The microbiome affects development and activity of the immune system, and may modulate immune therapies, but there is little direct information about this control in vivo. We studied how the microbiome affects regulation of human immune cells in humanized mice. When humanized mice were treated with a cocktail of 4 antibiotics, there was an increase in the frequency of effector T cells in the gut wall, circulating levels of IFN-γ, and appearance of anti-nuclear antibodies. Teplizumab, a non–FcR-binding anti-CD3ε antibody, no longer delayed xenograft rejection. An increase in CD8+ central memory cells and IL-10, markers of efficacy of teplizumab, were not induced. IL-10 levels were only decreased when the mice were treated with all 4 but not individual antibiotics. Antibiotic treatment affected CD11b+CD11c+ cells, which produced less IL-10 and IL-27, and showed increased expression of CD86 and activation of T cells when cocultured with T cells and teplizumab. Soluble products in the pellets appeared to be responsible for the reduced IL-27 expression in DCs. Similar changes in IL-10 induction were seen when human peripheral blood mononuclear cells were cultured with human stool samples. We conclude that changes in the microbiome may impact the efficacy of immunosuppressive medications by altering immune regulatory pathways.
Microbiota control immune regulation in humanized mice

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Introduction

Exposure to microbiota is required for the development of the immune system, immunologic tolerance, and effector function (1–3). Microbiota that lack the resilience and diversity needed to maintain this balance, possibly as a consequence of antimicrobial exposure, are postulated to contribute to the rise in autoimmune diseases, such as inflammatory bowel disease, in Western countries (4, 5). A prominent human symbiont, Bacteroides fragilis protects animals from colitis induced by Helicobacter hepaticus by producing polysaccharide A (PSA), which mediates the conversion of CD4+ T cells into Foxp3+ Tregs (6–8). Ingested antigens have been shown to induce tolerance. Peripheral Tregs that express RORγt and produce IL-10 have been found to be induced in the small intestine by dietary antigens (9).

In addition, immune effector cells may be activated by disturbances in the intestinal microbiota. Gastrointestinal infections can directly affect tolerance to commensals and activate microbiota-specific T cells that differentiate into inflammatory effector cells (10). Observational studies suggest that restricted diversity of the microbiota may be related to the progression of type 1 diabetes in those at risk (11–13). The composition of the microbiota may also modify responses to biologics and other therapeutics such as the checkpoint inhibitors used for cancer, anti–CTLA-4 or anti–PD-L1 mAbs (14–16). Despite the circumstantial evidence, direct cause/relationships between changes in the microbiota and modulation of human immune responses have not been demonstrated because it is difficult to directly study the effects of changing the microbiota on human immune responses.

Because of the intimate relationship between the microbiota, tolerance, and adaptive immune responses in the gut, we asked whether the microbiota are responsible for maintaining tolerance and its effects on the efficacy of anti-CD3 mAb in humanized mice. In previous studies in these mice, we described a
Mechanism whereby a non–FcR-binding anti-CD3 mAb (teplizumab) can induce tolerance by generating IL-10–producing cells in the gut, with regulatory function (17–19). Migration of the cells to the gut was needed for these events to occur, and therefore we tested whether the microbiome affected the immune regulatory effects of the mAb. We show that when humanized mice are treated with antibiotics there is breach of tolerance manifested by increased effector T cells in the lamina propria and development of anti-nuclear antibodies (ANAs), and the ability of teplizumab to prevent xenograft rejection was impaired. Biomarkers associated with efficacy of teplizumab in patients, such as release of IL-10 and expansion of CD8+ central memory (CD8CM) T cells were seen in humanized mice but not when antibiotics were given (20). The failure to induce regulatory mechanisms could not be attributed to a single microbial species and administration of individual antibiotics from the cocktail of 4 drugs did not induce the same effect. The cells affected directly by the change in microbiome were CD11b+CD11c+ cells, since direct culture of this subset with pellets from antibiotic-treated mice stimulated lower levels of IL-10 and IL-27 compared with pellets from non–antibiotic-treated mice. Moreover, when CD11b+CD11c+ cells that had been exposed to pellets from antibiotic-treated mice were added to cultures of T cells with teplizumab, activation of the T cells was increased. Likewise, stool samples from antibiotic-treated patients induced less IL-10 compared with patients not treated with antibiotics when cultured with peripheral blood mononuclear cells (PBMCs) from healthy donors. Our studies indicate that there is increased activation of T cells and reduced production of IL-10 when antigen-presenting cells (APCs) are exposed to microbiota from antibiotic-treated individuals and suggest that chronic exposure to the microbiome is needed to maintain tolerance. By modifying immune cells, changes in the microbiome may affect clinical responses to biologics.

