Immunity
Article

# Gender Bias in Autoimmunity Is Influenced by Microbiota

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## SUMMARY

**Gender bias and the role of sex hormones in autoimmune diseases are well established. In specific pathogen-free nonobese diabetic (NOD) mice, females have 1.3–4.4 times higher incidence of type 1 diabetes (T1D). Germ-free (GF) mice lost the gender bias (female-to-male ratio 1.1–1.2). Gut microbiota differed in males and females, a trend reversed by male castration, confirming that androgens influence gut microbiota. Colonization of GF NOD mice with defined microbiota revealed that some, but not all, lineages overrepresented in male mice supported a gender bias in T1D. Although protection of males did not correlate with blood androgen concentration, hormone-supported expansion of selected microbial lineages may work as a positive-feedback mechanism contributing to the sexual dimorphism of autoimmune diseases. Gene-expression analysis suggested pathways involved in protection of males from T1D by microbiota. Our results favor a two-signal model of gender bias, in which hormones**

et al., 1981). Supplementation of females with androgens leads to their protection from these diseases (Fox, 1992; Roubinian et al., 1978), and hormone therapy is used in human SLE patients (Kanda et al., 1997).

However, the hormonal influence on the sexual dimorphism of T1D in nonobese diabetic (NOD) mice appears to be sensitive to environmental influences: T1D incidence varies between institutions (Pozzilli et al., 1993) and even with time within the same institution (Table 1). Most importantly, germ-free (GF) NOD animals have much smaller differences in T1D incidence between genders: an independent rederivation of NOD/ShiLTJ mice into GF conditions resulted in remarkably similar incidence of T1D to that previously reported by our group (Table 1). Given the wide variation in T1D incidence and the female-to-male ratio of affected mice, these results lead to two conclusions: first, that the environmental settings and variations in commensal microbiota influence gender bias in NOD animals, and second, that the influence is likely affected by the composition of the microbiota. Thus, there likely exists an unknown interaction between known hormonal influences (Kovats and Carreras, 2008) and known microbial influences on T1D (Mathis and Benoist, 2012).

Three models can explain these results. Linear model A suggests that hormones regulate the microbes (either through

**Table 1. Gender Bias of T1D in NOD Colonies**

| Institution | Female (F) | Male (M) | Ratio F/M |
|---|---|---|---|
| SPF Facilities | | | |
| U of Chicago | 60% | 25% | 2.4 |
| Yale 2008 (Wen et al., 2008) | 90% | 70% | 1.3 |
| Taconic 2009[a] | 80% | 50% | 1.6 |
| Harvard 2011 (Kriegel et al., 2011) | 50%–60% | 10%–15% | 4.4 |
| 2002[b] | 75% | 50% | 1.5 |
| 2003[b] | 85% | 60% | 1.4 |
| 2004[b] | 90% | 45% | 2.0 |
| 2005[b] | 80% | 40% | 2.0 |
| 2006[b] | 65% | 35% | 1.9 |
| 2007[b] | 100% | 65% | 1.5 |
| 2008[b] | 90% | 85% | 1.1 |
| 2009[b] | 90% | 65% | 1.4 |
| Germ-Free Facilities | | | |
| Taconic 2008 (Wen et al., 2008) | 100% | 85% | 1.2 |
| U of Chicago 2012 | 95% | 84% | 1.1 |

Cumulative incidence of diabetes in NOD mice at 30 weeks of age.
[a]Taconic Farms NOD incidence study (http://www.taconic.com/user-assets/Documents/Products%20and%20Services/Animal%20Models/NOD_Onset_2009.pdf).
[b]T1DR-T1D repository at The Jackson Laboratory. Data available at http://type1diabetes.jax.org/gqc_incidence_studies.html.

Although colonized animals showed higher blood testosterone concentrations compared to GF animals, there was no strict correlation between the ability to induce testosterone and protection from T1D. Gene-expression analysis and genetic data suggested that at least one protective mechanism was mediated by a proinflammatory cytokine, interferon-γ (IFN-γ). Thus, our results favor a model in which signals from both hormones and microbes are integrated for prevention of the disease development.

## RESULTS

### Differences in Microbial Composition in Males and Females Are Driven by Hormones

Hormonal regulation of the microbe-controlling mechanisms predicts that commensal composition should be different

Second, principal component analysis (PCA) was performed to determine whether differences in abundance of bacterial families in pre- and postpubescent mice reflected gender bias (Figure 1B). The first and second principal components, explaining ~75%–80% of the total variance in both groups, only segregated the 10-week-old mice by gender and not the 4-week-old mice. The comparison of postpubescent males and females to prepubescent mice revealed that males deviated from the initially acquired microbiota with time, whereas the adult female microbiota stayed similar to the input microbiota of young mice (Figure 1C). We repeated the sequencing experiment with an independent group of animals and confirmed that postpubescent mice had more differences associated with gender compared to prepubescent mice (data not shown).

