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Prevention of Virus-Induced Type 1 Diabetes with Antibiotic Therapy

Naoko Hara,* Aimon K. Alkanani,* Diana Ir,† Charles E. Robertson,‡ Brandie D. Wagner,§ Daniel N. Frank,†,§ and Danny Zipris*

Microbes were hypothesized to play a key role in the progression of type 1 diabetes (T1D). We used the LEW1.WR1 rat model of Kilham rat virus (KRV)-induced T1D to test the hypothesis that the intestinal microbiota is involved in the mechanism leading to islet destruction. Treating LEW1.WR1 rats with KRV and a combination of trimethoprim and sulfamethoxazole (Sulfatrim) beginning on the day of infection protected the rats from insulitis and T1D. Pyrosequencing of bacterial 16S rRNA and quantitative RT-PCR indicated that KRV infection resulted in a transient increase in the abundance of Bifidobacterium spp. and Clostridium spp. in fecal samples from day 5- but not day 12-infected versus uninfected animals. Similar alterations in the gut microbiome were observed in the jejunum of infected animals on day 5. Treatment with Sulfatrim restored the level of intestinal Bifidobacterium spp. and Clostridium spp. We also observed that virus infection induced the expression of KRV transcripts and the rapid upregulation of innate immune responses in Peyer’s patches and pancreatic lymph nodes. However, antibiotic therapy reduced the virus-induced inflammation as reflected by the presence of lower amounts of proinflammatory molecules in both the Peyer’s patches and pancreatic lymph nodes. Finally, Sulfatrim treatment reduced the number of B cells in Peyer’s patches and downmodulated adaptive immune responses to KRV, but did not interfere with antiviral Ab responses or viral clearance from the spleen, pancreatic lymph nodes, and serum. The data suggest that gut microbiota may be involved in promoting virus-induced T1D in the LEW1.WR1 rat model. The Journal of Immunology, 2012, 189: 3805–3814.

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Abbreviations used in this article: BB, BioBreeding; CT, cycle threshold; KRV, Kilham rat virus; OTU, operational taxonomic unit; T1D, type 1 diabetes; Treg, regulatory T cell; VP2, viral protein 2.

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The Journal of Immunology

Type 1 diabetes (T1D) is a multistage, T cell-mediated autoimmune disease that involves the slow and progressive destruction of islet β cells, resulting in a complete loss of insulin secretion (1). How T1D is triggered is not yet known, but evidence from humans and animal models implicates environmental factors in the mechanism of disease initiation (2–4).

Emerging data suggest that intestinal microbiota play a crucial role in the development of the peripheral immune system and gut immunity in health and disease (5–7). The gut microbiome has been implicated in proinflammatory disorders. Indeed, alterations in the gut microbial composition have been detected in patients in the early stages of rheumatoid arthritis (8) and inflammatory bowel disease (9). In vivo experiments have indicated the involvement of the gut microbiota in a number of mouse models of autoimmune arthritis. For example, spontaneous ankylosing enthesopathy does not develop in germ-free mice (10). In contrast, disease was triggered in mice inoculated with a mixture of anaerobes, but not in animals inoculated with Lactobacillus or Staphylococcus spp. (10).

The intestinal microbiome has recently been associated with the development of T1D (11–18). Giongo et al. (13) documented a decline in Firmicutes and an increase in Bacteroidetes over time in individuals who were at risk for development of T1D. In healthy children, Giongo et al. found that Firmicutes increased, whereas Bacteroidetes declined over time. All of the changes detected in at-risk subjects were attributed to Bacteroides, and >20% of the changes within this genus were observed in the Bacteroides ovatus species (13). Whether these changes in the gut microbiome contribute to T1D progression or simply are a consequence of disease remains an open question (19). Other groups have demonstrated that gut bacteria are linked with T1D in the diabetes-prone BioBreeding (BB) rat (20) and NOD mouse (14, 21).

In this study, we used the LEW1.WR1 rat model of virus-induced T1D to delineate the mechanisms by which infectious agents promote development of T1D. Infection of rats with Kilham rat virus (KRV) leads to T1D within 2–4 wk after viral inoculation in 50% of the infected rats (22). LEW1.WR1 rats have normal T lymphocyte function and NOD mouse (14, 21).

The sequences presented in this article have been submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/sra) under accession number SRA052187.
immune system could play a role in the course of T1D in the LEW.1.WR1 rat model. In this study, we showed that infection of LEW.1.WR1 rats with KRV induces a transient alteration in the gut microbiome. We also demonstrate that viral infection activates the innate immune system in Peyer’s patches. When animals were treated with antibiotics, insulin and T1D were blocked, but viral clearance from the spleen, pancreatic lymph nodes, and serum was not affected. Our data suggest the involvement of the gut microbiome and innate immunity in the early course of virus-induced T1D and raise the possibility that modulating the composition of the gut bacteria may be used for diabetes prevention.

