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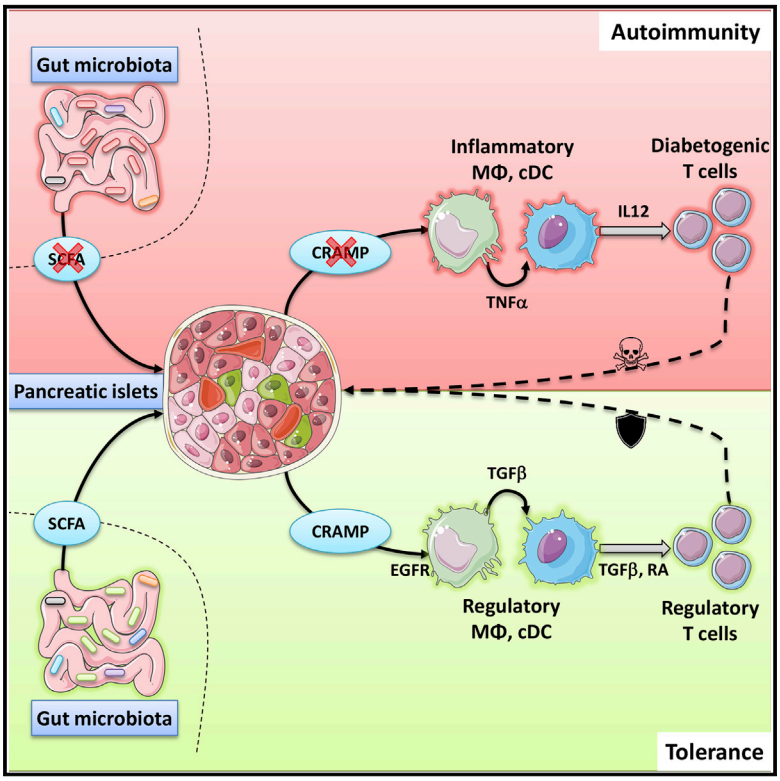
Immunity

Pancreatic β-Cells Limit Autoimmune Diabetes via an Immunoregulatory Antimicrobial Peptide Expressed under the Influence of the Gut Microbiota

Graphical Abstract



Highlights

- Pancreatic β-cells express the cathelicidin-related antimicrobial peptide (CRAMP)
- CRAMP is protective in the adult NOD mice, a model for type 1 diabetes
- CRAMP converts inflammatory into regulatory immune cells in the pancreas
- The gut microbiota via short-chain fatty acids governs CRAMP production by β-cells

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In Brief

It is unclear to what extent antimicrobial peptides expressed by epithelial cells influence immunity in peripheral tissues. Diana and colleagues show that the gut microbiota via short-chain fatty acids promote antimicrobial peptide CRAMP expression in pancreatic α- and β-cells, which protects against autoimmune diabetes in NOD mice

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Pancreatic β-Cells Limit Autoimmune Diabetes via an Immunoregulatory Antimicrobial Peptide Expressed under the Influence of the Gut Microbiota

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SUMMARY

Antimicrobial peptides (AMPs) expressed by epithelial and immune cells are largely described for the defense against invading microorganisms. Recently, their immunomodulatory functions have been highlighted in various contexts. However how AMPs expressed by non-immune cells might influence autoimmune responses in peripheral tissues, such as the pancreas, is unknown. Here, we found that insulin-secreting β-cells produced the cathelicidin related antimicrobial peptide (CRAMP) and that this production was defective in non-obese diabetic (NOD) mice. CRAMP administrated to prediabetic NOD mice induced regulatory immune cells in the pancreatic islets, dampening the incidence of autoimmune diabetes. Additional investigation revealed that the production of CRAMP by β-cells was controlled by short-chain fatty acids produced by the gut microbiota. Accordingly, gut microbiota manipulations in NOD mice modulated CRAMP production and inflammation in the pancreatic islets, revealing that the gut microbiota directly shape the pancreatic immune environment and autoimmune diabetes development.

