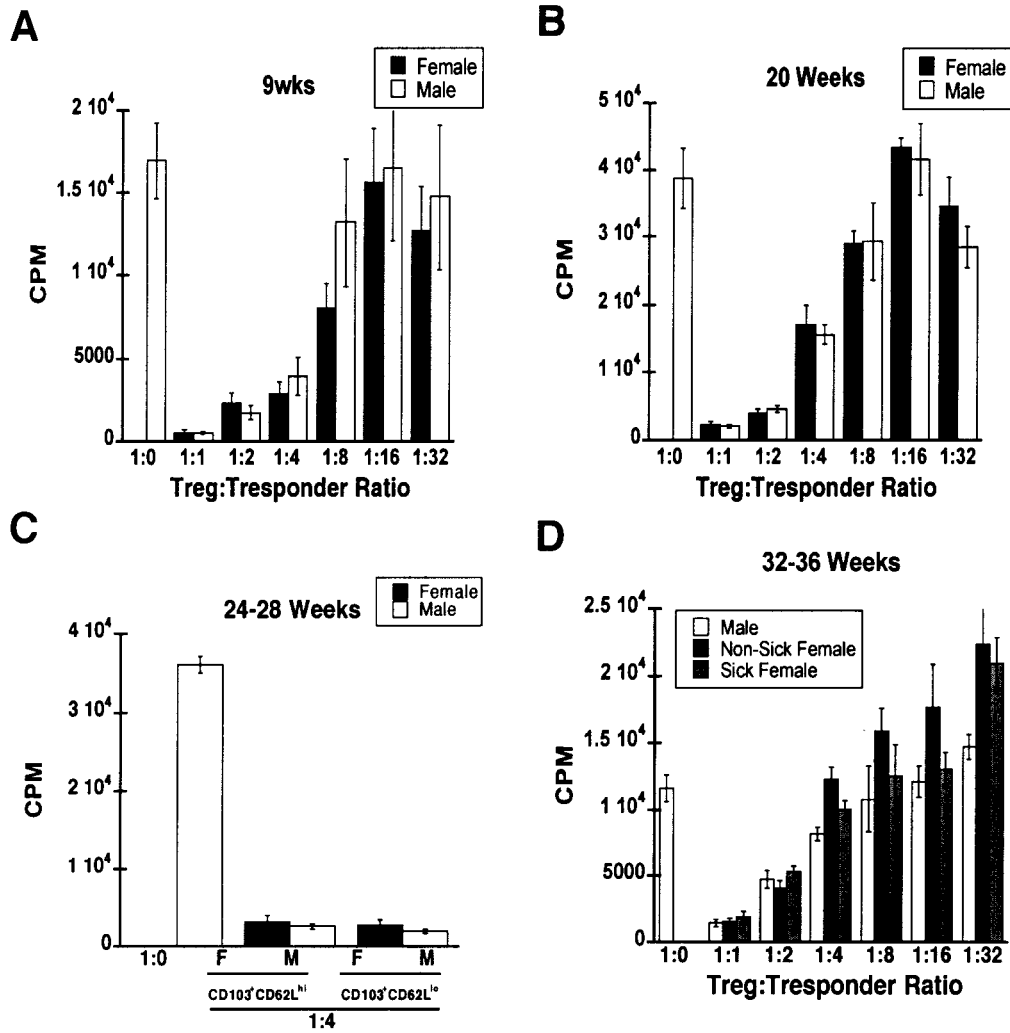


Figure 23. *In vitro* suppressive function of CD103⁺ Tregs from BWF1 mice. To test for regulatory function CD4⁺CD25⁺CD103⁺ cells were purified from (A) 9-week-old, (B) 20-week-old, (C) 24-28-week-old, and (D) 32-36-week-old sick female, non-sick female, and male BWF1 mice and varying numbers co-cultured with a constant number male CD4⁺CD25⁻ responder cells, male APC, and soluble anti-CD3 antibody. (A,D) A representative experiment of 3 performed is shown. (B) A representative experiment of 2 performed is shown. (C) 24-28-week-old CD4⁺CD25⁺CD103⁺ cells were further sorted into CD62L^{hi} and CD62L^{lo} populations. CPM is presented as mean ± SEM. Statistical

differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * $p < 0.05$, ** $p < 0.005$, and *** $p < 0.0005$.

Disease Time-line

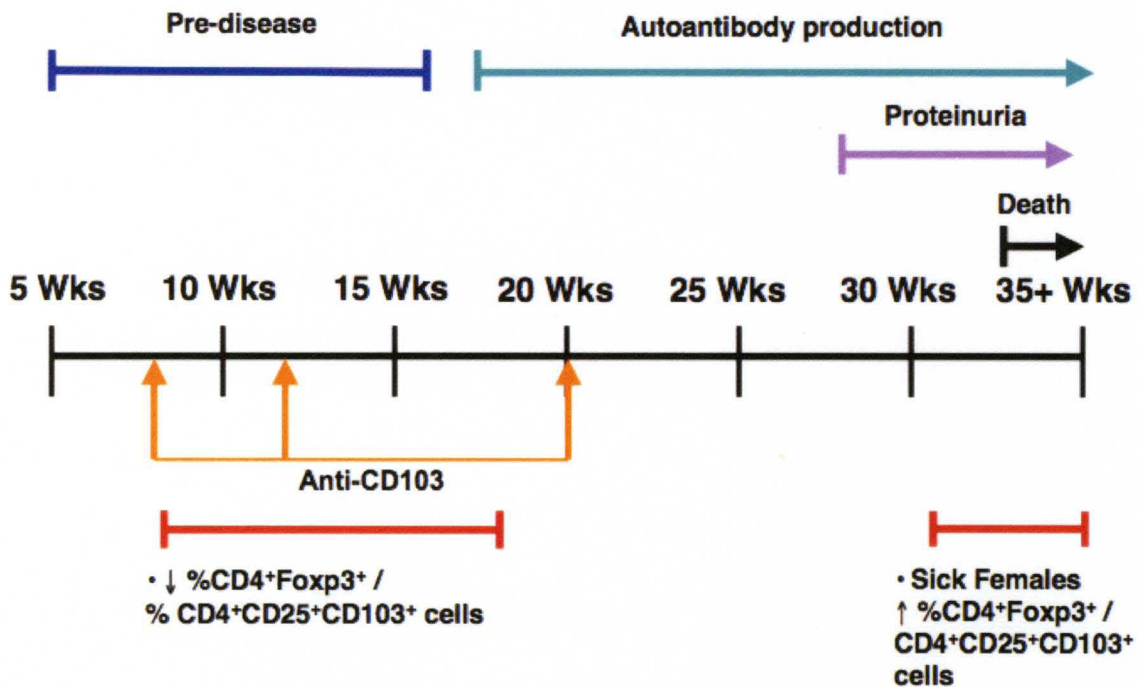


Figure 24. Disease timeline and anti-CD103 monoclonal antibody treatment in BWF1 mice. A schematic of lupus symptom development is shown, including initiation of autoantibody production and detection of proteinuria. The anti-CD103 monoclonal antibody treatment protocol is also shown (results are shown in Figure 26).