Materials and Methods

Animals and viruses

Specific pathogen-free LEW.1.WR1 rats of both sexes were obtained from Biomedical Research Models (Worcester, MA). Animals were bred and housed in a specific pathogen-free facility and maintained in accordance with the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, National Research Council, National Academy of Sciences, 1996) and the guidelines of the Institutional Animal Care and Use Committee of the University of Colorado Denver. KRV was propagated and titered as previously described (30).

Virus-induced diabetes, antibiotic treatment, and blood and lymphoid organ removal

Rats at 21–25 d of age were injected i.p. with 1 × 10^7 PFU KRV as previously described (31). The antibiotic Sulfatrim (1 mg/ml sulfamerthazole plus 0.2 mg/ml trimethoprim) was purchased from Hi-Tech Pharmaceutical (Amityville, NY). Neomycin sulfate (1 mg/ml), ampicillin (1 mg/ml), and metronidazole (1 mg/ml) were purchased from Sigma-Aldrich (St. Louis, MO). The drugs were administered to rats by dissolving them in drinking water. Rats were i.p. injected with KRV only, were infected with KRV and treated with antibiotics, or were treated with Sulfatrim only beginning on the day of virus inoculation to examine the effect of antibiotic therapy on the course of T1D. The animals were monitored for insulitis and disease development for 40 d postinfection or were sacrificed on day 5 for gene expression and flow studies. Spleens, pancreatic lymph nodes, Peyer’s patches, and sera were collected 5 or 40 d after virus infection. Sera were separated from blood cells immediately after blood removal and stored at −80°C until use. Pancreata were removed for insulitis evaluation 21 and 40 d after viral infection or after disease onset. Cells were assessed for insulin-positive and -negative lymphoid organs by staining the tissue through a 70-μm nylon mesh filter. In the case of the spleen, erythrocytes were lysed with NH4Cl. The cells were washed and resuspended in tissue culture media or PBS for further use.

Histological staining

Pancreatic tissue was fixed for 24 h in 10% neutral-buffered formalin, embedded in paraffin, cut (5–6 μm), and mounted on microscope slides. The tissue was stained with H&E.

Microbiome analyses

Bacterial profiles from rat fecal DNA samples were determined by broad-range PCR of 16S rRNA genes and phylogenetic sequence analysis. Amplicons of the 16S rRNA gene (32, 33) were generated via broad-range PCR (26 cycles) using 5′-bar-coded reverse primers (34). PCR yields were normalized using a SequalPrep kit (Invitrogen, Carlsbad, CA), pooled, lyophilized, and purified using a DNA Clean and Concentrator Kit (Zymo, Irvine, CA) (35). Pooled amplicons were provided to the Barbara Davis Center for Childhood Diabetes at the University of Colorado School of Medicine for pyrosequencing analysis. GS FLX Titanium sequencing was performed using Titanium chemistry (Roche Life Sciences, Indianapolis, IN).

Pyrosequencers were sorted into libraries by barcode and quality filtered using the software barbart (34). All pyrosequences were screened for nucleotide quality (bases at 5′ and 3′ ends with mean quality scores >20 over a 10-n window were deleted), ambiguous bases (sequences with >1 N were discarded), and minimum length (sequences <300 nt were discarded). The mean trimmed sequence length was ∼340 bp. The Infernal RNA alignment tool (36) was used to screen all sequences in terms of their fidelity to a covariance model derived from small subunit rRNA secondary structure models provided by the laboratory of Dr. Robin Gutell (37). Sequences that did not adequately match a bacterial covariance model were removed from all subsequent analyses. Chimera screening was performed using the software ChimeraSlayer (38), which requires that sequences be previously aligned with the software NAST-iEr (39). Putative chimeras and other sequences that could not be aligned by NAST-iEr were removed from subsequent analyses. Genus-level taxonomic classifications were produced by the RDP Classifier software, which performs naïve Bayesian taxonomic classification versus a training set (40). Pyrosequences were clustered into operational taxonomic units (OTUs) on the basis of taxonomic assignments. Ecological indices (41) of richness (e.g., Sobs, Schao), diversity (e.g., Shannon’s diversity [H0] and evenness [H0/Hmax]), and coverage (e.g., Good’s index) were computed with the software tool biodiv (42). These indices were estimated through bootstrap resampling (1000 replicates) and rarefaction of the OTU distributions obtained from each specimen. All 16S amplicon libraries were sequenced to >95% coverage. Pyrosequences were submitted to the National Center for Biotechnology Information short read archive and assigned the project accession number SRA052187.

DNA extraction and quantitative PCR analysis for the detection of gut bacteria

Bacterial DNA was recovered using the QIAamp DNA stool mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE). DNA from Lactobacillus, Bifidobacterium, Clostridium, and Bacteroides was detected by quantitative PCR analysis using previously published primers. The data were normalized to the total bacterial DNA in each sample using recently described primers and conditions (43).

