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Inhibition of Type 1 Diabetes Correlated to a Lactobacillus johnsonii N6.2-Mediated Th17 Bias

Kenneth Lau,* Patrick Benitez,* Alexandria Ardissone,* Tenisha D. Wilson,* Erin L. Collins,* Graciela Lorca,* Nan Li‡, Dhyana Sankar, † Clive Wasserfall, ‡ Josef Neu, † Mark A. Atkinson, ‡ Desmond Shatz, † Eric W. Triplett,* and Joseph Larkin, III*

Although it is known that resident gut flora contribute to immune system function and homeostasis, their role in the progression of the autoimmune disease type 1 diabetes (T1D) is poorly understood. Comparison of stool samples isolated from Bio-Breeding rats, a classic model of T1D, shows that distinct bacterial populations reside in spontaneous Bio-Breeding diabetes-prone (BBDP) and Bio-Breeding diabetes-resistant animals. We have previously shown that the oral transfer of Lactobacillus johnsonii strain N6.2 (LjN6.2) from Bio-Breeding diabetes-resistant to BBDP rodents conferred T1D resistance to BBDP rodents, whereas Lactobacillus reuteri strain TD1 did not. In this study, we show that diabetes resistance in LjN6.2-fed BBDP rodents was correlated to a Th17 cell bias within the mesenteric lymph nodes. The Th17 bias was not observed in the non-gut–draining axillary lymph nodes, suggesting that the Th17 bias was because of immune system interactions with LjN6.2 within the mesenteric lymph node. LjN6.2 interactions with the immune system were observed in the spleens of diabetes-resistant, LjN6.2-fed BBDP rats, as they also possessed a Th17 bias in comparison with control or Lactobacillus reuteri strain TD1–fed rats. Using C57BL/6 mouse in vitro assays, we show that LjN6.2 directly mediated enhanced Th17 differentiation of lymphocytes in the presence of TCR stimulation, which required APCs. Finally, we show that footpad vaccination of NOD mice with LjN6.2-pulsed dendritic cells was sufficient to mediate a Th17 bias in vivo. Together, these data suggest an interesting paradigm whereby T1D induction can be circumvented by gut flora-mediated Th17 differentiation. *The Journal of Immunology, 2011, 186: 3538–3546.

Type 1 diabetes (T1D) is due to a T cell (both CD4+ and CD8+ lymphocyte)-mediated destruction of insulin-producing β cells within the pancreas, resulting in the inability to self regulate blood sugar. Whereas genetic predisposition plays a significant role in T1D onset (1), a concordance rate between monozygotic twins of 30–50% (2–4) implies that environmental factors also play a significant role. For example, viral infections have been widely implicated as an environmental factor correlated with the progression of T1D (1). More recently, attention has been placed on resident gut microbiota as an environmental factor involved in the progression of T1D (5, 6). Given that resident gut microorganisms outnumber total human cells 10-fold (7) and affect immune system functions (5, 8), it is possible that modification of the resident gut microbial ecosystem can influence the onset of the autoimmune disease T1D. Moreover, it is tempting to speculate that modulation of the resident gut microbiota can potentially serve as a powerful therapeutic strategy in T1D, as previous probiotic health strategies have achieved success in humans for the treatment of allergies (9–11) and colitis (12, 13).

T and B cell-deficient rodents, bearing a T1D-promoting genetic background, fail to develop T1D. However, the transfer of diabetogenic CD4+ splenocytes is sufficient to confer T1D onset (14, 15). The ability to confer T1D through the adoptive transfer of diabetogenic CD4+ splenocytes underscores two important concepts, as follows: 1) CD4+ T cells play a crucial role in the onset of T1D, and 2) although the observed autoimmune pathology of T1D is the destruction of the insulin-producing islet cells, potentially diabetogenic CD4+ T cells are present systemically within the blood. IFN-γ–producing, Th1 CD4+ lymphocytes have been strongly implicated as a major contributor to T1D progression (16–18), whereas data suggest that Foxp3+ regulatory T cells inhibit T1D onset (19). The role of IL-17 (also referred to as IL-17A)–producing Th17 cells in the onset of T1D is poorly understood. Th17 lymphocytes, crucial for defense against extracellular bacterial pathogens (20–22), have recently been implicated as causal in a number of autoimmune diseases (23–26), including T1D (27, 28). However, recent evidence has also shown that IL-17–producing cells have a protective role in inflammatory bowel disease, a disease resulting from an aberrant immune response to commensal gut bacteria (29), and in T1D (30, 31). Moreover, although it is known that a splenic transfer of diabetogenic CD4+ T cells is sufficient to confer T1D onset, and that resident gut flora can modulate the systemic immune system, how resident gut flora can influence potentially diabetogenic CD4+ T cells is poorly understood.