Results

Modification of the microbiome leads to loss of tolerance in humanized mice. Immunodeficient NSG mice, reconstituted with human CD34+ stem cells, were treated with a cocktail of 4 antibiotics (neomycin, vancomycin, ampicillin, and metronidazole) at 12 weeks of age after screening. Within 2 weeks, the mice developed a megacecum (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/jci.insight.91709DS1). There was a reduced number of gut microbial species and changes in the distribution of the microbiota based on analysis of their fecal pellets: Eubacteria were depleted 24 ± 4.3-fold (mean ± SEM, n = 11) and segmented filamentous bacteria were depleted 34.7 ± 1.86-fold (Supplemental Table 1). Tenericutes spp. were the predominant phylum of bacteria remaining in pellets after antibiotic treatment (Figure 1A and B).

We analyzed immune cells in the tissues to determine the effects of the antibiotic treatment. We did not detect changes in the frequency of subsets of T, B, or NK cells in the spleen even in mice that had received antibiotics for up to 6 weeks (data not shown). There was a modest increase in the number of CD45+ cells in the small bowel (Figure 1C; 13,220 ± 265 vs. 29,330 ± 9,074, P = 0.038) but we did not identify lymphoctic infiltrates of graft-versus-host disease in the gut wall (Supplemental Figure 1B). In response to antibiotic treatment there was an increase in the frequency of CD4+ effector T cells (P = 0.040) in the gut wall and a decrease in naive cells (P = 0.019) with a similar pattern in gut-infiltrating CD8+ cells (P = 0.001; Figure 1 and D and E). In the gut wall, we also observed an increase in CD19+ cells following antibiotic treatment (Figure 1F, P = 0.026). The circulating levels of IFN-γ were significantly increased in antibiotic-treated mice, consistent with the signs of cell activation among the gut-infiltrating lymphocytes (Figure 1G).

Because of the activation of cells in the small bowel, we tested whether the mice had other signs of immune dysregulation. We detected ANAs in 5 of 6 mice treated with antibiotics versus 0 of 4 in humanized mice not treated with antibiotics (Figure 2, P = 0.048 by χ² test), indicating that the humanized mice develop signs of systemic immune activation when the microbiota are modified.

Modification of the microbiome impairs tolerance induced by anti-CD3 mAb. We previously showed that teplizumab, an non–FcR-binding anti-CD3 mAb, prevented xenograft skin rejection in humanized mice (17). Because this effect required migration and activation in the gut, we asked whether modification of the microbiome with antibiotics would affect these immunologic actions of teplizumab. We compared the rate of rejection of xenogeneic C56BL/6 skin grafts on mice that were treated or not with antibiotics. Similar to our previous experience, after a single dose of teplizumab the survival of xenogeneic skin grafts was prolonged on humanized mice not treated with antibiotics (P = 0.001, Figure 3A and Supplemental Figure 2). However, in mice treated with antibiotics, teplizumab treatment did not significantly delay graft rejection compared with mice treated with human Ig (hIg) (Figure 3B).
The differences in the rate of rejection were due to effects of antibiotic treatment, since the rates of rejection in hIg-treated mice were similar whether or not the mice had received antibiotics but different when the mice were treated with teplizumab \( (P = 0.03) \) (Figure 3, C and D).

