Cathelicidin antimicrobial peptide: a novel regulator of islet function, islet regeneration and selected gut bacteria

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ABSTRACT

Cathelicidin antimicrobial peptide (CAMP) is a naturally-occurring secreted peptide expressed in several organs with pleiotropic roles in immunomodulation, wound healing and cell growth. We previously demonstrated that gut Camp expression is up-regulated when type 1 diabetes (T1D)-prone rats are protected from diabetes development. Unexpectedly, we have also identified novel CAMP expression in the pancreatic β-cells of rats, mice and humans. CAMP was present even in sterile rat embryo islets, germ-free adult rat islets and neogenic tubular complexes. Camp gene expression was down-regulated in young BBdp rat islets before the onset of insulitis compared with control BBc rats. CAMP treatment of dispersed islets resulted in a significant increase in intracellular calcium mobilization, an effect that was both delayed and blunted in the absence of extracellular calcium. Additionally, CAMP treatment promoted insulin and glucagon secretion from isolated rat islets. Thus, CAMP is a promoter of islet paracrine signaling that enhances islet function and glucoregulation. Finally, daily treatment with the CAMP/LL-37 peptide in vivo in BBdp rats resulted in enhanced β-cell neogenesis and up-regulation of potentially beneficial gut microbes. In particular, CAMP/LL-37 treatment shifted the abundance of specific bacterial populations, mitigating the gut dysbiosis observed in the BBdp rat. Taken together, these findings indicate a novel functional role for CAMP/LL-37 in islet biology and modification of gut microbiota.
INTRODUCTION

Type 1 diabetes (T1D) results from autoimmune destruction of the pancreatic β-cells, leading to the loss of insulin secretion and subsequent hyperglycemia. The gut lumen contains large numbers of dietary antigens and microbes that impact the gut immune system and T1D incidence in diabetes-prone BBdp rats, NOD mice and humans (1-5). The innate immune system plays a critical role in maintaining immune tolerance to commensal bacteria and dietary antigens. Recently, our group demonstrated that innate immune host defense peptides in the gut are involved in protection from T1D in the BBdp rat (3). Specifically, Camp, the gene encoding the cathelicidin-related antimicrobial peptide (CRAMP), was up-regulated in the gut of the BBdp rat when T1D was prevented by feeding a low antigen diet. Additionally, CRAMP was expressed by anti-inflammatory M2 macrophages, consistent with protection from T1D development. CAMP/LL-37, the human homolog of cathelicidin, is a cationic molecule generated from the C-terminus of the hCAP18 precursor protein. CAMP/LL-37 and its rodent homolog, CRAMP, were traditionally thought of as antimicrobial peptides. However, a diverse range of additional biologically relevant functions has also been attributed to this peptide, including promotion of proliferation, cell migration, cell survival, cytokine release, angiogenesis, chemotaxis and wound healing (Reviewed in (6; 7)).

As CAMP is a secreted peptide, its immunomodulatory properties could theoretically confer local protection against autoimmune attack on the β-cell. Indeed, other host defense peptides, such as Reg3β, play a role in islet physiology, particularly in promoting islet neogenesis and β-cell survival (8; 9). Thus, we hypothesized that CAMP plays a role in pancreatic islet biology and T1D risk. Furthermore, as CAMP signals through a number of pathways that, in the islet, promote functions including glucose sensing and islet hormone
secretion, we sought to determine whether CAMP/LL-37 impacts islet function. Additionally, in human neutrophils, CAMP/LL-37 promotes calcium mobilization (10). Thus, because calcium mobilization plays a key role in islet hormone secretion, we hypothesized that CAMP/LL-37 treatment would enhance islet function through calcium-dependent mechanisms.

The current study utilized the well-characterized rat model of spontaneous T1D, the BBdp rat, and its diabetes-resistant control (BBc). Typically, the BBdp rat develops insulitis between 50 and 70 days of age and frank diabetes between 55 and 120 days (1). Using the BB rat, we expand our previous finding of CAMP in the gut (3), and for the first time, characterize its novel expression and role in the islets of Langerhans. We demonstrate that CAMP is expressed in β-cells and has a positive impact on islet function and regeneration as well as selected gut microbiota.
METHODS

Animal use, husbandry, and ethics

Inbred BBc and BBdp rats were housed in a barrier facility and received sterile food (Teklad Global 18% Protein Rodent Diet, Harlan, Montreal, QC, Canada) and water *ad libitum* beginning at weaning (23 days of age). Rats were anesthetized with 4% isoflurane (Abbott Laboratories, Montreal, QC, Canada) in oxygen and euthanized by exsanguination. Sterile/germ-free pancreas tissue was obtained from our previous study (3). Animals were maintained in accordance with the Canadian Council on Animal Care guidelines for care and use of laboratory animals. The studies were approved by the local ethics committee. Human pancreas tissue from a 65 year-old non-diabetic male subject was obtained by Dr. John Woulfe (Pathology Department, The Ottawa Hospital) with institutional ethics approval.