RNA extraction, cDNA synthesis, and quantitative RT-PCR

RNA extraction, cDNA synthesis, and quantitative RT-PCR were performed as previously described (29). A melting point analysis was performed in all cases to confirm the presence of the expected gene product. The standards used for the gene amplification were TOPO plasmid vectors (Invitrogen) expressing a ∼500-bp DNA fragment derived from the mRNA sequence of the gene of interest that includes the ∼100-bp sequence used for the PCR amplification. The primers used were synthesized by Life Technologies (Coralville, IA). Their sequences have been previously published (29). Data for the expression of KRV or proinflammatory molecules in pancreatic lymph nodes and Peyer’s patches were calculated as the ratio of gene expression to β-actin expression in the same sample of cDNA. Comparison of virus expression between serum samples was performed by calculating the n-fold difference in KRV abundance using the formula y = 2^−ΔCT, where ΔCT is the cycle threshold (CT) of sample 1 − CT of sample 2 and y is the n-fold difference in gene expression. The CT value was defined as the number of PCR cycles required for the fluorescence signal to exceed the detection threshold value. To determine the KRV transcript levels, we used the following primers: 5′-GGAAACGCTTACTCCGATGA-3′ and antisense 5′-AACGATGTCCTCCTCATTFF-3′. To determine IL-17A gene expression, we used 5′-ACTGAGACCCTGGAGCCTACCGTAAG-3′ and anti-sense 5′-AAAGTTATTGCTCCTGCGGTTGG-3′. The primers were validated by running the PCR product on an agarose gel.

Flow cytometry

Cells were resuspended in PBS containing 1% BSA and 0.1% sodium azide incubated with optimal concentrations of fluorochrome-conjugated Abs for 30 min at 4°C, washed, and fixed with 1% paraformaldehyde. A PerCP-conjugated anti-TCRαβ Ab (clone R73, mouse IgG1), a PE-conjugated anti-IL-2R a-chain Ab (CD25, clone OX-39, mouse IgG1), an allophycocyanin-conjugated anti-CD4 Ab (clone OX-35, mouse IgG2a), a PerCP-conjugated anti-CD8 a-chain Ab (clone OX-8, mouse IgG1), an FITC-conjugated anti-CD45R Ab (a marker of B cells, clone HIS24, mouse IgG2b), and appropriate isotype controls were purchased from BioLegend (San Diego, CA). An Efluor 450-conjugated mAb against Fopx3 (clone FJK-16s, rat IgG2a), and fixation and permeabilization buffers were purchased from eBioscience (San Diego, CA). Flow cytometry was performed using a Cyan ADP instrument (Beckman Coulter), and the results were analyzed with FlowJo software.

KRV-specific CDC8 IFN-γ+ T cells and detection of anti-KRV Abs

KRV-specific CDC8 IFN-γ+ and anti-KRV Abs were detected in the spleen on day 12 after viral infection as previously described (28). In brief, Y3–Ag 1.23 cells (Y3 cells; American Type Culture Collection) were infected with 0.5 × 10^9 PFU KRV in 4 ml complete DMEM for 18 h and used as
Anti-KRV Abs

Anti-KRV Abs were detected in samples that were plated onto 96-well ELISA plates coated with KRV (Charles River Breeding Laboratories, Wilmington, MA). Wells were blocked with 5% BSA (Bio-Rad, Hercules, CA) in PBS containing 0.05% Tween 20 (Sigma-Aldrich) for 1 h at 37°C and then washed twice with PBS-Tween 20. Serial dilutions of sera from uninfected or KRV-infected rats were then added to the wells in duplicate for 1 h at room temperature followed by three washes with PBS-Tween 20. Biotinylated goat anti-rat IgG (H and L chain-specific; Jackson ImmunoResearch, West Grove, PA) was then added to the wells. After incubation for 1 h at room temperature, the wells were washed three times and incubated with streptavidin-HRP (Jackson ImmunoResearch) for 30 min. The wells were then washed five times, and HRP was detected with o-phenylenediamine dihydrochloride (Sigma-Aldrich). Color development was terminated with 5 N HCl, and the plates were read at 492 nm.

Statistical analysis

Statistical comparisons of diabetes-free survival among groups were performed using the method of Kaplan and Meier. The Expcet sequence analysis software package (v.1.38; C.E.R. and D.N.F., unpublished software) was used for all microbiome statistical analyses. Changes in OTU prevalence and/or abundance between pairwise comparisons of treatment and control groups were analyzed using a two-part statistic (42). Null hypotheses were evaluated at $\alpha = 0.01$, $\alpha = 0.05$, and $\alpha = 0.10$. Comparisons between more than two groups were performed with a one-way ANOVA with Bonferroni’s multiple-comparison test. Comparisons between two groups were performed with unpaired $t$ test.