**Effects of anti-CD3 mAb on adaptive immune cells when the microbiota are modified.** The failure of teplizumab to induce tolerance during antibiotic treatment was not because of reduced lymphoid cells in the gut. The cell counts in the lamina propria 18 hours after mice received teplizumab were similar in mice that had been or had not been treated with antibiotics (Figure 4A). Kim et al. found that Tregs induced by dietary antigens expressed ROR\( \gamma \) (9). We analyzed ROR\( \gamma \) expression by reverse transcription PCR (RT-PCR) in the gut wall and found that the expression was reduced in the mice treated with the antibiotic cocktail (Figure 4B, \( P < 0.05 \)). We determined the frequency of Tregs in the gut and spleen by staining for CD4\(^+\)CD25\(^+\)CD127\(^{lo}\)Foxp3\(^+\) cells but the frequency of these Tregs was variable and we did not identify significant differences in these cells in the gut wall or peripheral sites in mice that had or had not received antibiotics before or after teplizumab treatment (data not shown).
We analyzed T cell subpopulations following teplizumab treatment in the spleen and mesenteric lymph nodes after teplizumab treatment. The mean fluorescence index (MFI) of CD127, which is reduced with T cell activation (21), was reduced in cells from the mesenteric lymph nodes in mice that were treated with antibiotics even without teplizumab treatment (Figure 5A, \( P = 0.016 \)) and unlike control mice, did not decrease further with the mAb (\( P = 0.02 \)).

In patients with type 1 diabetes treated with teplizumab, an increase in CD8^+CM T cells is a biomarker of responses to the drug (20, 22). CD8^+CM T cells were increased in the mesenteric lymph nodes of the humanized mice treated with teplizumab (Figure 5B, \( P = 0.049 \)) but not when they received antibiotics (Supplemental Figure 3, \( P = 0.03 \)). When splenic T cells were activated with PMA/ionomycin, there was a reduced frequency of IL-2–expressing CD4^+ and CD8^+ cells in mice treated with teplizumab but not when antibiotics were given (Figure 5, C and D, \( P = 0.037 \) and 0.015, respectively). In addition, the levels of p-STAT5 were increased after culture with IL-2 in mice treated with teplizumab and antibiotics, particularly among the CD8^+ T cells (Figure 5, E and F, \( P = 0.02 \)).

CCR4 expression is associated with migration to the skin and our previous studies have suggested that teplizumab may induce CCR4^+CD4^+ T cells with regulatory features such as IL-10 (19). We therefore analyzed the effects of antibiotic treatment on CCR4^+CD4^+ and CCR4^+CD8^+ T cells in the gut wall and spleen (Figure 6). In mice treated with antibiotics and teplizumab, the frequency of CCR4^+CD4^+ T cells was reduced in the spleen (\( P = 0.004 \)) and CCR4^+CD8^+ T cells in the gut and spleen (\( P = 0.027 \) and 0.03, respectively) compared with mice that had not been treated with antibiotics.

Antibiotic treatment modifies cytokine responses. IL-10 is an important mediator of tolerance in the gut after treatment with anti-CD3 mAb (17, 19, 23, 24). The levels of IL-10 in the serum of the humanized mice were increased following teplizumab treatment (\( P < 0.001 \)) but not when the mice were pretreated with antibiotics (Figure 7A). To determine whether treatment with a single antibiotic was responsible for this response to teplizumab, we measured the levels of IL-10 in the serum after mice were treated with teplizumab and with individual antibiotics or the pool of 4 antibiotics. Cladograms showing 16S sequencing are shown in Supplemental Figure 4 and the fold changes in Eubacteria and segmented filamentous bacteria are shown in Supplemental Table 1. The levels of IL-10 were lower in the mice that received anti-CD3 mAb and the combination of all 4 antibiotics compared with mice treated with teplizumab without antibiotics (Figure 7B, \( P = 0.034 \)), but the levels of IL-10 were not reduced when any of the single antibiotics were given with teplizumab.

Modification of dendritic cells by the microbiome. To determine whether qualitative differences in the pellets could account for the changes we observed in cytokine release and to identify which cells were responsible for the changes, we cultured splenocytes from humanized mice, not treated with antibiotics or teplizumab, with inactivated pellets from mice that had or had not received antibiotics. Equal numbers of bacteria were added to the cultures. Compared with pellets from control (no antibiotics) mice, the pellets from antibiotic-treated mice stimulated less IL-10 release by splenocytes (\( P = 0.04 \)), but there were no significant differences in the levels of IFN-\( \gamma \) or TNF (Figure 8, A–C).

