# Sex Differences in the Gut Microbiome Drive Hormone-Dependent Regulation of Autoimmunity

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Microbial exposures and sex hormones exert potent effects on autoimmune diseases, many of which are more prevalent in women. We demonstrate that early-life microbial exposures determine sex hormone levels and modify progression to autoimmunity in the nonobese diabetic (NOD) mouse model of type 1 diabetes (T1D). Colonization by commensal microbes elevated serum testosterone and protected NOD males from T1D. Transfer of gut microbiota from adult males to immature females altered the recipient's microbiota, resulting in elevated testosterone and metabolomic changes, reduced islet inflammation and autoantibody production, and robust T1D protection. These effects were dependent on androgen receptor activity. Thus, the commensal microbial community alters sex hormone levels and regulates autoimmune disease fate in individuals with high genetic risk.

Genome-wide studies have identified common polymorphisms associated with autoimmune disease risk, including causal variants implicated in immune regulation. These analyses have not addressed the impact of two other critical modifiers of autoimmunity: sexual dimorphism and environmental factors. The incidences of many autoimmune syndromes display a strong female bias (*1*–*3*), yet the mechanisms of sex-mediated immune regulation are poorly understood. Findings of incomplete concordance in monozygotic twins and a recent rise in autoimmune disease incidence in developed countries (*4*) indicate a causal role of environmental factors in disease. We have identified a direct interaction between sex hormones and microbial exposures and show that microbiome manipulations can provoke testosterone-dependent protection from autoimmunity in a genetically high-risk rodent model.

The nonobese diabetic (NOD) mouse displays spontaneous, immune-mediated pancreatic β cell destruction, causing type 1 diabetes (T1D) with complex genetic and environmental etiology (*5*, *6*). In NOD mice and humans, T1D is preceded by leukocyte infiltration of the pancreatic islets (insulitis) and production of autoantibodies (Aab) to islet antigens, including insulin (*7*–*10*). NOD T1D incidence displays a strong >2:1 female-to-male sex bias (*11*). Castration increases male T1D incidence (*12*), and androgen treatment confers protection to females (*13*); hence, testosterone regulation is implicated in T1D pathogenesis. Moreover, higher T1D incidence in NOD colonies is positively correlated with better hygiene status (*11*). Conversely, systemic immune challenge with bacterial antigens protects these animals from disease (*14*, *15*). Thus, the NOD model enables assessment of the roles of microbial colonization and sex in the regulation of spontaneous autoimmunity in high-risk individuals.

Male NOD mice housed in specific pathogen–free (SPF) conditions were protected relative to females ($P < 10^{-4}$; Fig. 1A and table S1), in agreement with previous reports (*11*, *16*, *17*). To assess the impact of commensal microbes on T1D

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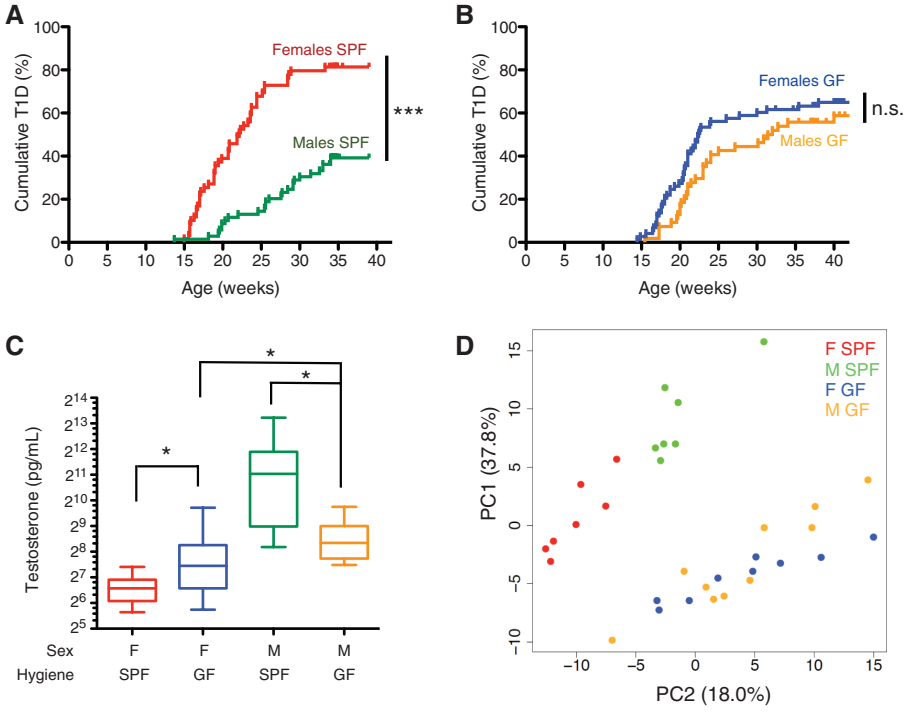