The Bio-Breeding diabetes-prone (BBDP) rat and the Bio-Breeding diabetes-resistant (BBDR) rat models are two well-
characterized rodent models of T1D. Whereas the BBDR rat model spontaneously develops T1D, the BBDR rat model is resistant to T1D onset unless influenced by an environmental change, such as viral infection with Kilham rat virus (32, 33). It is currently thought that the increased frequency of T1D in BBDR rats subsequent to Kilham rat virus infection is due to a shift in the balance of Th1 and Th2 lymphocytes (33). It is therefore possible that changes in the gut flora, an additional environmental agent, can also cause a shift in Th cell differentiation states, resulting in changes in T1D onset. Indeed, cesarean-derived BBDR rats experience accelerated onset of T1D (34). In addition, it has been shown that distinct microfloral bacterial populations reside within the gut of diabetes-prone (BBDP) and diabetes-resistant (BBDR) rats (35). Significantly, when BBDP (prose) rats were orally fed Lactobacillus johnsonii strain N6.2 (LjN6.2), isolated from BBDR (resistant) rats, they became resistant to the onset of T1D (36). In contrast, a second strain of Lactobacillus, Lactobacillus reuteri strain TD1 (LrTD1), failed to mediate resistance to T1D when administered under the same conditions. The mechanism, however, by which resistance to T1D was conferred remained a question. To specifically examine the capacity of LjN6.2 to modulate immune responses, lymph nodes and spleens were isolated from BBDP rats under the various bacterial feeding regimens and immunological parameters were assessed. In addition, we examined modulation of immune responses by LjN6.2 in vitro and in vivo, using C57BL/6 and NOD mice. Together, these results suggested that modification of dendritic cells (DCs) by oral feeding of LjN6.2 contributed to a Th17 lymphocyte bias. Moreover, these results suggest the possibility that oral feeding of LjN6.2 can confer resistance to T1D through sustained Th17 differentiation.

Materials and Methods

Animals

Bio-Breeding diabetes-prone (BBDP) rats (Biomedical Research Models, Worcester, ME), NOD mice, and C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME) were maintained in specific pathogen-free conditions at the Association for Assessment and Accreditation for Laboratory Animal Care–accredited University of Florida under the supervision of the Institutional Animal Care and Use Committee in strict accordance to approved protocols.

Bacteria administration to BBDP rats and diabetes onset determination

Postweaning, BBDP rats were administered (1 × 10^8 CFU) LjN6.2, LrTD1, or PBS control daily, through oral gavage, until diabetes onset or the culmination of the experiment as fully described in (36). Briefly, bacterial strains LjN6.2 and LrTD1, isolated from BBDR rats (35), were grown in deMan, Rogosa, and Sharpe broth (REMEL) at 37˚C for 16 h. Significant antibiotics were washed. Progenitor cells were subsequently incubated in RPMI 1640 (10-040-CV; Cellgro, Manassas, VA) containing 10% FBS (10-019-CV; Cellgro, Manassas, VA) supplemented with 25 mM HEPES (H1522; Sigma-Aldrich) and 1% antibiotic/antimycotic (30-004-CI; Cellgro) in 96-well round-bottom plates. After 72 h of incubation, cultures were pulsed with 0.5 mCi [3H]thymidine (GE Healthcare, Arlington Heights, IL) per well and harvested 16–18 h later. Lymphocytes isolated from C57BL/6 mice were coincubated with 4 µg/ml anti-CD3 (clone 17A2; eBioscience, San Diego, CA), 2 × 10^5 APCs, or 0.5 µg/ml anti-CD28 (clone 37.51; eBioscience) in the presence of LjN6.2 at various concentrations. To inhibit IL-6 signaling, lymphocytes were also coincubated with 0.5 µg/ml anti-CD28 (clone 37.51; eBioscience) in the presence of LjN6.2 at various concentrations, as indicated. Cell cultures were incubated in supplemented RPMI 1640 (10-040-CV; Cellgro, Manassas, VA) containing 10% FBS (10082-147; Life Technologies, Carlsbad, CA) and 1% antibiotic/antimycotic (30-004-CI; Cellgro) in 96-well round-bottom plates. After 72 h of incubation, cultures were pulsed with 0.5 µCi ([3H]thymidine (GE Healthcare, Arlington Heights, IL)) per well and harvested 16–18 h later. [3H]Thymidine incorporation was measured using a Beckman LS3801 Liquid Scintillation System.