Immunohistochemistry

Pancreas tissue was fixed in Bouin’s fixative and embedded in paraffin for histological analysis and immunohistochemistry as previously described (3). In separate experiments, anti-cathelicidin (Abcam, Toronto, Canada) and anti-insulin (DAKO Canada, Inc., Burlington, ON, Canada) primary antibodies were used. Sections were visualized at 400× magnification using an Axioplan 2 light microscope (Zeiss Canada, Mississauga, ON, Canada) equipped with a digital CCD color camera (QImaging, Burnaby, BC, Canada); image analysis was performed using Northern Eclipse software (Empix Imaging Inc., Mississauga, ON, Canada).
**Immunofluorescence**

Double immunofluorescence staining of CAMP with insulin, glucagon or somatostatin was performed on Bouin’s-fixed paraffin-embedded sections, as described above. Sections were incubated with primary antibodies either overnight at 4°C or 2 h at room temperature. In a separate study, sections were incubated with anti-cathelicidin (Abcam) and one of the following antibodies: anti-insulin (DAKO Canada, Inc.), anti-glucagon (DAKO Canada, Inc.) or anti-somatostatin (Cortex Biochem, Inc., San Leandro, CA, USA). Next, sections were incubated with Alexa-488-, Cy3-, Cy5- or FITC-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). Nuclei were counterstained with Hoechst (Sigma-Aldrich, Oakville, ON, Canada). Immunofluorescence was visualized using a Zeiss 510 Meta confocal microscope (Zeiss Canada) equipped with an argon laser at 488 nm (Alexa-488 and FITC), HeNe at 543 nm (Cy3), HeNe at 633 nm (Cy5) and a diode laser at 405 nm (Hoechst); 40×/1.30 oil or 63×/1.40 oil Plan-Apochromat objectives were used. Image analysis was performed using ZEN LE 2009 (Zeiss Canada).

**Islet isolation**

Islets were isolated from 30 day and 50-70 day BBc and BBdp rats as previously described (11). Briefly, collagenase (1 mg/ml in Hank’s buffered saline; Sigma-Aldrich, St. Louis, MO, USA) was infused into the pancreas through the bile duct. The pancreas was then digested for 8.5-9.5 min at 37°C. Islets were purified using a histopaque (Sigma-Aldrich) gradient and handpicked under a dissection microscope. Islets were cultured overnight in RPMI 1640 medium supplemented with 10% fetal bovine serum. For analysis of glucose-stimulated Camp expression, islets were cultured overnight in RPMI 1640 medium containing the indicated glucose concentration.
RNA isolation and qRT-PCR

The jejunum was isolated from BBc and BBdp rats, and Peyer’s patches were removed. RNA was isolated from purified islets and jejunum tissue using a Nucleospin RNA II RNA Isolation Kit according to manufacturer’s instructions (Machery-Nagel, Bethlehem, PA, USA) and reverse transcribed (M-MLV Reverse Transcriptase, Life Technologies, Carlsbad, CA, USA). Quantitative PCR was performed using TaqMan Gene Expression Assays (Life Technologies) and the ABI Prism 7500 Sequence Detection System. β-actin was used to normalize real-time expression using the Pfaffl method (12). PCR array profiling was performed using a Diabetes PCR Array (Qiagen, Toronto, ON, Canada) on islets isolated from 50-70 day old BBc rats that had been treated with human CAMP/LL-37 (10 µM; Anaspec, Fremont, CA, USA) or scrambled peptide (Scr; Anaspec) for 24 hours.

Western blot analysis

Islet protein was extracted and western blotting was performed as previously described (13). The presence of CRAMP in rat islets was detected by immunoreactivity to anti-CRAMP antibody (Abcam) with secondary antibody: horseradish peroxidase-conjugated anti-rabbit (Santa Cruz Biotechnology, Dallas, TX, USA). β-actin (Life Technologies) was used as a loading control with secondary antibody: horseradish peroxidase-conjugated anti-mouse (Abcam).

Measurement of cytoplasmic calcium

Analysis of intracellular calcium was performed as previously described (14). Briefly, dispersed islet cells from male BBc and BBdp rats (age 50-70 days) were loaded with fura-2 acetoxymethyl ester (4 µM in Krebs-Ringer buffer [KRB], Life Technologies) for 45 min at 37°C. Cells were stimulated with Scr or CAMP/LL-37 (10 µM) in the presence or absence of
extracellular calcium as indicated. Images and 340 nm:380 nm ratio were acquired using Stallion software (Intelligent Imaging Innovations, Gottingen, Germany). A positive response was defined as an individual cell that reached a minimum of 10% above the baseline ratio.