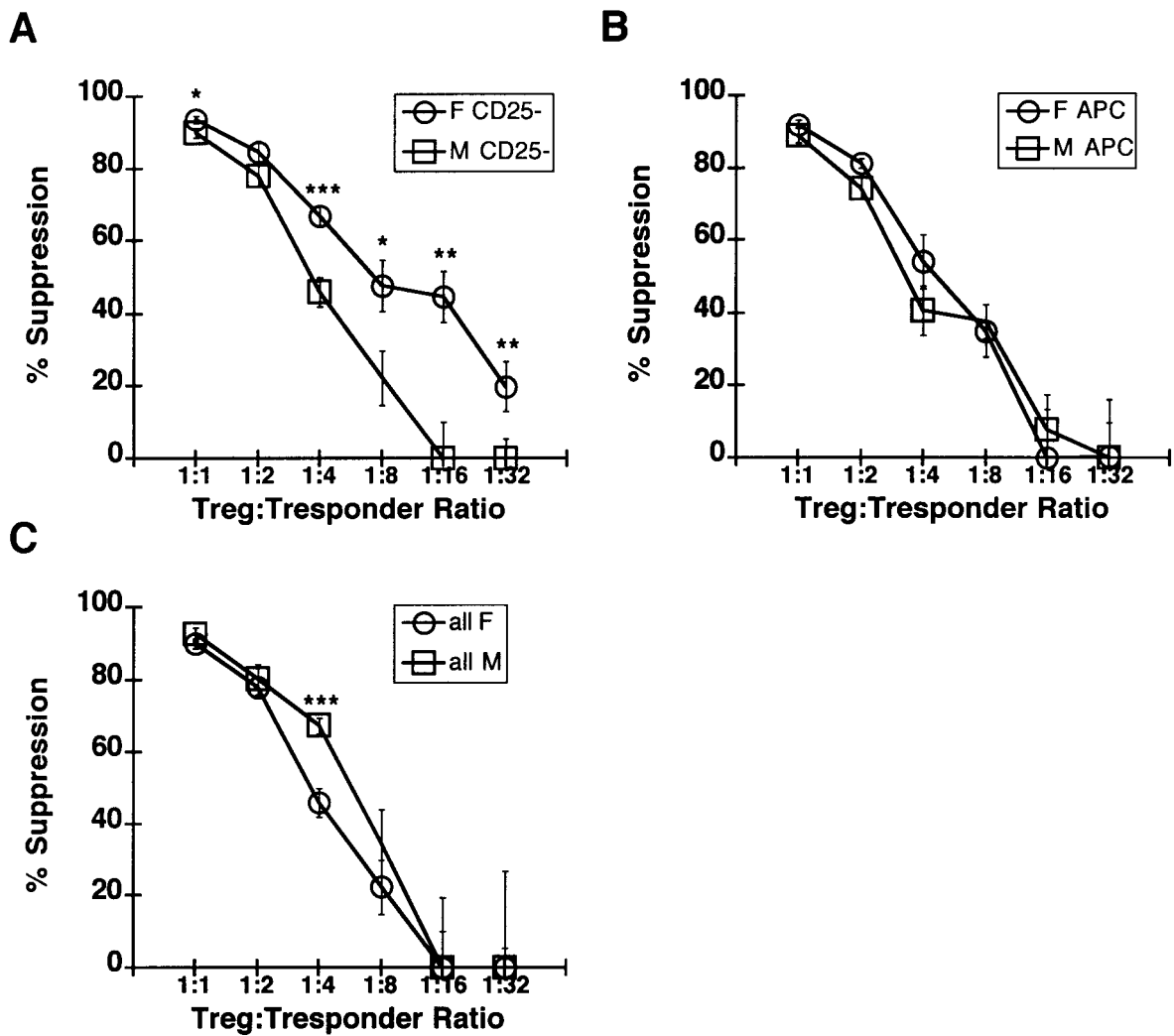


Figure 25. Regulatory function *in vitro* of cells from 9-week-old BWF1 mice. (A) To test for CD4⁺CD25⁻ responder cell sensitivity to suppression, CD4⁺CD25⁺ cells were purified from male 9-week-old BWF1 mice and varying numbers co-cultured with a constant number of 9-week-old female or male CD4⁺CD25⁻ responder cells, male APCs, and soluble anti-CD3 antibody. % suppression of positive control (responder cells alone) was calculated and the mean \pm SEM presented. (B) To test for the ability of APCs to support suppression, CD4⁺CD25⁺ cells were purified from male 9-week-old BWF1 mice and

varying numbers co-cultured with a constant number of male CD4⁺CD25⁻ responder cells, female or male APCs, and soluble anti-CD3 antibody. % suppression of positive control (responder cells alone) was calculated and the mean ± SEM presented. (C) To test for suppressive function with all female or all male cells, CD4⁺CD25⁺ cells were purified from female or male 9-week-old BWF1 mice and varying numbers co-cultured with a constant number of gender-matched CD4⁺CD25⁻ responder cells, gender-matched APCs, and soluble anti-CD3 antibody. A representative experiment of 3 performed is shown. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