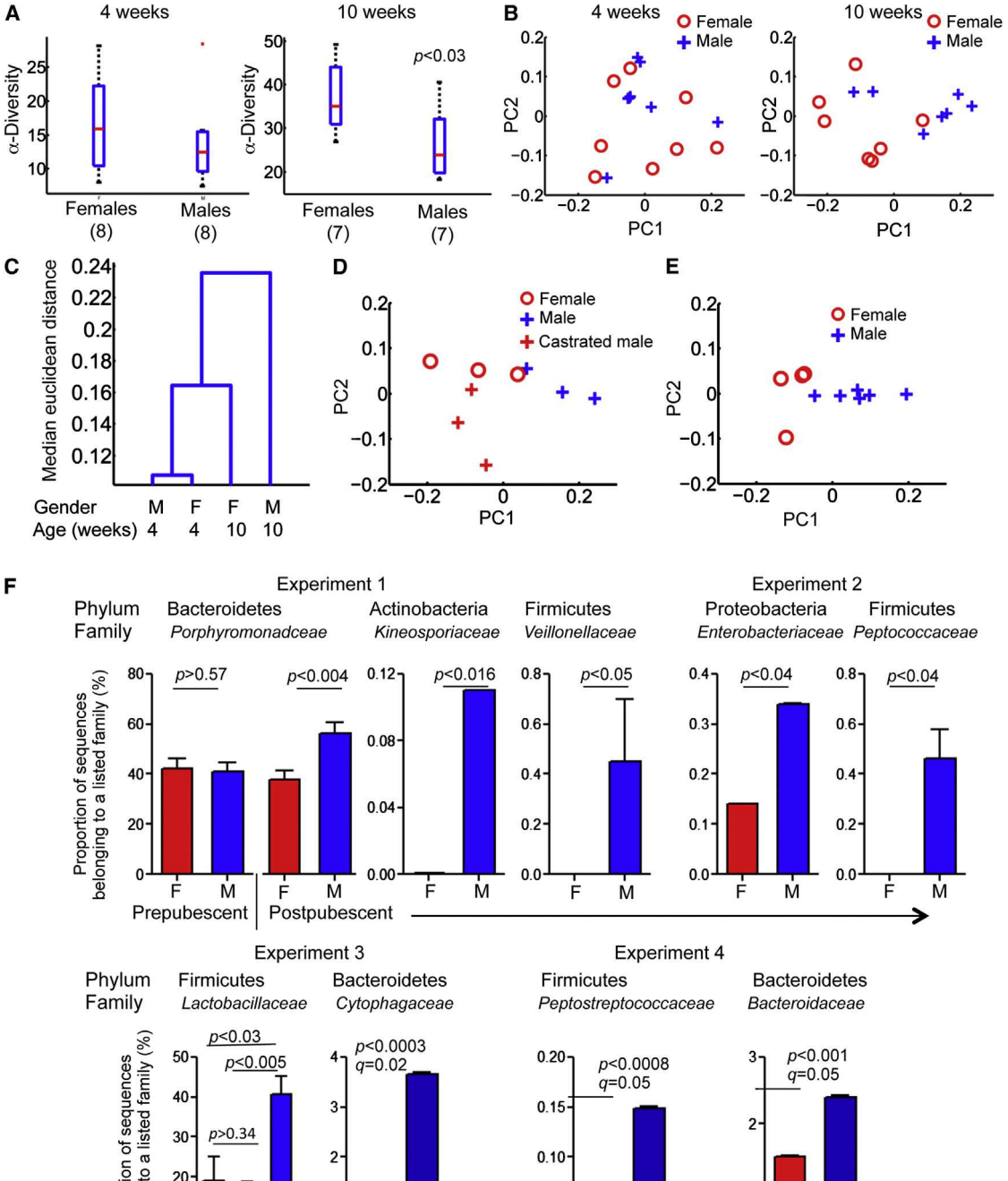
To test whether removal of the androgen source by castration would drive male microbiota closer to female microbiota, we sequenced 16S rRNA genes from microbiota of male, female, and castrated male littermates. Although the number of experimental animals was naturally limited by the size of the litter, the PCA analysis indicated that female and castrated male microbiota were closer to each other than to male microbiota (Figure 1D).

Finally, to eliminate possible adverse influences of the SPF environment on microbiota composition, we colonized GF mice with microbiota from an SPF female and compared male versus female microbiota after puberty. PCA analysis again distinctly segregated male and female microbiota (Figure 1E). The first and second principal components explained ~90% of the total variance in these experiments.

Thus, it is fair to conclude that male microbiota composition deviates more from the input microbiota and that this deviation is indeed hormone dependent.

### Input Microbiota Defines the Gender-Specific Changes in Microbiota

An important question is whether male hormones support or inhibit specific microbial lineages in a reproducible manner or whether the differences depend on the input microbiota (inherited from mother and coming from the environment). Thus, we compared the abundance of microbial families between adult males and females in four independent experiments. In the first experiment, an unbiased differential analysis of bacterial families showed an expansion of *Porphyromonadaceae* family (from the Bacteroidetes phylum) in males, which also corresponded to the largest coefficient in the first principal


A
4 weeks
10 weeks
α-Diversity
p<0.03
Females (8)
Males (8)
Females (7)
Males (7)
B
4 weeks
10 weeks
Female
Male
PC1
PC2
C
Median euclidean distance
Gender M F F M
Age (weeks) 4 4 10 10
D
Female
Male
Castrated male
E
Female
Male
F
Experiment 1
Phylum Bacteroidetes Actinobacteria Firmicutes
Family Porphyromonadceae Kineosporiaceae Veillonellaceae
Experiment 2
Proteobacteria Firmicutes
Enterobacteriaceae Peptococcaceae
Proportion of sequences belonging to a listed family (%)
p>0.57
p<0.004
p<0.016
p<0.05
p<0.04
p<0.04
Prepubescent
Postpubescent
Experiment 3
Phylum Firmicutes Bacteroidetes
Family Lactobacillaceae Cytophagaceae
Experiment 4
Firmicutes Bacteroidetes
Peptostreptococcaceae Bacteroidaceae
p<0.03
p<0.005
p>0.34
p<0.0003
q=0.02
p<0.0008
q=0.05
p<0.001
q=0.05

bacterial families differentially expressed between males and females, including *Cytophagaceae*, *Peptostreptococcaceae*, and *Bacteroidaceae* (Figure 1F).

Thus, the existence of a postpubescent gender bias in microbial diversity and representation of individual families became evident. However, the lack of consistent microbial variability between all experiments suggests that this variability is likely dependent on the input microbiota. This finding offers an explanation for the variation in T1D gender bias between animal facilities (Pozzilli et al., 1993) (Table 1) and makes two important predictions: (1) different microbes could be important for protection in males, and (2) some of the changes may be driven by the same mechanisms but are functionally irrelevant for protection from diabetes. The only definitive way to test these predictions was to use a gnotobiotic approach.

## Only Selected Microbes Support Gender Bias in Gnotobiotic NOD Mice

To address the requirement for specific microbiota for induction of gender bias, GF parents were inoculated with different microbes and bred within the isolators. Their progeny were either observed for T1D development or used at 12–13 weeks of age for analysis of the severity of mononuclear infiltration. The selection of microorganisms was based on the results of comparison of the male and female microbiota composition. The histopathology approach (Figures 2A and 2B) was adequate because it revealed a strong gender bias in SPF mice and a decrease in the gender bias in GF mice (Figures 2A and 2C) corresponding perfectly with the incidence of T1D in these groups. Representative images of islet infiltration are shown in Figure S2. First, we colonized GF mice with the probiotic mix VSL3 (Calcinaro et al., 2005), which is enriched in *Lactobacillaceae* and *Bifidobacteria*, because *Lactobacillaceae* were enhanced in males in one of the sequencing experiments. However, VSL3 did not reduce the severity of histopathology in males or females (Figure 2B); if anything, it was enhanced by VSL3. Thus, VSL3 was not capable of inducing the gender bias compared to the undefined SPF community.

