LETTERS

Innate immunity and intestinal microbiota in the development of Type 1 diabetes

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Type 1 diabetes (T1D) is a debilitating autoimmune disease that results from T-cell-mediated destruction of insulin-producing β-cells. Its incidence has increased during the past several decades in developed countries[1,2], suggesting that changes in the environment (including the human microbial environment) may influence disease pathogenesis. The incidence of spontaneous T1D in non-obese diabetic (NOD) mice can be affected by the microbial environment in the animal housing facility[3] or by exposure to microbial stimuli, such as injection with mycobacteria or various microbial products[4,5]. Here we show that specific pathogen-free NOD mice lacking MyD88 protein (an adaptor for multiple innate immune receptors that recognize microbial stimuli) do not develop T1D. The effect is dependent on commensal microbes because germ-free MyD88-negative NOD mice develop robust diabetes, whereas colonization of these germ-free MyD88-negative NOD mice with a defined microbial consortium (representing bacterial phyla normally present in human gut) attenuates T1D. We also find that MyD88 deficiency changes the composition of the distal gut microbiota, and that exposure to the microbiota of specific pathogen-free MyD88-negative NOD donors attenuates T1D in germ-free NOD recipients. Together, these findings indicate that interaction of the intestinal microbes with the innate immune system is a critical epigenetic factor modifying T1D predisposition.

Toll-like receptors (TLRs) are innate pattern-recognition receptors[6] involved in host defence[7], control over commensal bacteria and the maintenance of tissue integrity[8,9]. The role of the involvement of TLRs in organ-specific autoimmunity is not clear. The MyD88 adaptor protein is used by multiple TLRs (except TLR4 and TLR3, which can or must signal by means of TRIF (Toll/IL-1 receptor (TIR)-domain-containing adapter-inducing interferon-β), respectively) and other receptors (for example, interleukin-1 receptor, IL-1R). To test the contributions of these receptors to development of T1D in NOD mice, we examined the effect of *Myd88* gene disruption on disease incidence and progression. Two MyD88 knockout (KO) NOD strains were independently derived at the Jackson Laboratory and at Yale University. Both showed a loss of diabetes development during 30-week observation periods when housed under normal specific pathogen-free (SPF) conditions with continuous monitoring for the presence of mouse pathogens (Fig. 1). Because multiple TLRs signal through the MyD88 adaptor, follow-up studies were conducted in NOD mice lacking individual TLRs (TLR^{KO}). We found that TLR2 and TLR4 (as well as TLR3, data not shown) were dispensable for development of T1D (or protection from it by complete Freund's adjuvant; Supplementary Fig. 1) when deleted individually, in contrast to the effect of complete protection from diabetes associated with loss of MyD88 (Fig. 1).

These findings suggested that signalling through receptors that use the MyD88 adaptor is critical for T1D development, and that the autoimmune T cells would probably be affected systemically in $MyD88^{KO}$ NOD mice. Two types of experiments were performed to examine this hypothesis. First, splenocytes from pre-diabetic MyD88-sufficient and $MyD88^{KO}$ NOD mice were transferred into immunodeficient NOD/SCID (severe combined immunodeficient) animals. All recipients of control MyD88-sufficient splenocytes ($n = 5$), and four out of five recipients of $MyD88^{KO}$ NOD splenocytes, became diabetic, arguing against profound systemic tolerance of T cells in $MyD88^{KO}$ NOD mice. Second, an enzyme-linked immunospot (ELISPOT) analysis of interferon-γ (IFN-γ) production by T cells in response to four peptides known to be recognized by diabetogenic T cells[10–13] was performed (Fig. 2). T cells from spleens, mesenteric lymph nodes and pancreatic lymph nodes (MLNs and PLNs, respectively) were analysed. Spleens and MLNs of $MyD88^{KO}$ NOD mice contained activated precursors of the diabetogenic T cells, whereas their numbers were clearly reduced in PLNs (Fig. 2a). Because individual mice vary in their responses to different peptides[14], the overall reactivity to all four peptides is shown in Fig. 2a (see Supplementary Fig. 2 for primary data). The responses to a prevalent diabetes-associated peptide recognized by the 8.3-$CD8^+$ T cell clone[12,15] were found to be attenuated in the PLN of $MyD88^{KO}$ NOD mice in a statistically significant manner (Fig. 2b). In addition, adoptively transferred carboxy-fluorescein succinimidyl