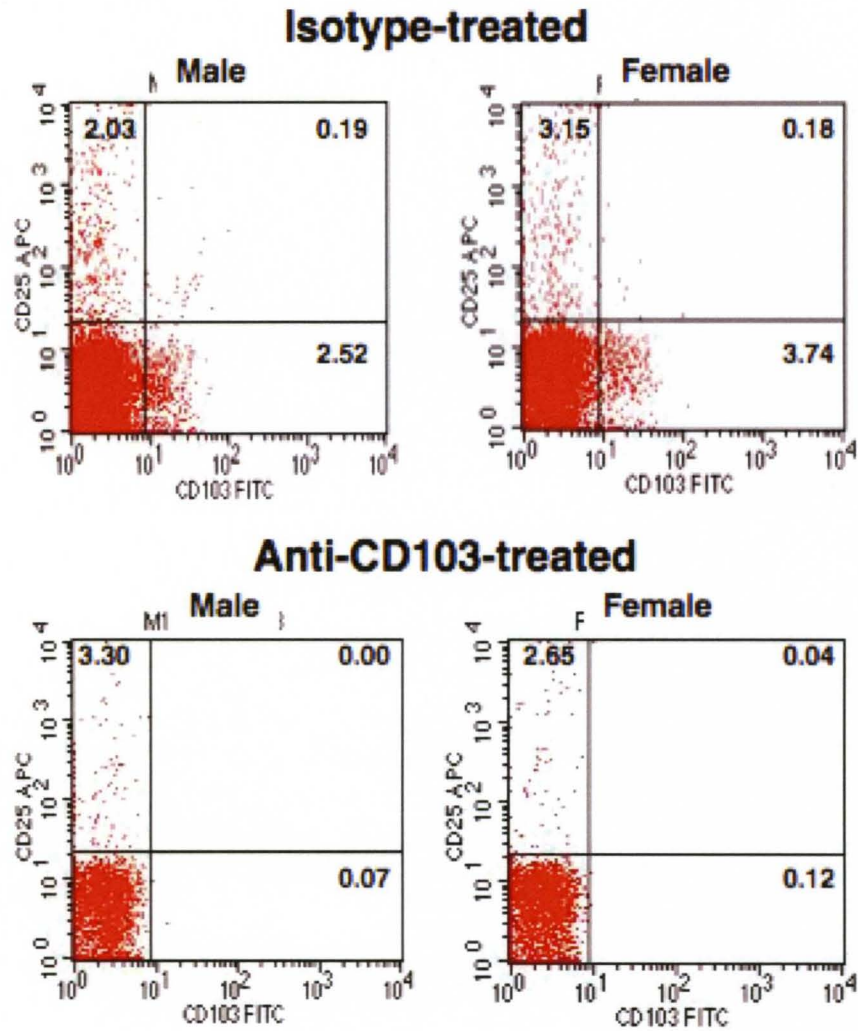
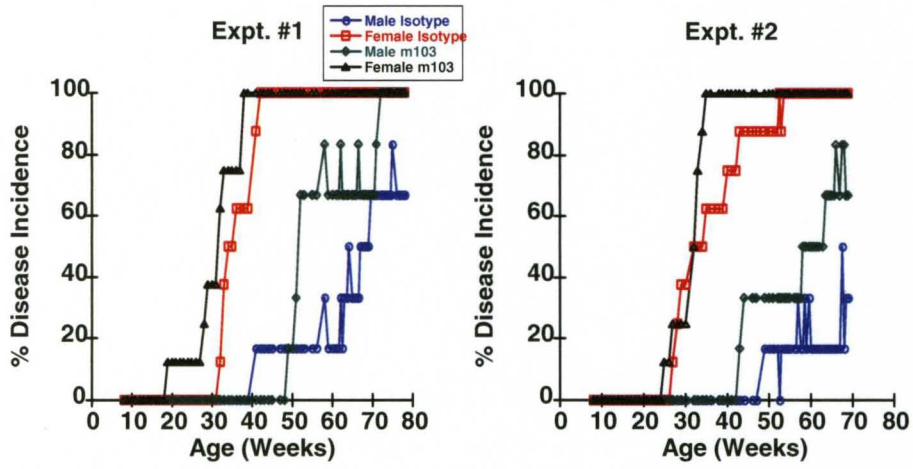
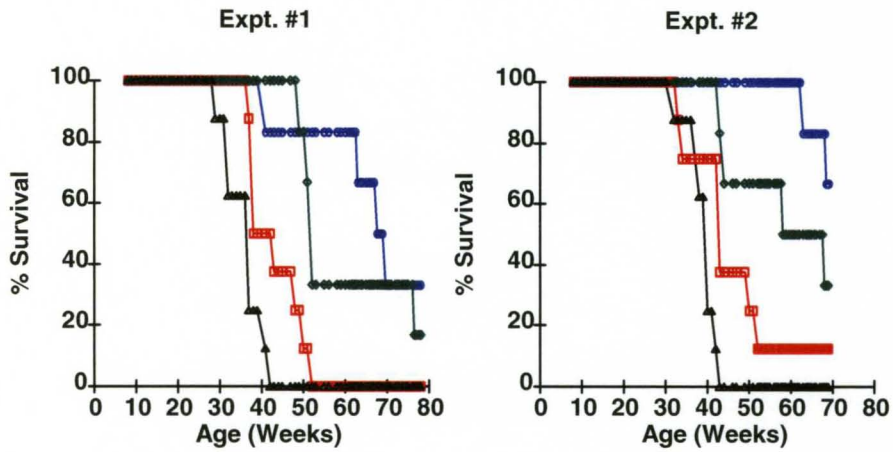
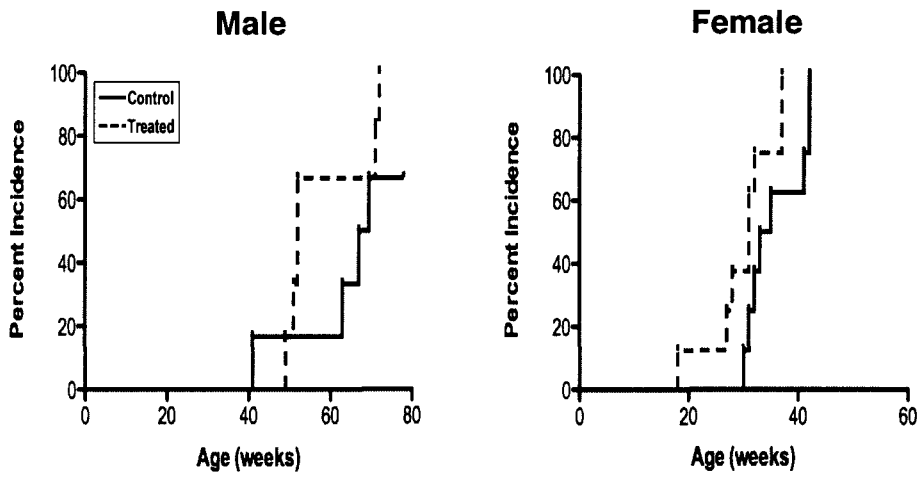


Figure 26. *In vivo* CD103-depletion in pre-diseased BWF1 mice. 8wk old female and male BWF1 mice were treated twice weekly with 0.5mg anti-CD103 for 4 weeks, then twice monthly until 20wks of age. Mice were considered sick when they had a proteinuria score of 3 for two consecutive weeks. Sample FACS figure comparing CD103 expression in the blood of anti-CD103-treated versus isotype-treated mice.

