Promotion of Autoimmune Diabetes by Cereal Diet in the Presence or Absence of Microbes Associated With Gut Immune Activation, Regulatory Imbalance, and Altered Cathelicidin Antimicrobial Peptide

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We are exposed to millions of microbial and dietary antigens via the gastrointestinal tract, which likely play a key role in type 1 diabetes (T1D). We differentiated the effects of these two major environmental factors on gut immunity and T1D. Diabetes-prone BioBreeding (BBdp) rats were housed in specific pathogen-free (SPF) or germ-free (GF) conditions and weaned onto diabetes-promoting cereal diets or a protective low-antigen hydrolyzed casein (HC) diet, and T1D incidence was monitored. Fecal microbiota 16S rRNA genes, immune cell distribution, and gene expression in the jejunum were analyzed. T1D was highest in cereal-SPF (65%) and cereal-GF rats (53%) but inhibited and delayed in HC-fed counterparts. Nearly all HC-GF rats remained diabetes-free, whereas HC-fed SPF rats were less protected (7 vs. 29%). Bacterial communities differed in SPF rats fed cereal compared with HC. Cereal-SPF rats displayed increased gut $CD3^+$ and $CD8\alpha^+$ lymphocytes, ratio of *Ifng* to *Il4* mRNA, and *Lck* expression, indicating T-cell activation. The ratio of $CD3^+$ T cells expressing the T_{reg} marker $Foxp3^+$ was highest in HC-GF and lowest in cereal-SPF rats. Resident $CD163^+$ M2 macrophages were increased in HC-protected rats. The cathelicidin antimicrobial peptide (*Camp*) gene was upregulated in the jejunum of HC diet–protected rats, and $CAMP^+$ cells colocalized with CD163. A cereal diet was a stronger promoter of T1D than gut microbes in association with impaired gut immune homeostasis. ***Diabetes*** **62:2036–2047, 2013**

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Despite extensive studies of the genetic and immune basis of human type 1 diabetes (T1D) during the past 50 years, neither cure nor prevention has been achieved (1). T1D occurs in individuals predisposed to β-cell–specific autoimmunity that is provoked by encounters with poorly understood environmental factors (2). Immune tolerance is impaired and islet regenerative capacity is limited, resulting in a deficit of β-cells. Thus, interventions that do not address the environmental component of this disease are likely to fail or have limited benefit, as has been the case with clinical trials to date (3). A major gap in our understanding of T1D is the extent to which environmental factors encountered in the gastrointestinal tract influence disease outcome.

The gastrointestinal tract is the nexus of interaction between self and nonself. A large collection of immune cells defends the gut against pathogens while regulatory cells dampen inappropriate immune responses against dietary components and commensal bacteria. Accumulating evidence suggests the gut is structurally and immunologically abnormal in a subset of individuals prone to T1D (2,4–6). For example, oral tolerance is frequently impaired in diabetes-prone rats (7) and humans (8). Gut inflammation (6) and leakiness are sometimes present, and correcting this in diabetes-prone rats by closing epithelial tight junctions (5) or feeding a protective diet prevents T1D (2). When the gut is inflamed and leaky, unusually high concentrations of microbial and dietary antigens can activate immune cells in the gut associated lymphoid tissue from whence they traffic to the pancreatic lymph nodes (9).

Although we begin life germ-free (GF), mammals are quickly colonized by a vast collection of microbes, the diversity of which is determined by ingested food (10). Cereal-based diets promote T1D in three rodent models of spontaneous T1D, NOD mice (11), diabetes-prone BioBreeding (BBdp) rats (2), and diabetes-prone LEW.1AR1/Ztm-*iddm* rats (12). In all three models, feeding a diet based on low-antigen hydrolyzed casein (HC) protects against T1D (2), and this may also be the case in humans (13). A newly diagnosed child fed a gluten-free diet has remained without insulin therapy for 20 months (14). Establishing oral tolerance early to a cereal diet or wheat proteins alone protects BBdp rats from T1D (15).

Thus, food has a major effect on expression of T1D (2), and recent evidence also implicates the gut microbiota

(16). The gut lumen contains large amounts of food molecules that affect gut immunity (7,8) and islet mass (17) and strongly influence the composition of the microbiota. Most studies have focused on diet or microbes, but not both. Gastrointestinal illnesses were linked to islet autoimmunity only in those exposed to wheat and barley in early childhood (18). Therefore, it is important to differentiate the contribution of dietary antigens and microbes to gut immune activation and T1D.

A major obstacle in understanding the importance of diet and gut inflammation to T1D is the overlap between the intestinal immune response elicited by dietary antigens and microbes, which confounds interpretation of diet-specific modulation of T1D. In the current study, we established a colony of GF BBdp rats and compared them with BBdp rats housed under standard specific pathogen-free (SPF) conditions. We asked whether the effects of diabetes-modifying diets on T1D incidence could be observed independently of microbes and whether certain aspects of the gut immune system are modulated by diet and/or microbial exposure.

RESEARCH DESIGN AND METHODS

Animals. Animal studies were approved by the local animal care committees. BBdp or control BB rats (BBc) were maintained in SPF or GF conditions and weaned onto the AIN93G HC-based diet or a cereal diet (NTP-2000 fed to SPF rats or irradiated C.R. Rodent 18% diet [PMI Nutrition International, Brentwood, MO] fed to GF rats) beginning at 23 days. GF status was confirmed by microbiological and immunological testing and by PCR analysis. Animals were monitored biweekly for T1D, defined as ≥11 mmol/L fasting blood glucose. Rats were killed within 24 to 48 h of T1D diagnosis or after ~134 days without T1D development.

Pancreas and washed gut tissues were fixed in Bouin's fixative for histological evaluation or were frozen for RNA isolation and gene expression analysis. Additional studies were performed using rats that were non-lymphopenic or that had in addition the *Gimap5* (lymphopenia) gene inserted (19). Except where noted, all analyses were performed on pancreas or jejunum of BBdp rats that remained asymptomatic until ≥134 days.

Human subjects. Jejunum biopsies were obtained from nondiabetic control subjects (patients with short stature, failure to thrive, gastroesophageal reflux disease, recurrent abdominal pain, anemia, n = 14 males) and 8 male patients with T1D aged between 8 and 16 years of age, with informed consent (20). All biopsy samples (range 1–7 per subject) on a single slide were analyzed (17).

Immunohistochemistry and morphometric analyses. Antibodies to the following markers were used: CD3 (Abcam), CD8α (BD Biosciences), CD4 (Abcam), Foxp3 (eBioscience), CD163 (rat; Santa Cruz Biotechnology), CD163 (human; Abcam), cathelicidin antimicrobial peptide (CAMP; Abcam), CD68 (AbD Serotec), and CD14 (Santa Cruz Biotechnology). Coded sections were analyzed using a Zeiss Axioplan2 microscope and Northern Eclipse morphometry software (Empix Imaging) (17). The numbers of $CD3^+$, $CD8\alpha^+$, and $CD4^+$ intraepithelial lymphocytes (IEL) were expressed as the number of IELs per 100 epithelial cells or lamina propria lymphocytes (LPL) per mm^2 mucosal area. $Foxp3^+$ cells, $CD163^+$ cells, and $CD68^+$ cells were expressed as the cell number per mm^2 mucosal area. Corresponding biotinylated secondary antibodies (DAKO Diagnostics) were applied, followed by incubation with streptavidin-conjugated horseradish peroxidase (DAKO Diagnostics) and 0.06% diaminobenzidine and 0.03% H_2O_2 as substrates; counterstain was hematoxylin. Immunofluorescence was performed using a Zeiss LSM 510 Meta confocal microscope and Alexa-488, Cy-3, and Cy-5-conjugated secondary antibodies (Life Technologies). Hoechst (Sigma) was used for nuclear staining.