Proliferation assays

Lymphocyte proliferation assays were performed, as previously described (38), with modifications. Total splenocytes (4 × 10^6) or 2 × 10^5 MACS-purified T lymphocytes were incubated with 4 µg/ml anti-CD3 (clone 17A2; eBioscience, San Diego, CA), 2 × 10^5 APCs, or 0.5 µg/ml anti-CD28 (clone 37.51; eBioscience) in the presence of LjN6.2 at various concentrations, as indicated. Cell cultures were incubated in supplemented RPMI 1640 (10-040-CV; Cellgro, Manassas, VA) containing 10% FBS (10082-147; Life Technologies, Carlsbad, CA) and 1% antibiotic/antimycotic (30-004-CI; Cellgro) in 96-well round-bottom plates. After 72 h of incubation, cultures were pulsed with 0.5 µCi ([3H]thymidine (GE Healthcare, Arlington Heights, IL)) per well and harvested 16–18 h later. [3H]Thymidine incorporation was measured using a Beckman LS3801 Liquid Scintillation System.

In vitro cytokine secretion analysis

Lymphocytes isolated from C57BL/6 mice were cocultured with 4 µg/ml anti-CD3, APCs, or 0.5 µg/ml anti-CD28 (BD Biosciences, San Diego, CA), or LjN6.2 (clone N6.2 or LrTD1) at various concentrations. To inhibit IL-6 signaling, lymphocytes were also cocultured with anti-CD3, APCs, and LjN6.2 in the presence or absence of an IL-6 signal-neutralizing Ab mixture containing the following: 0.2 mg/ml rabbit polyclonal to IL-6 (Abcam, Cambridge, MA), 2 µg/ml anti-mouse IL-6 (554398; BD Biosciences), and 10 µg/ml anti-mouse CD126 (554459; BD Biosciences) for 48 h. At various time points, 100 µl supernatant was removed from each well and replenished with fresh medium, as previously described (39). Cytokine ELISAs were subsequently performed on harvested supernatants. IFN-γ (555138; BD Biosciences) and IL-6 (555240; ELISA kits) were obtained from BD Biosciences. Capture mAb (555068) and detection mAb...
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(555067) for IL-17A were also obtained from BD Biosciences. Cytokine standard for IL-17 was purchased from eBioscience (14-8171-80).

Statistical calculations

Statistically significant differences were determined using GraphPad Prism software using an unpaired, two-tailed Student t test. Unpaired, two-tailed Student t tests were used in BBDP rat cytokine measurement experiments because distinctions between LjN6.2-fed and control rats were the primary focus [as described by Motulsky (40)].

Results

We have previously shown that BBDP rats fed LjN6.2 postweaning were resistant to onset of T1D when compared with PBS-treated control or LrTD1-fed BBDP rats (36). Eighty percent of LjN6.2 fed BBDP rats remained diabetes free in comparison with 40 and 20% of PBS-fed control, or LrTD1-fed BBDP rats, respectively. As previously described, BBDP rats were sacrificed at the onset of autoimmune diabetes or at the culmination of the experiment (140 d), and various organs were removed and stored in RNAlater (36) for further analysis.