Islet hormone secretion assays

Secretion assays were performed as previously described (11). Following overnight culture, isolated islets from 50-70 day old male BBc and BBdp rats were rinsed in KRB containing 2.8 mM glucose (insulin assays) or 5 mM glucose (glucagon assays) and allowed to equilibrate for 30 min. For analysis of glucose-stimulated insulin secretion (GSIS), islets were cultured for 1 hour in KRB containing 2.8 mM glucose and then transferred to KRB containing 16.7 mM glucose with CAMP/LL-37 or Scr control. One micromolar CAMP/LL-37 samples were supplemented with 9 µM Scr to control for amount of protein added. For analysis of glucagon secretion, islets were cultured for 1 hour in KRB containing 5 mM glucose and then transferred to KRB containing 2.8 mM glucose and 20 mM L-arginine with CAMP/LL-37 or Scr control. Assays were performed in quadruplicate (15 islets/sample). Insulin and glucagon were measured using Rat Insulin (Mercodia, Winston-Salem, NC, USA) and Rat Glucagon ELISA kits (Mercodia) according to the manufacturer’s instructions.

Analysis of extra-islet insulin+ duct cells

At 24 days of age, BBdp (n=13) and BBc rats (n=13) were intraperitoneally administered 1 mg/kg CAMP/LL-37 in PBS (n=13/group; AnaSpec) or PBS alone (n=13/group) daily for seven days. Following treatment, fecal samples were collected, flash frozen in liquid nitrogen and stored at -80°C. DNA was extracted from the fecal samples and used for metagenomic analysis as described below. Pancreas tissue was harvested, fixed in Bouin’s fixative and embedded in paraffin for histological and immunohistochemical analysis, as described above.
Insulin+ ductal cells were quantified using a ScanScope CS eSlide capture device and analyzed using ImageScope software (Aperio, Vista, CA, USA). The number of insulin-expressing cells localized to the ductal epithelium was normalized to total pancreas area and expressed as the number of duct-associated insulin+ cells/cm² pancreas.

Illumina sequencing based characterization of gut microbiota

Metagenomic DNA was extracted from fresh rat stool using the Fast DNA Spin Kit (MP Biomedicals, Solon, OH, USA) and the FastPrep machine (MP Biomedicals) as described previously (15). Construction of the 16S rRNA-V6 library for Illumina HiSeq 2500 sequencing was conducted as described previously (16; 17). Briefly, a two-step PCR strategy was followed to amplify the 16S rRNA-V6 region using universal 16S rRNA-V6 primers modified from Sundquist et al. (18) (Suppl. Table 1) and to introduce the barcode sequences using Illumina paired end sequencing adapters and flow cell adapters. The generated reads were submitted to the NCBI-Sequence Read Archive under accession number SRP059189. Paired-end sequences obtained by Illumina HiSeq 2500 were merged into longer reads using Fast Length Adjustment of Short reads (FLASh) software, without any mismatch in the overlap region that ranged from 10 to 80 nucleotides (19). Afterward, the fastq_quality_filter command from the Fastx toolkit (http://hannonlab.cshl.edu/) was used to filter the merged reads with a minimum quality score of 20 over 90% of the sequence. High quality reads were then sorted according to the forward and reverse barcode sequences with barcode trimming using the NovoBarCode software (Novocraft.com, Selangor, Malaysia). Next, the reads were clustered into operational taxonomic units (OTUs) using QIIME 1.7.0 (20) by following a closed-reference OTU picking workflow against the Greengenes reference set (release May 2013) and an average percentage of identity of 97%. The generated OTU table was used to summarize the taxonomy and to assess the alpha and
beta diversity within and among the samples using the default criteria of QIIME. A Mann-
Whitney two-tailed test was used for pairwise statistical comparisons of the relative abundances
of different taxa. To determine the microbial biomarkers of each group, the relative abundances
of different taxa computed by QIIME were analyzed using the LDA Effect Size (LEfSe)
algorithm (http://huttenhower.org/galaxy/) (21) following its default parameters.
RESULTS

CAMP is expressed in the islet β- and δ-cell but not in the α-cell

We recently demonstrated that when diabetes is prevented in the diet-protected, hydrolyzed casein-fed BBdp rat, Camp gene expression was significantly up-regulated in the gut, with expression co-localizing in CD163⁺ M2 macrophages (3). Surprisingly, CRAMP is also detectable in the rat β-cell (Fig. 1a). CRAMP is not expressed in the islet α-cell (Fig. 1b) but colocalizes with a subset of somatostatin-expressing δ-cells (Fig. 1c). In addition, CRAMP is expressed in the islets of embryos (Fig. 1d) and adult germ-free rats (Fig. 1e), suggesting that it is expressed even in sterile settings and has currently unrecognized functions in islet biology in addition to its immunomodulatory and antimicrobial properties. Consistent with a novel role in islet biology, CRAMP and CAMP/LL-37 are also expressed in mouse (Fig. 1f) and human islets (Fig. 1g), respectively.