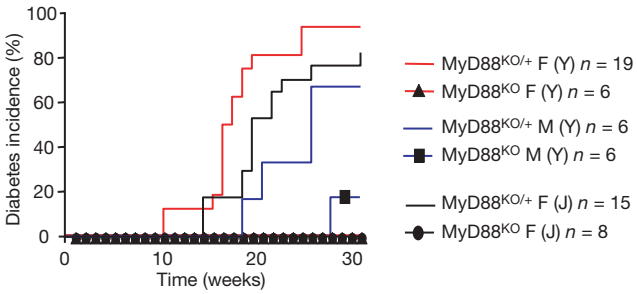

Figure 1 | MyD88-negative ($MyD88^{KO}$) mice are completely protected from development of type 1 diabetes. Incidence of diabetes in two independently derived $MyD88^{KO}$ NOD and heterozygous $MyD88^{KO/+}$ NOD stocks (J, Jackson Laboratory; Y, Yale University; 12 backcrosses to NOD). F, females; M, males; *n*, number of animals per group.

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ester (CFSE)-labelled naive CD4$^+$ T cells from mice carrying the diabetogenic T-cell receptor BDC2.5 proliferated in the PLN but not in other (mesenteric or skin-draining) lymph nodes of the NOD mice; however, their proliferation was clearly attenuated in the PLN of MyD88^{KO} mice (Fig. 2c).

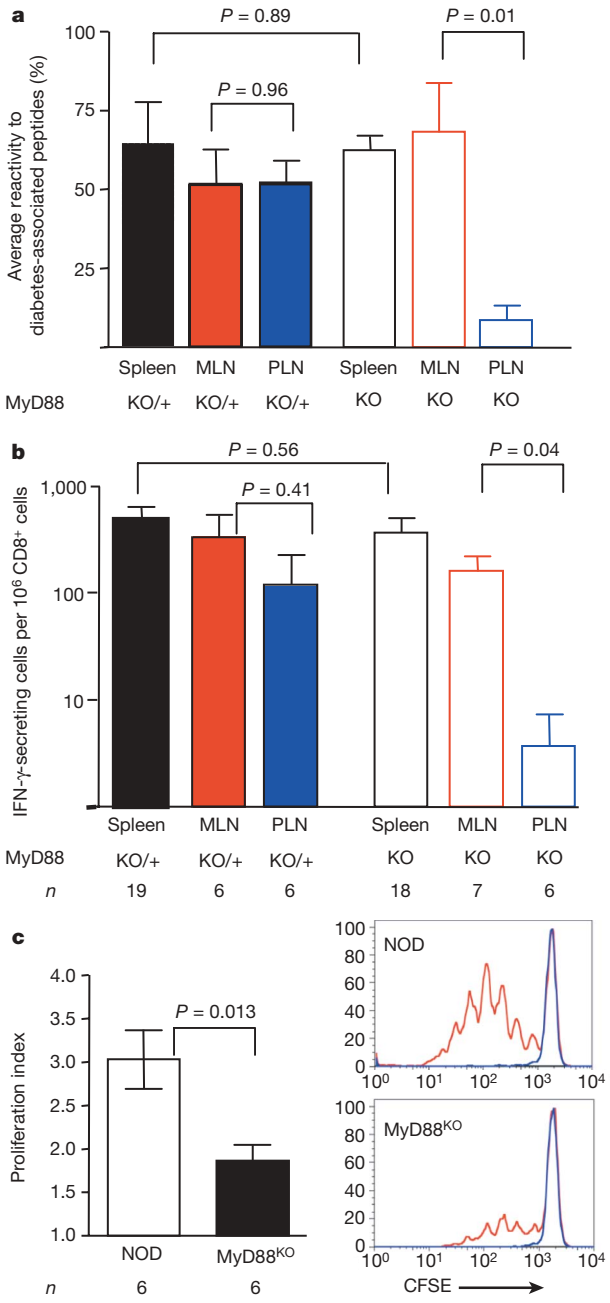

Figure 2 | MyD88 deficiency leads to local tolerance to pancreatic antigens. **a**, The overall reactivity of T cells from secondary lymphoid organs of MyD88-sufficient (filled bars) and MyD88-negative (open bars) NOD mice was calculated as a mean of the percentages of mice in the given group reacting to individual diabetes-associated peptides (see Supplementary Fig. 2c). Error bars, s.e.m. **b**, The frequency of CD8$^+$ T cells producing IFN-γ in response to peptide recognized by diabetogenic clone 8.3 was determined in the spleens, MLNs and PLNs of MyD88-sufficient (filled bars) and MyD88-negative (open bars) NOD mice. Error bars, s.e.m. **c**, Proliferation of CFSE-labelled BDC2.5 CD4$^+$ T cells, depleted of CD25$^+$ T cells, in the PLNs of SPF NOD and MyD88^{KO} NOD mice assayed on day 3 after intravenous injection. Representative CFSE dilution profiles are shown for BDC2.5$^+$-gated cells in PLNs (red) and control skin-draining lymph nodes (blue). *P* values in all experiments were determined using unpaired Student's *t* test. Error bars, s.e.m.; *n*, number of animals per group.