**Fig. 1.** Sex-biased autoimmunity in the NOD mouse depends on the microbiome. (**A** and **B**) The natural history of T1D in NOD/Jsd mice in SPF or GF conditions. (A) SPF females ($n = 59$) and SPF males ($n = 69$). ***$P < 0.0001$. (B) GF females ($n = 73$) and GF males ($n = 54$). $P = 0.2115$, log-rank (Mantel-Cox) comparisons of survival curves. (**C**) Serum testosterone concentrations were higher in GF versus SPF females ($P = 0.0248$) and lower in GF versus SPF males (asterisk represents significant differences between groups, $P = 0.0049$, Mann-Whitney test, $n \geq 10$ per group). Testosterone in GF male sera was higher than in GF female sera ($P = 0.0378$). Box plots display the median, 25th percentile, and 75th percentile; whiskers display minimum and maximum values. (**D**) PCA plot, generated by analysis of more than 180 serum metabolites in SPF females, SPF males, GF females, and GF males ($n \geq 6$ per group). Principal components PC1 and PC2, which explain 55.8% of the total variance observed, discriminate SPF female samples from male samples and discriminate both sexes in SPF housing from GF-housed females and males.

incidence, we rederived NOD mice into germ-free (GF) conditions and monitored GF cohorts by glucose testing. In contrast to SPF conditions (*18*), GF NOD males and females had similar T1D incidence ($P = 0.2115$; Fig. 1B and table S1). T1D incidence was stable under SPF conditions (fig. S1A) and was unaltered by feeding of a diet used in GF conditions (fig. S1B); therefore, the relative protection of males in the SPF setting appeared to be dependent on the presence of commensal organisms. GF NOD mice colonized with an altered Schaedler flora (ASF; eight species) displayed a sex-dependent trend ($P = 0.0878$), which suggests that this limited-diversity microbiota partially restored the sex bias in the T1D phenotype (table S1 and fig. S1C). To determine whether microbial colonization affected sex hormone levels, we quantified 17β-estradiol and testosterone in the serum of SPF and GF males and females. No hygiene-dependent effects were detected in 17β-estradiol (fig. S1D). In contrast, GF females displayed elevated testosterone relative to SPF females ($P < 0.05$; Fig. 1C), and GF males had lower levels relative to SPF males ($P < 0.05$; Fig. 1C), indicating that commensal colonization regulated testosterone production and/or usage.

Alterations in the composition and function of the gut microbiome exert mutualistic effects on host metabolism (*19*). Mass spectrometry–based metabolome analyses provide high sensitivity and reliable quantification of metabolites (*20*, *21*). To identify host metabolic changes induced by microbial colonization, we screened 183 serum metabolites using mass spectroscopy methods (*18*). Serum metabolite levels from SPF NOD males and females and from GF males and females were analyzed to identify metabolites that differentiated these four groups (tables S2 and S3). Principal components analysis (PCA) revealed a subset of glycerophospholipid and sphingolipid metabolites that accounted for a high proportion of total variability in this data set and showed distinct clustering of SPF males and SPF females (Fig. 1D). In contrast, these metabolites did not distinguish males and females in the GF state (Fig. 1D), revealing a role for microbiome exposure in regulating sex-specific features of the host metabolic profile. Therefore, under colonized settings, males and females either had distinct microbial communities that induced different hormonal and metabolic responses, or responded in a sex-specific manner to an identical community.