Gene expression by real-time PCR. RNA was extracted from frozen jejunum using TRIzol (Invitrogen) or Nucleospin RNA II RNA Isolation Kit (Macherey-Nagel). Quantitative PCR studies were performed using TaqMan Gene Expression Assays (Life Technologies). An ABI Prism 7000 Sequence Detection System was used to obtain threshold cycle (Ct) values; results are shown as fold-change ($2^{-\Delta\Delta Ct}$). Changes in gene expression are presented in relation to the low-antigen HC-GF group, which was arbitrarily set to one. For PCR array profiling, gene expression was analyzed using Innate and Adaptive Immune Response PCR arrays (SABiosciences/Qiagen) on samples from HC-SPF and cereal-SPF BBdp rats (n = 4 per group). Ct values were normalized with the average Ct values of five housekeeping genes.

Analysis of fecal bacteria. DNA was isolated from individual fecal pellets by grinding in sterile sand and liquid nitrogen and purified using the QIAamp DNA stool mini kit (Qiagen, Inc., Mississauga, ON). Quality was assessed using 0.8% agarose gels, and samples were stored frozen at −20°C. Fecal bacterial community profiling compared the V2-3 region of the 16S rRNA genes and was carried out by denaturing gradient gel electrophoresis (DGGE). Gels were digitized and then analyzed using BioNumerics software (BioNumerics, Brussels, Belgium). Band cross-sectional areas were expressed as relative intensity and analyzed by cluster analysis followed by non-metric multidimensional scaling analysis using Statistica software (Tulsa, OK).

Statistics. Survival analysis was performed using the log-rank test. Comparisons among sample populations were made using the Student t test or one-way ANOVA and least significant difference post hoc testing to determine the significance of differences between multiple means. The Mann-Whitney U test was performed for human samples. P values < 0.05 were considered statistically significant.

RESULTS

Feeding a cereal diet promotes T1D in the presence or absence of microbes. As we previously reported in SPF BBdp rats (2), feeding an HC diet increased age at onset and inhibited T1D development compared with a cereal diet (29 vs. 65%, $P < 0.005$; Fig. 1*A*). The HC diet was even more protective in GF BBdp rats, where T1D was almost completely prevented (7 vs. 53% in cereal-fed GF rats, $P < 0.005$). The main effect of microbes on T1D incidence was observed in HC-fed rats, in which disease was promoted by microbial exposure (29% HC-SPF vs. 7% HC-GF, $P < 0.05$). Cereal-fed rats housed in GF conditions displayed a delay in age at onset, but T1D incidence was not significantly different compared with cereal-fed SPF rats. Thus, in the absence of an antigenic diet (HC feeding), microbes can promote T1D in BBdp rats whereas microbial exposure did not significantly enhance T1D progression in cereal-fed animals. Overall, our results demonstrate that microbes were not required for T1D development and cereal antigens were the strongest environmental promoters of T1D in the BBdp model.

Body mass, pancreatic mass, and islet inflammation (insulitis) were not modified by microbes or diet in 130-day asymptomatic rats (data not shown). Total islet number was increased in GF rats compared with SPF rats (Fig. 1*B*), consistent with the delay in T1D onset in GF rats, regardless of diet. Under SPF conditions, rats fed an HC diet had a significantly increased β-cell mass compared with cereal-fed animals, consistent with our previous report (17). Cereal-fed rats raised in GF conditions displayed a larger β-cell mass than SPF cereal-fed rats. Among HC-fed rats, β-cell mass was not different between those raised in GF compared with SPF conditions. β-cell mass was lowest in cereal-SPF rats, and this was overcome by feeding the protective HC diet (HC-SPF). Serum insulin concentration was not different among the groups (data not shown).

Denaturing gradient gel electrophoresis of fecal samples from BBc and BBdp rats was performed to ascertain whether the bacterial communities were affected by diet. Nonmetric multidimensional scaling analysis revealed that BBdp rats fed a protective HC diet had a distinct bacterial profile compared with BBdp rats and BBc rats fed a standard cereal diet (Fig. 1*C*).

Influence of diet and microbes on gut T cells. Because gut inflammation has been reported in BBdp rats, NOD mice, and T1D patients, we evaluated the effect of diet and/or microbes on key gut immune cell populations. The highest numbers of $CD3^+$ and $CD8\alpha^+$ IEL and LPL occurred in cereal-SPF rats. HC-SPF rats displayed decreased $CD3^+$ and $CD8\alpha^+$ cell numbers compared with cereal-SPF rats (Fig. 2*A* and *B*). No significant diet-related