To have sufficient numbers of cells for this study, we used CD4^+ and CD8^+ T cells, B cells, and dendritic cells (DCs) sorted from PBMCs from healthy donors to determine which cells were affected by the modified microbiome, and cultured them with the pellets from untreated and antibiotic-treated mice (Figure 8D). The DCs, but not T or B cells, released high levels of IL-10 after culture with the
pellets ($P < 0.0001$, $P = 0.0008$), but less when the pellets were from antibiotic-treated mice ($P = 0.03$). The effects of the antibiotic-treated pellets on CD11b$^+$CD11c$^+$ cells were restricted to IL-10 ($P < 0.0001$) because release of IL-6, TNF, IL-1β, and IL-8 was not significantly different (Figure 8, E–I). We then tested whether there were similar effects of antibiotic treatment on responses to human microbiota. We cultured human PBMCs with heat-inactivated stool samples from 18 subjects receiving antibiotics and 11 subjects who were not receiving antibiotics and had not received them within the previous 3 months (Supplemental Table 2). We added an equivalent number of organisms ($2.2 \times 10^6$) to the cultures for 5 days. We tested each fecal sample against PBMCs pooled from 4 different individuals to minimize variability of the responder cells and compared cytokine levels in the supernatants. There was significantly reduced production of IL-10 by PBMCs cultured with the bacteria from stool samples from antibiotic-treated compared with control subjects (Figure 8J, $P = 0.0017$).

To determine whether the changes in DCs affected the activation of T cells, we isolated CD11b$^+$CD11c$^+$ cells from the PBMCs of healthy cell donors and cultured them overnight with equal numbers of bacteria from inactivated pellets from mice that had or had not received antibiotics. There was increased expression of CD86 on the CD11b$^+$CD11c$^+$ cells from the cultures with antibiotic-treated pellets ($P = 0.02$). We then added autologous T cells with teplizumab and measured activation of the T cells by expression of CD69 after overnight culture. When precultured with inactivated fecal bacteria from pellets from mice treated with antibiotics, there was increased expression of CD69 on CD4$^+$ ($P = 0.02$) and CD8$^+$ ($P = 0.04$) T cells compared with pellets from mice that had not been treated (Figure 9, B and C). Thus, pellets from the antibiotic-treated mice enhance the activation of T cells that are cocultured with CD11b$^+$CD11c$^+$ cells and teplizumab.

IL-27 has been shown to play an important role in IL-10 expression and development of Tregs that specialize to control Th1 cell–mediated immunity (25, 26). Several factors, including IL-10, TGF-β, and vitamin D have been shown to modulate DCs and induce tolerogenic DCs, which produce IL-27 (27). To understand the basis of changes in the DC population, we analyzed the expression of IL-27 by CD11b$^+$CD11c$^+$ cells that had been exposed to pellets from non–antibiotic-treated or antibiotic-treated mice (28, 29). Intracellular expression was similar in CD11b$^+$ macrophages but was reduced in CD11b$^+$CD11c$^+$ DCs that were cultured with pellets from antibiotic-treated compared with pellets from
mice not treated with antibiotics (Figure 10A, \( P = 0.02 \)). To determine whether soluble or insoluble products were involved, we suspended the pellets in PBS and cultured the CD11b+CD11c+ cells with the supernatants or insoluble material from these suspensions. There was significant reduction in the IL-27 expression in the CD11b+CD11c+ cells that were cultured with the supernatants from the pellets from the antibiotic-treated versus non–antibiotic-treated mice (Figure 10B, \( P = 0.012 \)).

**Discussion**

Our studies show that antibiotic treatment can modify regulation of the human adaptive immune system and break immunologic tolerance. When humanized mice were treated with antibiotics, there was reduced diversity and total bacterial load of the microbiota. Three observations show how the change in the microbiome affected immune tolerance. There was spontaneous development of autoantibodies (ANAs), and increased levels of IFN-\( \gamma \) in the circulation with activation of cells in the gut wall. Previously we found that teplizumab treatment prevented xenogeneic skin graft rejection in humanized mice by inducing T cells with regulatory function (17, 30), which we now show is modified by antibiotic treatment. The tolerogenic response to teplizumab requires migration to the gut and activation of cells at that location but the antibiotic treatment did not affect cell migration. Biomarkers of teplizumab action such as increased IL-10 in the serum and expansion of CD8CM T cells were not detected when antibiotics were given. The cocktail of 4 but not individual antibiotics affected IL-10 levels, suggesting that a single organism is not likely to be involved. CD11b+CD11c+ DCs appear to account for these effects since antibiotic treatment caused reduced IL-10 and IL-27 production by these cells when they were cultured with pellets from the antibiotic-treated mice. Soluble products produced by the microbiome account for the differences in IL-27 production by the DCs when mice are treated with antibiotics. Finally, we found a similar effect on IL-10 production by human PBMCs that were cultured with the microbiota from subjects that had or had not been treated with antibiotics, suggesting that these observations have relevance for immune responses in patients. Our studies suggest that modification of the microbiome by antibiotics can break tolerance through actions on DCs in the host. They suggest a mechanism whereby antibiotic use may foster autoimmunity and account for differences in individual responses to tolerogenic therapies.