Camp gene expression is down-regulated in the BBdp rat

Given that we observed an increase in Camp gene expression when diabetes was prevented in the BBdp rat gut, we hypothesized that CAMP may also be dysregulated in the BBdp β-cell and play a role in T1D. Compared with their BBc counterparts, BBdp rats display a 61% reduction in Camp mRNA levels (Fig. 2a, left panel) as early as postnatal day 30. This reduction precedes the onset of insulitis, suggesting that the deficiency in Camp expression may be a primary contributing factor to diabetes susceptibility rather than secondary to inflammation. Furthermore, although CRAMP is widely expressed in β-cells from healthy BBc (Fig. 1a) and non-insulitic BBdp rats (100 days old) (Fig. 2b), its expression in endocrine cells is decreased in animals with insulitis (100 days old) (Fig. 2b). It is important to note that in rats that have developed some degree of insulitis, CRAMP expression is dramatically reduced even in
individual islets that show only a moderate degree of insulitis (Fig. 2b). Moreover, the reduction in Camp gene expression in isolated islets is maintained following the onset of insulitis (Fig. 2a, right panel).

**CAMP/LL-37 treatment stimulates calcium mobilization and islet hormone secretion**

To elucidate the physiological context in which Camp gene expression is regulated in the healthy islet, we stimulated isolated islets with varying concentrations of glucose. Camp gene expression was up-regulated following treatment at 16.7 mM glucose compared with 2.8 mM glucose (Fig. 3a), suggestive of a role in glucoregulation. Additionally, our observation that CAMP is expressed in the β-cell, particularly in the sterile embryo, indicates a novel role for this peptide in islet function. In other cell types, CAMP has been shown to promote calcium mobilization (10), a critical step in insulin and glucagon secretion within the islet. Thus, we assessed the role of CAMP in calcium mobilization in dispersed primary islet cells. CAMP/LL-37 treatment stimulated a rapid and robust increase in cytoplasmic calcium levels that was similar in both BBc and BBdp cells (Fig. 3b) with 90.3% and 85.7% responders, respectively (Fig. 3c). To determine the source of the calcium signal, the response of dispersed islet cells to CAMP/LL-37 was analyzed in the absence of extracellular calcium. The absence of extracellular calcium delayed and blunted, but did not prevent, the calcium response to CAMP/LL-37 (Fig. 3b, d), indicating that CAMP acts via both intracellular and extracellular mechanisms.

As calcium mobilization promotes insulin (reviewed in (22)) and glucagon (23) secretion, we examined the effect of CAMP on islet hormone secretion in isolated BBc and BBdp islets. CAMP/LL-37 treatment dose-dependently enhanced insulin secretion at high glucose by approximately 1.6-fold and 2-fold following 1 µM and 10 µM CAMP/LL-37 treatment, respectively, compared with that of the scrambled control (Fig. 3e). Importantly, CAMP/LL-37
treatment improves GSIS in BBdp rat islets to a comparable degree as in BBc islets (Fig. 3f), indicating that the signaling pathways through which CAMP/LL-37 acts to improve insulin secretion are still functional in the diabetes prone rat. Additionally, when glucagon secretion was stimulated under low glucose conditions with arginine supplementation, CAMP/LL-37 treatment led to an additional ~8-fold increase in glucagon secretion that was comparable in both BBc and BBdp rats (Fig. 3g). Taken together, these results indicate a profound acute effect of CAMP on islet function and thus glucoregulation.

CAMP/LL-37 treatment promotes β-cell regeneration

To investigate the chronic effects of CAMP/LL-37 treatment on β-cell gene expression and neogenesis, we screened for changes in gene expression in key β-cell markers using diabetes-specific gene expression PCR arrays. Treatment of isolated islets with CAMP/LL-37 for 24 hours led to changes in gene expression in glucose metabolism and insulin signaling pathways as well as neogenic markers (Fig. 4a). Given these findings, we assessed the role of CAMP in β-cell neogenesis from ductal precursors. Interestingly, CRAMP$^+$ cells were also detected in pancreatic ducts (Fig. 4b), sites of postnatal β-cell neogenesis, as well as in neogenic tubular complexes (Fig. 4c) (8), consistent with CRAMP presence in mature islets (Fig. 1) and indicative of a role for this factor in β-cell regeneration. To investigate whether CAMP stimulates neogenesis from the pancreatic ducts, rats were treated with CAMP/LL-37 or saline daily for one week. Remarkably, CAMP/LL-37 treatment significantly enhanced the number of duct-associated extra-islet insulin$^+$ clusters in BBdp but not BBc rats, indicative of enhanced β-cell neogenesis in the diabetes-prone cohort (Fig. 4d). This was not accompanied by changes in islet cell number or β-cell mass (data not shown). Additionally, CAMP/LL-37$^+$ duct-associated cells were observed in human pancreatic tissue (Fig. 4e).
CAMP/LL-37 treatment in BBdp rats shifts the abundance of specific microbiota