Because the anti-diabetogenic effect of MyD88 deficiency was localized to PLNs, it became clear that there was no systemic suppression of immune activation in SPF MyD88^{KO} NOD mice, making our initial conclusions about the requirement of MyD88 signalling for initiation of T1D an oversimplification.

PLNs drain both the pancreas and the intestine, and they are an important compartment in which islet-specific T cells are activated[16,17]. Because MyD88 signalling could be critical for the interactions of the host with the gut microbiota, we explored the hypothesis that the T1D resistance of MyD88^{KO} NOD mice could be driven by their intestinal microbiota. First, we treated SPF MyD88^{KO} NOD mice with a broad-spectrum antibiotic (Sulfatrim) throughout their lifetime. Antibiotic-treated MyD88^{KO} NOD animals developed T1D at higher rates than untreated MyD88^{KO} NOD mice (Fig. 3a versus Fig. 1), although they did not quite reach the incidence observed in control MyD88$^{KO/+}$ NOD mice. Thus, to fully eliminate any residual microbes, we re-derived NOD and MyD88^{KO} NOD animals as germ-free. T1D development in our germ-free MyD88-sufficient NOD mice was similar to that previously reported[18] and was not notably different from that in mice raised in our SPF facilities (Fig. 3b and Supplementary Figs 3 and 4). Thus, initiation of autoimmunity is genetically programmed and not affected by the presence of microbiota in immunocompetent SPF NOD animals in high-health-standard facilities.

Most importantly, in contrast to our finding that MyD88^{KO} NOD mice raised under SPF conditions did not develop T1D, germ-free MyD88^{KO} NOD mice robustly developed diabetes (Fig. 3b and Supplementary Fig. 3). This finding clearly shows that neither IL-1R nor MyD88-dependent TLRs are required for activation of an anti-islet T-cell response (similar to MyD88^{KO}, autoimmune regulator (AIRE)-deficient mice[19]). The efficient T-cell priming observed in germ-free MyD88^{KO} NOD mice does not confirm a previous report

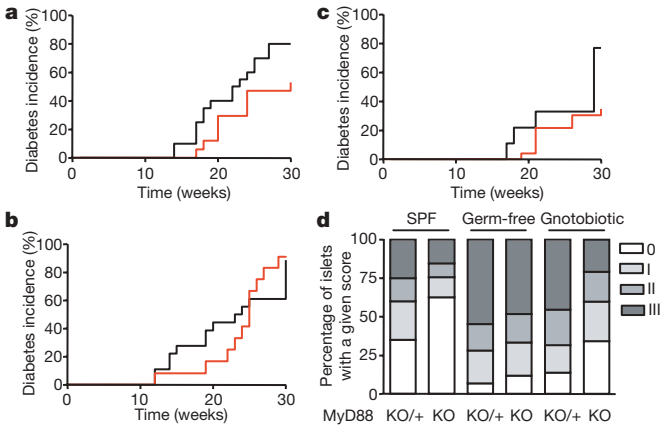

Figure 3 | MyD88-negative NOD mice are protected from diabetes by the gut microbiota. **a**, Diabetes incidence in SPF MyD88^{KO} NOD (J) females (red trace, $n = 16$) and control heterozygous MyD88$^{KO/+}$ NOD littermates (black trace, $n = 24$) treated with a broad-spectrum antibiotic from birth. **b**, Diabetes incidence in germ-free MyD88^{KO} NOD (red trace, $n = 12$) and control MyD88$^{KO/+}$ (black trace, $n = 18$) mice. Incidence is shown for male mice; 100% of female MyD88^{KO} NOD and MyD88$^{KO/+}$ NOD female germ-free mice became diabetic (Supplementary Fig. 1). **c**, Diabetes incidence in gnotobiotic male MyD88^{KO} NOD (red trace, $n = 23$) and control MyD88$^{KO/+}$ NOD (black trace, $n = 9$) mice colonized with a consortium of six bacterial strains (ASF 361, 519, 356, 492, 502 and 500; see Supplementary Information for descriptions). The incidence of diabetes in gnotobiotic MyD88^{KO} NOD mice was significantly different from the incidence in germ-free MyD88^{KO} NOD animals ($P < 0.001$) and in gnotobiotic MyD88$^{KO/+}$ NOD mice ($P < 0.05$; Kaplan–Meier test). **d**, Histological scores of islet destruction in SPF, germ-free and ASF-colonized MyD88$^{KO/+}$ NOD and MyD88^{KO} NOD mice. Mice in all groups were males, except for the SPF MyD88^{KO} NOD group, which included both genders.

that suggested that TLR2 signalling, which is MyD88-dependent, is required for T-cell priming in NOD mice[20].