Results

Treatment with Sulfatrim prevents insulitis and virus-induced T1D in LEW1.WR1 rats

We tested the hypothesis that gut bacteria are involved in virus-induced T1D in the LEW1.WR1 rat model. LEW1.WR1 rats at 21–25 d of age were injected with $1 \times 10^7$ PFU KRV and given Sulfatrim in their drinking water on the day of viral inoculation. The cells were cultured at 37°C in an atmosphere of 95% air and 5% CO$_2$ for 5 h and then stained with a PE-conjugated anti-rat CD8 mAb. The cells were then fixed and permeabilized using the Cytofix/Cytoperm kit (BD Pharmingen) and incubated with an FITC-conjugated anti-rat IFN-γ mAb (clone DB-1; BioLegend) or its isotype control (mouse IgG1; BioLegend). The cells were analyzed by flow cytometry as described earlier. A total of 140,784 high-quality pyrosequencing reads were generated for all 24 samples (Table I). The median Goods coverage score, a measure of completeness of sequencing, was $\approx 99\%$, indicating that the depth of sequencing was sufficient to fully describe the biodiversity of the samples. In addition, no differences were observed in the number of genera detected (i.e., genus richness) in treated compared with untreated animals.

We detected a total of 9 phyla and 96 genera in fecal samples from naive LEW1.WR1 rats. The most abundant phylum-level bacterial taxa identified were those of Bacteroidetes and Firmicutes; neither
was statistically different in treated versus untreated animals, as evaluated by both prevalence and abundance (Table I). In contrast, the phylum Actinobacteria (e.g., high G-C Gram-positive microorganisms) was significantly increased in prevalence and abundance in KRV-infected versus uninfected animals (p < 0.05). Analysis of bacterial communities at the genus level indicated that the median percentage abundance of the Firmicutes Anaerostipes spp. and Anaeroplasmata was ~9- to 11-fold elevated in infected versus uninfected animals (p < 0.05). A more modest but significant increase was noted for the Firmicute Clostridium spp. (p < 0.05 versus uninfected). A trend toward increased abundance of Bifidobacterium spp. and a reduction in Anaerovorax spp. was also detectable in infected versus uninfected animals (p < 0.1).

Administering rats with KRV plus Sulfatrim led to a significant reduction in the prevalence and abundance of Verrucomicrobia and Akkermansia spp. (p < 0.05 versus KRV only). A trend toward reduced prevalence and/or abundance of bacterial communities such as Clostridium spp., Anaeroplasmata spp., Bifidobacterium spp., and Parasutterella spp. was detected in animals treated with KRV plus Sulfatrin or Sulfatrin only versus KRV only. Taken together, these data raise the possibility that alterations in gut bacterial communities are involved in the course of virus-induced T1D in the LEW1.WR1 rat model.

Validation of the high-throughput pyrosequencing data

We used quantitative PCR analysis to validate the observations made using the 454 pyrosequencing approach. We also addressed in greater depth the possibility that the alterations observed in gut bacterial communities are associated with KRV infection. For this purpose, groups of animals were left untreated (n = 3–7), were infected with KRV only (n = 5–7), were treated with KRV plus Sulfatrin (n = 7–9) beginning on the day of infection, or were treated with Sulfatrin only (n = 3–7). Fecal samples were collected on day 5 postinfection and on day 12, which is 2 d before the time when TID is first observed in infected LEW1.WR1 rats (28). We evaluated the abundance of bacterial genera that were found by 16S pyrosequencing to be altered post-KRV infection or Sulfatrin therapy (Bifidobacterium spp. and Clostridium spp.), or previously shown to be most abundant in the diabetes-resistant BB rat model (Lactobacillus spp. and Bacteroides spp.; see Ref. 18).

The data presented in Fig. 2A confirm that infection with KRV significantly increased the abundance of the Bifidobacterium spp. and Clostridium spp. genera compared with untreated animals (p < 0.01), and administering animals with KRV plus Sulfatrin reduced the level of these bacteria (p < 0.001 versus KRV only). The data also indicate that infection with KRV or treatments with KRV plus Sulfatrin do not alter the abundance of Lactobacillus spp. and Bacteroides spp.

Table I. Percentage prevalence and median abundance of bacterial communities in KRV-infected LEW1.WR1 rats

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Uninfected</th>
<th>KRV</th>
<th>KRV plus Sulfatrin</th>
<th>Sulfatrin</th>
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</thead>
<tbody>
<tr>
<td><strong>Phylogeny</strong></td>
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<td><strong>Firmicutes</strong></td>
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<td><strong>Bacteroidetes</strong></td>
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<td><strong>Parabacteroides</strong></td>
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<td><strong>Deferribacteres</strong></td>
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<td><strong>Anaerostipes</strong></td>
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<td><strong>Coprococcus</strong></td>
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<td><strong>Anaerovorax</strong></td>
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<td><strong>Lactobacillus</strong></td>
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<td><strong>Bifidobacterium</strong></td>
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<td><strong>Anaerovorax</strong></td>
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<td><strong>Lactobacillus</strong></td>
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The numbers in bold represent differences with p values <0.1 or <0.05. Phylogeny of bacterial phyla and genera, assigned by 16S rRNA pyrosequence analysis.

Prevalence of 16S rRNA pyrosequences for bacterial clade and treatment group.

Phylogeny of bacterial phyla and genera, assigned by 16S rRNA pyrosequence analysis.