Similar to the islet, we observed a significant reduction in Camp gene expression in the BBdp gut compared with the BBc gut (Suppl. Fig. 1). Although the direct consequences of the reduction in gut CAMP are currently unknown, targeting gut microbes in susceptible subjects could be an effective strategy for T1D modulation (24; 25). In healthy individuals, intestinal microbiota play a critical role in normal physiological processes, including nutrient digestion and proper immune function; however, it has been previously reported that the gut microbiota are altered in individuals with diabetes (reviewed in (26)). To investigate whether treatment with this antimicrobial peptide could ameliorate this phenomenon, fecal pellets were collected following saline or CAMP/LL-37 treatment for 16S rRNA-based bacterial analysis. Untreated BBdp rats displayed a clear shift in abundance in populations of gut microbiota compared with BBc rats (Fig. 5a and b). Although in vivo LL-37 treatment did not globally impact the gut microbiome (Fig. 5a), it did result in a shift in abundance in specific bacterial populations towards control BBc levels. In particular, CAMP/LL-37-treated BBdp rats displayed an increased abundance of several related taxa from the same phylogenetic lineage, including Actinobacteria (phylum), Coriobacteria (class), Coriobacteriales (order), Coriobacteriaceae (family) and Adlercreutzia (genus) (Fig. 6). In addition, CAMP/LL-37 treatment in BBc rats induced the expression of probiotic species, including Lactobacillus (Suppl. Fig. 2). In summary, CAMP/LL-37 injections in young BBdp rats upregulated several related taxa that were naturally more abundant in the diabetes-resistant BBc strain. Thus, potentially beneficial modification of microbiota in BBdp rats was achieved by exogenous administration of CAMP/LL-37.
DISCUSSION

We demonstrate for the first time that CAMP is expressed in the pancreatic β-cells in rats, mice and humans. β-cell CRAMP expression was decreased in diabetes-prone BBdp rats before onset of insulitis, consistent with a role in T1D susceptibility. Furthermore, treatment of isolated rat islets with the human CAMP/LL-37 peptide stimulated islet hormone secretion, likely enhancing intra-islet communication and glucoregulation. Finally, in diabetes-prone rats, we demonstrated that CAMP/LL-37 promotes islet regeneration and shifts the abundance of specific bacterial populations towards control BBc levels.

The observation that Camp gene expression is reduced in the BBdp rat prior to the onset of insulitis (Fig. 2) is suggestive of a primary role in T1D development, rather than a secondary effect of increased islet inflammation. Furthermore, in rats with inflamed islets, there was a decrease in CRAMP expression in most islets.

CAMP could have a positive effect on trophic and regenerative processes in the pancreas. In particular, we observed that daily treatment for 7 days enhanced β-cell regeneration from ductal precursors (Fig. 4d) in BBdp but not BBc rats. The observation that this occurred in BBdp but not BBc rats is consistent with a previous study from our group indicating that BBdp rats display signs of neogenesis more frequently than control rats, possibly as a response to early diabetes-promoting changes in the islet microenvironment (27). Thus, CAMP/LL-37 treatment may be further potentiating this phenomenon. As patients with T1D suffer from significant loss of β-cells, the ability to enhance β-cell regeneration would be therapeutically valuable. In addition, treatment with CAMP/LL-37 stimulated acute islet hormone secretion at least partially through calcium-dependent mechanisms (Fig. 3). The finding that CAMP/LL-37 treatment promotes intracellular mobilization of calcium is consistent with a previous report in human
neutrophils (10). These authors found that exclusion of calcium from the medium completely blocked this effect. In contrast, when extracellular calcium was excluded from dispersed islet cells, the cells displayed a delayed and blunted calcium response, suggesting that CAMP/LL-37 affects both extracellular and intracellular stores. This difference between the two studies indicates that the mechanism of CAMP/LL-37 action on calcium mobilization could be tissue-specific. The receptors through which CAMP/LL-37 acts in the islet remain to be elucidated. In other organs CAMP/LL-37 is known to signal through several receptors (7) including the epidermal growth factor receptor, insulin-like growth factor receptor, chemokine (C-X-C motif) receptor 2, P2Y purinoceptor 11, P2X purinoceptor 7 and toll-like receptors, all of which are expressed in the islet.