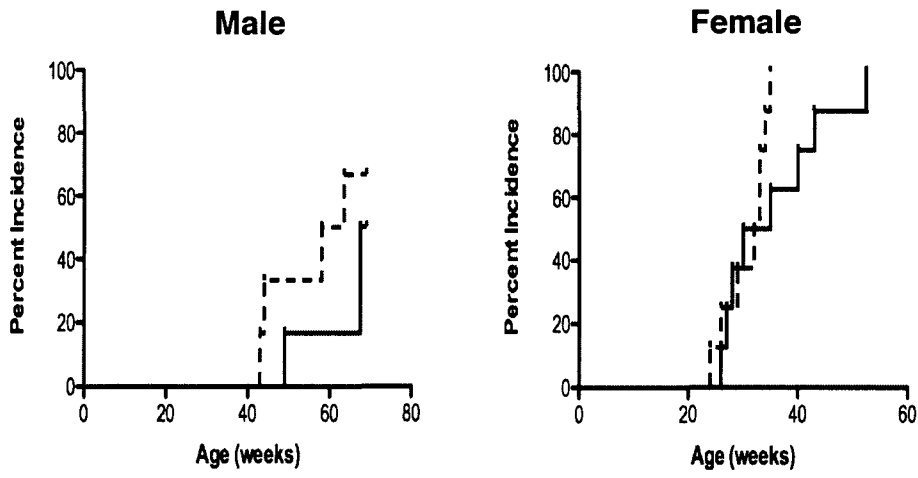
A**B**

C

Expt. #1



Expt. #2



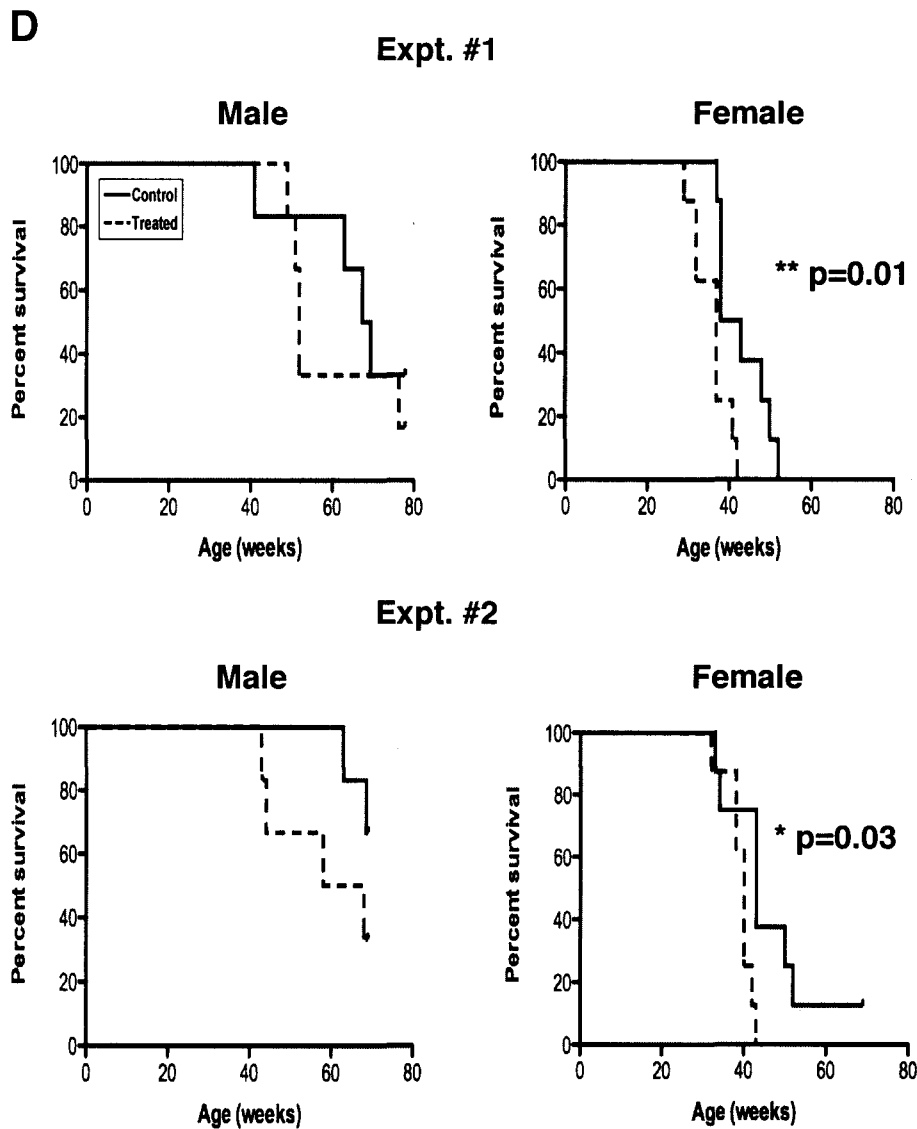


Figure 27. Disease incidence and survival in CD103-depleted BWF1 mice. Eight-wk-old female and male BWF1 mice were treated twice weekly with 0.5mg anti-CD103 for 4 weeks, then twice monthly until 20 wks of age. Mice were considered sick when they had a proteinuria score of 3 for two consecutive weeks. (A) Percent disease incidence. (B) Percent survival. (C) Kaplan-Meier disease incidence curves. (Experiment #1: control vs treated male Chi square 1.266, $p=0.26$; control vs treated female Chi square 3.023,

p=0.08; and Experiment #2: control vs treated male Chi square 1.009, p=0.32; control vs treated female Chi square 1.812, p=0.18) (D) Kaplan-Meier survival curves. (Experiment #1: control vs treated male Chi square 0.5225, p=0.47; control vs treated female Chi square 6.682, ** p=0.01; and Experiment #2: control vs treated male Chi square 1.950, p=0.1626; control vs treated female Chi square 4.795, * p=0.03).

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male				70.0 ± 11.3	39.0 ± 12.0	26.6 ± 2.0	
Non-Sick Female				64.0 ± 7.1	*68.0 ± 1.0	*50.8 ± 2.6	
Sick Female				54.4 ± 11.3	28.7 ± 9.6	27.2 ± 9.2	

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	24.2 ± 5.2	17.2 ± 1.2	29.5 ± 9.4	29.5 ± 9.4	18.7 ± 5.7	39.6 ± 23.5	
Non-Sick Female	24.8 ± 2.4	22.7 ± 1.8	31.6 ± 4.1	31.6 ± 4.1	47.5 ± 16.4	20.6 ± 2.5	
Sick Female	46.9 ± 17.4	133.1 ± 74.3	40.9 ± 7.0	40.9 ± 7.0	14.9 ± 7.3	101.4 ± 52.8	

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	68.4 ± 6.4	68.4 ± 14.5		66.8 ± 2.1	84.7 ± 3.8	81.7 ± 24.8	
Non-Sick Female	77.8 ± 9.1	71.4 ± 20.9		97.2 ± 10.0	178.3 ± 78.0	112.3 ± 23.4	
Sick Female	181.0 ± 56.3	**216.5 ± 41.9		***133.0 ± 7.1	166.2 ± 51.7	175.2 ± 51.5	

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male				52.9 ± 4.7	47.6 ± 19.3	51.1 ± 11.9	
Non-Sick Female				56.0 ± 6.1	41.4 ± 6.8	57.0 ± 10.2	
Sick Female				46.6 ± 7.0	9.7 ± 6.5	117.9 ± 97.3	

Table 10. Thymic weights and cell numbers in thymus, lymph nodes and spleen of 32-36-week-old BWF1 mice. Thymus, lymph nodes and spleen were collected from 32-36wk old sick female, non-sick female, and male BWF1 mice and weights or cell numbers determined. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Table 11A: LN MFI CD4⁺Foxp3⁺							
	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	9187.4 ± 289.6	725.4 ± 24.4	992.6 ± 121.2		496.5 ± 120.6	467.8 ± 36.3	164.8 ± 7.2
Non-Sick Female	8966.8 ± 225.4	733.2 ± 30.8	862.2 ± 85.6		438.8 ± 14.7	521.2 ± 27.0	148.1 ± 4.6
Sick Female	8915.2 ± 733.2	756.7 ± 19.3	648.2 ± 37.9		373.8 ± 24.3	473.6 ± 23.1	153.0 ± 4.9

Table 11B: Spleen MFI CD4⁺Foxp3⁺							
	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	7043.0 ± 254.8	643.5 ± 17.2	*651.7 ± 13.7		337.3 ± 10.1	542.5 ± 103.9	123.6 ± 1.6
Non-Sick Female	7549.5 ± 274.5	662.2 ± 16.7	621.1 ± 14.1		362.2 ± 25.1	398.7 ± 22.9	133.9 ± 2.6
Sick Female	7317.0 ± 408.2	694.4 ± 17.6	562.7 ± 23.3		370.8 ± 8.6	477.2 ± 22.9	139.4 ± 7.3

Table 11. Mean fluorescence intensity of Foxp3 on CD4⁺ cells in 32-36-week-old BWF1 mice. Cells from lymph nodes (LN) and spleen of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4 and anti-Foxp3 antibodies and analyzed by FACS[®]. Labeled CD4⁺ cells were gated and analyzed for the per cell expression of Foxp3 in (A) LN and (B) spleen. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