In the second microbiota comparison experiment, *Enterobacteriacae* family (phylum *Proteobacteria*) was found amplified in males (Figure 1F). Colonization of GF mice with a proteobacterium that was cultured from males (Figure S2B) and had 16S rRNA gene sequence ''similar to *E. coli* and *Shigella*'' (*SECS*) led to a clear decrease in histopathology in males (Figure 2B).

Finally, we tested whether segmented filamentous bacteria tions, another single bacterium, SFB, was capable of supporting the gender bias.

Because both genders of myeloid differentiation primary response gene 88 (MyD88)-negative NOD mice are protected under normal conditions (Wen et al., 2008), it was likely that the protection offered by the gender-bias mechanism is different from the protection offered by the microbiota in MyD88-negative mice. Thus, we colonized GF MyD88-deficient NOD mice with SFB (Figure 2E). Gender bias was preserved in MyD88-deficient mice colonized with SFB. The data argue that MyD88 is dispensable for the gender bias and that distinct bacteria trigger the two protective mechanisms.

## Microbiota Elevates Androgens to the Threshold Required for Protection

Earlier studies have established that both testosterone (Makino et al., 1981) and microbes (Wen et al., 2008) were involved in the gender bias of T1D in NOD mice. Now that the gender-specific differences in microbiota were found to be androgen dependent (Figure 1D), it was critical to determine whether the ability of microbiota to induce gender bias correlated with its ability to increase androgen concentration. Thus, we measured serum testosterone in SPF and GF mice, as well as in gnotobiotic mice. The elevated amounts of testosterone correlated with colonization with conventional microbiota and with SFB and *SECS*. At the same time, colonization with VSL3 did not cause a rise in testosterone concentration (Figure 3A). Thus, it would seem that the linear model B supports the observed T1D progression. However, it was critical to determine whether a testosterone threshold necessary for protection could be reached and whether higher testosterone provided better protection. When the severity of islet infiltration was plotted against testosterone concentration in the same mice (Figure 3B), it became clear that after serum testosterone reached a certain concentration (less than 2 ng/ml), a further increase was not required for protection against T1D by microbes capable of providing a protective signal.

## Gene-Expression Analysis Reveals Possible Signaling Networks Involved in the Gender Bias of T1D

To address the question of which signaling pathways may be affected by both gender and microbiota, we compared gene-expression patterns in the pancreatic lymph nodes (PLN), critical for T1D development (Gagnerault et al., 2002; Höglund et al., 1999; Turley et al., 2005). Only one group out of four (two genders

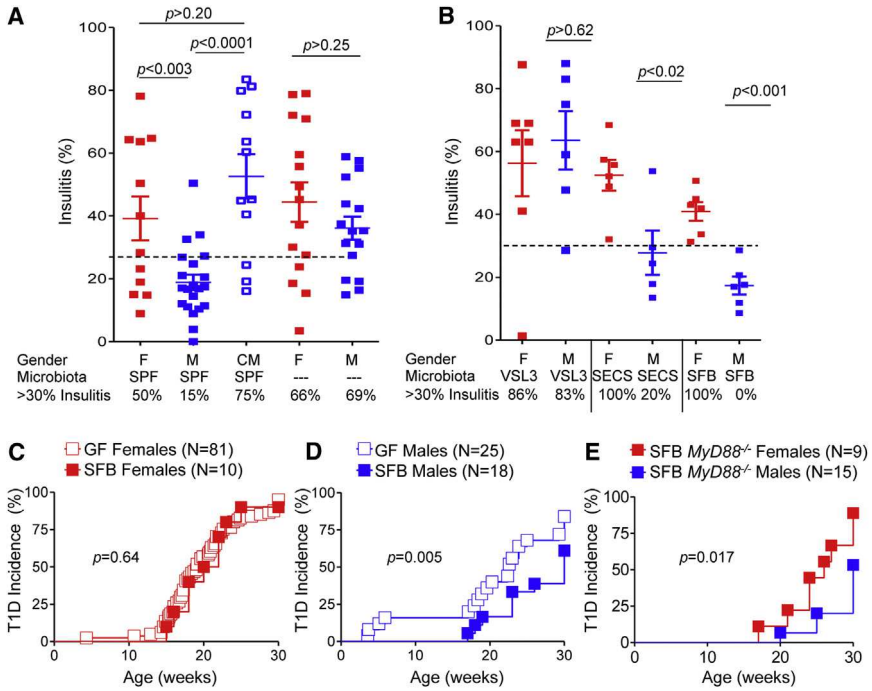



**Figure 2. Influence of Microbial Lineages on the Gender Bias in T1D Development in NOD Mice**

(A) Histopathology (percentage of islets with infiltrates beyond peri-insulitis) in 13-week-old mice from male and female SPF NOD mice, from males castrated at 4 weeks of age, and from GF male and female mice. Data are represented as mean insulitis score ± SEM, and 30% infiltration was chosen as an arbitrary threshold.

(B) Histopathology in 13-week-old gnotobiotic NOD mice reconstituted with indicated microbiota. Data are represented as mean insulitis score ± SEM. Germ-free mice were reconstituted by natural acquisition of microbes from parents infected by gastric gavage during breeding.

(C) Diabetes incidence in NOD GF female mice and in gnotobiotic NOD female mice monocolonized with SFB.

(D) Diabetes incidence in NOD GF male mice and in gnotobiotic NOD male mice monocolonized with SFB. The differences between SFB-colonized and GF males were significant ($p = 0.005$), as well as between SFB-colonized females and males ($p = 0.002$).