The observation that CAMP/LL-37 promotes both insulin and glucagon secretion has important implications for intra-islet communication. CAMP may be acting directly as a novel autocrine and paracrine factor in the islet. Furthermore, CAMP action in the islet may also indirectly promote signaling by enhancing secretion of both hormones, further modulating hormone secretion in response to changes in nutrients and thus providing a novel mechanism of intra-islet regulation. However, we demonstrate that high glucose stimulates the expression of Camp, indicating that CAMP action is sensitive to circulating glucose levels. At high glucose concentrations, glucagon secretion is effectively turned off, suggesting that in vivo, the primary role of CAMP is to specifically enhance insulin secretion to more efficiently regulate blood glucose levels. Thus, CAMP may be sensitizing the islet to changes in glycemia, thereby improving glucoregulation.

It is worth noting that the effects of CAMP/LL-37 treatment on calcium mobilization and islet hormone secretion are comparable in BBc versus BBdp rats, indicating that the islet CAMP
signaling pathways remain intact in a rodent model of T1D. Additionally, an impaired glucagon response to hypoglycemia is a significant concern in patients with T1D (28-30). Thus, more efficient glucose sensing and glucagon secretion could also aid in the prevention of dangerously low blood glucose levels in addition to contributing to the reduction in glycemic levels. Furthermore, in vivo, CAMP expression, secretion and/or action may be context-dependent. Therefore, it will be interesting to investigate the dynamics of CAMP and islet hormone secretion in response to glucose and/or other nutrients.

Although BBdp rats lacking microbes can ultimately develop T1D (3), altered bacterial composition is associated with diabetes (31-33). CAMP administration intra-colonically prevented colitis in mice, an effect associated with decreased bacteria and immune cell infiltration (34), demonstrating the concomitant microbe-modifying and immune-modifying effects of this peptide. We demonstrated for the first time that short-term CAMP/LL-37 treatment results in a shift in abundance in specific bacterial populations towards control BBc levels. Thus, CAMP/LL-37 likely plays a role in intestinal homeostasis. In particular, one of the most upregulated genera was the Aldercreutzia bacteria. The sole member of this genus, Aldercreutzia equolifaciens, is an equol-producing bacterial strain. Though little is currently known about this species, previous work has indicated that production of equol by gut microbiota can have beneficial metabolic impacts, including improved leptin:BMI profiles (35), antioxidant activity (36) and protection from β-cell death (37). In addition, comparison of BBc and BBdp rats demonstrated an alteration in the composition of microbiota, many of which are taxonomically related, in the BBdp rat. Interestingly, CAMP/LL-37 treatment is associated with an enhancement of bacteria that are naturally enriched in the BBc rat microbiome. The observation that CAMP/LL-37 treatment augments many taxonomically related species that are
naturally abundant in BBc rats suggests that this peptide has a targeted rather than a global impact on the gut milieu. Whether modulation of the microbiota associated with CAMP/LL-37 treatment will have a direct impact on T1D treatment and/or prevention remains to be clarified. It is conceivable that, despite changes in the gut microbiota, any impact of CAMP/LL-37 on T1D susceptibility could be modulated primarily through islet-specific and/or other mechanisms (3).

CAMP has a number of pleiotropic roles; however, it is worth noting that this property may be a limitation for CAMP/LL-37 administration in vivo. Previous studies have implicated CAMP/LL-37 in some autoimmune diseases, including skin disorders (38-42). LL-37 levels do not decline during successful treatment of psoriatic plaques and, in fact, increase when lesions are treated with topical vitamin D3 analogues (41). Furthermore, previous studies have indicated that LL-37 treatment may promote ovarian (43) and lung (44) cancer. In contrast, however, it may have a preventative effect on colon cancer (45) and the ability to sensitize multi-drug resistant cancer cells to chemotherapeutic agents (46). Additionally, LL-37 treatment promotes healing of gastric and venous ulcers (47; 48). Future studies assessing the impact of CAMP/LL-37 administration on the treatment and/or prevention of T1D should analyze the potential off-target effects of CAMP/LL-37 treatment.