INTRODUCTION

Antimicrobial peptides (AMPs) are evolutionarily conserved peptides found in almost all plants and animals. In mammals, they are expressed by epithelial cells under steady-state conditions and by infiltrating immune cells during inflammation (Gallo and Hooper, 2012). AMPs are cationic polypeptides with the ability to kill microorganisms by disrupting the integrity of their membrane (Zasloff, 2002). Apart from their antimicrobial activity, AMPs have many functions in immunity, causing either pro-inflammatory or anti-inflammatory effects according to the model being investigated (Mansour et al., 2014). Among AMPs, the role of the cathelicidins (human LL-37 and mouse CRAMP) has been particularly documented in autoimmune diseases such as atherosclerosis, small-vessel vasculitis, systemic lupus erythematous, and psoriasis (Döring et al., 2012; Kessenbrock et al., 2009; Lande et al., 2011; Lande et al., 2007). One common cause of these diseases is an excessive production of cathelicidin by neutrophils, then forming complexes with self-nucleic acids. These immune complexes target plasmacytoid dendritic cells (pDCs) via TLR7 and TLR9 and induce a deleterious production of type I interferon (IFN) (Kahlenberg and Kaplan, 2013). We have also highlighted that the same mechanism occurs during the initiation of autoimmune diabetes in young non-obese diabetic (NOD) mice (Diana et al., 2013). Indeed, around the age of weaning, neutrophils transiently infiltrate the pancreas and produce cathelicidins activating IFN-α-secreting pDCs. However, the concentration of cathelicidins has been shown to be reduced in the serum of patients with autoimmune type 1 diabetes (T1D) compared with healthy subjects suggesting an additional role of cathelicidins in T1D (Brauner et al., 2014). Regarding the various potential source of cathelicidins in peripheral tissues (non-immune versus immune cells) and the opposing immunomodulatory roles of cathelicidins (pro- versus anti-inflammatory), we addressed the question of whether cathelicidins are produced by non-immune cells in the pancreas and might influence the pancreatic immune environment in the context of autoimmune diabetes in NOD mice.

RESULTS

CRAMP Is Secreted in Pancreatic Islets and Its Secretion Is Faulty in Female NOD Mice

To address whether the cathelicidin CRAMP was expressed in pancreatic islets, we evaluated by ELISA the ex vivo secretion

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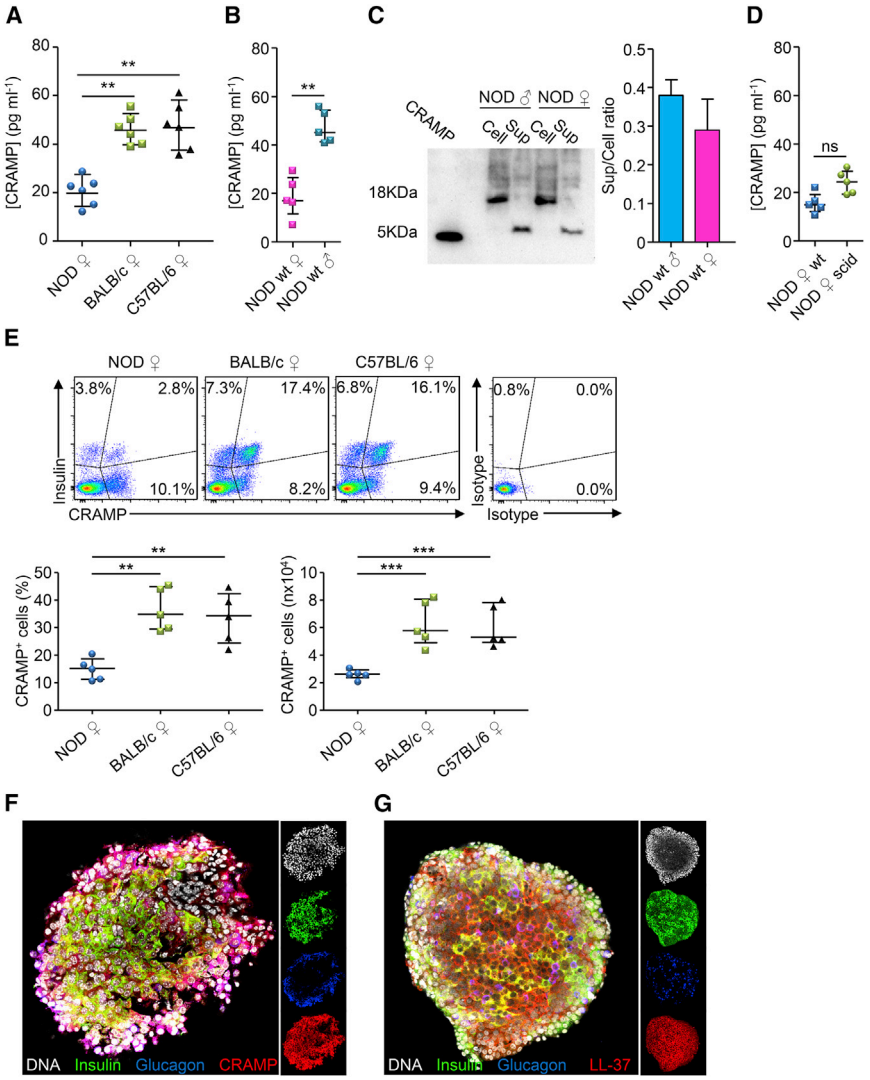