To test for gut microbiome composition differences between the sexes and for changes that might accompany maturation, we sequenced bacterial 16*S* ribosomal RNA (rRNA) libraries prepared from cecal contents of SPF-housed NOD males and females at weaning (3 weeks), puberty (6 weeks), and adulthood prior to T1D onset (14 weeks). PCA was applied to these data to detect age- and sex-specific phenotypes. Although weanling NOD males and females were indistinguishable from each other, sex-specific differences in microbiome composition became evident at puberty and were most apparent in adult mice

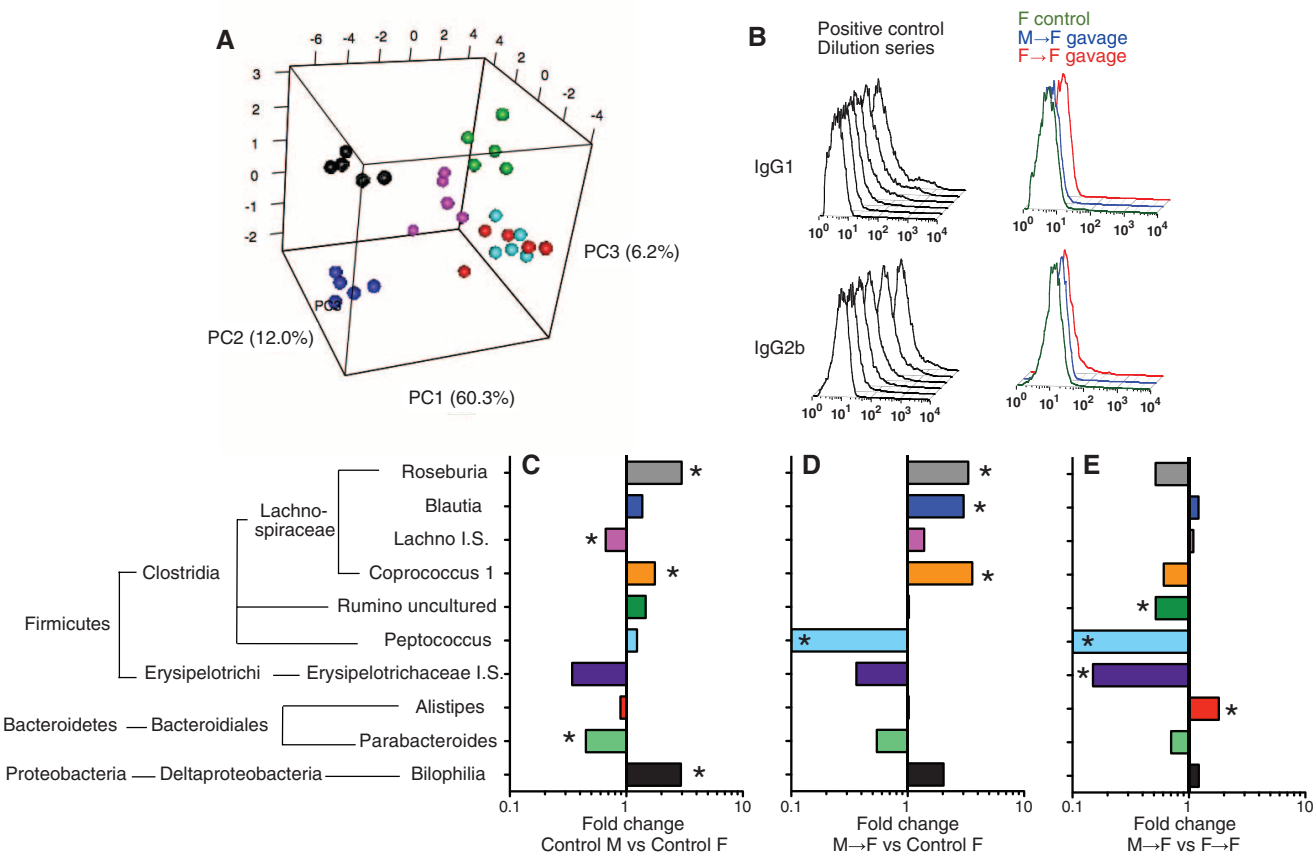


**Fig. 2.** Sex-specific microbiome profiles emerge after puberty, and cecal microbiome transplantation can stably alter the microbiome of the host without inducing systemic immune priming. (**A**) 16*S* bacterial rRNA sequencing was used to define the microbiome profiles of NOD male and female SPF mice at various developmental time points. PCA was used to compare the top 30 differentially abundant bacterial families across six different groups ($n = 5$ per group): 3-week-old males (turquoise), 3-week-old females (red), 6-week-old males (pink), 6-week-old females (green), 14-week-old males (blue), and 14-week-old females (black). These groups were separated by principal components PC1 to PC3, collectively explaining 78.5% of the total variance. (**B**) Systemic immune priming against the commensal microbiome was evaluated in M→F, F→F, and unmanipulated female NOD mice (right histograms). No microbiome-dependent differences in commensal immunoglobulin G1 (IgG1) or IgG2b titers were detected among these groups ($n > 10$ per group; see fig. S2). Control mice were inoculated systemically with a bacterial isolate (black traces). (**C** to **E**) 16*S* sequencing comparisons of control males versus females at 14 weeks of age (C), female recipients of male microbiome (M→F) versus unmanipulated females (D), and female recipients of male microbiome (M→F) versus female recipients of female microbiome (F→F) (E) ($n = 5$ per control group, $n = 10$ per gavaged group). Bacterial genera shown represent those found to be significantly different in at least one of these pairwise comparisons [*$P < 0.05$, two-part statistic (*35*); see table S6].