(E) Diabetes incidence in MyD88-negative NOD gnotobiotic mice monocolonized with SFB. n represents the number of mice per group. p values for incidence were determined with Kaplan-Meier statistics, for histopathology by Student's t test. See also Figure S2.

network of signaling pathways with the STRING database (Szklarczyk et al., 2011). The signatures of the two specific pathways became apparent: IFN-γ and interleukin-1β (IL-1β) (Figure 4C). Because PLNs are complex organs, we asked what cell type was critical for the gene-expression pattern that we have detected. For that, we generated a gene abundance profile comparing the expression of about 40 genes with the gene-expression patterns from a panel of 96 cell types from the BioGPS murine RNA Gene Expression Atlas (http://biogps.gnf.org). Hierarchical clustering associated this expression pattern with the monocyte-macrophage cell lineage (Figure 5A; Figure S3). Additionally, an upregulation of Chitinase-3-like protease 1 gene (*Chi3l-1*) suggested a more abundant presence of the tolerogenic "alternatively activated" or M2 macrophages (Gordon and Taylor, 2005; Loke et al., 2002; Raes et al., 2002) in SPF males. The representation of macrophages with M2 surface markers was compared between SPF males and females by

loss of IFN-γ signaling removes the gender bias from T1D (Figure 6A). NOD mice with targeted deletions of genes encoding IFN-γ, IFN-γ-receptor-1, and of the downstream signaling molecule STAT4, all had similar incidence of T1D in females and males at 30 weeks of age. During the same time period in the same facility, NOD mice negative for IL-12, IL-10, and IL-4 continued to exhibit gender-biased autoimmunity (Figure 6A). Genetic deletion of *Casp1*, which is necessary for the cleavage of inactive pro-IL-1β (and of pro-IL-18), has been reported to lead to higher incidence in males compared to males derived from the same backcrossing experiment (Schott et al., 2004). The female incidence was not affected by the lack of Caspase-1. These results strongly support our findings of potential involvement of IFN-γ and IL-1β in the gender-biased protection from T1D.

## IFN-γ Production in Regional Lymph Nodes Is Enhanced in NOD Males

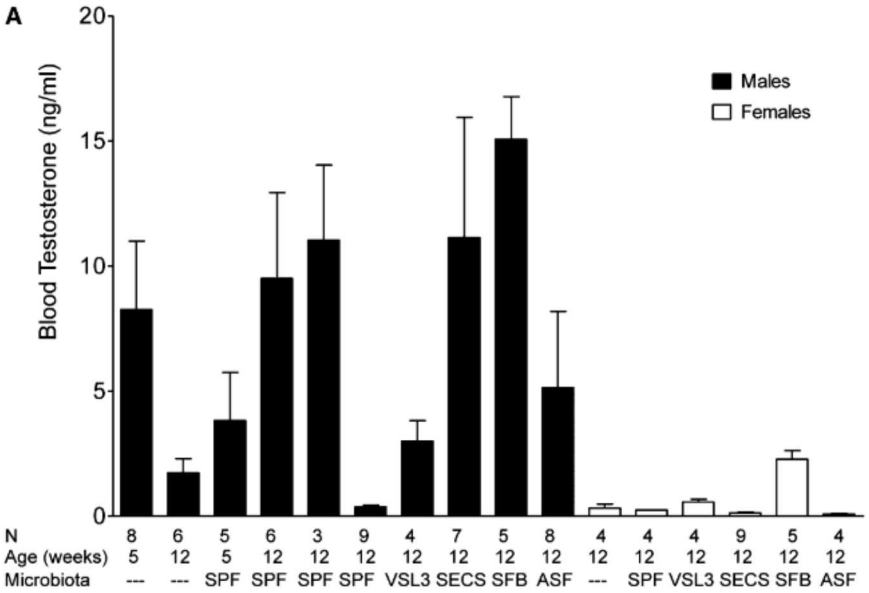



**Figure 3. Microbiota and Blood Testosterone Concentrations**

(A) The blood testosterone concentrations of the 13-week-old GF, SPF, and gnotobiotic males and females of indicated ages. Mean testosterone concentration ± SEM. Blood samples were collected between 10 am and 12 pm. The following abbreviations are used: C, castrated; MC, mock-castrated males.

(B) Islet histopathology in mice that demonstrated no gender bias (GF and VSL3 populated, open squares) and that demonstrated gender bias (SPF and *SECS* populated, black squares) plotted against blood testosterone concentrations. Data compiled from four experiments.

Lymph node cells were activated with phorbol myristate acetate (PMA) and ionomycin in vitro and stained for surface markers and intracellular IFN-γ. Male PLNs (as well as mesenteric nodes, but not spleens; Figure S4) contained significantly more IFN-γ-producing T cells than PLNs of female and castrated male mice (Figure 6D). Enhanced production of IFN-γ by T cells in males could result from direct signaling by hormones and/or microbes or reflect the influence of these signals on antigen-presenting cells. provide necessary signals to host cells that also receive a second signal from androgens. Together, these signals control the gender bias in T1D development in NOD mice.

## DISCUSSION

The importance of the intestinal commensal microbiota for development of T1D has been clearly demonstrated in NOD mice lacking the MyD88 signaling adaptor (Wen et al., 2008). Importantly, GF NOD mice used in these experiments lose the commonly observed gender bias (enhanced T1D development in females). Thus, it became clear that hormones and microbiota interact to modify the course of the disease progression (Figure S5). We sought to test the three models explaining the mechanisms behind the gender bias. Sequencing of 16S rRNA genes from male and female mice revealed that the microbiota can be gender-biased and that the adult female microbiota is more similar to the microbiota of prepubescent mice of both genders than the male microbiota. Thus, puberty affects the male microbiota composition, which becomes less diverse than the female microbiota. Importantly, comparison of male, female, and castrated male microbiota demonstrated that sex hormones rather than X chromosome-associated factors were