IL-17A is associated with diabetes resistance

Because enteropathy is a common characteristic of BBdd rats (41–43), and gut flora modification conferred T1D resistance to the BBDP rats. As can be seen in Fig. 2, significantly higher levels of IL-17A and IL-23R mRNA were present in the mesenteric lymph nodes of LjN6.2-fed rats when compared with either LrTD1-fed or PBS-treated BBDP rats. Moreover, the higher levels of IL-17 and IL-23R could be directly correlated with diabetes resistance, as diabetes-resistant LjN6.2-fed rats (denoted by the black dots) also possessed the highest levels of the two cytokines (Fig. 2). In addition, it is interesting to note that the one LjN6.2-fed rat that became diabetic (red dot) possessed the lowest levels of IL-17A and IL-23R among his cohort. Furthermore, the one nondiabetic LrTD1-fed rat possessed the highest levels of IL-17A and IL-23R among his respective cohort. Statistical differences in two additional Th17-associated cytokines, IL-17F and IL-22, were not observed (Supplemental Fig. 1). As Foxp3+ regulatory T cells have been associated with T1D disease resistance (19), we also examined the transcription factor Foxp3. Foxp3 expression was higher in the mesenteric lymph nodes of nondiabetic versus diabetic rats, but statistical distinctions could not be made between LjN6.2-fed and control BBDP rats (Supplemental Fig. 1). Previous studies have shown age-dependent increases in regulatory T cells (44, 45). Despite a small sample size (n = 2 mice per group), we observed a trend toward increased Foxp3 expression with age in PBS-fed control rats that became diabetic (data not shown). Because the control rats became diabetic despite increases in Foxp3 levels, we con-

![Table I. Primer sequences and annealing temperatures](http://www.jimmunol.org/)

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<tr>
<td>IL-17A R (mouse)</td>
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LjN6.2 mediates lymphocyte skewing to Th17 phenotype

We next analyzed whether there was a correlation between the observed Th17 bias and the LjN6.2 feeding, which conferred diabetes resistance to the BBDP rats. As can be seen in Fig. 2, significantly higher levels of IL-17A and IL-23R mRNA were present in the mesenteric lymph nodes of LjN6.2-fed rats when compared with either LrTD1-fed or PBS-treated BBDP rats. Moreover, the higher levels of IL-17 and IL-23R could be directly correlated with diabetes resistance, as diabetes-resistant LjN6.2-fed rats (denoted by the black dots) also possessed the highest levels of the two cytokines (Fig. 2). In addition, it is interesting to note that the one LjN6.2-fed rat that became diabetic (red dot) possessed the lowest levels of IL-17A and IL-23R among his cohort. Furthermore, the one nondiabetic LrTD1-fed rat possessed the highest levels of IL-17A and IL-23R among his respective cohort. Statistical differences in two additional Th17-associated cytokines, IL-17F and IL-22, were not observed (Supplemental Fig. 1). As Foxp3+ regulatory T cells have been associated with T1D disease resistance (19), we also examined the transcription factor Foxp3. Foxp3 expression was higher in the mesenteric lymph nodes of nondiabetic versus diabetic rats, but statistical distinctions could not be made between LjN6.2-fed and control BBDP rats (Supplemental Fig. 1). Previous studies have shown age-dependent increases in regulatory T cells (44, 45). Despite a small sample size (n = 2 mice per group), we observed a trend toward increased Foxp3 expression with age in PBS-fed control rats that became diabetic (data not shown). Because the control rats became diabetic despite increases in Foxp3 levels, we con-
cluded that Foxp3 induction was not the primary mechanism mediated by LjN6.2. Interestingly, we did observe lower levels of Foxp3 in LrTD1-fed BBDP rats than either LjN6.2 or PBS control rats, which could partially account for their increased rate of diabetes onset compared with controls (Supplemental Fig. 1) (36).

In summary, these results showed that feeding LjN6.2 to BBDP rats is correlated to a Th17 lymphocyte bias within the gut-draining mesenteric lymph nodes, which most likely does not involve changes in Foxp3 levels.