To show directly that T1D development in germ-free MyD88^{KO} NOD mice was indeed a consequence of the lack of a microbiota, adult germ-free MyD88$^{KO/+}$ NOD mice were colonized with a consortium of bacterial species contained in the altered Schaedler flora (ASF)[21] and intercrossed. After introducing the ASF cocktail, polymerase chain reaction (PCR) assays revealed that six of the eight species colonized the animals (based on sampling of caecal contents; Supplementary Information and Supplementary Fig. 5). The ASF-colonized MyD88^{KO} NOD mice exhibited a significant reduction in the incidence of diabetes (Fig. 3c): only 34% of males became diabetic at 30 weeks of age, compared to >80% of germ-free MyD88^{KO} NOD males (Fig. 3b), whereas 70% of ASF-colonized MyD88$^{KO/+}$ NOD males developed diabetes with kinetics similar to disease development in SPF MyD88$^{KO/+}$ NOD mice (Fig. 3c versus Fig. 1).

A histological analysis of pancreata from SPF, germ-free and ASF-colonized MyD88^{KO} NOD mice was performed to compare the effects of the microbiota on T-cell-mediated destruction of the islets of Langerhans. The islets of SPF MyD88^{KO} NOD mice were less infiltrated compared to islets of SPF MyD88$^{KO/+}$ NOD mice. Moreover, germ-free MyD88^{KO} NOD mice had considerably increased islet infiltration, which was moderated by the introduction of the ASF (Fig. 3d, histological grading shown in Supplementary Fig. 6). Although, the overall effect of ASF on diabetes development was not fully penetrant, these results suggest that bacterial lineages normally present in the gut can modify T1D progression.

We found that MyD88 signalling is critical for development of T1D, and postulated that it is needed for control over component(s) of the microbiota that otherwise (in MyD88^{KO} mice) can protect against development of T1D. To test how MyD88-dependent innate immunity shapes the composition of the gut microbiota, we used a culture-independent, 16S ribosomal RNA gene-sequence-based approach to characterize the microbial communities of SPF MyD88$^{KO/+}$ NOD and MyD88^{KO} NOD littermates. Because MyD88 deficiency affects T1D development at the early stages (Supplementary Fig. 7), experimental female mice were housed individually and killed at 8 weeks of age. DNA was isolated from their caecal contents, bacterial 16S rRNA genes were amplified by PCR, and the resulting full-length amplicons were sequenced ($n = 36$ mice; total of 7,223 16S rRNA gene sequences; average of 201 sequences per animal; average sequence length of 1,310 nucleotides).