The activated T cells that were found in the mice treated with antibiotics may not be susceptible to the action of teplizumab or cells induced by teplizumab. In clinical studies, we have shown expansion of CD8CM T cells that show transcriptional profiles of tolerance such as decreased expression of the IL-7 receptor and increased expression of IL-10 (20). Cell tracing studies showed that in vitro the major source of these cells is the naive CD8+ T cell pool, which was decreased in the antibiotic-treated mice and therefore may account for the failure of the biologic. Our clinical studies also showed that teplizumab induced CCR4+IL-10+CD4+ T cells in the peripheral blood, which several investigators have shown have regulatory function (19, 31–34). CCR4+CD4+ and CCR4+CD8+ cells were reduced in frequency by antibiotic treatment, suggesting that the microbiome was needed for their induction. Therefore, in addition to activation of T cells which may render them resistant to regulation, the antibiotic treatment also affected cells that can mediate immune regulation.

**Figure 4.** Effects of antibiotic treatment on cells in the gut wall after teplizumab treatment. (A) The number of CD2+ T cells in the lamina propria was determined 18 hours after treatment with teplizumab in mice that had or had not received antibiotics (Abx). (B) Gene expression of ROR\( \gamma \)t was analyzed by RT-PCR in gut cells 18 hours after treatment with anti-CD3 mAb in mice that had or had not received antibiotics. There was a significant reduction in the expression of ROR\( \gamma \)c in the antibiotic-treated mice. * \( P = 0.035 \), Student’s t test; each symbol represents an individual mouse.
We identified CD11b⁺CD11c⁺ cells as the sensor cells for the microbiome. Rather than a single organism, our findings with individual antibiotics and cultures with supernatants and insoluble material from the pellets suspended in PBS suggest that soluble products account for the effects on IL-27 production. Further studies and correlations between the presence or absence of bacteria and loss of IL-10 in the humanized mice will be needed to determine whether specific families of organisms are responsible. One possibility is that the metabolites derived from commensal bacteria such as short-chain fatty acids (SCFAs), which include butyrate, propionate, and acetate that have the potential to change the epigenome of host cells that control development and function.
of Tregs in the gut, are no longer produced (35, 36). Interestingly, in mice, *Bifidobacterium breve* was shown to induce IL-10– and IL-27–producing Tr1 cells in the colon via a TLR2/MyD88 pathway, consistent with our findings of activation with the pellets from the antibiotic-treated mice (37). Eubacteria can produce butyrate, which induces gene expression changes in DCs leading to suppression of IL-12, IL-6, and expression of RELB, which contributes to Treg differentiation (38, 39). Butyrate has also been linked to IL-10 production through the activation of the niacin receptor Gpr109a (40). Although further studies to directly demonstrate an association between these organisms together or individually and IL-10 production are needed, these observations suggest that the normal microbiome maintains tolerance of human cells by the balance of metabolites that are produced.

An alternative explanation may also account for our findings about the activation properties of the CD11b+CD11c+ cells and the microbiome. Rather than the absence of an inducer of immune regulation the absence of an immune activator may be involved. Watson and Kim described the phenomenon of “endotoxin tolerance” in which stimulation of immune cells with LPS induces a refractory state to a repeated immune challenge (41). Vatanen et al. found that LPS from different strains of commensals differed in their ability to induce this state and may account for susceptibility or resistance to autoimmune diabetes in genetically similar individuals from Russia and Finland (13). The failure to induce a state like endotoxin tolerance is consistent with the enhanced T cell activation when CD11c cells were cultured with pellets from the antibiotic-treated mice. In line with this notion is also the fact that IL-10, serum levels of which are higher in non–antibiotic-treated mice and which is more highly expressed by CD11b+CD11c+ cells in response to fecal samples of untreated mice, is known to block induction of TLR4 by LPS (42).