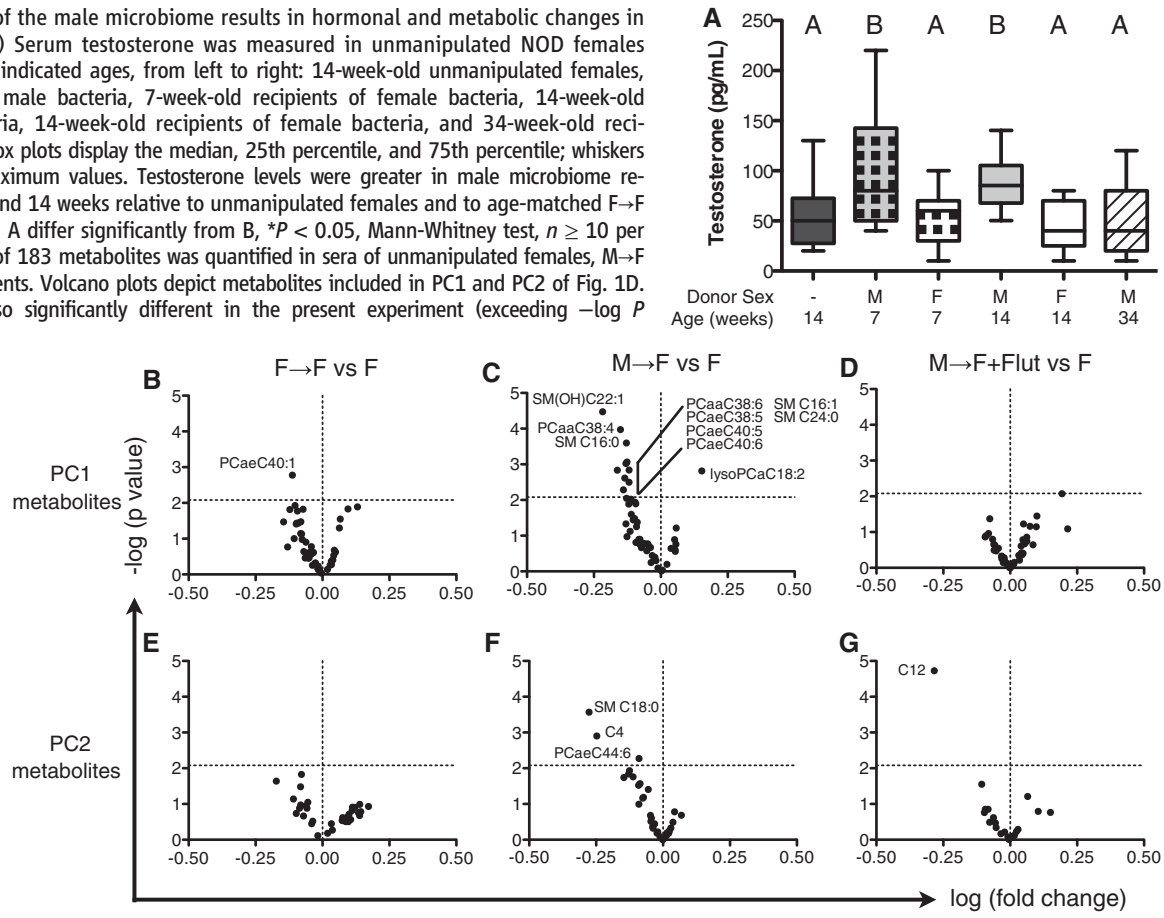


**Fig. 3.** Transplantation of the male microbiome results in hormonal and metabolic changes in the female recipient. (**A**) Serum testosterone was measured in unmanipulated NOD females and gavage recipients at indicated ages, from left to right: 14-week-old unmanipulated females, 7-week-old recipients of male bacteria, 7-week-old recipients of female bacteria, 14-week-old recipients of male bacteria, 14-week-old recipients of female bacteria, and 34-week-old recipients of male bacteria. Box plots display the median, 25th percentile, and 75th percentile; whiskers display minimum and maximum values. Testosterone levels were greater in male microbiome recipients at both 7 weeks and 14 weeks relative to unmanipulated females and to age-matched F→F recipients (groups marked A differ significantly from B, $*P < 0.05$, Mann-Whitney test, $n \geq 10$ per group). (**B** to **G**) A panel of 183 metabolites was quantified in sera of unmanipulated females, M→F recipients, and F→F recipients. Volcano plots depict metabolites included in PC1 and PC2 of Fig. 1D. Metabolites that were also significantly different in the present experiment (exceeding −log *P* threshold of 2.08) are labeled. Comparisons of metabolite levels in F→F versus F [(B) and (E)] and M→F versus F [(C) and (F)] are shown ($n \geq 5$ per group). The metabolomic assay was also applied to M→F recipients that had also been implanted with continuous-release pellets containing the AR antagonist flutamide [(D) and (G)].

(Fig. 2A). Given their similar housing conditions (*18*), these data indicated that sexual maturation was a major determinant of the cecal microbiome community structure. Because the relative T1D protection of males depended on commensal colonization (Fig. 1, A and B) and because mature males and females harbored distinct cecal microbiota, we asked whether manipulating the microbiota of young SPF females by transfer of cecal contents from adult donors provoked changes in microbiome composition, hormonal status, and metabolic status of the recipients.

Female NOD weanlings were gavaged twice with diluted cecal contents from either adult NOD male or NOD female donors. Systemic exposures to microbial agents can protect NOD mice from T1D (*14*, *22*). Therefore, we tested whether gavage had unintentionally caused systemic priming that resulted in antibodies specific for the inoculated bacteria (*23*) (Fig. 2B). Positive control sera for this assay were prepared from mice systemically primed by intravenous injection with commensal bacteria. Low-titer commensal antibody responses were observed in control and gavaged females (Fig. 2B and fig. S2) relative to systemically primed controls. Therefore, the gavage transfer protocol did not induce systemic immune priming against cecal bacteria.