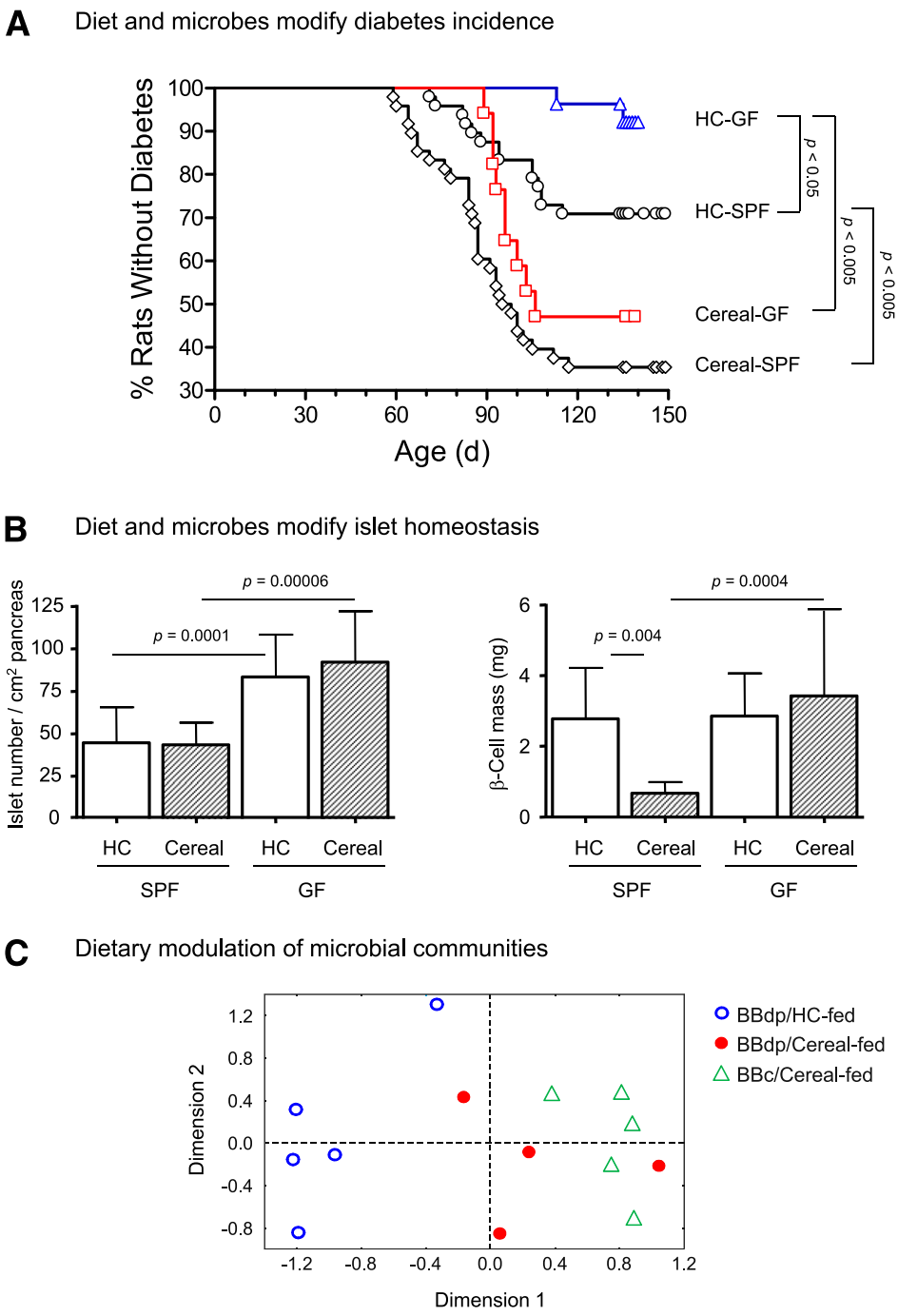

FIG. 1. Feeding a cereal diet promotes T1D in BBdp rats in the presence or absence of microbes—diabetes incidence, islets, and bacterial communities. BBdp rats were maintained under SPF or GF conditions, fed an HC diet or cereal diet, and monitored for T1D development. (*A*) Kaplan-Meier plot shows percentages of nondiabetic rats: HC-GF group (n = 27), HC-SPF group (n = 48), cereal-GF group (n = 17), and cereal-SPF group (n = 48). (*B*) Islet number and β-cell mass are shown in asymptomatic 130-day BBdp rats fed an HC diet (open bars) or cereal diet (hatched bars) and housed under GF or SPF conditions. Data represent mean ± SD; n = 5–7/group. *P* values obtained using ANOVA, followed by least significant difference post hoc test. (*C*) Nonmetric multidimensional scaling diagram shows microbial community distribution in BBc rats fed cereal (open triangles), BBdp rats fed cereal (filled circles), or HC (open circles). The figure shows the relative statistical differences between bacterial communities derived from each sample on an arbitrary two-dimensional surface. Similar community profiles are closer together in two-dimensional space. (A high-quality color representation of this figure is available in the online issue.)

differences in the numbers of $CD3^+$ or $CD8\alpha^+$ IEL or LPL were observed in GF animals. Among cereal-fed rats, the numbers of $CD3^+$ and $CD8\alpha^+$ IEL and LPL were larger in SPF rats compared with GF rats. Also, more $CD3^+$ cells were found in HC-SPF than in HC-GF animals. Thus, $CD3^+$ and $CD8\alpha^+$ lymphocytic infiltration was promoted by microbial exposure. The number of $CD4^+$ cells was higher than expected and the overall pattern differed from CD3 and CD8α labeling, possibly due to other cell populations being labeled (Fig. 2*C*).

Foxp3 is a master transcription factor that directs the differentiation and function of regulatory T cells ($Foxp3^+$ T_{reg}) (21). $Foxp3^+$ cells were exclusively localized to the lamina propria (Fig. 3*A*), and confocal microscopy

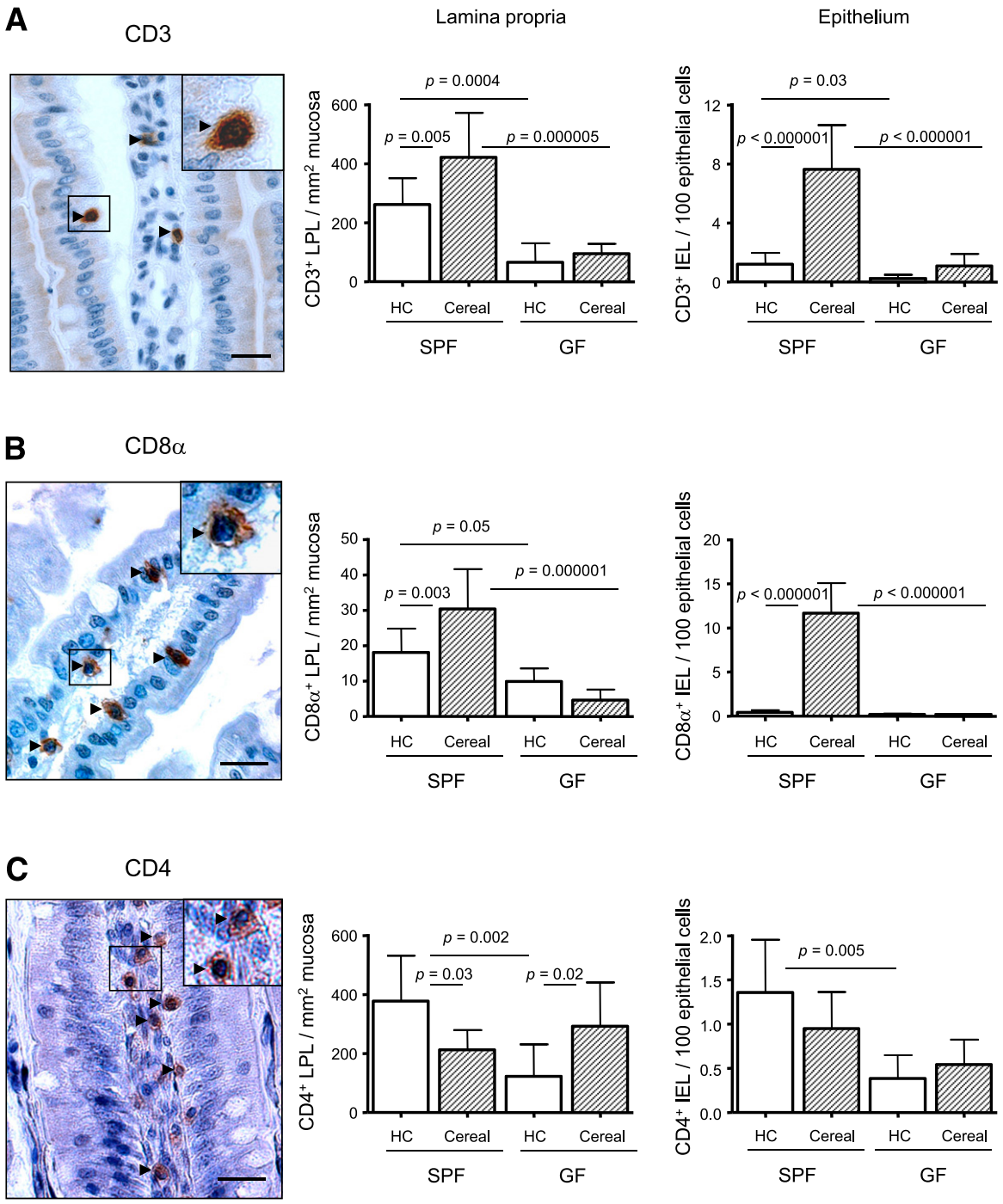

FIG. 2. Influence of diet and microbes on gut T cells. BBdp rats were maintained under SPF or GF conditions and fed an HC diet (open bars) or cereal diet (hatched bars). (*A*) Representative image displays CD3$^+$ (arrowheads) IEL and LPL in BBdp jejunum (bar = 20 μm); number of CD3$^+$ LPL/mm^2 mucosa and CD3$^+$ IEL/100 epithelial cells in 130-day asymptomatic BBdp rat jejunum. (*B*) Representative image displays CD8α$^+$ cells (arrowheads) in BBdp jejunum (bar = 20 μm); number of CD8α$^+$ LPL/mm^2 mucosa and CD8α$^+$ IEL/100 epithelial cells. (*C*) Representative image displays CD4$^+$ cells (arrowheads) (bar = 20 μm); number of CD4$^+$ LPL/mm^2 mucosa and CD4$^+$ IEL/100 epithelial cells. Data represent mean ± SD; *n* = 5–7 per group. *P* values obtained using ANOVA, followed by least significant differences post hoc test. (A high-quality color representation of this figure is available in the online issue.)

confirmed nuclear expression in CD3$^+$ lymphocytes (data not shown). Unlike total CD3$^+$ cells, the number of Foxp3$^+$ cells did not decline under GF conditions. The most protective low-antigen combination of GF housing and HC feeding resulted in the highest number of Foxp3$^+$ cells (Fig. 3*B*), and this difference became significant when expressed as a percentage of jejunal CD3$^+$ (Fig. 3*C*) or CD4$^+$ LPL (Fig. 3*D*). A much lower percentage of Foxp3$^+$ cells was observed in the most diabetes-promoting, high-antigen situation of SPF housing and/or cereal feeding (Fig. 3*C* and *D*). Thus, the dietary influence on Foxp3$^+$ T_{reg} proportion was apparent under sterile conditions, with protective HC feeding resulting in enrichment of regulatory lymphocytes.