In summary, our data indicate a novel role for CAMP in islet function, regeneration and partial correction of the gut dysbiosis in the BBdp rat. Importantly, by stimulating both insulin and glucagon secretion, CAMP may be acting as a novel regulator of intra-islet communication thereby enhancing overall islet function and glucoregulation. Furthermore, changes in expression in diabetes-prone rats indicate that CAMP may be playing a role in T1D susceptibility. Future studies that address the role of CAMP/LL-37 in the prevention and/or treatment of diabetes are warranted.
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AUTHOR CONTRIBUTIONS

L.D.P. designed the studies and performed gene expression, calcium mobilization and islet hormone secretion studies, analyzed the data and wrote the manuscript. C.P. designed the studies, performed the immunohistochemical staining, analyzed the data and wrote the manuscript. C.E.E. designed the studies and performed gene expression, calcium mobilization and islet hormone secretion studies and analyzed the data. R.V. assisted with islet studies, performed gene expression analyses and analyzed the data. G-S.W. performed the islet neogenesis study and analyzed the data. W.M. and T.A. performed the gut microbiota analysis, analyzed the data and wrote the manuscript. A.S. designed and directed the gut microbiota studies. F.W.S. designed and directed the study, analyzed the data and wrote the manuscript. All authors read and approved 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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Figure 1. CAMP is expressed in the pancreatic β-cell but not the α-cell. Representative dual-labeling immunohistochemistry images for CRAMP (red) and islet hormones (green) are displayed. (a) Insulin (rat islet), (b) glucagon (rat), (c) somatostatin (rat), (d) insulin (sterile rat embryo) and (e) insulin (adult germ-free rat). Representative immunohistochemistry images for (f) CRAMP (NOD mouse islet) and (g) CAMP/LL-37 (nondiabetic human islet). Bars represent 20 µm. Hoechst staining is shown in blue.

Figure 2. Camp gene and CRAMP protein expression are down-regulated in the diabetes prone BBdp rat. Quantification of relative (a) Camp gene expression in BBc versus BBdp isolated rat islets at 30 days and 55 days of age as noted. BBc: n=3; BBdp: n=3. Data represent the mean ± SEM. *p<0.05 by Student’s t-test. (b) Representative immunohistochemistry images of CRAMP+ cells in the control BBc, non-inflamed non-diabetic BBdp and insulitic non-diabetic BBdp islet (100 days old). The lower right panel depicts an islet without insulitis that was identified in the same animal with infiltrating immune cells in other islets. Although this islet did not display overt signs of insulitis, CRAMP expression has been lost.

Figure 3. CAMP/LL-37 treatment stimulates calcium mobilization and islet hormone secretion in rat islets. (a) Quantification of relative Camp gene expression following treatment with glucose, as indicated. *p<0.05 vs. 2.8 mM, **p<0.01 vs. 2.8 mM, †p<0.05 vs. 5 mM by one-way repeated measures ANOVA with post hoc Tukey’s multiple comparison test. (b) Representative calcium mobilization from BBc and BBdp dispersed islet cells treated with CAMP/LL-37 or scrambled CAMP/LL-37 peptide in the presence or absence of extracellular...
calcium as indicated. (c) Proportion of cells responding and (d) latency to respond to the indicated treatments. Experiments were repeated with a minimum of 3 islet preparations with 11 cells each. **p<0.001, ****p<0.0001 using chi squared test with post hoc pairwise comparison with Bonferroni correction (% responders) or one-way ANOVA (latency to respond). (e) Dose-response effect of CAMP/LL-37 treatment on GSIS in islets isolated from BBc rats. Scr: n=3; 1 µM: n=3; 10 µM: n=3. *p<0.05 vs. respective Scr (at 16.7 mM glucose) using two-way repeated measures ANOVA with posthoc pairwise comparison with Sidak’s multiple comparisons test. (f) Comparison of the effect of CAMP/LL-37 treatment on GSIS in BBc versus BBdp isolated rat islets. BBc: n=4 per condition; BBdp: n=4 per condition. *p<0.05 vs. respective Scr (at 16.7 mM glucose) using two-way repeated measures ANOVA and posthoc pairwise comparison with Sidak’s multiple comparisons test. (g) Comparison of the effect of CAMP/LL-37 treatment on glucagon secretion in BBc versus BBdp isolated rat islets. n=3 rats per condition for each strain. *p<0.05 vs. respective Scr (at 2.8 mM glucose + arginine) using two-way repeated measures ANOVA with posthoc pairwise comparison with Sidak’s multiple comparisons test.

Figure 4. CAMP/LL-37 treatment promotes islet regeneration in vivo. (a) Gene expression fold changes in isolated islets treated with 10 µM CAMP/LL-37 for 24 hours compared with islets treated with scrambled CAMP/LL-37 control peptide. p-values calculated using Student’s t-test. Representative immunohistochemistry images for CRAMP expression in (b) rat pancreatic ductal cells and (c) rat neogenic tubular complexes. CAMP⁺ cells are indicated by black arrows. A tubular complex is displayed in the box with CAMP⁺ cells indicated by red arrows. (d) Duct-associated insulin⁺ cells/pancreas area in CAMP/LL-37 treated versus saline treated BBc and BBdp rats as described in “Materials and Methods.” BBc: Saline: n=6; CAMP/LL-37: n=7;
BBdp: Saline: n=7; CAMP/LL-37: n=6. *p<0.05 vs. saline-treated using Student’s t-test. (e)

Representative immunohistochemistry image for CAMP expression in human duct-associated cells. CAMP⁺ cells are indicated by black arrows.