To evaluate the impacts of gavage transfers on the recipients' microbiomes, we performed 16*S* rRNA sequencing of cecal samples from age-matched unmanipulated male and female controls (Fig. 2C) and from female recipients of adult male (M→F) or adult female (F→F) microbiota. Introduction of either adult male or female microbiota stably altered the recipient microbiome relative to unmanipulated female controls (Fig. 2, D and E). Some bacterial genera that were differentially represented in unmanipulated females relative to M→F gavage recipients also distinguished unmanipulated males and females (e.g., *Roseburia*, *Blautia*, *Coprococcus 1*, *Parabacteroides*, and *Bilophilia*; Fig. 2, C and D, and tables S4 and S6), whereas other differences resulting from gavage of male microbiota were not "male-typic" (e.g., *Peptococcus* and *Lachno I.S.*; Fig. 2, C and D, and tables S4 and S6). Statistical analysis indicated excellent coverage of the biodiversity among these samples (fig. S3A). Thus, gavage of male microbiota into SPF-colonized weanling females altered their microbiota to a third state, distinct from both unmanipulated males and females. Although durable for ≥11 weeks, these gavage-induced changes were no longer evident at 34 weeks of age (fig. S3B).

Given our observations that microbial colonization status was correlated with testosterone levels (Fig. 1C), we analyzed serum testosterone in female gavage recipients of either male or female microbiota. Strikingly, at ages 7 and 14 weeks, but not at 34 weeks, the M→F gavage recipients displayed significantly increased testosterone levels relative to unmanipulated adult females and to age-matched F→F recipients (Fig. 3A). The magnitude of testosterone increase produced by M→F transplant was lower than observed values in unmanipulated, age-matched SPF males (compare Fig. 1C). To determine whether this moderate elevation affected fecundity, we paired cohorts of M→F gavage recipients and unmanipulated females with stud males. Litter size and time to birth of a first litter did not differ between the two groups, which suggests that the male microbiota–dependent increase in testosterone did not impair fertility (table S6). These data show that transfer of male microbiota into young females conferred sustained testosterone elevation compatible with normal breeding behavior in recipient females.