The proinflammatory T_H1 cytokine gene, *Ifng* was upregulated by the cereal diet in GF rats (Fig. 4*A*). Microbes

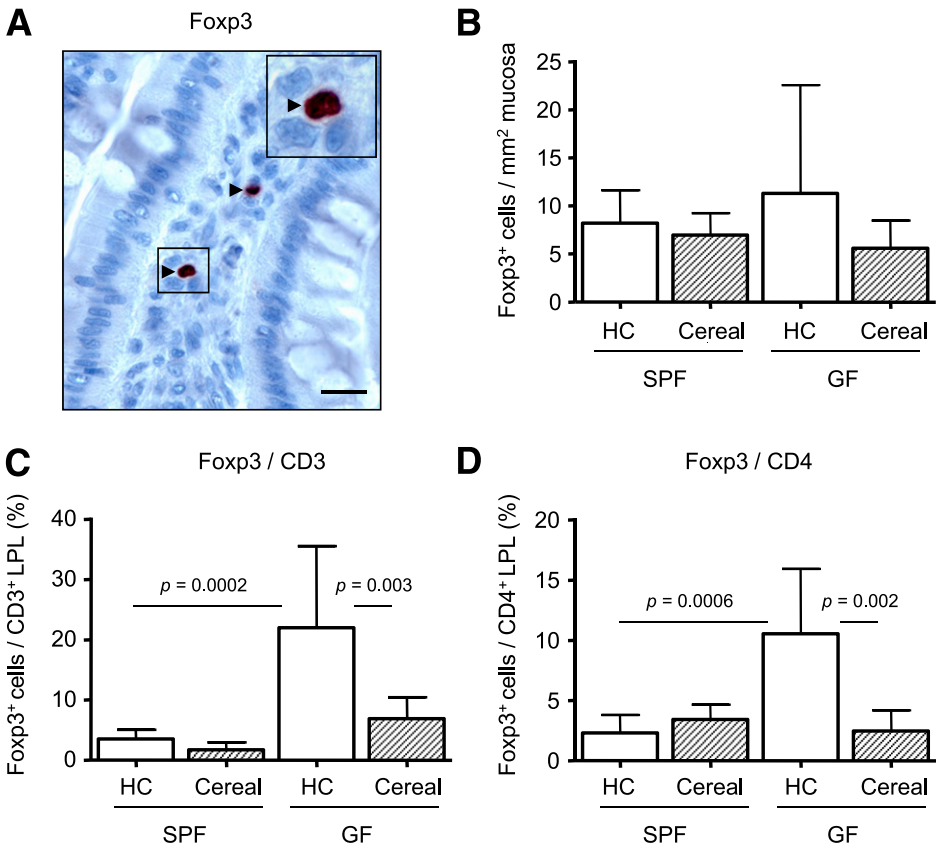

FIG. 3. Regulatory Foxp3$^+$ T cells. (*A*) Representative image displaying Foxp3$^+$ cells (arrowheads) in BBdp jejunum (bar = 20 μm). (*B*) Number of total Foxp3$^+$ cells/mm^2 mucosa. Number of Foxp3$^+$ cells/mm^2 mucosa normalized to the number of CD3$^+$ (*C*) and normalized to CD4$^+$ LPL/mm^2 mucosa in BBdp rat jejunum (*D*). Data represent mean ± SD; *n* = 7–9 rats per group. *P* values obtained using ANOVA, followed by least significant differences post hoc test. (A high-quality color representation of this figure is available in the online issue.)

increased *Ifng* expression in HC-SPF rats versus HC-GF rats, but expression was similar in cereal-SPF and cereal-GF rats. There were no differences in *Il17a* gene expression among the four groups (Fig. 4*B*), and *Il15* expression was largest in cereal-GF rats (Fig. 4*C*). Under GF conditions, the cereal diet stimulated upregulation of the hallmark T_H2 cytokine, *Il4*, compared with the HC diet (Fig. 4*D*). The ratio of *Ifng/Il4* was highest in cereal-SPF rats and was significantly different from the HC-SPF and cereal-GF groups (Fig. 4*G*), reflecting enhanced cereal-associated T_H1 polarization in the gut immune system, as reported previously (7). The highest expression of *Il10* and *Tgfb1* was in SPF compared with GF rats, regardless of diet (Fig. 4*E* and *F*). The ratio of *Ifng/Il10* was highest in cereal-GF rats and low in the other three groups (Fig. 4*H*). The ratio of *Ifng/Tgfb1* was highest in cereal-GF rats and was relatively low in the other three groups (Fig. 4*I*). The data suggest that overall, antigenic stimulation by microbial exposure and/or cereal feeding upregulated cytokine gene expression, with *Ifng* expression predominating over various counter-regulatory cytokines in cereal-fed rats.

Increased frequency of CD163$^+$ macrophages in diet-protected rats. Tissue-resident CD163$^+$ (M2) macrophages are immunosuppressive, have lower levels of major histocompatibility complex (MHC) class II and other markers of classically activated macrophages (22), and contribute to maintenance of a hyporesponsive state. We observed CD163$^+$ cells mainly in the lamina propria and also in a smaller subset in the epithelial compartment (Fig. 5*A*). In a previous analysis of cereal-fed SPF BBdp rats, we observed a deficiency in gut CD163$^+$ macrophages compared with BBc rats (Fig. 5*B*). In the current study of BBdp rats only, feeding the protective HC diet increased the number of CD163$^+$ cells compared with BBdp rats fed a cereal-based diet (Fig. 5*C*). No dietary differences were observed under GF conditions. Gene expression analysis was consistent with these data (Fig. 5*D*).

The *Hmox1* gene for the rate-limiting cytoprotective heme oxygenase-1 (HO-1) enzyme in the CD163 pathway showed highest expression in the most protected animals lacking antigen exposure, HC-GF (Fig. 5*E*). The number of CD68$^+$ macrophages was unaffected by diet or microbial status (data not shown). These data suggest the influence of the environment on macrophages occurs primarily through changes in the M2 subset of macrophages, in keeping with the HC-associated decrease in the *Ifng/Il4* ratio (Fig. 4*G*), which favors polarization of M2 macrophages (23). To investigate the potential relevance of these results to patients, we performed preliminary analysis of gut biopsy specimens from a small group of males newly diagnosed with T1D. This analysis revealed lower numbers of CD163$^+$ macrophages in the jejunum compared with control individuals (Fig. 5*F* and *G*).