We next examined cytokines responsible for Th17 lymphocyte differentiation, which are predominantly produced by APCs. Lymphocyte Th17 differentiation is dependent upon TCR stimulation in the presence of IL-6, and then sustained through IL-23 production (46). Indeed, both IL-6 and IL-23 message levels within the mesenteric lymph nodes were significantly higher in LjN6.2-fed BBDP rats than in control or LrTD1-fed BBDP rats (Fig. 2). Similar to results observed with T cell-associated Th17 factors, the highest levels of IL-6 and IL-23 were associated with the nondiabetic LjN6.2-fed rats (Fig. 2, black dots). In addition, the mesenteric lymph nodes of the one nondiabetic LrTD1-fed rat possessed the highest level of IL-23 among its respective cohort. Similar to the results seen with IL-17 and IL-23R, no statistical differences were observed between IL-6 and IL-23 in the axillary lymph nodes (data not shown). Together, these data show that cytokines IL-6 and IL-23, which are involved in both the induction and sustenance of Th17 cells, were higher in the mesenteric lymph nodes of LjN6.2-fed rats than either LrTD1-fed or PBS-treated control BBDP rats.

**Th17 bias observed within the spleen**

Numerous studies have shown that diabetogenic T lymphocytes can accumulate within the spleen of diabetes-prone animals, as the transfer of splenocytes from a diabetic rodent is sufficient to transfer T1D to a T cell-deficient rodent bearing a T1D-susceptible
background (14, 15). We next determined whether there were distinctions present within the splenocytes of rats treated with LjN6.2 in comparison with untreated, or LrTD1-fed BBDP rats. Fig. 3 shows that significantly higher levels of IL-17A were present in the spleens of LjN6.2-fed BBDP rats in comparison with either control or LrTD1-fed rats. In contrast, Fig. 3 shows that there was no statistical difference in the levels of IFN-γ or Foxp3 within the spleens of any of the rat feeding groups. In contrast to the mesenteric lymph nodes, there was no statistical difference between the mRNA levels of Th17 induction-related proteins IL-6, IL-23, or IL-23R (Fig. 3 and data not shown).

The lack of IL-23R message is most likely due to sufficient surface expression of the IL-23R protein or possibly due to decreases in message expression without sufficient stimulation from IL-6 and/or IL-23. These data show that a Th17 bias was observed within the spleens of diabetes-resistant LjN6.2-fed BBDP rats, suggesting accumulation within the spleen rather than specific induction in the spleen.

**LjN6.2 can directly mediate Th17 bias in vitro**

To assess the capacity of LjN6.2 to directly modulate immune responses, a mixture of T lymphocytes and APCs was incubated with varying concentrations of LjN6.2. As can be seen in Fig. 4A, LjN6.2 failed to mediate significant leukocyte proliferation in comparison with anti-CD3–treated positive control. Although coculture of LjN6.2 could not mediate significant leukocyte proliferation, LjN6.2 was sufficient to mediate production of IL-6 in a dose-dependent fashion. Of note, higher concentrations of LjN6.2 mediated higher production of IL-6 than anti-CD3–treated control leukocytes. However, the highest concentrations of LjN6.2 induced apoptosis of leukocytes (K. Lau and J. Larkin, unpublished observations), and are reflected by no background proliferation or IL-6 production at the highest concentrations of LjN6.2 (Fig. 4A).

Although coinubcation of leukocytes with LjN6.2 was sufficient to mediate IL-6 production, coinubcation of LjN6.2 was insufficient to mediate IL-17 production. As can be seen in Fig. 4A, at no concentration of LjN6.2 was IL-17 production observed, in contrast to anti-CD3–treated leukocytes, which both proliferated and produced IL-17. In summary, these data show that incubation of LjN6.2 was sufficient to mediate the production of IL-6 in a dose-dependent fashion, but was incapable of mediating leukocyte proliferation or IL-17 production.

Because an increase in IL-6 and IL-17 production was observed in response to feeding LjN6.2 to BBDP rats (in contrast to LrTD1), but only an IL-6 (not IL-17) increase when leukocytes were incubated with LjN6.2 in vitro, we hypothesized that increased IL-17 production required both LjN6.2 incubation and stimulation of T cells through the TCR. To test this hypothesis, anti-CD3–activated mouse splenocytes were incubated in vitro with LjN6.2 or LrTD1 for various times and cytokine production was assessed. In contrast to either anti-CD3–treated controls alone or LrTD1- incubated splenocytes, IL-6 production was clearly evident in splenocytes incubated with a combination of anti-CD3 and LjN6.2 after 24 h (Fig. 4B). Moreover, at both 36 and 48 h, there was significantly more IL-6 produced by splenocytes incubated with LjN6.2 than either LrTD1 treated or control. Significantly, ~2-fold more IL-17A was produced by splenocytes incubated with LjN6.2 after 48 h than LrTD1 (Fig. 4B). The production of IL-17 was IL-6 dependent, as Abs shown to block IL-6 signaling (47, 48) were capable of reducing the production of IL-17 by 30% (Fig. 4C).