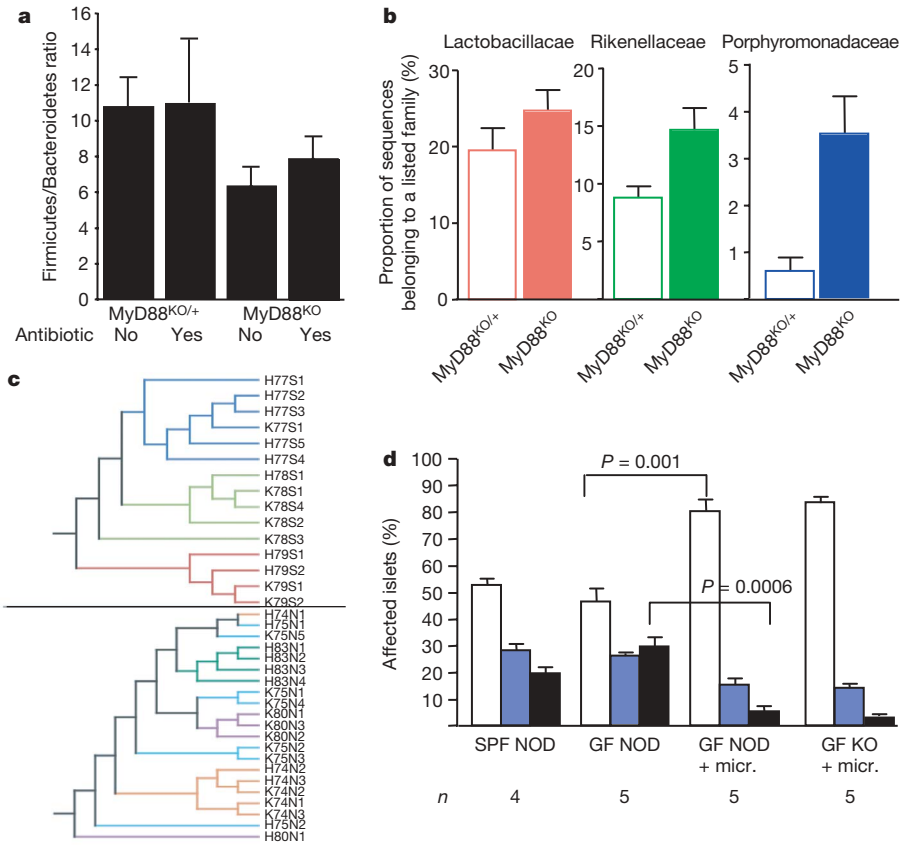

Figure 4 | MyD88 deficiency leads to specific changes in the composition of intestinal microbiota. a, Ratio of Firmicutes to Bacteroidetes in the caecal microbiota of MyD88$^{KO/+}$ NOD and MyD88^{KO} NOD mice who were or were not treated with the antibiotic Sulfatrim. Mean and s.e.m. is shown. Untreated MyD88^{KO} NOD mice had, on average, a significantly lower Firmicutes/Bacteroidetes ratio than all other mice combined (one-tailed *t*-test, $t = -2.31$, $P = 0.013$). When comparing post-hoc the effect of Sulfatrim in MyD88^{KO} NOD mice only, no significant difference in Firmicutes/Bacteroidetes ratios was observed ($t = 0.85$, $P = 0.20$). **b**, Abundance of members of three different bacterial families in the caecal contents of MyD88$^{KO/+}$ NOD and MyD88^{KO} NOD mice (not treated with Sulfatrim). Mean and s.e.m. Each of the three families is enriched in MyD88^{KO} NOD mice (Lactobacilliaceae $t = -1.54$, $P = 0.07$; Rikenellaceae $t = 2.74$, $P = 0.007$; Porphyromonadaceae $t = 2.1$, $P = 0.03$; one-tailed *t*-test). **c**, Clustering of mouse caecal bacterial communities using the unweighted UniFrac metric; $n = 7,223$ sequences. Diversity, not abundance, of bacteria was taken into consideration. Top panel, Sulfatrim-treated litters; bottom panel, untreated litters. Line colours indicate families. Each label is a mouse: H stands for MyD88$^{KO/+}$ NOD. K stands for MyD88^{KO} NOD. The number is the common mother; S and N designate exposure to Sulfatrim or no Sulfatrim, respectively. The final number represents an individual animal. **d**, Histological examination of the pancreata of 8-week-old males from SPF NOD, germ-free (GF) NOD, as well as germ-free NOD and germ-free MyD88^{KO} NOD (KO) strains exposed from birth to microbiota (micr.) of an SPF MyD88^{KO} NOD female. The percentages (mean and s.e.m.) of affected islets with no infiltration (open bars), periinsulitis (blue bars) and true infiltration (combined grades II and III) are shown. *P* values were obtained using unpaired Student's *t* test. *n*, number of animals per group.

In concordance with previous findings in mice and humans[22–24], two bacterial divisions (phyla)—the Firmicutes and the Bacteroidetes—dominated the distal gut (caecal) microbiota of mice from all groups (80.7% and 16.9% of all sequences, respectively). The remainder of the microbiota was composed of divisions commonly encountered at lower abundance in the mouse and human gut: Verrucomicrobia, Proteobacteria, Actinobacteria and the candidate phylum TM7 (ref. 25). Furthermore, close relatives of ASF strains were detected in SPF NOD mice.

Analysis of the caecal microbiota of MyD88$^{KO/+}$ NOD versus MyD88^{KO} NOD mice showed significant differences. Antibiotic-free MyD88^{KO} NOD mice had, on average, a significantly lower Firmicutes/Bacteroidetes ratio compared to all other groups (one-tailed *t*-test $t = -2.31$, $P = 0.013$; Fig. 4a). Antibiotic treatment of animals eliminated the statistically significant difference in Firmicutes/Bacteroidetes ratio (Fig. 4a). A change of Firmicutes/Bacteroidetes ratio may be important by itself because it can influence the efficiency of processing of otherwise indigestible complex polysaccharides in the diet[26,27]. Diet-related changes in diabetes in germ-free NOD mice have been observed[28]; these effects could be related to the components of the diet *per se* or attributed to the presence of microbial products in the feed.