Upregulation of *Camp* in diet-protected BBdp gut associated with CD163$^+$ M2 macrophages. To gain a better understanding of dietary modulation of immune factors under standard housing conditions, we screened for diet-modifiable immune factors in the jejunum of conventionally housed SPF rats using focused immune-associated PCR arrays. Only candidates that were differentially

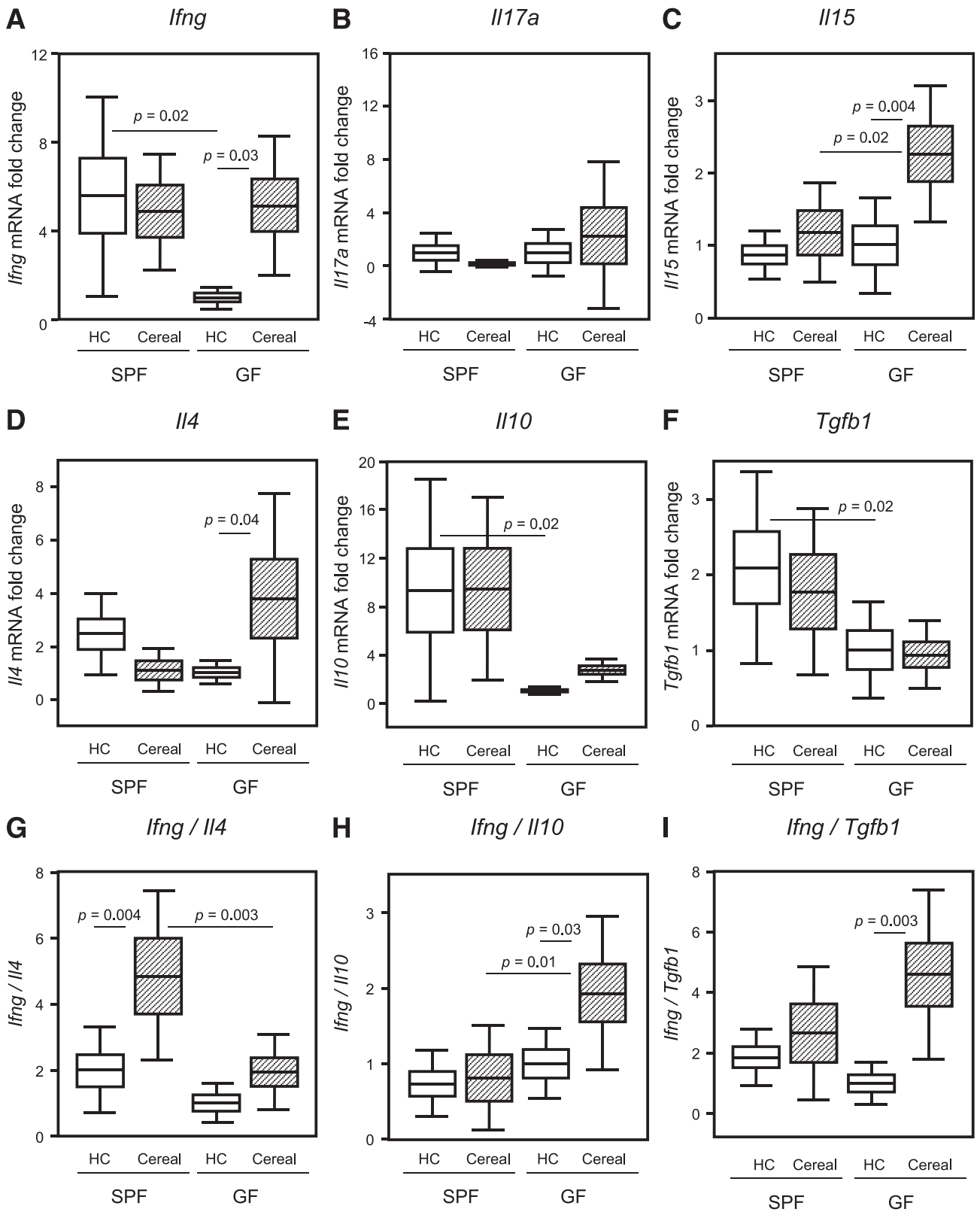

FIG. 4. Cytokine gene expression. Frozen jejunum samples were obtained from BBdp rats maintained under SPF or GF conditions and fed an HC diet (open bars) or a cereal diet (hatched bars). RNA was isolated, and gene expression analyses were performed by using reverse transcription, followed by quantitative RT-PCR. The ΔC_T value was obtained by subtracting the Ct value of β-actin from the gene of interest, and the triplicate ΔC_T values were averaged for each animal. The ΔC_T value of the standard Wistar Furth (WF) rat was subtracted from each animal to obtain the $\Delta\Delta C_T$ value. Results are shown as relative amounts $2^{-\Delta\Delta CT}$ using an age-matched cereal-fed WF rat as a standard; genes of interest were normalized to expression of β-actin. The average value obtained from the HC-GF group was standardized to 1 and averages from other groups were standardized by the same factor. Data represent mean ± SEM (boxes) ± SD (whiskers); *n* = 5–7 per group. *P* values obtained using ANOVA, followed by least significant differences post hoc test.

expressed ± twofold with $P < 0.05$ were explored; results are presented as a volcano plot (Fig. 6*A*). *Il1f6* expression was 2.78-fold upregulated in HC-fed rats compared with cereal-fed rats ($P = 0.004$; Fig. 6*B*). *Il1f6* encodes the cytokine interleukin 1 family member 6 (IL-1F6). The IL-1F6 cytokine has been implicated in cutaneous inflammation (24) and induced antimicrobial expression, including β-defensins and CAMP (25). Interestingly, the other significantly upregulated gene in HC-fed BBdp rats was *Camp*. *Camp* expression was 2.37-fold upregulated in HC-fed rats compared with cereal-fed rats ($P = 0.03$; Fig. 6*B*). CAMP is a multifunctional antimicrobial effector and immunomodulatory host defense factor (26). In addition, consistent with increased $CD3^+$ and $CD8\alpha^+$ cells in gut of cereal-fed rats (Fig. 2), the expression of *Lck*, a T-cell signaling molecule, was 2.43-fold downregulated in HC-fed rats compared with cereal-fed rats ($P = 0.003$; Fig. 6*B*). $CAMP^+$ cells were detected in the epithelium and lamina propria of the SPF jejunum (Fig. 6*C*). $CAMP^+$ cells were also present in gut of sterile embryos and GF BBdp rats (Fig. 6*C*), suggesting CAMP is not only antimicrobial but also has additional functions. Additional confocal analyses revealed that CAMP colocalized with $CD163^+$ and $CD14^+$ cells in the epithelium and lamina propria (Fig. 6*D*), but not with the macrophage marker CD68 (Fig. 6*C*). This suggests that CAMP could be an effector peptide or product of M2 macrophages. These findings reveal that feeding a protective HC diet is associated with antimicrobial gene upregulation in $CD163^+$ macrophages in the small intestine.

Gut inflammation in BBdp rats is not due to lymphopenia or diabetes risk MHC. We previously reported that BBdp rats display enteropathy that precedes T1D and is not present in BBc rats (6). To determine whether BBdp enteropathy is linked to major BBdp diabetes risk loci, we evaluated the presence of enteropathy in various congenic rat strains. Jejunal sections of congenic ACI.1^u and ACI.1^u*lyp/lyp* rats were analyzed at 49, 60, 82, and 259 days. No distinct morphological abnormalities were observed in the gut tissue from these congenic animals (Fig. 7). Inflammation was absent in nonlymphopenic rats congenic for the diabetes risk MHC II allele, RT1^u (Wistar Furth, BB control, ACI.1^u) nor was it present in ACI.1^u*.lyp/lyp* rats that possess the RT1^u allele and the *Gimap5* mutation. Therefore, the presence of enteropathy in BBdp rats was not attributable to *Gimap5*-associated lymphopenia or RT1^u, the rat diabetes risk MHC.

DISCUSSION

The relationship among diet, microbiota, gut immunity, and diabetes is poorly understood and, at times, contradictory. Some studies report no relation between early infections or vaccinations and risk of T1D (27), and a large nationwide study of Danish children found no association with antibiotic use (28). MyD88-deficient NOD mice were protected from T1D under SPF conditions but not GF conditions, further illustrating the complex role of microbes in T1D (16).

In the present study, a cereal-based diet was the major diabetes-promoting factor in both SPF and GF conditions. This clearly illustrates that despite dietary differences in gut bacterial profiles (Fig. 1*C*), diet has a stronger influence than microbes on T1D development in these animals. An earlier report also found that microbes were not essential for development of T1D in the cereal-fed BB rat (29). The nearly complete protection afforded by the HC diet in GF conditions was partially inhibited in the presence of microbes (SPF-HC). Thus, microbes promote T1D, but to a lesser extent than cereal diets. Among HC-fed rats, microbes also promoted infiltration of T cells (Fig. 2) and decreased the proportion of T_{regs} (Fig. 3). It is possible that any effect of bacteria would be most prominent in the colon (30). Immune system changes in the jejunum may only be part of the picture, and a more detailed examination of the influence of microbial and dietary antigenic load along the entire length of the gastrointestinal tract would also be informative.