Table 12A: LN CD4⁺CD25⁺CD103⁺ Cell #s (x10⁶)							
	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	0.050 ± 0.014	0.084 ± 0.022	*0.083 ± 0.031	0.122 ± 0.078	0.076 ± 0.017	0.416 ± 0.343	
Non-Sick Female	0.054 ± 0.009	0.122 ± 0.048	0.086 ± 0.016	0.090 ± 0.029	0.585 ± 0.392	0.095 ± 0.014	
Sick Female	0.273 ± 0.139	3.282 ± 1.995	0.863 ± 0.325	0.403 ± 0.144	0.110 ± 0.056	2.095 ± 1.211	

Table 12B: Spleen CD4⁺CD25⁺CD103⁺ Cell #s (x10⁶)							
	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	*0.133 ± 0.032	**0.261 ± 0.105		***0.105 ± 0.029	0.343 ± 0.113	1.343 ± 0.610	
Non-Sick Female	0.193 ± 0.038	0.509 ± 0.300		0.266 ± 0.060	2.632 ± 2.049	2.051 ± 0.524	
Sick Female	1.051 ± 0.401	3.986 ± 1.196		1.246 ± 0.280	2.097 ± 1.043	5.885 ± 2.483	

Table 12. Numbers of CD4⁺CD25⁺CD103⁺ cells in 32-36-week-old BWF1 mice. Cells from lymph nodes (LN), spleen, and thymus of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4, anti-CD25, and anti-CD103 antibodies and analyzed by FACS[®]. The total numbers of CD4⁺CD25⁺CD103⁺ cells are shown in (A) LN, (B) spleen, and (C) thymus. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	56.2 ± 1.4	46.6 ± 1.2	*46.5 ± 2.1	45.0 ± 5.9	49.6 ± 6.0	49.6 ± 3.7	57.4 ± 1.9
Non-Sick Female	54.4 ± 2.1	46.7 ± 3.1	54.9 ± 1.4	46.6 ± 3.4	51.5 ± 1.8	54.7 ± 2.3	61.3 ± 1.1
Sick Female	**47.2 ± 1.7	45.5 ± 2.8	51.5 ± 2.2	37.6 ± 4.2	49.8 ± 2.9	44.9 ± 3.9	52.2 ± 4.0

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	**30.7 ± 0.5	26.0 ± 1.1	*28.7 ± 0.9	25.7 ± 0.7	28.3 ± 2.1	29.6 ± 0.6	25.9 ± 1.4
Non-Sick Female	35.3 ± 0.9	28.7 ± 1.7	35.6 ± 0.8	27.3 ± 2.6	32.7 ± 3.6	31.4 ± 1.7	29.5 ± 0.9
Sick Female	34.5 ± 0.6	28.1 ± 2.9	35.6 ± 2.2	22.3 ± 0.7	28.9 ± 1.4	29.4 ± 2.1	27.2 ± 3.5

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	10.2 ± 0.5		18.0 ± 0.7	8.2 ± 0.3		17.4 ± 4.1	
Non-Sick Female	8.9 ± 0.6		13.0 ± 1.6	8.2 ± 0.2		17.3 ± 3.0	
Sick Female	**36.3 ± 8.2		5.3 ± 0.7	**13.7 ± 2.0		**36.8 ± 4.2	

Table 13. Percentages of CD4⁺ cells in 32-36-week-old BWF1 mice. Cells from lymph nodes (LN), spleen, and thymus of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4 antibody and analyzed by FACS®. Labeled T cells were gated and analyzed for the percentages of cells expressing CD4 in (A) LN, (B) spleen, and (C) thymus. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	9.890 ± 1.784	7.039 ± 0.663	11.347 ± 3.888		8.435 ± 3.409	15.878 ± 7.704	
Non-Sick Female	10.367 ± 1.357	9.419 ± 0.981	14.266 ± 2.844		18.999 ± 5.751	10.371 ± 1.505	
Sick Female	14.726 ± 5.476	48.773 ± 25.196	17.188 ± 1.531		5.647 ± 2.600	36.911 ± 17.721	

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male	14.194 ± 1.491	14.865 ± 2.688			18.991 ± 1.703	23.254 ± 6.562	
Non-Sick Female	15.445 ± 2.009	15.735 ± 3.201			37.557 ± 15.696	33.051 ± 5.299	
Sick Female	29.019 ± 9.438	**50.41 2 ± 10.460			33.886 ± 8.833	47.835 ± 13.878	

	Expt. #1	Expt. #2	Expt. #3	Expt. #4	Expt. #5	Expt. #6	Expt. #7
Male			2.373 ± 0.389			7.231 ± 1.136	
Non-Sick Female			1.609 ± 0.538			8.678 ± 0.709	
Sick Female			**0.271 ± 0.059			28 030 ± 20.190	

Table 14. Numbers of CD4⁺ cells in 32-36-week-old BWF1 mice. Cells from lymph nodes (LN), spleen, and thymus of 32-36-week-old sick female, non-sick female, and male BWF1 mice were labeled with anti-CD4 antibody and analyzed by FACS[®]. The total numbers of CD4⁺ cells are shown in (A) LN, (B) spleen, and (C) thymus. Statistical differences were analyzed using a student's t test or ANOVA and the Tukey-Kramer multiple comparisons test. * p<0.05, ** p<0.005, and *** p<0.0005.

DISCUSSION

Regulatory T cells have been shown to be important in controlling autoimmune disease (123, 124). Females are generally more susceptible to autoimmune disease, including systemic lupus erythematosus (SLE), in which women are nine times more likely to develop disease than men (1). We hypothesized that gender differences in regulatory T cell populations are associated with differences in disease susceptibility. We used a spontaneous mouse model of SLE, (NZBxNZW)F1 (BWF1), in which females predominantly develop disease, to investigate gender differences in regulatory T cell populations. We also compared Treg populations in autoimmune-prone BWF1 mice to other autoimmune-prone and more resistant strains.

Strain differences in regulatory T cell percentages correlate with disease susceptibility.

Strains of mice with different genetic backgrounds have varying susceptibility to disease. Previous studies have found differences in regulatory T cell populations, either in number or function, between “more” and “less susceptible” strains (244, 254, 255). In a 2005 study by Howard and colleagues, the authors describe C57BL/6 mice as more susceptible to autoimmune disease than BALB/c mice and show that C57BL/6 mice have significantly lower percentages of CD4⁺CD25⁺ Tregs in the thymus and peripheral

lymphoid organs than BALB/c mice (254). We also found that BALB/c mice had a higher percentage of Tregs (although we used the more specific Treg marker, Foxp3, to distinguish Tregs) than C57BL/6 mice in thymus, LN, and spleen (Figure 2A). Whereas it is true that autoimmune disease can be induced in C57BL/6 mice (i.e. MOG-induced EAE (261)), it can also be induced in BALB/c mice (i.e. collagen-induced arthritis (262)), and by comparison to other strains, such as the highly autoimmune-prone strains, BWF1 and SJL mice, the C57BL/6 and BALB/c strains of mice rank extremely low on the autoimmune disease susceptibility scale. We, therefore, believe that it is significant that lupus-prone BWF1 mice had dramatically lower percentages of Tregs than the other three strains studied (Figure 2A). With regard to functional differences in Tregs between strains, Chen et al. found apparent differences in Treg suppressive function between C57BL/6 and BALB/c mice *in vitro* (254). When cultured with syngeneic CD4⁺CD25⁻ responder cells, C57BL/6 CD4⁺CD25⁺ Tregs did not suppress as well as CD4⁺CD25⁺ BALB/c Tregs, and the difference in suppressive function between C57BL/6 and BALB/c Tregs was due to the increased resistance of C57BL/6 CD4⁺CD25⁻ responder cells to suppression (254). We found no differences in the suppressive ability of CD4⁺CD25⁺ Tregs between any of the four strains studied when co-cultured with either syngeneic or C57BL/6 CD4⁺CD25⁻ responder cells (Figure 1). Furthermore, C57BL/6 responder cells seemed to be as sensitive to suppression as responder cells from any of the three other strains. We cannot account for these differences other than to suggest that there may be differences in protocol or mouse colony conditions. Morel and colleagues also found CD4⁺CD25⁻ resistance to suppression in NZB2410 mice, a congenic mouse model on a C57BL/6 background that expresses the lupus susceptibility locus *Sle1a* of