Regardless of the cause, the changes in the microbiome lead to DC activation and increase the activation of T cells when cultured with teplizumab. Although teplizumab is non–FcR binding, in previous studies we have shown that the effects of the mAb on T cells requires the presence of APCs (18).

There are limitations to our findings. The antibiotic treatment affects the microbiota at other locations as well as the gut, and these effects, as well as direct effects of the drugs on immune cells, may be involved. Studies from other investigators suggest this is not the case, since immunologic effects that were seen in antibiotic-

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**Figure 6. CCR4+CD4+ and CCR4+CD8+ T cells in the gut wall and spleen.** The expression of CCR4 on CD4+ cells in the gut (A) and spleen (B) and on CD8+ cells in the gut (C) and spleen (D) was measured by flow cytometry in cells that were harvested 18 hours after mice that had (black bars) or had not (open bars) been treated with hlg or teplizumab (Tep). *P < 0.05, **P < 0.01 by 2-way ANOVA (n = 7-10 mice/group). Abx, antibiotics.
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treated mice were similar to gnotobiotic-treated mice (14–16), and our studies with pellets from the antibiotic-treated mice and human samples suggest that the direct effects of the antibiotics on the gut flora rather than the host are responsible. Although we did not detect effects of antibiotic treatment on gut migration of T cells, we cannot exclude the possibility that failure to induce tolerance was associated with enhanced migration of effector cells to the skin grafts, which may be modulated when the microbiota are present. Because this humanized mouse model system does not have a thymus, studies of the function of Tregs are not optimal in this model. In addition, some tolerance mechanisms that may be important even when the microbiome is altered differ in the humanized mice from those in normal humans. Central tolerance and peripheral ligands such as PD-L1 and other immune inhibitory receptors may not cross react between mouse and human cells.

In summary, our studies show that the microbiome is needed to maintain tolerance by human immune cells. Our findings highlight the critical role of the microbiota in this setting since teplizumab restored tolerance when autoimmunity was induced by other means (i.e., anti–CTLA-4 mAb) (30). These observations may have broad application to understanding the basis for autoimmune diseases and the increased frequency of these ailments that have been associated with the avoidance of infections and use of antibiotics in Western countries (43). In addition, our studies suggest a mechanism that could account for individual differences in responses to biologics. Further studies from clinical trials may help to determine whether consideration of the microbiota can be used to personalize treatments for immune therapies.

**Methods**

**Humanized mice.** NOD/SCID IL-2γc−/− (NSG) mice were purchased from The Jackson Laboratory, and bred and housed in our animal facility under specific pathogen–free conditions. CD34+ cells were isolated from 11 different donors and purified with magnetically labeled microbeads conjugated to anti–human CD34. The cells (1 × 10^5) were injected intrahepatically into irradiated (1 Gy) NSG pups within 48 hours of birth. The reconstitution of the mice was determined in peripheral blood at 12 weeks of age. Mice that showed at least 50% of hCD45+ cells in the peripheral blood were used for studies.

**Antibiotic treatment.** A cocktail of vancomycin (0.5 g/l), ampicillin (1 g/l), neomycin (1 g/l), and metronidazole (1 g/l) was added to the drinking water. The antibiotic-containing drinking water was continued until the time of sacrifice.

Fecal microbiota from the mice were collected before and after 2 weeks of antibiotic treatment and frozen at −80°C. Fecal pellets were weighed and bacterial DNA was isolated using a PowerSoil DNA isolation kit (Mo Bio). Equivalent amounts of DNA were used for analysis of eubacterial and segmented filamentous bacterial contents by quantitative PCR using the SyberGreen protocol. V4-directed 16S rRNA...
gene sequencing was performed as described (44). Briefly, amplicons were generated using dual-barcoded primers (45), pooled, and sequenced using 2 × 250 bp chemistry on an Illumina MiSeq instrument. Paired-end reads were assembled and clustered into operational taxonomic units (OTUs) at 97% identity using an open-reference method. Taxonomy was assigned using the Greengenes gg_13_5_otus reference database (46) and OTUs rarefied to 20,000 sequences per sample and singletons excluded prior to further analysis.