Snell-Bergeon et al. (18) suggest that diet-induced gut inflammation could be a prerequisite for gut pathogen–induced islet autoimmunity. Interestingly, we observed more overlap between the bacterial profiles of cereal-fed BBdp rats and cereal-fed BBc rats compared with HC-fed BBdp rats. This suggests that HC feeding did not afford protection from T1D in SPF rats by shifting the bacterial profile toward the control strain profile. Nonetheless, the microbiota are different in BBdp rats that develop diabetes (31–33), and administering protective bacteria can prevent T1D (34). When BBdp Wor/Gro rats were treated with antibiotics and fed an HC diet from weaning, T1D was completely prevented (31), in agreement with the present results (Fig. 1) and consistent with the proposition that gut-derived environmental antigenic load is a key determinant of T1D promotion. The HC diet can change the gut microbiota (33), and this may contribute to the diabetes-protective effect. Thus, diet and microbes could have synergistic effects on gut immunity, islet homeostasis, and T1D incidence.

Under SPF conditions, there were increases in the numbers of $CD3^+$ and $CD8\alpha^+$ LPL and IEL in rats fed a cereal diet compared with an HC diet, demonstrating that under normal conditions, cereal-feeding drives T-cell infiltration in the small intestine of BBdp rats. Although the cytotoxic nature of IEL in BBdp rats is unclear, patients with celiac disease, Crohn's disease, and ulcerative colitis are known to have increased densities of these cells that may mediate tissue damage in the small bowel (35), potentially increasing gut leakiness. When small intestinal biopsy samples from children with T1D were cultured with gliadin, the numbers of $CD3^+$ IEL and activated lamina propria $CD25^+$ cells were increased in T1D gut (20). These data indicate that inflammation is present in the jejunum of patients with T1D and suggest that the small intestine of subjects with T1D is immunologically more responsive to cereal antigens, consistent with our findings.

Ifng was upregulated by microbes and cereal diet, whereas *Il10* and *Tgfb* were upregulated primarily by microbes. In GF rats, the cereal diet upregulated *Il15*, *Il4*, *Ifng/Il10*, and *Ifng/Tgfb1*. IL-15 is a proinflammatory cytokine that is implicated in celiac disease (35) and has recently been shown to promote T1D in NOD mice (36). In the HC-GF group, which had essentially no exposure to environmental antigens, gene expression for *Ifng*, *Il4*, and *Il10* was low, corresponding with the decreased number of T cells in this group (Fig. 2). A combined effect of microbes and cereal-feeding on proinflammatory T_H1 polarization was evident from the *Ifng/Il4* ratio, which was highest in the cereal-SPF group but low in the cereal-GF group. This ratio was decreased by HC feeding, further demonstrating an inhibition of the usual T_H1 bias in the gut of BBdp rats (37). Thus, cereal antigens promote T_H1-biased gene expression in the small intestine, as suggested previously (37). The induction of small intestinal T-cell

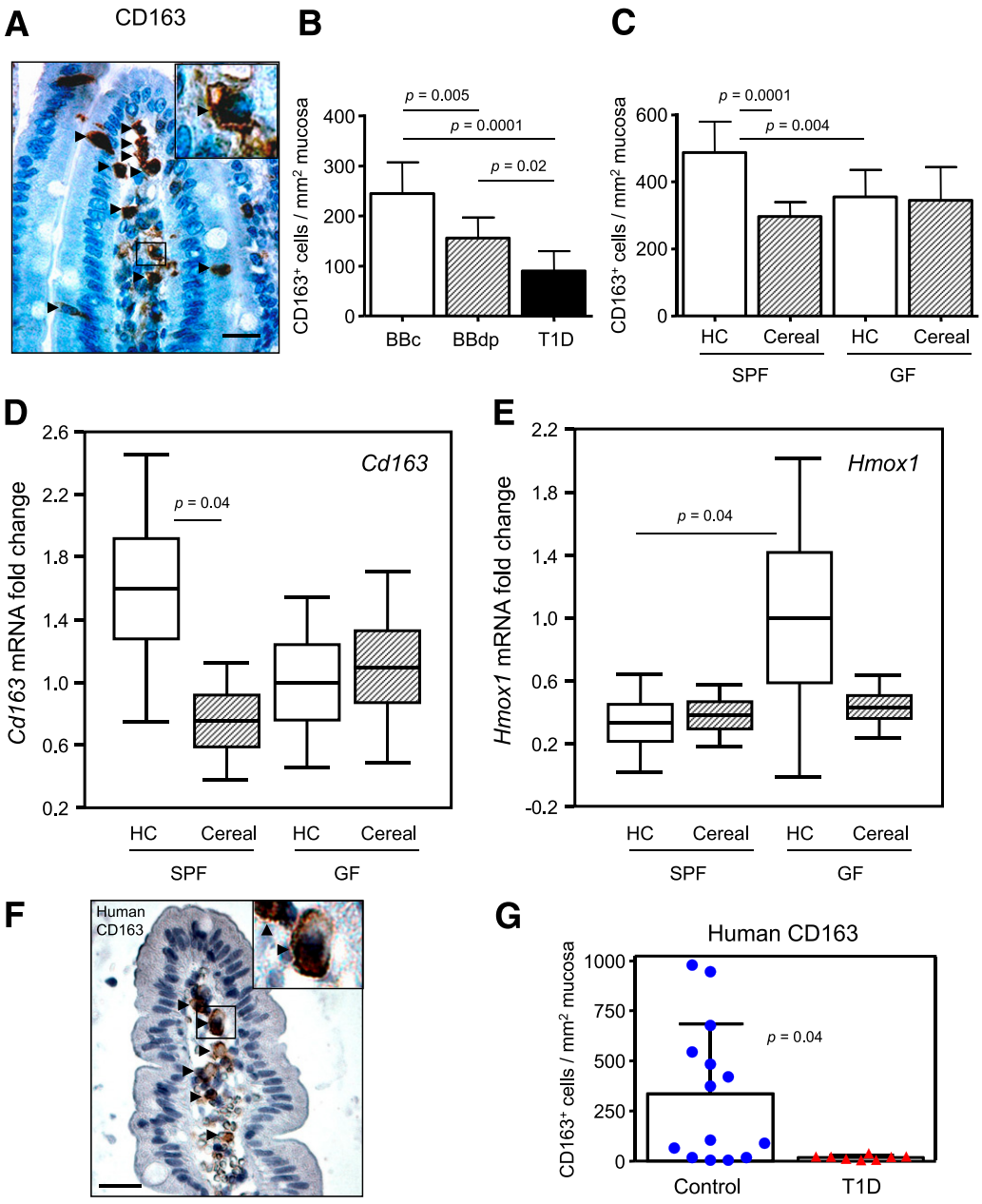

FIG. 5. CD163$^+$ M2 macrophages in BBdp jejunum. BBdp rats were maintained under SPF or GF conditions and fed an HC diet (open bars) or cereal diet (hatched bars). (*A*) Representative image displays CD163$^+$ cells (arrowheads) in BBdp jejunum (bar = 20 μm). (*B*) Number of CD163$^+$ cells/mm^2 mucosa in jejunum of 130-day control BBc, 130-day asymptomatic BBdp, and ~100–130-day overt diabetic BBdp rats, *n* = 6–8. (*C*) Number of CD163$^+$ cells/mm^2 mucosa in 130-day asymptomatic BBdp rat jejunum. Data represent mean ± SD; *n* = 7–9 rats per group. Gene expression of *Cd163* (*D*) and *Hmox1* (*E*) was analyzed using quantitative RT-PCR. Data represent mean ± SEM (boxes) ± SD (whiskers); *n* = 5–7 per group. (*B–E*) *P* values obtained using ANOVA, followed by least significant differences post hoc test. (*F*) Image of CD163$^+$ cells (arrowheads) in gut biopsy from a non-T1D control subject (10-year-old male) with gastroesophageal reflux disease (bar = 25 μm). (*G*) Number of CD163$^+$ cells/mm^2 mucosal area in the jejunum of control subjects (filled blue circles; *n* = 14) or patients with T1D (red triangles; *n* = 8). Data represent mean ± SD. *P* value obtained using Mann-Whitney *U* test. (A high-quality color representation of this figure is available in the online issue.)

signaling in the cereal-SPF group was further reflected by increased expression of *Lck*, the most strongly expressed cereal-induced gene on the PCR array. *Lck* encodes tyrosine kinase/p56, a lymphocyte-specific protein involved in the initiation of T-cell activation (38). The involvement of T_H17 in BB diabetes is controversial (39,40). However, our data demonstrate no diet- or microbe-induced differences in jejunum *Il17a* gene expression (Fig. 4*B*).