Analysis of IFN-γ and IL-10 production revealed no significant differences based on the distinct bacteria incubations with respect to time (data not shown). Together, these data show that incubation with LjN6.2 directly increased the production of both IL-6 and IL-17A. In addition, the increased production of IL-17 mediated by LjN6.2 required T lymphocyte stimulation through the TCR. Moreover, these data show that distinct bacterial strains, even in the same genera, can diversely affect overall immune response.

**LjN6.2-mediated bias is dependent upon APCs**

Although APCs are potent producers of IL-6 (49), CD3+ lymphocytes also have the capacity to produce IL-6 (50). To determine whether LjN6.2 could mediate a Th17 bias through direct interaction with T cells or required APCs, we next measured the capacity of LjN6.2 to mediate IL-6 and IL-17 production in the presence or absence of APCs. MACS-purified CD3+ lymphocytes (>95% pure) were incubated, with graded doses of LjN6.2 and anti-CD3 in the presence or absence of APCs for 72 h. Under
conditions in which lymphocytes were not incubated with APCs, anti-CD28 was added in their place, as it has been shown to provide necessary costimulation to lymphocytes (51). At 48 h, an aliquot of supernatant was removed from cultures, replaced with fresh media, and used for cytokine analysis. At 72 h, proliferation was assessed by overnight incubation with \[^{3}H\]thymidine incorporation. As can be seen in Fig. 5, although some IL-6 could be produced in the lymphocyte-only cultures, robust IL-6 production was only observed under culture conditions containing APCs (Fig. 5). The IL-6 production increased in a dose-dependent manner, with increasing LjN6.2 concentrations. IL-17 production did not occur in the absence of anti-CD3 stimulation (see Fig. 4A), but could be produced with anti-CD3 in an APC- and LjN6.2-independent manner (Fig. 5). Coincubation of lymphocytes with increasing concentrations of LjN6.2 did not mediate increases in IL-17 production (Fig. 5). However, when lymphocytes were co-incubated with APCs and LjN6.2, significant increases in IL-17 production were observed. In contrast, proliferation was not affected by either the presence of APCs or the concentration of LjN6.2 (Fig. 5). In summary, these data show that although anti-CD3-activated lymphocytes could proliferate and produce some IL-6 and IL-17, robust LjN6.2-mediated Th17 differentiation required APCs.

**NOD mice receiving LjN6.2-pulsed BMDC vaccinations possess a Th17 bias**

Although it is known that DCs have the capacity to extend projections through the tight junctions of mucosal epithelium cells and sample the bacterial contents of the lumen (52), other contributing factors such as a leaky intestinal barrier or cytokines produced by epithelial cells (5) may also indirectly mediate Th17 differentiation. To determine whether direct interactions of LjN6.2 with DCs could mediate a Th17 bias in a diabetes-prone model, we generated BMDCs from NOD mice. BMDCs, identified as CD11c+ with upregulation of MHCII and CD80 (data not shown), were pulsed with LjN6.2 in the presence of purified T lymphocytes. LjN6.2-pulsed BMDCs, in the absence of other APC populations, were sufficient to mediate increased production of IL-6 and IL-17 (Fig. 6A).

It has been previously shown that footpad vaccination of tolerogenic BMDCs could prevent onset of T1D in NOD mice (53).
To determine whether a vaccination of LjN6.2-pulsed BMDCs (LjN6.2–DC vaccination) could mediate a Th17 bias in the absence of bacterial feeding, we next administered three footpad LjN6.2–DC vaccinations or PBS control vaccinations into NOD mice once per week beginning at 9 wk of age. Two weeks after the last injection, the 13-wk-old NOD mice receiving either the LjN6.2–DC vaccination or control were sacrificed, and the production of IL-6 and IL-17 was assessed. Increases in the production of IL-6 and IL-17 were observed in the spleens of mice receiving the LjN6.2–DC vaccination by both message (Fig. 6B) and protein production by splenocytes incubated for 48 h without any additional treatment (Fig. 6C). Moreover, treatment of ex vivo splenocytes from LjN6.2-pulsed BMDC-treated NOD with anti-CD3 yielded greater amounts of IL-17 (Fig. 6C). Significantly, these data show that DCs, pulsed with LjN6.2, are sufficient to mediate a Th17 bias in vitro and in vivo in NOD mice.