To characterize further the changes in the gut microbiota imposed by MyD88 deficiency, 16S rRNA genes were classified taxonomically to the family level (using the Ribosomal Database Project Classifier[29]). The proportion of sequences in each family was determined for individual mice, averaged and compared across treatments. The representation of three bacterial families was increased significantly in the microbiota of antibiotic-free SPF MyD88^{KO} NOD mice compared to the SPF NOD animals: the Lactobacillaceae (Firmicutes), Rikenellaceae and Porphyromonadaceae (both Bacteroidetes; Fig. 4b). Interestingly, the VSL3 probiotic mix, containing four species of Lactobacillaceae, affects diabetes[30] in NOD mice.

Gut microbial communities are known to be inherited from the mother[22]: this was also the case in these experiments, where clustering of 16S rRNA genes was strongly influenced by shared mothers (Fig. 4c).

To show that the changes in the intestinal microbiota of MyD88^{KO} NOD animals were responsible for attenuation of T1D development, the newborn progeny of germ-free NOD mice were exposed to SPF MyD88^{KO} NOD females and allowed to mature to 8 weeks of age, after which time their pancreata were removed and analysed histologically (Fig. 4d). Islet infiltration was significantly reduced in germ-free NOD animals exposed to microbiota from SPF MyD88^{KO} mouse compared to germ-free NOD mice (increased percentage of intact islets ($P = 0.001$) and reduced percentage of infiltrated islets ($P = 0.0006$)).

Although the precise mechanism of induction of local tolerance by the microbiota remains to be elucidated (see Supplementary Fig. 8), the finding that the normal intestinal microbiota can alleviate progression of autoimmune diabetes in a MyD88-independent manner provides a different perspective about disease pathogenesis. Moreover, knowledge-based use of live microbial lineages or microbial products could present new therapeutic options for T1D in the future.

METHODS SUMMARY

Mice. B6 mice carrying MyD88 and TLR mutations were backcrossed 10–12 times to NOD/LtJ (at The Jackson Laboratory) or to NOD/Caj (at Yale University) mice and intercrossed to produce KO and heterozygous animals. Germ-free animals were re-derived from NOD/LtJ females impregnated by MyD88^{KO} NOD males and kept germ-free at Taconic Farms, the University of Chicago and Washington University in St Louis. ASF was introduced to germ-free MyD88^{KO} NOD mice by adding caecal contents from donor mice to sterile drinking water. Wild-type germ-free mice were colonized with microbiota from SPF MyD88^{KO} NOD animals by co-housing germ-free NOD females and newborn progeny with SPF MyD88^{KO} NOD females.

Histopathology of diabetes. Damage to the islets was scored in a blinded fashion, and graded as follows: 0, no visible infiltration; I, periinsulitis; II, insulitis with <50%; and III, insulitis with >50% islet infiltration (Supplementary Fig. 6). At least 100 islets in each group of 5 to 12 animals were scored. In microbiota transfer experiments, 20 sections per pancreas cut at 40-μm intervals (≥10 islets per section) were examined and scored (combining grades II and III).

ELISPOT analysis. 6×10^5 splenocytes alone, or 2×10^5 lymph node cells mixed with 4×10^5 irradiated splenocytes from B6.NOD-(D17Mit21-D17Mit10)/LtJ (B6.g7) mice, per well of 96-well plates pretreated with anti-IFN-γ antibodies were incubated overnight (16–18 h) with corresponding peptides. Lymphocytes were stained with antibodies against CD4 and CD8 to calculate the frequency of peptide-specific cells per 10^6 CD4$^+$ or CD8$^+$ T cells.

Sequence and phylogenetic analysis. 16S rRNA gene sequences were edited and assembled into consensus sequences using PHRED and PHRAP, aided by XplorSeq[23], and bases with a PHRAP quality score of <20 were trimmed. Sequences were aligned using the NAST online alignment tool (http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi), and checked for chimaeras using the online Greengenes server (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) with a window size of 300 and using the NAST-aligned sequences[31].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions L.W. designed and supervised experiments at Yale University; R.E.L. performed analysis of 16S rRNA sequences of the gut microbiota; P.Yu.V. analysed T1D development in germ-free and microbiota-colonized mice; P.B.S. performed ELISPOT analysis; L.A. and A.C.S. established and characterized mutant mouse strains at The Jackson Laboratory and at The University of Chicago; C.H., F.S.W. and L.W. characterized mutant mouse strains at Yale University; G.L.S. was involved in performance of regulatory-T-cell-based assays; J.A.B. designed and supervised the T cell assays; J.I.G. helped with design and interpretation of gut microbial ecology studies and oversaw the microbiota transfer experiments; A.V.C. conceived and designed the project, and wrote the manuscript with substantial critical contributions from L.W., R.E.L., F.S.W., J.A.B. and J.I.G.