Foxp3$^+$ T_{reg} cells play a central role in the inhibition of autoimmunity and suppression of physiological immune responses (41). In GF conditions, the proportion of Foxp3$^+$ T_{reg} cells was decreased in cereal-fed rats compared with HC-fed rats. Strikingly, the proportion of Foxp3$^+$ T_{reg} cells was lowest in the cereal-SPF rats and highest in the HC-GF rats, reflecting the highest and lowest external antigen load and the highest and lowest T1D incidences, respectively. In addition, HC-GF rats displayed a significantly increased proportion of Foxp3$^+$ T_{reg} cells compared with HC-SPF rats, which paralleled the delay in age of onset in GF rats. Consistent with this, Peyer's patches of BALB/c mice fed a gluten-containing diet displayed a smaller proportion of Foxp3$^+$ T cells than mice fed a gluten-free formula (42).

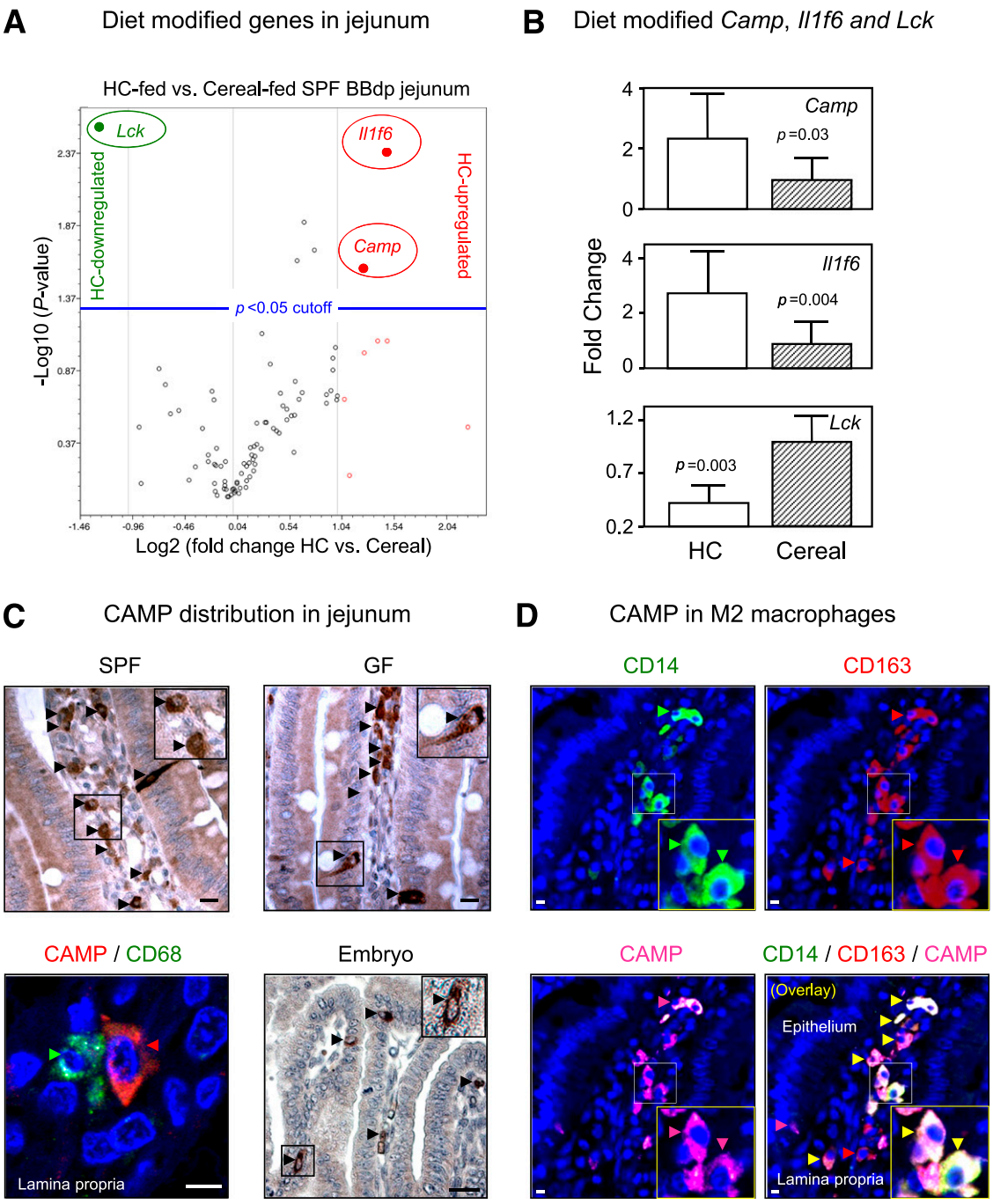

FIG. 6. Gene expression profiling of jejunum identifies *Camp* upregulation in HC diet-protected rats associated with CD163$^+$ M2 macrophages. BBdp rats were maintained under SPF conditions and fed an HC diet or a cereal diet and screened for a panel of innate and adaptive immune factors by PCR array analysis. (*A*) Results presented as a volcano plot of gene expression in HC-fed rats relative to cereal-fed rats; circled candidates were HC downregulated (green circle) or HC upregulated (red circles) at least twofold, with *P* values < 0.05. *P* values were obtained using the Student *t* test. (*B*) Highlighted significant results from the screen indicating *Camp* and *Il1f6* as significant HC upregulated genes; *Lck* as a significant HC downregulated gene. (*C*) Representative images (*left panels*) display CAMP$^+$ cells (arrowheads) in lamina propria and epithelium of 130-day asymptomatic BBdp jejunum (bar = 20 μm), followed by a double immunofluorescence confocal microscopy image displaying CAMP$^+$ cell (red arrowhead) adjacent to CD68$^+$ macrophage (green arrowhead) in lamina propria of a 130-day asymptomatic BBdp rat (bar = 5 μm). CAMP$^+$ cells (arrowheads) in sterile 130-day GF adult BBdp jejunum and BBdp embryonic gut (*right panels*). (*D*) Confocal microscopy image displays multiple CAMP$^+$ cells (Cy3/magenta arrowhead) in the lamina propria of 130-day asymptomatic GF BBdp jejunum colocalizing with both CD14 (Alexa 488/green arrowhead) and CD163 (Cy5/red arrowhead); nuclei labeled with Hoechst (bars = 5 μm). The yellow arrowheads designate CAMP$^+$CD14$^+$CD163$^+$ cells. (A high-quality color representation of this figure is available in the online issue.)