**Discussion**

T1D is a CD4+ T cell-mediated autoimmune disease, which eventually results in the inability of the body to regulate and use blood sugar. Although it is clear that T1D onset consists of a genetic component, it is likely that environmental factors, such as diet, gut flora, and chemical exposure, also play a role in the onset of T1D. The BBDP rat spontaneously develops autoimmune diabetes, and is a well-accepted rodent model of human T1D. Our previous studies showed that LjN6.2 feeding to BBDP rats conferred resistance to T1D, in contrast to control or LrTD1-fed rats (36). Our current study shows that lymphocytes present in BBDP rats fed LjN6.2 possessed a Th17 bias, as indicated by increased IL-17 and IL-23R expression levels, which was not present in control or LrTD1-fed BBDP rats. In addition, Th17 phenotype differentiating and sustaining cytokines, IL-6 and IL-23, respectively, were significantly higher within the mesenteric lymph nodes of LjN6.2-fed BBDP rats than control BBDP rats. This Th17 bias was observed in the gut-draining mesenteric lymph nodes, but not in the armpit-associated axillary lymph nodes. Moreover, the Th17 bias was observed within the spleens of LjN6.2-fed BBBDP rats and in NOD mice receiving footpad LjN6.2–DC vaccinations. Our in vitro assays showed that LjN6.2, in contrast to LrTD1, mediated a significant increase in IL-17 production in a time-dependent manner. The LjN6.2-mediated Th17 bias was dependent on the presence of APCs, as denoted by significant increases in IL-6 and IL-17 production in vitro.
Moreover, the LjN6.2–DC vaccinations were sufficient to mediate a Th17 bias in vivo, strongly suggesting that DCs are the APCs mediating the Th17 bias. Together, these data show a strong correlation between LjN6.2-mediated Th17 bias and resistance to T1D onset.

A correlation between IL-17 production and gut flora has already been established as germfree rodents produce reduced amounts of IL-17 in the lamina propria (54). Significantly, germfree, diabetes-prone rodents experience an accelerated frequency of T1D onset in comparison with rodents housed under specific pathogen-free conditions (6, 34). In this study, we have shown that LjN6.2 can directly mediate a Th17 bias in vitro using the nondiabetes-prone C57BL/6 and the spontaneously diabetic NOD mouse models. Moreover, LjN6.2 feeding or LjN6.2–DC vaccinations were sufficient to mediate a Th17 bias in the spleens of two spontaneous models of diabetes, the BBDDP rats and the NOD mouse. In contrast, feeding with LrTD1 failed to mediate enhanced IL-17 production and, likewise, did not confer T1D resistance to BBDDP rats. It was elegantly shown that distinct microbial species can differentially regulate IL-17 production within the gut (54). Specifically, Gram (+) bacterial strains possessed a significant advantage over Gram (−) bacteria in mediating a Th17 phenotype (54). It is currently unclear why two Lactobacillus strains of bacteria (LjN6.2 and LrTD1) have diverse capacities to mediate IL-17 production. It is clear, however, that different gut-inhabiting microbial species can have differing capacities to mediate a Th17 bias.

Although it is clear that changes in gut flora can mediate changes in the immune system throughout the body (55), how this relates to the onset or prevention of T1D is poorly understood. We and others have shown that changes in gut flora can modulate T1D onset in both the NOD mouse (6, 56) and BBDDP rat model (36). Our current study shows that a Th17 bias is present in both the mesenteric lymph node and the spleen of BBDDP rats conferred resistant to T1D onset by LjN6.2 feeding, in contrast to PBS- or LrTD1-fed BBDDP rats. Moreover, the spleens of NOD mice receiving LjN6.2–DC vaccinations also possessed a Th17 bias. As the spleen is responsible for immunological events occurring within the blood, the presence of Th17 cells within the spleen strongly suggests crosstalk between the lymph nodes, blood, and the spleen. Moreover, these data suggest that immune events that occur in various lymph nodes can also affect immune events more distal. It is well established that splenocyte transfer from a diabetic rodent into a nondiabetic recipient is sufficient to confer T1D onset (14, 15). Significantly, these data suggest that lymphocyte immune responses can be modulated through adaptations of gut flora in a DC-dependent manner, which could then possibly skew prediabetogenic lymphocytes away from mediating T1D onset.