Prevalence comparison of KRV versus Sulfatrin: *p < 0.1, † † p < 0.05; comparison of uninfected versus KRV: † † † p < 0.05; comparison of KRV versus Sulfatrin: *p < 0.1; comparison of KRV plus Sulfatrin versus Sulfatrin: ‡ ‡ ‡ ‡ p < 0.1.

Median values for treatment group.
spp. and *Bacteroides* spp. In contrast with that observed on day 5, the analysis of fecal samples from day 12 demonstrated similar levels of bacteria (Fig. 2B). Fig. 2C further shows that the altered abundance of *Bifidobacterium* and *Clostridium* could also be detected in the jejunum of 5-d-infected animals ($p < 0.001$ versus uninfected). These observations validate the pyrosequencing observations and lend support to the hypothesis that KRV induces transient alterations in the intestinal microbiome.

*Sulfatrim alters the B lymphocyte compartment in the pancreatic lymph nodes and Peyer’s patches*

We have previously shown that the infection of LEW1.WR1 rats with KRV alters the cellular composition of the pancreatic lymph nodes shortly postinfection (46). We tested the possibility that virus-induced T1D and Sulfatrim-induced disease prevention are associated with altered lymphocyte cell numbers in the pancreatic lymph nodes and Peyer’s patches. We were particularly interested in addressing regulatory T cells (Tregs), because the gut microbiome has recently been implicated in the development and function of this cell subset (47). Animals were left untreated ($n = 4–22$), were infected with KRV ($n = 3–12$), were infected with KRV and treated with Sulfatrim ($n = 8–17$), or were treated with Sulfatrim only ($n = 3–20$) beginning on the day of viral inoculation, to investigate our hypothesis. Peyer’s patches and pancreatic lymph nodes were harvested 5 d later, and cells were counted and analyzed by flow cytometry for the expression of CD4, CD8, and CD45R (a marker of B lymphocytes in the rat), as well as the coexpression of CD4 and Foxp3 (a marker of Tregs). The data presented in Fig. 3 demonstrate that infection with KRV did not alter the absolute number of CD4 and CD8 cells in the pancreatic lymph nodes or Peyer’s patches on day 5 postinfection compared with uninfected animals. However, a 9-fold and a 2-fold increase in the number of B lymphocytes and CD4$^+$Foxp3$^+$ cells, respectively, were detected in the pancreatic lymph nodes of infected animals when compared with uninfected rats ($p < 0.001$ and $p < 0.05$, respectively). In contrast with the pancreatic lymph nodes, treatment with KRV resulted only in reduced numbers of B cells and CD4$^+$Foxp3$^+$ cells in Peyer’s patches, but this reduction did not reach statistical significance when compared with uninfected rats ($p > 0.05$). Treating animals with KRV plus Sulfatrim led to a decrease in the number of B cells in the pancreatic lymph nodes ($p < 0.01$ versus KRV only) and Peyer’s patches ($p < 0.01$ versus uninfected, $p < 0.001$ versus Sulfatrim only). Unlike pancreatic lymph nodes, the absolute number of CD4$^+$Foxp3$^+$ cells in Peyer’s patches was diminished when compared with uninfected animals ($p < 0.01$). Taken together, our data imply that the mechanism whereby Sulfatrim reverses the virus-induced development of T1D does not involve elevated number of Tregs but could involve alterations in the B cell compartment in the pancreatic lymph nodes and Peyer’s patches.

*Antibiotic therapy reduces KRV-induced inflammation in the pancreatic lymph nodes and Peyer’s patches*

We have previously demonstrated that infection with KRV induces a robust activation of the innate immune system in the pancreatic lymph nodes and pancreatic islets on day 5 postinfection (26, 29, 46). We hypothesized that the KRV-induced inflammation is likely to play a key role in the development of T1D (25), as reflected by alterations in the expression of proinflammatory mediators in the pancreatic lymph nodes and Peyer’s patches. To investigate this hypothesis, we assessed the transcripts for IRF-7, CXCL-10,
IL-17A, and IL-6 in untreated rats (n = 3–7) and rats that were infected with KRV and treated with Sulfatrim on day 5 post-virus infection (n = 6–7), or rats that were treated with Sulfatrim only (n = 3–7).

Levels of transcripts for KRV capsid viral protein 2 (VP2) were also assessed on day 5 postinfection. The presence of KRV VP2 mRNA is indicative of viral replication (48, 49). The data presented in Fig. 4 indicate that transcripts for KRV VP2 were detectable in the pancreatic lymph nodes and Peyer’s patches from KRV- and KRV+Sulfatrim-treated animals, but not in the same tissues from naive rats or animals that received Sulfatrim only.

The data presented in Fig. 4 indicate that transcripts for KRV VP2 were detectable in the pancreatic lymph nodes and Peyer’s patches from KRV- and KRV+Sulfatrim-treated animals, but not in the same tissues from naive rats or animals that received Sulfatrim only.