The effect of M→F microbiome transfer on recipient testosterone levels suggested that this manipulation might exert broader metabolic influence. Therefore, we performed a serum metabolomics analysis of 14-week-old unmanipulated males and females and of M→F and F→F recipient females, with a focus on metabolites that had distinguished the sexes under SPF and GF conditions (Fig. 1D and tables S2 and S3). Transfer of male, but not female, microbiota lowered serum concentrations of glycerophospholipid and sphingolipid long-chain fatty acids (Fig. 3, B, C, E, and F), demonstrating that the sex of the microbiome donor determined metabolic out-

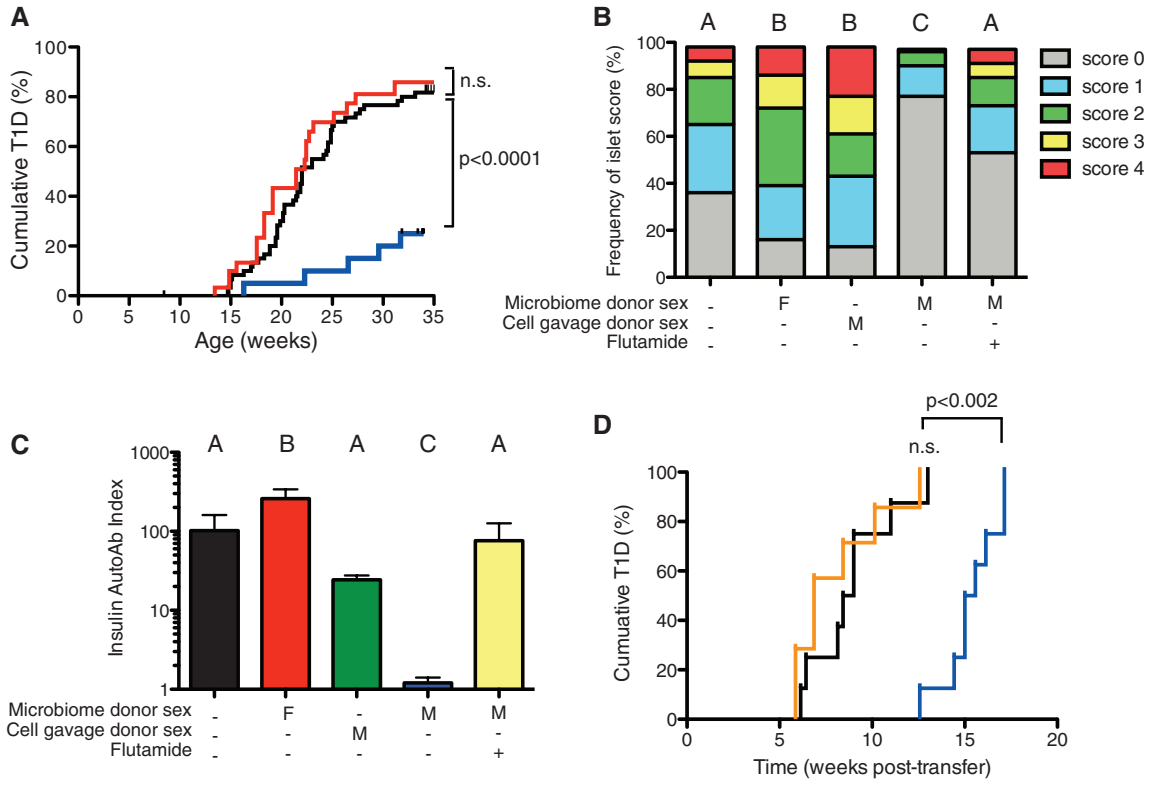


**Fig. 4.** Gavage of female NOD pups with male NOD-derived intestinal microbiome results in T1D protection, decreased insulitis severity, and decreased insulin Aab titer by an androgen-dependent mechanism. Female NOD weanlings were gavaged with cecal bacteria from either adult NOD males or adult NOD females. (**A**) T1D was assessed in unmanipulated NOD females (black, $n = 59$), F→F female recipients (red, $n = 30$), and M→F recipients (blue, $n = 38$). T1D survival curves did not differ between F and F→F groups. M→F recipients were protected from T1D relative to unmanipulated females ($P < 0.0001$, log-rank test). (**B**) Insulitis severity was assessed by established protocols (*18*) in (from left to right) unmanipulated females, recipients of the female microbiome (F→F), or recipients of the male microbiome (M→F), as well as a M→F group that simultaneously received a subcutaneous pellet secreting the AR antagonist flutamide. As a control for possible effects of male cells contaminating the gavage inoculum, females gavaged at weaning with male cells only were also included (middle histogram bar). Different letters indicate significantly different insulitis score distributions by $\chi^2$ test (Bonferroni-corrected $\alpha = 0.005$, $n \geq 5$ biological replicates per group). (**C**) Insulin Aab titer was assessed as another preclinical T1D-related phenotype. Different letters indicate significantly different group means by Mann-Whitney test (Bonferroni-corrected $\alpha = 0.005$, $n \geq 5$ per group). Error bars indicate SEM. (**D**) As a test for possible effects of microbiome manipulation and changes in host hormonal and metabolic status on T cell diabetogenicity, $10^7$ purified splenic T cells were prepared from unmanipulated NOD females (black), M→F gavage recipients (blue), or M→F gavage recipients treated with flutamide to antagonize AR signaling (orange), then transferred to 4- to 5-week-old female NOD.*SCID* recipients by intravenous injection. Recipients were monitored for hyperglycemia ($n > 7$ per group). The latency of T1D in NOD.*SCID* recipients of T cells isolated from M→F gavage recipients was greater than in recipients of T cells isolated from either unmanipulated females or from M→F gavage recipients that had been treated with flutamide ($P < 0.002$, log-rank comparisons of survival curves).