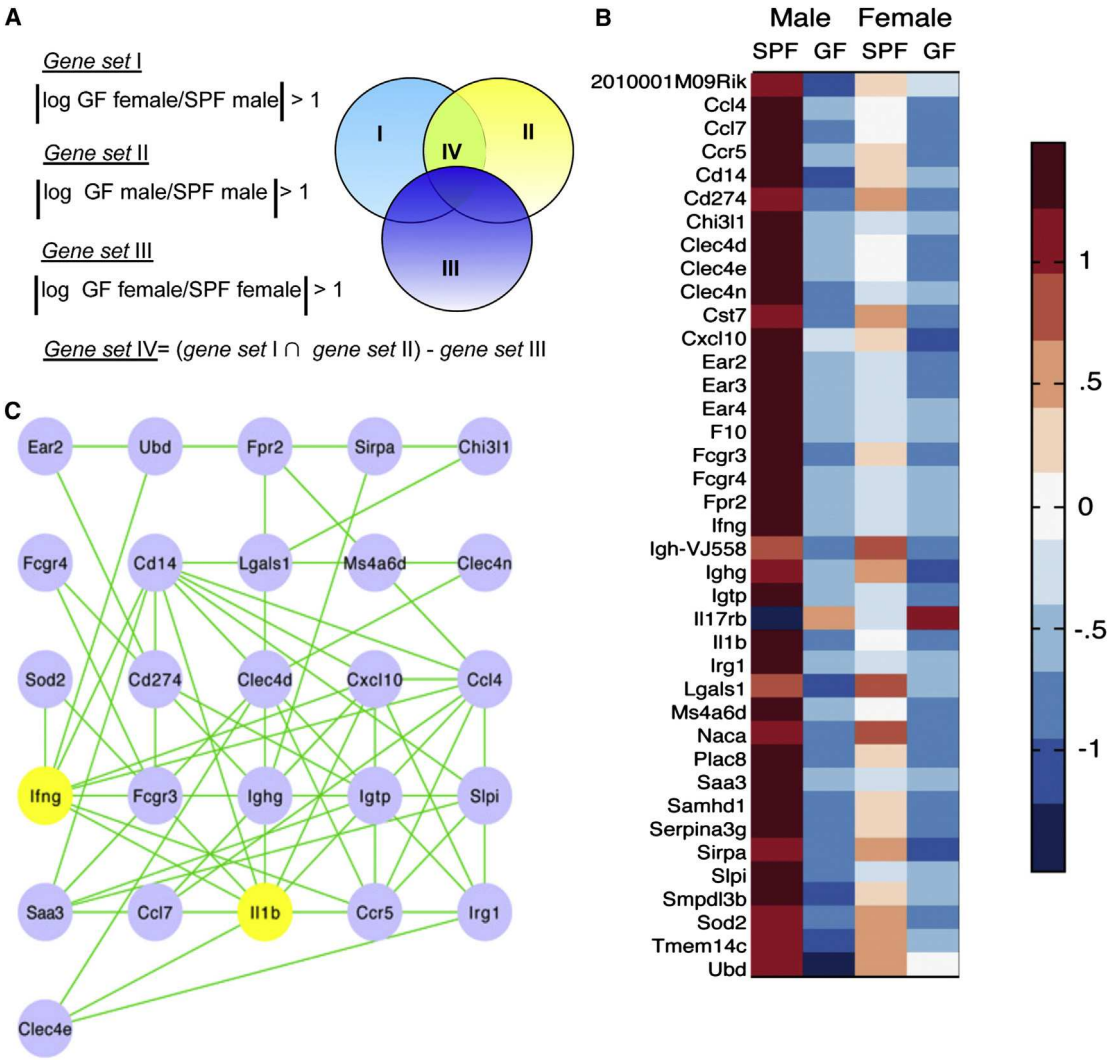


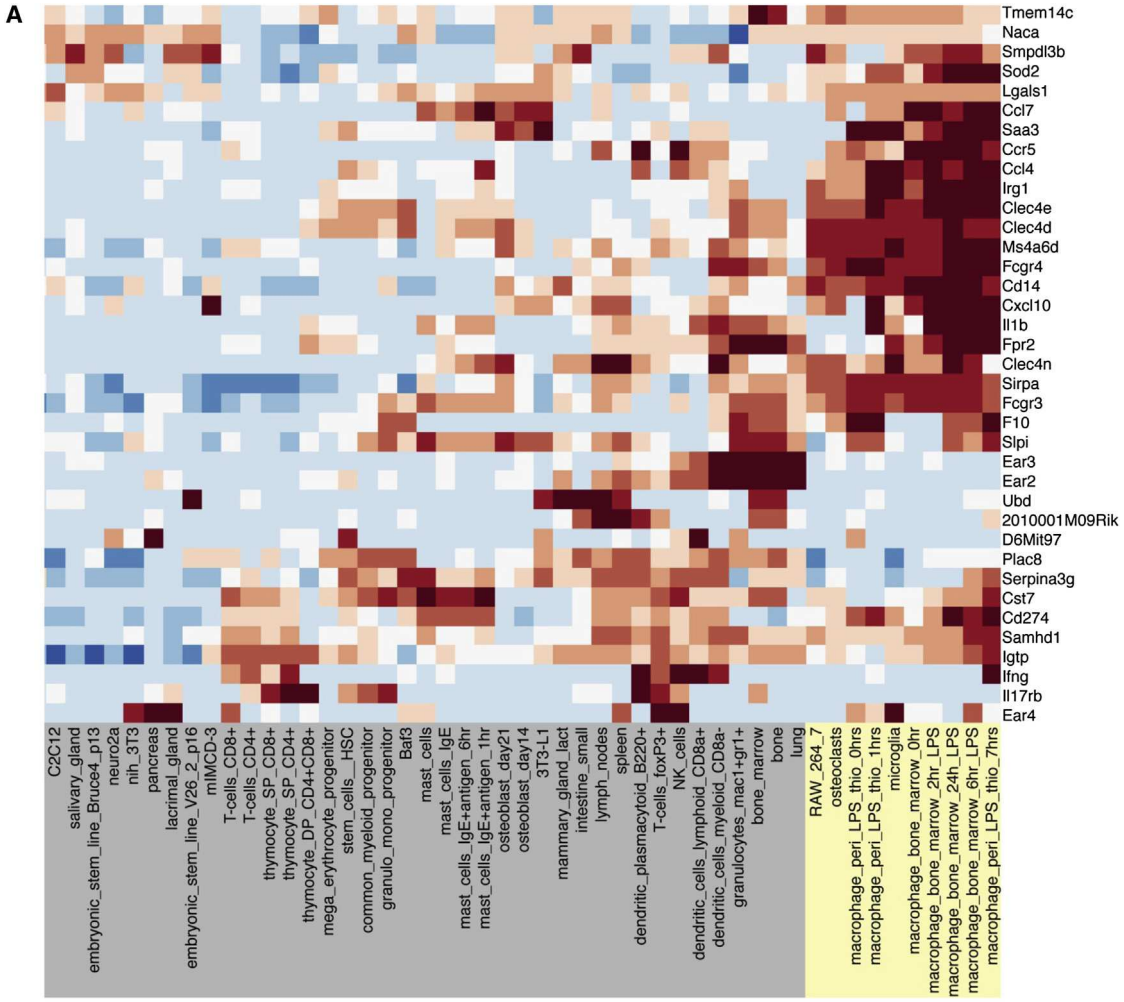

**Figure 4. Analysis of the Changes in Gene Expression Driven by Microbes and Gender**

(A) SPF and GF NOD males and females were used as donors of the PLN. Of the four groups only one (SPF males) is protected from T1D. The logic of arrival at the gene set IV, specific to this group, is shown.