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The data presented in Fig. 4 demonstrate increased levels of transcripts for CXCL-10 (p < 0.01), IRF-7 (p < 0.01), and IL-6 (p < 0.05), but not IL-17A (p > 0.05), in the Peyer’s patches from infected versus untreated rats. In the pancreatic lymph nodes, we observed increased levels of transcripts for CXCL-10 (p < 0.01), IRF-7 (p < 0.001), IL-17A (p < 0.001), and IL-6 (p < 0.05) in infected rats compared with uninfected rats. Treating the animals with Sulfatrim for 5 d beginning on the day of KRV infection led to a reduction in the levels of CXCL-10 and IRF-7 in Peyer’s patches (p < 0.05 for both genes versus animals treated with Sulfatrim only). Reduced IL-6 transcript levels were detected in the Peyer’s patches from rats that were infected with KRV and treated with Sulfatrim, but this reduction was not statistically significant. Reduced levels of transcripts for CXCL-10 (p < 0.05), IL-17A (p < 0.001), and IL-6 (p < 0.05) were observed in the pancreatic lymph nodes from animals treated with KRV and Sulfatrim versus animals that received KRV only. Collectively, the data raise the possibility that virus-induced proinflammatory responses in Peyer’s patches along with other lymphoid organs (46) may be part of disease mechanisms. The findings also suggest that the mechanism of Sulfatrim-induced disease prevention could involve downregulation of the virus-associated inflammation in the pancreatic lymph nodes and Peyer’s patches.

**KRV-specific adaptive immune responses in the spleen are reduced in antibiotic-treated rats**

Because we found that Sulfatrim downregulated the virus-induced innate immune responses and subsequent anti-islet autoimmunity, we tested the possibility that disease prevention may involve an
altered adaptive immune response. For this purpose, we analyzed the effect of Sulfatrim on the induction of virus-specific T cells. Rats were left untreated, were infected with KRV only, were infected with KRV and treated with Sulfatrim, or were treated with Sulfatrim only for 12 d (n = 3–9/group). The data presented in Fig. 5A and 5B show that the proportion of KRV-specific CD8+IFN-γ+ cells in the spleens of naive LEW1.WR1 rats or rats treated with Sulfatrim only was low (0.16 ± 0.02 and 0.16 ± 0.1% of the total CD8 cells, respectively). Infection with KRV increased the proportion of CD8+IFN-γ+ cells to 3.6 ± 1.2% (p < 0.001 versus naive rats), whereas infection with KRV plus treatment with Sulfatrim led to a reduction in the proportion of KRV-specific CD8+IFN-γ+ cells to 2.0 ± 0.9% (p < 0.05 versus KRV only and p < 0.05 versus uninfected animals).

Sulfatrim therapy does not compromise KRV-specific humoral immunity and viral clearance

Because Sulfatrim suppressed the anti-KRV T cell response and innate immune responses, we analyzed its effect on anti-KRV humoral responses and viral clearance. For evaluating the humoral response, rats were left untreated, were administered with KRV only, were treated with KRV plus Sulfatrim, or were fed with Sulfatrim only for 14 and 40 d beginning on the day of virus inoculation (n ≥ 3). For assessing virus transcript expression, we used a similar treatment regimen as described earlier, but animals were sacrificed on days 5 and 40 postinfection (n ≥ 3). The spleens, pancreatic lymph nodes, and sera were harvested on days 5 and 40 postinfection. The data shown in Fig. 6A demonstrate that the level of anti-KRV Abs in uninfected LEW1.WR1 rats was below detection levels. Infection with KRV induced a substantial anti-KRV Ab response on days 14 and 40. Rats that were infected with KRV and treated with Sulfatrim produced similar levels of anti-KRV Abs as those observed in animals infected with KRV only.

Next, we addressed the ability of the Sulfatrim-treated rats to clear the virus from the spleen and pancreatic lymph nodes. cDNA was generated from total RNA and was used to quantitate the KRV transcripts by quantitative PCR. The data presented in Fig. 6B show that the transcripts for KRV were absent in the spleens, pancreatic lymph nodes, and sera of all groups of rats, indicating that Sulfatrim treatment did not impair viral clearance.

FIGURE 4. KRV and proinflammatory gene expression in Peyer’s patches and pancreatic lymph nodes from Sulfatrim-treated rats. LEW1.WR1 rats were left untreated, were injected with 1 × 10^7 PFU KRV, were given 1 × 10^7 PFU KRV plus Sulfatrim, or were administered Sulfatrim only, as indicated in the figure. RNA was extracted from the pancreatic lymph nodes and Peyer’s patches 5 d postinfection, and the expression levels of the indicated genes were assessed using quantitative RT-PCR. Results are expressed as the mRNA expression of the gene of interest relative to the expression of β-actin. Statistical analyses were performed using an ANOVA with Bonferroni’s multiple-comparison adjustments. PLN, Pancreatic lymph nodes. *p < 0.001, **p < 0.01, ***p < 0.05.