Author Information 16S rRNA sequences obtained from microbiota of SPF NOD and MyD88^{KO} NOD mice treated and not-treated with antibiotic were deposited in GenBank under accession numbers EU450891–EU458113. Reprints and permissions information is available at www.nature.com/reprints.
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METHODS

Mice. TLR4-negative C57BL/10ScN mice were purchased from the NIH. TLR2 and MyD88^{KO} B6 mice were a gift from S. Akira. TLR2^{KO}-carrying animals at Yale were obtained as an early backcross to NOD/LtJ through the generosity of M. O. Li. KO/+ and KO animals used for diabetes experiments were F_2 of extensively backcrossed KO/+ mice: this strategy was designed to reduce the impact of non-NOD genetic loci possibly present elsewhere in the genomes of these mice.

SPF mice were fed with autoclaved NIH 5K52 diet (see http://www.labdiet.com) or Harlan Teklad 7012 diet (see http://www.teklad.com/standardrodentdiet/r7012.asp), whereas germ-free mice received an autoclaved NIH 31 chow (see http://www.teklad.com/pdf/7017.pdf).

DNA isolated from NOD animals carrying the targeted mutations was subjected to genome-wide screening using the Illumina Sentrix Universal BeadChips. The analysis was performed at University of Texas Southwestern Medical Center in Dallas (for details see http://microarray.swmed.edu/s_illumina.html and http://www.illumina.com). In addition, we used the PCR-based Amplifluor SNP analysis platform[32] at Kbiosciences with primers distinguishing NOD and 129 alleles in the regions flanking the targeted genes (*Tlr2*, *Tlr4* and *Myd88*) on chromosomes 3, 4 and 9, respectively[32]. The combined data from these two approaches showed that the genetic boundaries for the 129 or B6 sequences still present in the flanking regions between positions for telomeric (t) and centromeric (c) ends were as follows: TLR2^{KO} NOD (J): t, 46624359/48004616; c, 91071226/93995376. TLR2^{KO} NOD(Y): t, 70607621/74327277; c, 121126022/122002332. TLR4 NOD (J): t, 47358427/51035410; c, 70467819/74403260. MyD88 NOD (J): t, 108690630/109861337; c, 117891342/123174839. MyD88 NOD (Y): t, 115090216/117891342; c, 122337931/123174839.

In addition TLR2^{KO} NOD (Y) mice had a non-NOD segment on chromosome 11 (32946888–43847855). However, TLR2^{KO} NOD (J) mice carried the NOD sequence in the same segment.

Positions of *Idd* (insulin dependent diabetes susceptibility) loci were determined according to the T1D database (https://dil.t1dbase.org/page/Welcome/display/species/Mouse). Accordingly, in TLR2^{KO} NOD (J) mice the 129 sequences flanking *Tlr2* gene could not be segregated from *Idd17*, whereas in NOD TLR2^{KO} (Y) mice these sequences also included *Idd10* and *Idd18*. These loci were found to affect T1D development in NOD × (NOD × 129/SvJ.H2^{g7})F_1 backcrosses (E. Leiter, personal communication). In the same cross, no T1D-affecting genes were found on chromosome 9 harbouring the *Myd88* gene.

Antibiotic treatment. Sulfatrim (JA Webster) was delivered through drinking water according to the manufacturer's instructions. Breeding mice were kept on Sulfatrim through mating and nursing, and the progeny were kept on the drug until the end of the observation period.

Complete Freund's adjuvant injections. Three-week-old female mice were injected with 25–30 µl of a 1:1 mixture of complete Freund's adjuvant (Difco Laboratories) and saline into the hind footpads. Diabetes development was monitored up to 30 weeks of age.

Diabetes monitoring. Mice were tested for diabetes weekly, starting at 10 weeks of age, by measuring urine glucose with Diastix strips (Bayer); this was confirmed by testing for blood glucose $>2.5\ g\,l^{-1}$ ($13.9\ mmol\,l^{-1}$).