(B) Heatmap of expression of the genes from set IV. The intensity of the color corresponds to the strength of expression relative to the mean expression across all conditions.

(C) Gene set IV organized in a network with the STRING database. Genes encoding IFN-γ and IL-1β are highlighted in yellow.

Interestingly, VSL3 has been previously reported to be protec- made it clear that many microbial lineages are capable of


A
Tmem14c
Naca
Smpdl3b
Sod2
Lgals1
Ccl7
Saa3
Ccr5
Ccl4
Irg1
Clec4e
Clec4d
Ms4a6d
Fcgr4
Cd14
Cxcl10
Il1b
Fpr2
Clec4n
Sirpa
Fcgr3
F10
Slpi
Ear3
Ear2
Ubd
2010001M09Rik
D6Mit97
Plac8
Serpina3g
Cst7
Cd274
Samhd1
Igtp
Ifng
Il17rb
Ear4
C2C12
salivary_gland
embryonic_stem_line_Bruce4_p13
neuro2a
nih_3T3
pancreas
lacrimal_gland
embryonic_stem_line_V26_2_p16
mIMCD-3
T-cells_CD8+
T-cells_CD4+
thymocyte_SP_CD8+
thymocyte_SP_CD4+
thymocyte_DP_CD4+CD8+
mega_erythrocyte_progenitor
stem_cells_HSC
common_myeloid_progenitor
granulo_mono_progenitor
Baf3
mast_cells
mast_cells_IgE
mast_cells_IgE+antigen_6hr
mast_cells_IgE+antigen_1hr
osteoblast_day21
osteoblast_day14
3T3-L1
mammary_gland_lact
intestine_small
lymph_nodes
spleen
dendritic_plasmacytoid_B220+
T-cells_foxP3+
NK_cells
dendritic_cells_lymphoid_CD8a+
dendritic_cells_myeloid_CD8a-
granulocytes_mac1+gr1+
bone_marrow
bone
lung
RAW_264_7
osteoclasts
macrophage_peri_LPS_thio_0hrs
macrophage_peri_LPS_thio_1hrs
microglia
macrophage_bone_marrow_0hr
macrophage_bone_marrow_2hr_LPS
macrophage_bone_marrow_24h_LPS
macrophage_bone_marrow_6hr_LPS
macrophage_peri_LPS_thio_7hrs
B
F4/80+ TFRC+ Cells (%)
p=0.005
Female
Male
Max (%)
CD206 MFI
p=0.02
Female
Male
CD206 + cells (%)
p=0.04
Female
Male

similar to a recently published experiment (Markle et al., 2013), in which ASF does not significantly protect gnotobiotic NOD males from T1D. However, ASF was perfectly capable of increasing blood testosterone concentration in associated male mice to an average of 5 ng/ml. Thus, a consortium with a poor ability to induce the gender bias supports enhancement of testosterone.

Plotting islet pathology versus testosterone concentration suggested that when the threshold is achieved, it is the nature of the microbial stimulus that matters, once again supporting the two-signal hypothesis and suggesting that androgen enhancement by microbiota is not enough to explain how the gender bias works.

The ability of microbes to regulate hormones and of hormones to change microbial diversity cannot be simply discarded and must be taken into account. In a modified gender bias model, hormone enhancement and maintenance of a gender-biased microbiota provide a regulatory feedback loop needed to maintain the gender bias of T1D protection. It is also likely that in complex microbial communities, the functions of hormone enhancement and provision of regulatory signals to the immune system could be divided between different members of the community.

What are the protective mechanisms that are induced by hormones and microbes? Gene-expression analysis has revealed possible signaling mechanisms that are involved in the process. Two points need to be made clear: (1) these mechanisms are likely just the tip of the iceberg of gender bias, and (2) not all connections shown by this experiment are necessarily meaningful. Moreover, the differences detected by microarray analysis may reflect both microbiota-induced changes and changes induced by a different course of T1D development.

Genetic experiments usually provide the most definitive results. In the case where the microbiota is involved as an epigenetic factor influencing disease development, carefully controlled experiments are needed. Because the role of IFN-γ in the gender bias has been supported by simultaneous observation (at the same time and in the same facility) of the three genetically modified mouse strains relevant to IFN-γ signaling with mice carrying targeted mutations that did not support the gender bias, it is very likely that IFN-γ is central to at least one of the protective mechanisms.

Furthermore, lymph nodes of male NOD mice produced more IFN-γ compared to female lymph nodes and also contained more IFN-γ producing T cells. How could excessive IFN-γ be (Klöting et al., 2004) and of *tlr4* (Dong et al., 2012) have preserved the gender bias, indicating that although CD14 was strongly expressed in SPF males, this enhancement was not meaningful. The other cytokine revealed by gene-expression analysis was IL-1β, also a proinflammatory cytokine. The role of IL-1β is still unclear, because IL-1-receptor requires MyD88 for signaling, whereas gender bias seems to be MyD88-independent. It is, however, possible that several MyD88-dependent and -independent mechanisms controlled by hormones exist to support the gender bias of autoimmunity. The involvement of Caspase-1 in gender bias (Schott et al., 2004) also points at the inflammasome involvement.

It remains to be determined whether the mechanisms that are induced in a gender-biased manner can be used for treatment of non-gender-biased diseases. This is especially important because T1D in humans is not gender biased, although the disease itself is likely a constellation of diseases that needs to be stratified to reveal the bias. Finally, it is essential to determine whether gender bias in other autoimmune disorders is dependent on microbiota.