Although Th17 cells can promote insulin and pancreatic inflammation (27, 28), progression to T1D only occurs after conversion of Th17 cells to Th1 (57, 58). These studies strongly suggest that distinct changes occur during the conversion of Th17 cells to Th1 cells, which are critical to diabetogenesis. In addition, these studies implicate that retention of the Th17 differentiation state may inhibit T cell conversion to the diabetogenic phenotype, thus preventing or significantly delaying the onset of T1D. Moreover, these data suggest that Th17 cells may not be intrinsically diabetogenic, but have the capacity to become diabetogenic in the absence of intervention. We have previously shown that LjN6.2 feeding to BBDDP rats was sufficient to confer resistance to T1D onset, despite insulitis (36). Our current study shows that lymphocytes present in BBDDP rats, which became resistant to T1D onset through LjN6.2 feeding, possessed a Th17 bias in the spleen and mesenteric lymph nodes. Future studies will include analysis of the differentiation state of lymphocytes present in the pancreas and pancreatic lymph node (pLN) of diabetes-prone rodents with LjN6.2 conferred diabetes resistance. Analysis of the pLN will be of particular importance as the pLN drains lymphocytes from both the pancreas and the mesenteric lymph nodes (59). However, even in the absence of analysis of lymphocytes present in the pancreas and the pLN, our current study suggests that it is possible that the LjN6.2 feeding to BBDDP rats inhibited the differentiation of lymphocytes to a diabetogenic state, by sustaining the Th17 phenotype. On a larger scale, these data suggest that it is highly possible that gut flora composition is one environmental factor that can contribute significantly to the onset of autoimmune disease T1D.

Several groups have shown that enteropathy precedes T1D in BBDDP rats (41–43), suggesting a close correlation between intestinal health and T1D onset. Therefore, a more comprehensive understanding of the interrelationship between gut flora and immune system function, in the context of T1D onset, is critical in devising prevention strategies. Mediating changes in the ratio/number of certain gut bacteria strains, as a T1D prevention strategy, is a very tempting possibility. Gut bacterial modulation of autoimmune response offers significant possible advantages in that once human T1D resistance strains of bacteria are identified, oral administration would likely be regarded as a safe strategy, as presumably these strains would already be present within the gut flora of T1D-resistant persons.

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Disclosures

The authors have no financial conflicts of interest.

References


gamma-dependent regulatory circuits in immune inflammation highlighted in 


Fig. S1

A

IL-17F

IL-22

Relative Units

0 200 400 600 800

PBS LjN6.2 LrTD1

Relative Units

0 200 400 600 800 1000

PBS LjN6.2 LrTD1

B

Foxp3

Foxp3

Relative Units

0 500 1000 1500 2000 2500

Diabetic Non-Diabetic

Relative Units

0 500 1000 1500 2000 2500

PBS LjN6.2 LrTD1

*
Supplemental Figure Legend

Figure S1.  LjN6.2 mediated resistance to T1D onset is not mediated by cytokines IL-17F, IL22 or regulatory T cells.  A. Graphs showing RNA message levels of IL-17F and IL22 from mesenteric LN of BBDP rats fed either LjN6.2, LrTD1, or untreated. Relative levels of expression were determined by quantitative PCR using specific primers (Table 1) as indicated and β-actin 1 as a housekeeping gene. Each dot represents an individual BBDP rat. Means are indicated by bars within each graph. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.005 using unpaired Student’s T test.  B. top: Graph showing Foxp3 RNA message level in the lymph nodes of diabetic and non-diabetic BBDP rats. Bottom: Graphs showing Foxp3 RNA message levels from mesenteric LN of BBDP rats fed either LjN6.2, LrTD1, or untreated.