The decreased proportion of Foxp3$^+$ T_{reg} cells in cereal-GF rats compared with HC-GF rats could be an important permissive factor contributing to proinflammatory reactivity to cereal antigens under sterile conditions. The relative increase in Foxp3$^+$ T cells in HC-GF rats was not accompanied by increased gene expression for *Tgfb1* and *Il10*. However, the lowest number of lamina propria CD4$^+$ T cells was observed in HC-GF rats (Fig. 2*C*), by a factor of two- to threefold, yet the levels of *Tgfb1* in these animals were not different from cereal-GF rats and only half that of

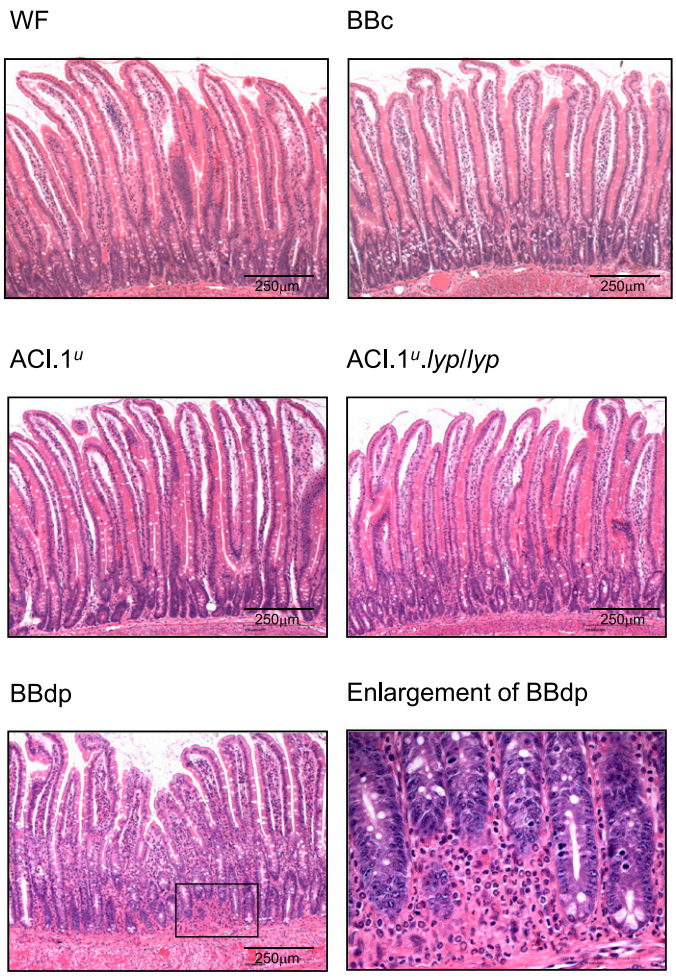

FIG. 7. Enteropathy in BBdp rat is not attributable to diabetes risk MHC or *Gimap5* mutation. Jejunum sections were from 60–80-day non–diabetes-prone congenic rats (Wistar Furth, BBc, ACI.1^{u}, and ACI.1^{u}.*lyp*/*lyp*) and BBdp rats fed cereal diets. Hematoxylin and eosin–stained gut sections displayed crypt hyperplasia, villus atrophy (flattened, shorter), and immune cell infiltration in BBdp rats but not in the non–diabetes-prone rats. All the non–diabetes-prone rats have the diabetes risk MHC, and the ACI.1^{u}.*lyp*/*lyp* rats also have the *Gimap5* (lymphopenia) gene but none displays enteropathy, suggesting these genes are not involved in enteropathy and gut inflammation.(A high-quality color representation of this figure is available in the online issue.)

both SPF groups. We speculate that on a cell-per-cell basis, the synthesis of *Tgfb1* was increased in the HC-GF rats, further consistent with the high proportion of Foxp3^{+} T cells being the source of *Tgfb1* (Fig. 3).

The largest reservoir of tissue-resident regulatory macrophages in the body occurs in the lamina propria of the intestine (43). These cells suppress inflammation (44) and express CD163, a scavenger receptor for haptoglobin-hemoglobin complexes. CD163 is inducible by IL-4 and IL-10 (45), and differentiation of regulatory CD163^{+} macrophages is promoted by Foxp3^{+} T_{reg} (46). Thus, development and maintenance of an immunoregulatory state in the gut depends on a constant interplay between tolerogenic subsets of antigen-presenting cells and T cells. The number of resident CD163^{+} M2 macrophages was inversely related to T1D risk, highest in control, diabetes-resistant BBc and lowest in overt diabetic BBdp rats and was increased in the diet-protected HC-SPF BBdp rats. The HC diet–induced increase in CD163^{+} cells required the presence of microbes. CD163 expression was highest when the ratio of *Ifng*/*Il4* was low, consistent with a downregulation of T_H1 inflammation by M2 macrophages. In addition, the *Hmox1* gene was downregulated in antigen-exposed groups and was highest in the most protected (low-antigen) HC-GF group, suggesting additional environmental sensitivity of the CD163/HO-1 pathway (47). These findings are in keeping with a recent report that IL-4/IL-10/TGF-β–induced M2 macrophages prevented T1D in NOD mice (48). Furthermore, our preliminary data suggest there can be fewer CD163^{+} cells in the gut of some newly diagnosed T1D patients, a finding that requires confirmation.

The increase in small intestinal *Camp* gene expression in HC diet-protected BBdp rats and colocalization of CAMP and CD163 proteins suggests that antimicrobial peptide production is a function of M2 macrophages that could promote an anti-inflammatory state in the gut. Rats and humans possess only one cathelicidin gene (49). CAMP has been reported to be produced by neutrophils, monocytes, macrophages, and epithelial cells in various tissues (50). The most extensively characterized function of CAMP is microbicidal activity, but additional roles have been described, including regulation of inflammation, growth, chemotaxis, tissue repair, wound healing, apoptosis regulation, and angiogenesis (49). CAMP has been implicated in inflammatory bowel diseases, as decreased CAMP expression has been reported in patients with Crohn's disease (51). CAMP-deficient mice displayed a more pronounced form of colitis compared with control mice treated with dextran sodium sulfate (52). In addition, intracolonic treatment with synthetic CAMP prevented dextran sodium sulfate colitis, an effect associated with a decreased number of fecal bacteria, enhanced mucin production, and suppression of neutrophil infiltration (53). Thus, decreased *Camp* expression, partly from CD163^{+} M2 macrophages, could favor chronic inflammation induced by environmental antigens.

Therefore, in BBdp rats, a cereal diet promoted T1D in the presence or absence of microbes. Cereal feeding was associated with increased T-cell infiltration, a T_H1 cytokine bias, and deficiencies in anti-inflammatory CD163^{+} M2 macrophages that were partly corrected in SPF animals fed a protective HC diet. PCR array screening of SPF BBdp rat jejunum further revealed *Camp* as a novel HC diet-upregulated factor. CAMP colocalization with CD163 suggests it may be a new marker of M2 macrophages and highlights a potential role for this antimicrobial peptide in T1D modulation. Importantly, the upregulation of CD163 in HC-SPF animals suggests that this protective effect required the presence of microbes, further emphasizing the complex interaction between diet and microbes in the gut of diabetes-prone animals. We speculate that diet-induced T1D depends on an increased load of antigens from diet and/or microbes crossing a leaky gut epithelial barrier and activating β-cell–specific immunity. This immune activation was dampened when animals were fed an essentially antigen-free HC diet, particularly under GF conditions. Microbes could modify this process because the HC diet resulted in a different bacterial profile in parallel with increased CD163 macrophages that produce antimicrobial CAMP. This study highlights that development of T1D is a heterogeneous, environmentally driven process, particularly in humans, and is likely to occur by several different pathways.

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C.P., G.-S.W., D.E.L., and J.A.C. designed and performed the experiments, analyzed the data, and wrote the manuscript. B.S., C.E., M.Mo., S.P.J.B., and M.L.K. designed and performed the experiments and analyzed the data. C.R.K. contributed materials and analysis tools. M.Ma. and R.T. contributed human samples and helped write the manuscript. P.P. contributed congenic animals, assisted in the design of the study, and helped write the manuscript. F.W.S. conceived and designed the study, analyzed the data, and wrote the manuscript. F.W.S. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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