Figure 8. Effects of antibiotic treatment on pellets and cells from humanized mice. (A–C) Splenocytes from mice that had not been treated with antibiotics (Abx) were cultured with pools of pellets from mice that had not or had been treated with antibiotics. The levels of IFN-γ, IL-10, and TNF were measured in the supernatants after 5 days and compared by paired t test. *P = 0.04, n = 8 cell donors/group. (D) Subsets of human PBMCs were isolated by magnetic beads and cultured with pellets from mice that had or had not been treated with antibiotics (n = 5 each). There was increased IL-10 release when the DCs were cultured with pellets from both groups of mice but reduced release with pellets from the antibiotic-treated mice. *P = 0.03, ***P = 0.0008, ****P < 0.0001, 2-way ANOVA with paired comparisons. (E–I) DCs were isolated from the PBMCs from healthy donors and cytokine levels were measured after 5 days in culture with a pool of pellets from mice that had or had not been treated with antibiotics. Each symbol represents a DC cell donor. Culture with the pellets increased production of all cytokines (P < 0.0001) compared with the cultures without pellets (Ctrl) (P = 0.002 by ANOVA). (J) Human stool samples of antibiotic-treated patients and healthy controls were heat inactivated and cultured with PBMCs that were pooled from 4 healthy cell donors. The level of IL-10 in the supernatant after culture for 3 days is shown. Each symbol represents a stool sample donor. **P = 0.002, ****P < 0.0001 by Student’s t test.
Skin grafts. Mouse full-thickness skin xenografts from C57B6/J RAG–/– mice were transplanted on the dorsum of the recipient humanized mice. The next day either hlg or teplizumab (humanized, non–FcR-binding anti-CD3 mAb, gift of MacroGenics, Inc, Rockville, Maryland, USA) (5 μg) was given i.p. Some mice were sacrificed after 18–22 hours, others after 10 days, and the organs and sera were collected. Dressings were removed under anesthesia at day 7. The skin grafts were monitored daily and the day of rejection was assessed by 2 observers and defined as the day when the graft was no longer present.

ANAs. ANA testing was performed with HEp-2 slides that were incubated with a 1:40 dilution of serum samples collected from both control and experimental mice. The serum was incubated with the slides for 1 hour at room temperature in a humidifying chamber. Excess unbound antibodies were removed by washing for 5 minutes in PBS. The slides were incubated with secondary antibody at a 1:100 dilution in PBS for 1 hour, after which they were washed in PBS for 5 minutes and mounted. Images were rendered using an Olympus BX40 microscope. Without knowledge of the mouse donor, they were scored from +1 to +4, depending on the intensity of staining for ANA.

Immune cell phenotypes. Cells were isolated from spleen, peripheral blood, and mesenteric lymph node following tissue homogenization. RBCs in peripheral blood and spleen were lysed using ACK lysis buffer (Lonza). The entire small intestine was resected and the cells were isolated by digesting the gut with collagenase for 20 minutes followed by 2 washes with sterile cold PBS. The immune cells were purified on a Ficoll density gradient and counted using BD Trucount beads during flow analysis with a FACSAria (BD Biosciences). T cell phenotypes were determined by flow cytometry using the following antibodies purchased from Biolegend and BD: CD45 (HI30) APC, anti–mouse CD45 (30-F11) Pacific Blue, CD19 (SJ25C1) PE, CD3 (OKT-3) FITC, CD4 (RPA-T4) APC-H7, CD8 (RPA-T8) PerCP-Cy5.5, CD45RO (UCHL-1), CCR7 (G043H7) PE, CD45RA (H100) APC-H7, CD103 (Ber-ACT8), CCR4 (L291H4), and CD2 (RPA-2.10). T cell memory phenotypes were identified as naive (CD45RO–CCR7+), central memory (CD45RO+CCR7+), effectors (CD45RO+CCR7–), and effector memory RA (CD45RO–CCR7–). Tregs were identified with CD2 (RPA-2.10), CD4 (RPA-T4), CD25 (2A3), CD127 (A019D5), and Foxp3 (259D). The phenotypes of CD11b+ and CD11c+ cells (identified with ICRF44 and Bu15, respectively) were determined using CD86 (IT2.2) APC. CCR4 (ab1664) antibody was purchased from Abcam. For intracellular cytokine staining, splenocytes or 1 × 10⁶ PBMCs were stimulated with PMA/ionomycin for 6 hours in the presence of GolgiStop and stained with intracellular antibodies by using a BD Fix/Perm kit.