comes in the recipient. A recent report that many of these same long-chain fatty acids differed between human males and females (*24*) suggests evolutionary conservation of the sex-dependent effects we observed. Thus, in agreement with phylogenetic analysis of microbiome composition (Fig. 2, C to E), M→F gavage produced a metabotype distinct from that of both unmanipulated females and males.

To determine whether the testosterone elevation observed in M→F females caused the metabotype changes, we repeated the M→F gavage of weanling females and also treated these recipients with the androgen receptor (AR) antagonist flutamide, using a 60-day implant formulation. Blockade of AR signaling attenuated all of the male microbiome–specific changes in female host metabolites (Fig. 3, D and G), demonstrating that testosterone elevation caused by male microbiome transfer was critical for the generation of downstream host metabolomic phenotypes.

Because transfer of male microbiota altered recipient female hormonal and metabolic profiles, we asked whether these changes also altered the course of autoimmunity. We established cohorts of NOD females that were either unmanipulated or gavaged at weaning with male or female microbiota, followed by blood glucose monitoring. Relative to either unmanipulated females or F→F gavage recipients, M→F recipients were strongly protected from T1D (Fig. 4A, $P < 0.0001$). Diabetes onset is preceded by progressive insulitis (*5*, *6*). Thus, insulitis was assessed at 14 weeks of age in separate cohorts of unmanipulated females, F→F gavage recipients, and M→F recipients with or without flutamide treatment. Because intestinal epithelial cells are continuously soughed into the gut lumen and are likely present in the M→F gavage inoculum, we performed an in vivo cytotoxicity assay (*25*) to verify that gavage transfer of male cellular antigens did not induce an immune response in female recipients (fig. S4). As an additional control for potential effects of male antigens in cecal preparations, another cohort of females was gavaged with sterile male cells only. Gavage of male cells did not protect against insulitis progression, in accord with the in vivo cytotoxicity assay; this finding indicates that oral delivery of male antigens did not confer an immune response in this model (Fig. 4B). In contrast, female recipients of male microbiota were protected from invasive insulitis relative to both unmanipulated and F→F recipients, and the latter displayed slightly greater insulitis severity than unmanipulated controls (Fig. 4B). Moreover, insulitis protection conferred by M→F microbiome transfer was lost when AR signaling was antagonized by flutamide (Fig. 4B), demonstrating that testosterone activity was essential to the protection from islet inflammation.