FIGURE 5. KRV-specific CD8+IFN-γ+ cells in the spleen. Rats were either left untreated, were infected with 1 × 10^7 PFU KRV, were infected with KRV and treated with Sulfatrim, or were treated with Sulfatrim only, as described in Materials and Methods. Twelve days postinfection, spleen cells from individual animals were harvested and cultured in the presence of KRV-infected Y3 APCs for 4 h. Spleen cells were surface stained with mAbs against CD8 and intracellularly labeled with an mAb against IFN-γ. The horizontal (IFN-γ) and vertical (CD8) axes indicate the fluorescence intensity. Representative flow cytometry images and frequencies of IFN-γ+ cells out of the total CD8+ cells in the spleens are shown in (A) and (B), respectively. The proportion of CD8+ cells out of the total IFN-γ+ cells is shown in the upper right quadrant of each flow panel. Statistical analyses were performed using an ANOVA with Bonferroni’s multiple-comparison adjustments. *p < 0.001, ***p < 0.05.
pancreatic lymph nodes, and sera of uninfected animals. Similar levels of KRV transcripts were observed in the spleens and pancreatic lymph nodes from 5-d infected rats as compared with rats injected with virus plus Sulfatrim. However, the level of KRV transcripts in the serum from KRV- plus Sulfatrim-treated rats was higher than that detected in the serum from animals injected with KRV only. Taken together, the data suggest that Sulfatrim therapy does not interfere with the anti-KRV humoral response or viral clearance from the spleen, pancreatic lymph nodes, and serum, but may alter virus expression in the serum early post-infection.

Discussion
In this study, we demonstrate that virus-induced T1D in the LEW1.WR1 rat correlates with transient changes in the composition of gut bacteria most notably by an increase in the abundance of Bifidobacterium and Clostridium shortly after viral infection. We also show that administration of a broad-spectrum antibiotic prevents insulitis and islet destruction via mechanisms that may involve downmodulation of innate immunity responses in the pancreatic lymph nodes and Peyer’s patches. Overall, the data raise the hypothesis that intestinal bacteria could be involved in the course of virus-induced T1D in the LEW1.WR1 rat model.

We demonstrated that an oral treatment with Sulfatrim, but not ampicillin, metronidazole, or neomycin sulfate, protected animals from T1D. Because the antibiotics used in our studies target different groups of bacteria (44, 45), our observations may suggest that not all bacteria are equally involved in the anti-islet autoimmune response leading to T1D. This is in agreement with an earlier report showing that different types of bacteria could be involved in different immune responses (47). For example, oral treatment with neomycin, but not ampicillin, vancomycin, or metronidazole, suppressed the CTL response against the influenza virus (50).

A number of our experiments indicate that Sulfatrim therapy suppresses innate and adaptive immune responses on day 5 post-KRV infection. First, we observed a reduction in the absolute number of B lymphocytes in the pancreatic lymph nodes and Peyer’s patches. We have previously shown that B lymphocytes are involved in the proinflammatory response leading to T1D, because these cells are robustly activated by KRV and produce high levels of proinflammatory cytokines (27). Second, the proportion of anti–KRV-specific T cells in the spleens of Sulfatrim- plus KRV-treated animals on day 5 was lower compared with the controls. This finding is reminiscent of previous data demonstrating that antibiotic therapy reduced Th1 responses in a mouse model of Crohn’s disease (51). Finally, the transcript levels of proinflammatory cytokines and
chemokines in the pancreatic lymph nodes and Peyer’s patches were reduced in the animals infected with KRV and treated with Sulfatrim. These observations are consistent with the hypothesis that the innate immune system plays a key role in the mechanism leading to T1D in humans and in animal models (25, 26, 28, 29, 52). In the LEW1.WR1 rat, infection with KRV leads to a robust innate proinflammatory response in the spleen and pancreatic lymph nodes, and a reduction in the inflammation suppresses the development of virus-induced T1D (26, 29, 46). It remains to be seen whether and how pancreatic islets are involved in the mechanism of disease prevention. In any case, our data demonstrate that Sulfatrim treatment blocks the development of insulitis.

Our data suggest that the mechanism by which Sulfatrim down-regulates the innate proinflammatory responses in the pancreatic lymph nodes and Peyer’s patches and prevents disease is unlikely to involve reduced viral expression, because the levels of KRV transcripts in the pancreatic lymph nodes, Peyer’s patches, and serum from rats treated with antibiotics were either comparable or were increased compared with those observed in animals treated with virus only. Our data also imply that disease prevention does not involve increased numbers of CD4^+Foxp3^+ cells in Peyer’s patches. We cannot exclude the possibility that the mechanism of Sulfatrim-induced disease prevention is mediated, at least in part, by its direct effect on immune cells (51). However, in vitro studies have demonstrated that Sulfatrim does not alter the proliferative response of T cells cultured in the presence of anti-CD3 and anti-CD28 Abs (data not shown).