## EXPERIMENTAL PROCEDURES

### Mice

NOD/ShiLtJ (The Jackson Laboratory, Bar Harbor, ME) mice were kept under SPF and GF conditions at The University of Chicago Animal Resource Center. GF status was monitored by aerobic and anaerobic fecal cultures and PCR amplification of bacterial 16S rRNA genes from fecal DNA as previously described (Kane et al., 2011).

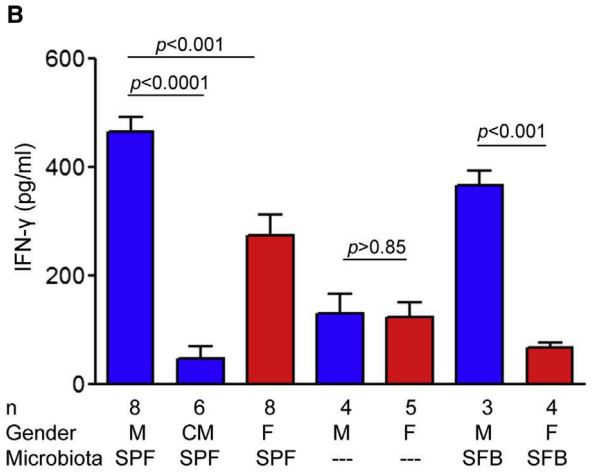
Gnotobiotic NOD mice were derived from GF mice by introduction of a specified bacterial community via gastric gavage to the parents in a separate isolator. Bacteria were transferred to the progeny naturally from the mother, and the efficiency of colonization of the progeny has been confirmed by PCR for 16S rRNA genes specific for the colonizing lineages. VSL3 mix containing *Bifidobacterium breve*, *B. longum*, *B. infantis*, *Lactobacillus acidophilus*, *L. plantarum*, *L. casei*, *L. bulgaricus*, and *Streptococcus thermophilus* was a generous gift from Dr. Claudio De Simone (VSL Pharmaceuticals, Inc, Gaithersburg, MD). ASF (Dewhirst et al., 1999) was obtained from Taconic Farms (Hudson, NY). SFB were kept frozen as cecal contents obtained from SFB monocolonized mice, defrosted, and used to colonize GF mice. *SECS* bacterium was introduced to GF male and female mice at the time of weaning by gavage of 100 μL of overnight cultures. Mice were housed under specific-pathogen-free or germ-free conditions at The University of Chicago. All experiments were performed in accordance with the institutional and national guidelines.

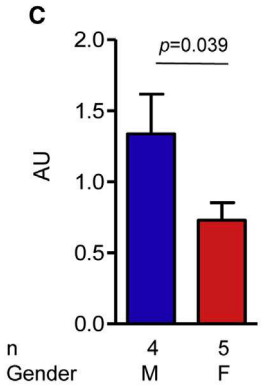
### Surgery

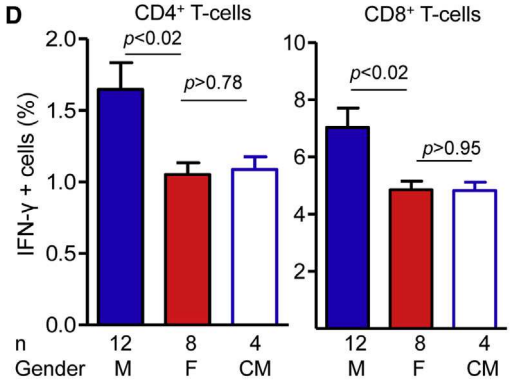
Gonads were excised from 4-week-old males anesthetized with ketamine and xylazine combination (100 mg/kg and 5 mg/kg, respectively) with the Chance

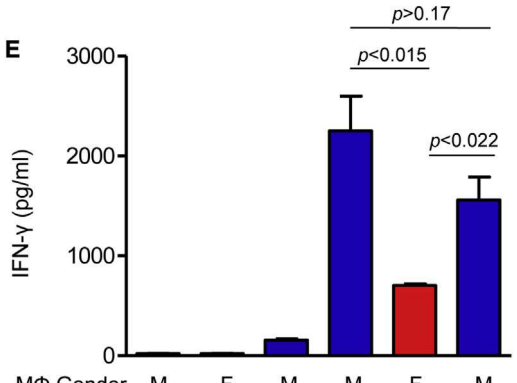
A

| Gene Knockout | Female | Male | Ratio F/M |
|---|---|---|---|
| *Ifng* | 70% | 65% | 1.1 |
| *Ifngr1* | 50% | 50% | 1 |
| *Stat4* | 45% | 40% | 1.1 |
| *Stat6* | 90% | 55% | 1.6 |
| *Il4* | 70% | 45% | 1.6 |
| *Il10* | 40% | 15% | 2.7 |
| *Casp1* | 95% | 75% | 1.3 |
| Controls | 95% | 40% | 2.4 |



### RNA Isolation and Reverse Transcription-PCR (RT-PCR)

One microgram of total RNA recovered from pancreatic lymph nodes of 10-week-old mice by homogenizing samples in Trizol (Invitrogen, Carlsbad, CA) was reverse transcribed with SuperScript III First-Strand kit (Invitrogen) and Oligo-dT primers. SYBR Green (Bio-Rad, Hercules, CA) real-time PCR was performed in 20 μL reactions with 2 μL of complementary DNA. The following primers were used: GAPDH sense 5′-AACGACCCCTTCATTG AC-3′, GAPDH antisense 5′-TCCACGACAT ACTCAGCAC-3′, IFN-γ sense 5′-AACGCTACACACTGCATCT-3′, IFN-γ antisense 5′-GAGCTCATTGAATGC TTGG-3′. AB StepOnePlus system (Applied Biosystems, Foster City CA) and StepOne software were used.

For IFN-γ intracellular staining, lymph node and spleen cells were activated in vitro with PMA and Ionomycin for 4 hr, permeabilized, and stained as described (Kriegel et al., 2011).