Stat5 phosphorylation in T cells. Constitutive and IL-2–induced STAT5 phosphorylation was evaluated in CD2+CD4+ or CD8+ splenocytes after stimulation with IL-2 in vitro (100 U/well). Splenocytes were starved overnight in a serum-free medium (AIM-V from Invitrogen) followed by incubation with 100 U recombinant human IL-2 per 10⁶ cells for 20 minutes at 37°C. The cells were stained, fixed, and permeabilized as described previously (30) and then stained with anti–p-STAT5 (Y694)-Alexa 488 (BD Biosciences, catalog 612598, clone 47). The relative expression of p-STAT5 (MFI) was determined by flow cytometry.

Figure 9. DCs cultured with antibiotic-treated pellets enhance T cell activation with anti-CD3 mAb. CD11c+ cells were isolated from PBMCs and cultured with pellets as described in the Methods. (A) The expression of CD86 on CD11b+CD11c+ DCs cultured in pellets from antibiotic-treated mice was increased. *P = 0.02 by multiple comparison from ANOVA. (B) Autologous T cells were then added to the cultures with teplizumab and the expression of CD69 was measured on the CD4+ and CD8+ T cells after overnight culture. There was increased expression of CD69 on CD8+ T cells that had been cultured with cells and antibiotic-treated pellets. *P < 0.05, ****P < 0.0001 by 2-way ANOVA with paired comparisons. Abx, antibiotics.
**Serum cytokine measurements.** Human cytokines were measured with an ultrasensitive Milliplex xMAP assay (Millipore). The lower limit of detection levels for these assays was 1 pg/ml. The data were log transformed for statistical analysis.

**Cultures with total fecal pellets, supernatants, or pellets of fecal samples from humanized mice.** Freshly collected fecal pellets from humanized mice that had or had not been treated with antibiotics (5 mice each) were pooled, dissolved in sterile PBS, and heat inactivated for 30 minutes at 95°C. The number of bacteria was determined by their optical density; 2.2 × 10⁶ bacteria, their supernatants, or their pellets (following centrifugation at 6,797 g for 15 minutes) were used to stimulate 2 × 10⁵ splenocytes or PBMCs from humans, cultured in 200 μl RPMI 1640 media (Invitrogen) supplemented with 10% FCS. After overnight culture or 5 days of culture, levels of cytokines in the culture supernatants were determined by a Milliplex xMAP assay. The samples were tested against a panel of 4 cell donors.

**Cultures with human stool samples.** Human stool samples were collected and frozen from hospitalized patients that had or had not been treated with antibiotics in the past 3 months. The samples were dissolved in PBS and heat inactivated as described above. Equal numbers of bacteria (2.2 × 10⁶), determined by OD, were added to cultures.

**Statistics.** Unless indicated, all data are presented as the mean ± SEM. In general, individual symbols represent individual mice from 3–4 independent experiments, each with 3–6 mice/group. Comparisons between groups were performed with the indicated statistical tests. All analyses were done with GraphPad Prism software (version 6.0). A P value of less than 0.05 was considered statistically significant. Student’s t tests were 2 tailed.

**Study approval.** IACUC approval was obtained for all studies. Written informed consent was given for donation of stool samples. The studies with human samples were approved by the IRBs at Yale University, New Haven, CT and the Albert Einstein College of Medicine, Bronx, NY.

**Author contributions**
EG, NKV, MM, SM, and KCH performed experiments, analyzed data, and wrote the manuscript. SD, JCR, and RT performed experiments and collected data. PPH and BL performed experiments and analyzed data, analyzed data, and wrote the manuscript, RT collected and analyzed data. SMV, JDHM, MK, ALG, BCH, and CC analyzed data and wrote the manuscript.

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