Our observation that intestinal bacteria may promote T1D in the LEW1.WR1 rat model is different from the observations made in the NOD mouse by Wen and colleagues (14). The latter group found that specific pathogen-free NOD mice deficient for MyD88 do not develop T1D, and disease prevention is dependent on the presence of commensal microbes. This group further observed that NOD mice deficient for MyD88 that lack commensal bacteria or were fed with Sulfatrim develop disease, implying that the gut microbiota is involved in diabetes prevention. The reason for the difference between the LEW1.WR1 rat and the NOD mouse is unclear and could be species related. It does not involve major differences in the composition of the gut bacteria, as we found that the gut microbiome in the LEW1.WR1 rat is similar to that of mice and humans, and is composed of two major bacterial phyla, Bacteroidetes and Firmicutes, and a few other less abundant types of bacteria. In any case, our findings are compatible with the overall notion that unlike the NOD mouse, microbial infection and TLR-induced innate activation promote anti-islet autoimmunity in the LEW1.WR1 model (22). Our data are also in agreement with previous reports demonstrating that antibiotic treatment (17) and the administration of Lactobacillus (20) prevent spontaneous disease in the diabetes-prone BB rat. Amelioration of autoimmunity with antibiotics has also been demonstrated in animal models of colitis (53) and Crohn’s disease (51, 54).

The finding that alterations in gut bacteria have distal effects on innate immunity in the pancreatic lymph nodes is in agreement with previous data demonstrating that manipulating gut microbiota in the intestine with antibiotics can regulate immune responses in the respiratory mucosa (36). Indeed, it has recently been recognized that the gut microbiota can impact immunity beyond the intestine (reviewed in Ref. 36). For example, mice deficient for GPR43, a receptor that recognizes acetate and propionate, the products of fiber metabolism by intestinal bacteria, have exacerbated inflammation in the KxB/N arthritis model and in a model of allergic airway inflammation (55). The finding that infection with KRV leads to increased Bifidobacterium in the intestine raises the possibility that virus-induced T1D may involve alterations in the balance between bacterial groups, resulting in the downregulation of protective mechanisms or, conversely, the upregulation of proinflammatory innate pathways culminating in anti-islet T cell responses. It remains to be determined whether and to what extent the virus-induced alterations in the microbiota are directly involved in the pathogenesis of T1D.

Several caveats constrain the interpretation of our data. We have not yet established a cause-and-effect relationship between the altered microbiome and virus-induced T1D. Second, we are currently unable to rule out the possibility that the altered gut microbiome is rather a consequence of the anti-islet autoimmune process initiated by KRV. In addition, the mechanism by which KRV infection modulates commensal bacteria, the specific bacterial strain(s), and how exactly they are involved in virus-induced T1D remain to be identified. One possibility is that T cells specific for Ags expressed by subdominant bacteria that expand after KRV infection cross-react with a yet unknown β-cell Ag(s) leading to islet destruction. One other plausible explanation is that the KRV-induced alterations in the microbiome could result in a more robust innate immunity response that drives the upregulation of autoreactive T cells. In any case, our observations may suggest that altered gut microbiota shortly after KRV infection could be important but clearly not the only factor involved in the mechanism leading to islet destruction. We are currently unable to determine whether the alterations in the abundance of bacterial communities observed in virus-infected animals are a consequence of a specific increase or decrease in bacterial genera. It is tempting to speculate that intestinal microbes cooperate with KRV to promote proinflammatory responses in the pancreatic lymph nodes and Peyer’s patches. Recent data have documented that mechanisms of cooperation between viruses and microbiota in the intestine exist. Indeed, it has been shown that immunity against mouse mammary tumor virus (56) and systemic pathogenesis caused by enteric viruses (57) are dependent on interactions between the viruses and the microbiota.

In summary, our data show that antibiotic therapy can suppress the virus-induced anti-islet responses in the LEW1.WR1 rat via the downmodulation of the innate immune system. A better understanding of the role of the gut microbiome in the early course of T1D could lead to the design of therapies that promote a more favorable gut microbial balance that can better regulate autoimmunity and prevent islet destruction.

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Disclosures
The authors have no financial conflicts of interest.

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Corrections


In Fig. 1A, the y-axis was labeled incorrectly. The correct y-axis label is “Percent Diabetes-Free”. The correctly labeled Fig. 1A is shown below. The legend for Fig. 1 was correct as published and is shown below for reference.

**FIGURE 1.** Kaplan–Meier analysis of KRV-induced T1D in LEW1.WR1 rats treated with Sulfatrim. LEW1.WR1 rats at 21–25 d of age of either sex were injected i.p. with $1 \times 10^7$ PFU KRV. Animals were either left untreated or were given antibiotics in the drinking water as described in Materials and Methods. Rats were tested for diabetes for 40 d after viral inoculation. Diabetes was defined as the presence of plasma glucose concentrations $>250$ mg/dl (11.1 mmol/l) on 2 consecutive days. Survival was analyzed using the Kaplan–Meier method. Statistical analyses among groups were performed using the log-rank test (A). Shown are paraffin sections of tissues from treated and untreated LEW1.WR1 rats stained with H&E (B).

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