Figure 5. Different rat strains exhibit different gut microbial profiles and CAMP/LL-37 alters the BBdp gut microbiota. (a) Principal coordinate analysis (PCoA) of the gut microbiota of saline-treated BBc strain against BBdp strain treated with either saline or CAMP/LL-37 based on un-weighted UniFrac distances (left) and weighted UniFrac distances (right) conducted by QIIME 1.7. (b) Histogram of the LDA effect size scores and cladogram of differentially abundant taxa between saline-treated BBc and BBdp strains. (c) Histogram of the LDA effect size score and cladogram of differentially abundant taxa between saline-treated and CAMP/LL-37-treated BBdp rats. The relative abundances of the bacterial taxa obtained from the analysis of the Illumina reads were analysed by Kruskal-Wallis test and the identified differential taxa were then used to construct a Linear Discriminant Analysis (LDA) model from which these taxa were ranked according to the effect size with which they differentiate the tested groups. Letters in brackets before the taxa names represent their position on the corresponding cladogram.

Figure 6. CAMP/LL-37 treatment restores the relative abundance of Coriobacteria in BBdp rats to the same level as in BBc rats. Changes in relative abundances of Actinobacteria (a), Coriobacteria (b), Coriobacteriales (c), Coriobacteriaceae, (d) and Adlercreutzia (e) in saline-treated BBc and BBdp rats either treated with saline or CAMP/LL-37. Data represent the mean ± SEM. Mann-Whitney two-tailed test was applied for statistical pairwise comparison (*p<0.05; **p<0.01).
Figure 1.

a. Rat islet - Insulin  
b. Rat islet - Glucagon  
c. Rat islet - Somatostatin  
d. Sterile rat embryo  
e. Adult germ-free rat  
f. NOD mouse  
g. Non-diabetic human
Figure 2.

a. BBc and BBdp 30 days

![Graph showing relative islet cell expression for BBc and BBdp at 30 days.]

b. BBc (100 d), BBdp (100 d), non-inflamed, non-diabetic

![Images showing histological sections of BBc and BBdp conditioned for 30 and 55 days.]

BBdp (100 d), inflamed, non-diabetic

![Images showing histological sections of inflamed BBdp conditioned for 30 and 55 days.]
Figure 4.

a. Table:

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b. Graph showing -Log10(p-value) vs -Log2(Fold Change LL37/Saline) for various genes.

c. Images showing duct-associated insulin+ cells/cm² pancreas.

d. Bar graph showing duct-associated insulin+ cells/cm² pancreas for Saline and LL-37 conditions.

e. Additional microscopic images.
Figure 5.

a. 

b. 

LDA SCORES (log_{10})

C. 

LDA SCORES (log_{10})
Figure 6.

(a) p_Actinobacteria

(b) c_Coriobacteria

(c) o_Coriobacteriales

(d) f_Coriobacteriaceae

(e) g_Adlercreutzia

Relative abundance

BBc Saline BBdp Saline BBdp LL-37

** ns

Saline Saline LL-37

** ns

Saline Saline LL-37

** ns

Saline Saline LL-37

** ns

Saline Saline LL-37

*** ns

Saline Saline LL-37

** ns

Saline Saline LL-37

*** ns

Saline Saline LL-37
Supplementary Table 1. Primers used for constructing the 16S rRNA-V6 Illumina library. The nucleotides in bold represent the barcode sequences.

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Supplementary Figure 1. Camp gene expression is down-regulated in the diabetes prone BBdp rat gut. Quantification of relative Camp gene expression in BBc versus BBdp gut at 50-70 days of age. BBc: n=6; BBdp: n=3. Data represent the mean ± SEM. *p<0.05 by Student’s t-test.
Supplementary Figure 2. Alteration of BBc gut microbiota by CAMP/LL-37 treatment. The relative abundance of bacterial taxa obtained from the analysis of Illumina reads was analyzed using Kruskal-Wallis test, and the identified differential taxa were then used to construct a Linear Discriminat Analysis (LDA) model from which these taxa were ranked according to effect size with which they differentiate from the tested groups. (a) Histogram of the LDA effect size score and cladogram of differentially abundant taxa between saline-treated and CAMP/LL-37-treated BBc rats. (b) Change in relative abundances of *Bacilli*, *Lactobacillales*, *Lactobacillaceae* and *Lactobacillus* between saline-treated and CAMP/LL-37-treated BBc rats. Data represent the mean ± SEM. Mann-Whitney two-tailed test was applied for statistical pairwise comparison (*p<0.05).