### Flow Cytometry Analysis

Lymph nodes and spleens from 10- to 13-week-old mice were manually disrupted and either used as suspensions or incubated with Collagenase (0.2 mg/mL) (Type II, Invitrogen) and DNase (0.15 mg/mL) (Roche) for 30' at 37°C and passed through a nylon mesh to release macrophages. Cells were stained with directly conjugated antibodies to TFRC-APC (Transferrin receptor), F4/80-PerCP, and CD11b-PE or CD11b-PECy7 in combination with anti-CD206-PE, CD4-PeCy7 (all from eBioscience, San Diego, CA) and CD8-Pacific Blue (Biolegend, San Diego, CA). Anti-mouse IFN-γ XMG1.5-APC antibodies were from BD Biosciences. Cells were analyzed with a FACS Canto or LSR-II flowcytometers (BD Biosciences), and the data were analyzed with FlowJo software (V. 9.6.1, Tree Star, Ashland, OR). For macrophage analysis, all gates were established such that 1%–2% of cells stained with isotype controls were positive and dead cells were excluded by calcein blue staining (eBioscience).

### T Cell Activation In Vitro

Peritoneal macrophages were isolated 4 days after intraperitoneal administration of 1.5 ml of thioglycolate (Difco Laboratories, Detroit MI). Macrophages were plated in Click's medium at a density of $5 \times 10^4$ cells per well of a 96 well flat-bottom plate, allowed to settle and attach, and washed with PBS to remove nonadherent cells. Macrophages were stimulated for 18 hr with heat-killed *SECS* at a ratio of approximately 1:25. Wells were washed with Click's medium prior to addition of $7.5 \times 10^4$ purified G9C8 T cells and 3 μg/ml of cognate peptide InsB15-23.

### Testosterone Measurements

Serum testosterone concentrations were determined with a rat/mouse testosterone ELISA kit (IBL America, Minneapolis, MN).

### Statistical Analysis

Statistical analysis of histology scoring, serum testosterone concentration, and IFN-γ concentration was performed with Prism 5 (GraphPad). Results are expressed as means ± SEM. The statistical difference between two groups was determined by Student's t test. For multiple groups, the statistical difference was determined with one-way ANOVA. T1D incidence data was analyzed by Kaplan-Meier with Prism 5 (GraphPad). A p value $< 0.05$ was considered statistically significant.

and plating on LB and MacConkey agar, followed by incubation at 37°C for 20 hr. A single colony from a MacConkey plate was subcultured and frozen stocks prepared in 10% Glycerol and stored at −80°C for long-term storage. This clone was classified by colony PCR by amplification and sequencing of its 16S rDNA with universal bacterial primers 8F: 5′-AGAGTTTGATCCTGG CTCAG-3′, and 1392R: 5′-ACGGGCGGTGTGTAC-3′. The bacterium was classified using the Michigan State University Ribosomal Database Project classifier function (http://rdp.cme.msu.edu/).

### Gene-Expression Analysis

PLNs were isolated from 9- to 10-week-old GF and SPF male and female NOD mice (three mice per group). RNA was extracted with guanidinium-cesium chloride gradient ultracentrifugation (Chirgwin et al., 1979). RNA quality was assessed with agarose gel electrophoresis and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto CA). Samples were analyzed with Illumina MouseRef 8 BeadChip (Illumina, San Diego, CA) array at the Functional Genomics Facility core at the University of Chicago.

Sets of genes with expression changes greater than 1 log-fold were identified with and without permutation testing. The combinatorial gene-selection model identified a signature of 39 genes (gene set IV) regulated in a male-specific and germ-dependent manner, which were examined in the context of known and predicted gene-gene interactions with the STRING database (Szklarczyk et al., 2011) and identified a large connected subnetwork with *Il1b* and *Ifng* serving as hubs based on the number of interacting gene partners.

To determine cell-specific expression pattern by our gene signature, we generated a gene-abundance profile by using a panel of 96 different cell types and conditions available from the BioGPS murine RNA Gene Expression Atlas (http://biogps.gnf.org) (Lattin et al., 2008; Wu et al., 2009). Microarray data were deposited in GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/) database under accession number GSE49467.

### Molecular Identification of Bacteria with 16S Sequencing

#### 16S Sequencing of Samples from 4-, 10-, and 13-Week-Old Mice

Cecal contents of male and female mice were collected with sterile instruments into cryovials and snap frozen in liquid nitrogen until processing. Samples were collected from three age groups of mice: prepubescent newly-weaned 4-week-old mice, postpubescent 10-week-old mice, and another group of 13-week-old mice.

DNA was extracted from cecal samples, and the V4 region of the 16S rRNA gene was amplified and sequenced on a 454 Genome Sequencer FLX Titanium platform (Roche Diagnostics and Beckman Coulter Genomics). Sequencing was performed at Argonne National Laboratory and at Research and Testing Laboratory (RTL), Lubbock, TX, as previously described (Dowd et al., 2008). On average, 10,000 sequences per sample were acquired. Libraries were separated by exact matches to barcode tags and deposited to MG-RAST. The standard MG-RAST processing pipeline was used to (1) remove artificial replicate sequences produced by sequencing artifacts, (2) remove contaminant mouse sequences with DNA level matching to the mouse genome, and (3) filter sequence reads based on length and on the number of ambiguous base calls with default settings. Sequences were deposited in Metagenomics Analysis Server (MG-RAST, http://

families in males and females was tested with a one-sided t test. We controlled for multiple hypothesis testing with the q value method by adjusting the p value to reflect the false discovery rate (Benjamini and Hochberg, 1995; Storey, 2002).

Hierarchical clustering of 16S sequencing of cecal samples from 4-week-old and 10-week-old male and female mice were performed with the Euclidean distance metric and clustering by average linkage. We standardized the data by calculating the median count of microbes at the family level for all samples in a group. The hierarchical clustering analysis allowed us to visualize similarity between pairwise comparisons of the four groups. The results are displayed in a dendrogram, which shows the linkage points at increasing degree of dissimilarity.

## ACCESSION NUMBERS

Microarray data were deposited in GEO (Gene Expression Omnibus, http://www.ncbi.nlm.nih.gov/geo/) database under accession number GSE49467. 16S sequences were deposited in Metagenomics Analysis Server (MG-RAST, http://metagenomics.anl.gov) database under the following accession numbers: mgp1501, mgp1470, mgp2995, and mgp4244.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2013.08.013.

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