BWF1 mice (115). They found that high levels of IL-6 produced by bone marrow-derived DCs rendered CD4⁺CD25⁻ responder cells resistant to suppression by Tregs (115). In our study, we did not see any evidence of resistance of BWF1 responders to suppression by Tregs or evidence that the female BWF1 APCs were any more capable of conferring any kind of resistance onto the responders (Figure 14B & 14C, 25B). The discrepancies in the results between the studies could be due to 1) the APCs that we tested in the BWF1 model were endogenous spleen cells and not the bone marrow-derived DC that Morel and colleagues tested and 2) the mouse model used by the Morel group was a congenic strain of BWF1 that was primarily on a C57BL/6 background.

While other groups have looked at regulatory T cell populations in different strains of mice, and even compared autoimmune-prone and less autoimmune-prone strains of mice, they have not systematically studied the CD103⁺ Treg subset in these mouse strains. CD103⁺ Tregs are a very potent subset of Tregs that are thought to have a memory/effector phenotype and are found in both mice and humans (207-210). These CD103⁺ Tregs are better suppressors both *in vitro* and *in vivo* than CD103⁻ Tregs (149, 207, 211). We found that both autoimmune-prone strains, BWF1 and SJL, have lower percentages of CD4⁺CD25⁺CD103⁺ Tregs in LN than the less autoimmune-prone strains, C57BL/6 and BALB/c (Figure 3A). Again we found no difference in the ability of CD4⁺CD25⁺CD103⁺ Tregs to suppress syngeneic CD4⁺CD25⁻ responder cells between strains *in vitro* (Figure 3D). These data suggest that lower Treg ratios may be important in disease susceptibility.

Reddy et al. have reported that while SJL mice, which are susceptible to PLP 139-151-induced EAE, and B10.S mice, which are resistant to PLP 139-151-induced EAE,

have similar percentages of CD4⁺CD25⁺ Tregs, B10.S mice have a 3X higher PLP 139-151 antigen-specific Treg to antigen-specific effector T cell ratio than SJL mice, (263). When CD4⁺CD25⁺ Tregs in B10.S mice were eliminated using a depleting CD25 antibody, antigen-specific effector T cells were uncontrolled and able to proliferate, causing these mice to develop EAE (263). These data support the hypothesis that reduced percentages of Tregs in BWF1 mice, when compared to other mouse strains, may be unable to control autoreactive T cell proliferation and expansion well enough to prevent disease. We found no significant differences in homeostatic proliferation of CD4⁺Foxp3⁻ effector T cells *in vivo* between strains in young mice (Figure 5B). However, since we do not have the tools to analyze antigen-specific T cells in wildtype mice, we cannot rule out the possibility that autoreactive T cells are proliferating more in autoimmune-prone mice.

Gender differences in regulatory T cell percentages in autoimmune-prone and less autoimmune-prone mouse strains.

Women are more susceptible to many autoimmune diseases than men (1). This is especially true for systemic lupus erythematosus (49, 50). Females have a stronger immune response in general compared to males. They produce higher antibody titers in response to antigens (6), have more activated T cells (7), and produce more IFN- γ in response to antigens (10). In fact, inflammatory cytokine production is increased when CD4⁺ T cells are treated with estradiol and decreased when treated with the androgen *in vitro* (11, 12). Exposure to estrogens, either produced naturally in females or present in estrogenic compounds such as oral contraceptives and fertilizers, has been associated with an increased risk of autoimmune disease (13). Androgens have been shown to be

protective in some autoimmune disease settings. Treatment of SJL mice with adoptively transferred EAE with DHT 90-day release pellets resulted in less severe and eventually remitting disease (31). Castration of male non-obese diabetic (NOD), a mouse model of type 1 diabetes and (NZBxNZW)F1 (BWF1) mice resulted in disease similar to that seen in females (40, 68). Androgen treatment of female NOD or BWF1 mice protects these mice from disease (39, 68). The BWF1 mouse model of lupus mirrors human disease in two ways: first, disease develops spontaneously, and second, BWF1 mice also have a female bias for development of disease (66, 67). We evaluated 9-week-old female and male BWF1 mice, which do not yet exhibit disease symptoms (e.g., circulating dsDNA antibodies and proteinuria) to investigate gender differences in Treg populations. We also looked at female and male Treg populations in age-matched mice from three other strains, another autoimmune-prone strain, SJL, and two less autoimmune-prone strains, C57BL/6 and BALB/c. We found that of all four strains studied, only BWF1 females had lower percentages and numbers of CD4⁺Foxp3⁺ Tregs than males in the periphery (Figure 9A & 9B). CD4⁺CD25⁺ regulatory cells have been shown to be important in disease prevention in BWF1 mice, since depletion of these cells by anti-CD25 antibody treatment (i.e. Treg depletion) *in vivo* significantly accelerates disease (174). In another mouse model of lupus, NZM2328, removal of Tregs through pre-pubertal thymectomy also accelerated disease and adoptive transfer of CD25⁺ Tregs from non-sick mice could rescue from disease (175). These data support our hypothesis that the reduction we see in CD4⁺Foxp3⁺ Tregs in female BWF1 mice when compared to males may be an important contributing factor to their increased disease susceptibility.

Females of all four strains we studied had lower percentages and numbers of CD4⁺CD25⁺CD103⁺ Tregs in the periphery (Figure 10A, Table 7). This is interesting and may suggest that the potent CD4⁺CD25⁺CD103⁺ subset of Tregs is sensitive to hormonal influences. We found no differences in either CD4⁺CD25⁺ or CD4⁺CD25⁺CD103⁺ Treg function *in vitro* between young female and male BWF1 mice (Figure 8, 10D), suggesting that it may be the decreased ratio of Tregs:Effector cells and not a defect in inherent suppressive function that increases female disease incidence.

Natural Tregs develop in the thymus through positive selection on a high affinity TCR (185). In all four strains studied, we saw no differences between female and male thymic percentages or numbers of either CD4⁺Foxp3⁺ Tregs or the CD4⁺CD25⁺CD103⁺ subset of Tregs (Figure 11), indicating that there was no defect in thymic production of these cells. It is true that Tregs can develop extra-thymically through conversion in the periphery (190, 191), and it would be interesting to compare female and male conversion both *in vitro* and *in vivo*, especially in the BWF1 mice, in the future to determine whether female BWF1 mice, or BWF1 mice in general compared to other mouse strains, have a defect in conversion.