Insulin-specific Aab, a second autoimmune phenotype in prediabetic NOD mice and humans, was quantified in the same cohorts of mice examined for insulitis. Relative to either unmanipulated females or F→F recipients, M→F recipients displayed lower Aab titers (Fig. 4C). In agreement with our insulitis data (Fig. 4B), Aab titers were not significantly decreased in M→F flutamide-treated mice or in recipients of male cell gavage only (Fig. 4C). Collectively, these results show that male microbiome transfer to females drove testosterone-dependent attenuation of autoimmune phenotypes and protection from T1D. To test whether the diabetogenic

potential of T cells was altered by these microbiome manipulations, we assessed the ability of T cells from unmanipulated and from M→F recipient females (with or without flutamide) to transfer T1D to lymphocyte-deficient, T1D-resistant NOD.*SCID* (severe combined immunodeficient) recipients. T cells from unmanipulated NOD females transferred T1D to 100% of NOD.*SCID* recipients within 13 weeks (Fig. 4D). In contrast, T cells from M→F mice were delayed in their ability to transfer T1D ($P < 0.002$; Fig. 4D). T cells from M→F + flutamide-treated mice transferred T1D with equivalent kinetics to the T cells from unmanipulated females (Fig. 4D). Thus, testosterone was a key mediator of male microbiota effects on the female metabolome and of the autoimmune response evident in insulitis progression, Aab production, and the capacity of T cells to transfer diabetes to NOD.*SCID* recipients. Additional studies are needed to define the pro-autoimmune mechanisms conferred by the female intestinal microbiome that likely involve other hormone-regulated pathways.

Our results reveal that alteration of the gut microbiome composition in early life potently suppresses autoimmunity in animals at high genetic risk for disease. Recent human data demonstrate that puberty and pregnancy shape the intestinal microbiota, provoking metabolic changes that may favor fertility and reproduction (*26*, *27*). Similar to our findings in the NOD model, sex hormones may also modulate sexual dimorphism in human autoimmune diseases. The female-to-male bias in rheumatoid arthritis and multiple sclerosis incidence declines with older age at onset, coincident with a decline in testosterone (*28*, *29*). In contrast, human T1D is not sex-biased, perhaps because the peak age at onset precedes puberty, with a recent rapid rise in incidence reported in children under 5 (*30*, *31*). Our data demonstrate that microbiome alterations in young, commensally colonized mice conferred testosterone and metabolite changes sufficient to oppose genetically programmed autoimmunity while preserving fertility.

Evidence of intestinal dysbiosis in autoimmune disease patients is emerging (*32*, *33*). As shown here and in a recent study of autoimmune demyelination (*34*), rodent models identify microbiome alterations as a causal factor and not merely a consequence of autoimmune disease. Improved prospective identification of children at high risk for autoimmunity through the use of genetic and immune markers could facilitate the testing of nonpathogenic microbial therapies in disease prevention and treatment.


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**Supplementary Materials**

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Materials and Methods
Figs. S1 to S4
Tables S1 to S6
References (*36*–*58*)


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# Interferon-ε Protects the Female Reproductive Tract from Viral and Bacterial Infection


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**The innate immune system senses pathogens through pattern-recognition receptors (PRRs) that signal to induce effector cytokines, such as type I interferons (IFNs). We characterized IFN-ε as a type I IFN because it signaled via the Ifnar1 and Ifnar2 receptors to induce IFN-regulated genes. In contrast to other type I IFNs, IFN-ε was not induced by known PRR pathways; instead, IFN-ε was constitutively expressed by epithelial cells of the female reproductive tract (FRT) and was hormonally regulated. *Ifn*-ε–deficient mice had increased susceptibility to infection of the FRT by the common sexually transmitted infections (STIs) herpes simplex virus 2 and *Chlamydia muridarum*. Thus, IFN-ε is a potent antipathogen and immunoregulatory cytokine that may be important in combating STIs that represent a major global health and socioeconomic burden.**


Type I interferons (IFNs) are crucial in host defense because of their antipathogen actions and ability to activate effector cells of the innate and adaptive immune responses (*1*, *2*). The type I IFN locus contains genes encoding 13 IFN-α subtypes, IFN-β, and IFN-ω (*3*) whose promoters contain acute response elements [such as interferon regulatory factors (IRFs) and NF-κB in IFN-β], which ensure rapid induction of these genes by pattern-recognition receptor (PRR) pathways (*4*, *5*). This locus also contains a gene, which we previously designated *IFN*-ε, but whose function has remained uncharacterized.

Interferon-ε shares only 30% amino acid homology to a consensus IFN-α sequence and to IFN-β. Therefore, we first demonstrated that