FACS analysis. Lymph node and splenic cells were stained with antibodies directly coupled to fluorochromes against CD4, CD25 and FOXP3 (all from e-Biosciences) according to the manufacturer's protocols. For transfer experiments, T cells were treated with 0.5 µM CFSE (Invitrogen). Lymph node cells containing CFSE$^+$ cells were counterstained with anti-CD4 and anti-BDC2.5 clonotype[33] followed by rat anti-mouse IgG2b–biotin (Becton-Dickinson) and streptavidin–PE (Becton-Dickinson). Data were acquired using FACSCalibur or LSR II (Becton-Dickinson) flow cytometers. Data analysis was performed using FlowJO (Tree Star Inc.) and ModFit LT (Verity Software House) software. Proliferation of CFSE$^+$ cells was measured by using ModFit and expressed as a 'proliferation index' (defined as the sum of the cells in all generations divided by the computed number of original parent cells theoretically present at the start of the experiment).

ELISPOT analysis. An IFN-γ ELISPOT antibody pair was purchased from Pharmingen. MultiScreen plates (96 wells) were from Millipore. Peptides used were from the insulin B chain (InsB amino acids 15–23) (ref. 10), or mimic peptides recognized by islet-specific TCRs AI-4 (ref. 34), 8.3 (ref. 15) and BDC2.5 (ref. 35). Plates were treated and spots revealed according to the manufacturer's protocol. Background dots in the wells with the same T cells incubated without peptides were subtracted.

Transfer of T cells. CD4$^+$ T cells were isolated from spleens and lymph nodes of BDC2.5 transgenic NOD mice using anti-CD4$^+$ magnetic beads and MACs technology (Miltenyi Biotech). CD25$^+$ T cells were removed either by treating with anti-CD25 antibodies and rabbit complement (Pel-Freeze) or by treatment with anti-CD25 biotinylated antibodies followed by anti-biotin magnetic beads. Recipient mice were injected intravenously with $1–2 \times 10^6$ CFSE-labelled cells. PLNs, MLNs and skin-draining nodes were collected on day 3 and analysed by flow cytometry. No proliferation was observed in either mesenteric or skin-draining lymph nodes.

16S-rRNA-sequence-based studies of the caecal microbiota. SPF MyD88^{KO} NOD grandmothers of experimental animals were bred to SPF MyD88^{KO} NOD males without exposure to Sulfatrim. Their daughters were separated into two groups and harem-mated with SPF MyD88$^{KO/+}$ NOD males with or without antibiotic. The female progeny were weaned, housed individually to prevent exchange of their gut microbiota, and maintained either with or without Sulfatrim treatment until they were 8 weeks of age. Caecal contents from experimental mice (8–11 mice per group) were quickly removed, snap-frozen in liquid nitrogen and stored at −80 °C.

DNA was extracted from frozen caecal contents using a bead-beating/phenol–chloroform extraction protocol[36]. 16S rRNA amplicons were obtained as described[23] using bacteria-specific forward (5′-AGAGTTTGATCC TGGCTCAG-3′) and a universal reverse (5′-GACGGGCGGTGWGTRCA-3′) primer[37]. Amplicons were gel-purified (Qiagen), cloned into TOPO TA pCR4.0, and transformed into *Escherichia coli* TOP10 cells (Invitrogen). For each sample, 384 colonies were selected: inserts were sequenced using vector-specific primers and the internal 16S rRNA primer 907R (5′-CCGTCAATTCCTTTRAGTTT-3′), and an ABI 3730xl capillary DNA sequencer.

Putative non-chimaeric sequences (not flagged as chimaeric using the on-line tool Bellerophon (http://greengenes.lbl.gov/cgi-bin/nph-bel3_interface.cgi) with parameters setting that included a 'parent to fragment threshold' of 90; and a 'divergence threshold' ratio of 1.1) >800 bp in length were named according to the mouse genotype, antibiotic treatment and the mother's identity: sequences derived from mice that were heterozygous (KO/+) for the MyD88 gene have prefixes beginning in 'H', in contrast to 'K' for sequences derived from MyD88^{KO} mice; the middle two digits of the prefix refer to the mother's identity; antibiotic treatment is denoted as 'S' and no treatment as 'N'; and the last digit of the prefix refers to a specific mouse sibling.

Sequences were added to an existing Arb alignment (http://greengenes.lbl.gov/Download/Sequence_Data/Arb_databases/prokMSA_old.arb) using the parsimony insertion tool[38] and tree (tree_1250_nochimera), which was used for clustering mouse communities using the online version of UniFrac (http://bmf.colorado.edu/unifrac/)[39,40].

Statistical analyses. Statistical significance for sequence classification results was determined by one-tailed *t*-test implemented in Excel (version 11.0, Microsoft).

Statistical significance of ELISPOT and regulatory T cell staining results was determined by unpaired Student's *t*-test. Diabetes incidence curves were compared by Kaplan–Meier survival curve statistics using GraphPad Prism version 4.00c for OS X, GraphPad Software.

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