We looked at proliferation *in vivo* of CD4⁺Foxp3⁺ Tregs, CD4⁺CD25⁺CD103⁺ Tregs, and CD4⁺Foxp3⁻ non-Tregs from 9-week-old mice. The only strain with gender differences in proliferation ratios of CD4⁺Foxp3⁺:CD4⁺Foxp3⁻ T cells was the BWF1 strain (Figure 12D). Female BWF1 mice had a lower CD4⁺Foxp3⁺:CD4⁺Foxp3⁻ proliferation ratio than BWF1 males in the LN because the Foxp3⁻ cells were proliferating more and not because the Foxp3⁺ cells were proliferating less in females (Figure 12). So the question becomes, are the Foxp3⁻ effector cells proliferating in

female BWF1 mice because there are too few Foxp3⁺ Tregs to control them or are the Tregs dysfunctional thus allowing the Foxp3⁻ effector cells to proliferate and creating the imbalance in the Foxp3⁺:Foxp3⁻ ratio. Perhaps by looking at earlier time-points in BWF1 mice we may be able to distinguish between these two possibilities.

Hormones impact the development of autoimmune disease. It has been shown that castration of male BWF1 mice induces disease comparable to female disease (68). Estrogen treatment of castrated male or ovariectomized BWF1 mice increases disease severity while androgen treatment protects from disease (68). Though we don't understand the mechanism, our lab has also found that androgen-treated BWF1 females have increased Treg percentages, both CD4⁺CD25⁺ and CD4⁺CD25⁺CD103⁺ Treg subsets, comparable to levels seen in age-matched males, and that castrated male Treg percentages are comparable to percentages found in females (unpublished data). We hypothesize that androgens in males increase the numbers (perhaps by increasing survival or conversion/induction of these cells), and possibly the function, of Tregs by comparison to females, and it is through this mechanism, at least in part, that androgens protect males from the development of autoimmune disease even when they have a genetic predisposition for disease, as in BWF1 mice.

Regulatory T cell populations change with disease progression.

Regulatory T cell populations have been shown to be important in systemic lupus erythematosus (SLE) in both humans and mice (160, 161). In humans, some studies have found reduced Treg percentages and function in SLE patients (162-164), whereas other studies have found reduced percentages, but not function of Tregs (165, 166). Still others

have found increased Treg percentages in SLE patients (167-169). The discrepancy in data on Treg percentages and function in humans may be due to the fact that human SLE patients present with a wide array of symptoms for which they receive a variety of different treatments that could impact Treg populations. Also, the distinction between active and inactive SLE patients is not made clear in the literature, so different research groups may define them differently and, thus find different results. Another important factor when looking at human Tregs is that activated $CD4^+$ T cells in humans transiently express Foxp3 (138) and some Foxp3⁺ cells may actually be the IL-17-producing Foxp3⁺ cells described by Sakaguchi and colleagues (171), so any study defining Tregs as $CD4^+Foxp3^+$ may also include some activated T cells. It is, therefore, still not clear whether defects, either quantitative or qualitative, in Tregs in lupus patients are common and whether they play a role in pathogenesis. As more research tools become available, this issue will become clearer. In the meantime, mouse studies are critical to fill in the gaps in our knowledge.

In mice, there has been a great deal of research directed at depleting or increasing Treg populations and determining the impact on disease. As mentioned earlier, Treg depletion using an anti-CD25 monoclonal antibody in BWF1 mice accelerated disease (174). Removal of Tregs through pre-pubertal thymectomy in lupus-prone NZM2328 mice also accelerated disease and mice could be rescued from disease through adoptive transfer of $CD4^+CD25^+$ from non-sick mice (175). Increasing Treg populations, whether through adoptive transfer of Tregs or through treatment with tolerizing peptides, could delay or prevent lupus development in lupus-prone mice (176-179). These data together highlight the importance of Tregs in lupus development.

In our facility, approximately 50% of female BWF1 mice develop severe kidney disease by 32 weeks of age while 0% of males have developed disease at this age (Figure 13). Autoantibodies can be detected in the serum of females beginning at about 16 weeks of age and we begin measuring proteinuria in female BWF1 mice at about 28 weeks of age (Figure 24). When we compared Treg populations in sick female, non-sick female, and male 32-36-week-old BWF1 mice, we found that sick females had significantly higher percentages of both CD4⁺Foxp3⁺ and the CD4⁺CD25⁺CD103⁺ cells (Figure 15A, 16A). This was surprising because, as stated above, many groups have found that decreased Treg percentages are associated with acceleration and severity of disease and increased Treg percentages are associated with protection from disease. Interestingly, these sick female Tregs, both CD4⁺CD25⁺ cells and the CD4⁺CD25⁺CD103⁺ subset, suppressed as well *in vitro* as non-sick female and male Tregs (Figure 14A, 16D). Morel and colleagues found that CD4⁺CD25⁻ were resistant to suppression in NZB2410 mice, a congenic mouse model on a C57BL/6 background that expresses the lupus susceptibility locus *Sle1a* (115). However, when we compared CD4⁺CD25⁻ susceptibility to suppression, we found no differences between sick female, non-sick female, and male susceptibility to suppression *in vitro* (Figure 14B). Morel and colleagues also found that increased IL-6 production by bone marrow-derived DC *in vitro* rendered CD4⁺CD25⁻ responder cells resistant to suppression by CD4⁺CD25⁺ Tregs (115). However, we found no difference in the ability of APCs to support *in vitro* suppression between sick females and age-matched non-sick females and males (Figure 14C). Although these data do not rule out the possibility that Tregs in sick female mice function abnormally *in vivo*, it does

indicate that neither the Tregs, the CD4⁺CD25⁻ responders nor the APC have inherent defects that are detectable *in vitro*.

It is difficult to determine where in the body Treg suppressive function is needed to control/prevent lupus. Lupus presents with a wide variety of symptoms and immune defects making the identification of the important sites where suppression is needed *in vivo* very difficult (82). It has been shown, however, that T cell infiltration into the kidney is absolutely required for development of end-stage kidney disease (91), so the kidney, and particularly the kidney-draining LN, may represent important sites for Treg suppressive function. Thus, we examined the kidney-draining LN to determine whether sick female Tregs were able to traffic to this organ. We found that, as in the periphery, sick females had higher percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ Tregs in kidney-draining LN (Figure 19). We next looked at CD62L expression, since this molecule must be downregulated before T cells can traffic out of the LN. We did not find that sick female Tregs expressed any more CD62L than non-sick female and male Tregs (Figure 20). In the future, it will be necessary to investigate other Treg trafficking molecules (e.g., chemokines) expressed by sick female Tregs in comparison to age-matched non-sick females and males.

We could not explain the abundance of Tregs in sick female mice at 32-36 weeks of age, so we decided to investigate Treg populations in BWF1 mice from 9 to 32-36 weeks of age to see how they change with age. As discussed earlier, we found that 9-week-old females had lower percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ Tregs compared to age-matched males (Figure 9A 10A, 21, 22). By 20 weeks of age, female and male Treg percentages were not significantly different, however female

percentages still trended lower (Figure 21, 22). At 24-28 weeks of age, female and male BWF1 mice had similar percentages of Tregs, and by 32-36 weeks of age sick females had significantly higher Treg percentages than age-matched non-sick females and males (Figure 15A, 16A, 21, 22). These data suggested that 9 weeks may be an important period in time during which gender differences can influence disease development. Accordingly, we examined female and male 9-week-old mice in greater depth. As discussed previously, 9-week-old female BWF1 mice have decreased percentages of both CD4⁺Foxp3⁺ and CD4⁺CD25⁺CD103⁺ Treg subsets when compared to age-matched males (Figure 9A, 10A). These female Tregs suppress as well as male Tregs *in vitro*, however the CD4⁺CD25⁻ effector cells proliferate more *in vivo* in females than males, suggesting Tregs may be unable to control effector cell proliferation/activation *in vivo* (Figure 8, 12B). We had found that there were no differences in CD4⁺CD25⁻ resistance to suppression *in vitro* between 32-36-week-old sick female, non-sick female, and male mice (Figure 14B). However, there was a possibility that young 9-week-old female CD4⁺CD25⁻ cells were resistant to suppression at this critical period of time, and for this reason, compared CD4⁺CD25⁻ resistance to suppression *in vitro* between 9-week-old females and males. We did not find that female CD4⁺CD25⁻ responder cells were more resistant to suppression than males, in fact, if anything they were more susceptible to suppression *in vitro* (Figure 25A). We also found no difference between the ability of 9-week-old female and male APCs to support suppression *in vitro* (Figure 25B). These data suggest that it may be a decreased ratio and not a defect in suppressive function that underlies gender differences in disease susceptibility.

To add further support to our hypothesis that the pre-disease 9-week-old time-point is important in setting up gender differences in disease development later on, we depleted CD103⁺ cells in these young mice. Mice were treated with depleting antibody twice a week from 8-12 weeks of age then given a maintenance dose twice a month from 12-20 weeks of age. We found that CD103-depletion decreased time to disease onset and accelerated death in both female and male BWF1 mice (Figure 27). These results suggest a role for CD103⁺ cells in protection from disease, as depletion of these cells induced disease in males, and males don't normally develop full-blown disease without castration.

Other immune cells express CD103 besides the potent CD4⁺CD25⁺CD103⁺ subset of Tregs. A small percentage of regulatory CD8⁺ cells that express CD103 have been reported (220, 221). However, cytotoxic CD8⁺ T cells express CD103 as well (222-224). A subset of DCs found primarily in the gut, also expresses CD103 (226). These DCs induce a gut-homing phenotype on T cells (226) and are thought to mediate conversion of CD4⁺CD25⁻ effector cells to CD4⁺CD25⁺ Tregs in the gut (228, 229). A subset of CD103⁺ DCs is also found in the skin (230). A small invariant NKT cell subset found in LN and skin express CD103 and have been found to produce IL-17 (231). We have not yet ruled out the possibility that depletion of any of these cell types may play a role in disease development in our CD103-depleted mice. However, depletion of cytotoxic CD8⁺ T cells or IL-17-producing NKT cells would presumably reduce inflammation and increase time to disease as well as survival. The CD8⁺CD103⁺ regulatory T cells represent a very small percentage and have been reported to play a role in a model of tolerance induced *in vivo* referred to as anterior chamber-associated immune deviation (ACAID) and it is not clear whether they exist naturally (221). Therefore, we would not

expect depletion of these cells to have an appreciable impact on disease in BWF1 mice. Depletion of CD11c⁺CD103⁺ DCs using anti-CD103 antibody in a model of *Bordetella pertussis* infection causes decreased inflammatory cellular infiltration in the lung and delayed bacterial clearance (264) suggesting that again, if anything, these DCs are involved in inflammation and not tolerance. This is also true for the prolongation of MHC-mismatched islet allograft survival after depletion of CD103⁺ cells (265). CD103-deficient mice on a C57BL/6 background have reduced T cell numbers in the gut (217) and develop skin lesions (218), and although they are more susceptible to adoptive transfer of colitis, they do not develop colitis spontaneously (227). Therefore, depletion of CD103⁺ DCs in the gut and skin could potentially cause mice to develop colitis or skin lesions, however, we saw no evidence of either in our CD103-depleted BWF1 mice. Taken together, these data suggest that the majority of immune cells other than Tregs that express CD103 mediate inflammatory rather than tolerogenic responses and it is more likely that their depletion would, therefore, have a positive, rather than the negative effect that we see on prevention of disease. The only tolerogenic CD103⁺ populations appear to reside in the skin and gut, and should have a local and not a systemic effect, and in fact, we did not even see any evidence of a local (no skin or gut lesions) effect in our anti-CD103-treated mice. Therefore, we believe that it is depletion of the CD4⁺CD25⁺CD103⁺ cells that is affecting disease incidence and mortality in anti-CD103 antibody-treated BWF1 mice.

In summary, these data suggest that the proportion of regulatory T cells in the peripheral lymphoid organs of mice plays an important role in disease susceptibility very early in the disease process, but not after disease is already established. The data also

suggest that factors associated with gender play an important role in the development and/or maintenance of the potent CD4⁺CD25⁺CD103⁺ Treg population in mice, in general, and very likely contribute to the propensity for females to develop autoimmune disease (or the protection of males from disease) through this mechanism. Future work will focus on other potential critical periods of time during disease development and on how altering Treg populations can impact disease. Further investigation will also focus on potential environmental factors and trafficking issues in newly sick female BWF1 mice that could be limiting the effectiveness of their abundant Treg populations and what influence hormones might have on this system.

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LIST OF ABBREVIATIONS

ACAID	Anterior Chamber-Associated Immune Deviation
APC	Antigen Presenting Cell
BCG	Bacillus Calmette-Guérin
BWF1	(NZBxNZW)F1
CNS	Central Nervous System
Cr2	Complement receptor 2
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein 4
DC	Dendritic Cell
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DNA	Deoxyribonucleic Acid
dsDNA	double-stranded Deoxyribonucleic Acid
DTH	Delayed-Type Hypersensitivity
EAE	Experimental Autoimmune Encephalomyelitis
EAU	Experimental Autoimmune Uveitis
ER	Estrogen Receptor
Fc γ R	Fc-gamma Receptor
GITR	Glucocorticoid-Induced TNFR-Related Protein

GVHD	Graft-Versus-Host Disease
HLA	Human Leukocyte Antigen
HMGB1	High-Mobility Group Box 1
ICOS	Inducible Costimulatory Molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked Syndrome
IRF4	Interferon Regulatory Factor 4
Kid-LN	Kidney-draining LN
MBP	Myelin Basic Protein
MHC	Major Histocompatibility Complex
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
Naip1	NLR Family Apoptosis Inhibitory Protein 1
NOD	Non-Obese Diabetic
Pbx1	Pre-B-cell Leukemia Homeobox 1
PPD	Purified Protein Derivative
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
Sh2d1b	SH2 Domain Containing 1B
SIRP- α	Signal-Regulatory Protein Alpha
SLAM	Signaling Lymphocytic Activation Molecule

SLE	Systemic Lupus Erythematosus
SNF1	(NZBxSWR)F1
TCR	T Cell Receptor
TGF- β	Transforming Growth Factor Beta
T _H	T Helper
TNF	Tumor Necrosis Factor
Treg	Regulatory T cell
TSP-1	Thrombospondin-1

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- 2004-2005 Lab Technician
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ABSTRACTS:

1. **Tucker CF**, Nebane-Ambe D, Parnell SA, Manirarora JN, Alard P, and Kosiewicz MM. Sex differences in regulatory T cells in a mouse model of systemic lupus erythematosus. Research! Louisville, 2007. (Poster Presentation)
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