Figure 1. CRAMP Is Secreted in Pancreatic Islets and Its Secretion Is Faulty in Female NOD Mice

(A–D) Pancreatic islets from 8-week-old mice were cultured overnight before CRAMP measurement in the supernatant by ELISA (A, B, D) or Western blot (C). Data are the median ± interquartile range (A, B, D) or are representative (C) from five or six independent experiments with two pooled mice per group in each experiment.

(E) Islet cells were isolated, surface stained for CD45, and intracellularly stained for insulin and CRAMP, and analyzed by flow cytometry. Data are the frequency and absolute number of positive cells among the CD45$^-$ population and are representative or the median ± interquartile range from five independent experiments with two pooled mice per group in each experiment.

(F and G) Isolated islets from male NOD mice (F) or male healthy subject (G) were cytospinned, fixed, and intracellularly stained for insulin (green), glucagon (blue), CRAMP or LL-37 (red), and DNA (gray). Data are representative from eight independent experiments. **p < 0.01, ***p < 0.001.

of CRAMP by islets isolated from 8-week-old female NOD, BALB/c, and C57BL/6 mice. CRAMP was detected in the supernatant of short-term cultured islets from all mouse strains analyzed. However, its secretion was unexpectedly lower in the NOD strain compared to the non-autoimmune strains (Figure 1A). The production of CRAMP was also lower in the islets from female NOD mice compared to their male counterparts (Figure 1B), suggesting a role for CRAMP in diabetes development since male NOD mice develop a reduced incidence of the disease. By Western blot, we observed that both the cytoplasmic pro-form (18KDa) and the secreted mature form (5KDa) of CRAMP were produced by the islets of male and female NOD mice (Figure 1C). We observed a similar ratio between the pro- and the mature forms in male and female mice excluding a defect in the post-translational processing of the peptide in female NOD mice (Figure 1C).

CRAMP Is Expressed by Endocrine Cells in Mouse and Human Pancreatic Islets

As cathelicidins have been shown to be produced by both immune and non-immune cells in the intestine or the skin (Gallo and Hooper, 2012), we investigated the cellular source of CRAMP in the pancreatic islets of 8-week-old prediabetic NOD mice. We have previously identified neutrophils as a source of CRAMP in the islets of young NOD mice; however, neutrophils infiltrated the islets only transiently around weaning period (Diana et al., 2013). We also excluded a role for infiltrating lymphoid cells since islets from WT and lymphoid cell-deficient scid NOD mice secreted similar amount of CRAMP (Figure 1D). By flow cytometry, we observed that the CD45$^+$ infiltrating immune cells did not express CRAMP in adult NOD mice, while the CD45$^-$ non-immune cells did (Figure S1A). Among the non-immune cells, insulin$^+$ β-cells expressed CRAMP together with insulin$^-$ cells, and the frequency and number of CRAMP$^+$CD45$^-$ cells in the islets was lower in NOD mice compared to the non-autoimmune mice (Figure 1E). By microscopy, we further observed that CRAMP co-localized with both glucagon- and insulin-producing cells, supporting that both α- and β-cells constitutively produced CRAMP in the mouse adult islets (Figure 1F). LL-37, the human ortholog of CRAMP, was also expressed by α- and β-cells in human islets from healthy donors (Figure 1G). We failed to detect CRAMP expression by α- or β-cells in 4-week-old mice regardless the strain analyzed (Figure S1C). These data suggested that in the NOD mice CRAMP was sequentially produced in the islets, first by neutrophils in young mice (Diana et al., 2013) and then by endocrine cells in adult mice. Moreover, ex vivo, we observed that cathelicidin colocalized with citrulline in activated neutrophils while we failed to detect citrulline expression by β-cells (Figure S1D). As citrullination alters the immunomodudatory roles of cathelicidin (Kilsgård et al., 2012; Koziel et al., 2014), we hypothesized that CRAMP

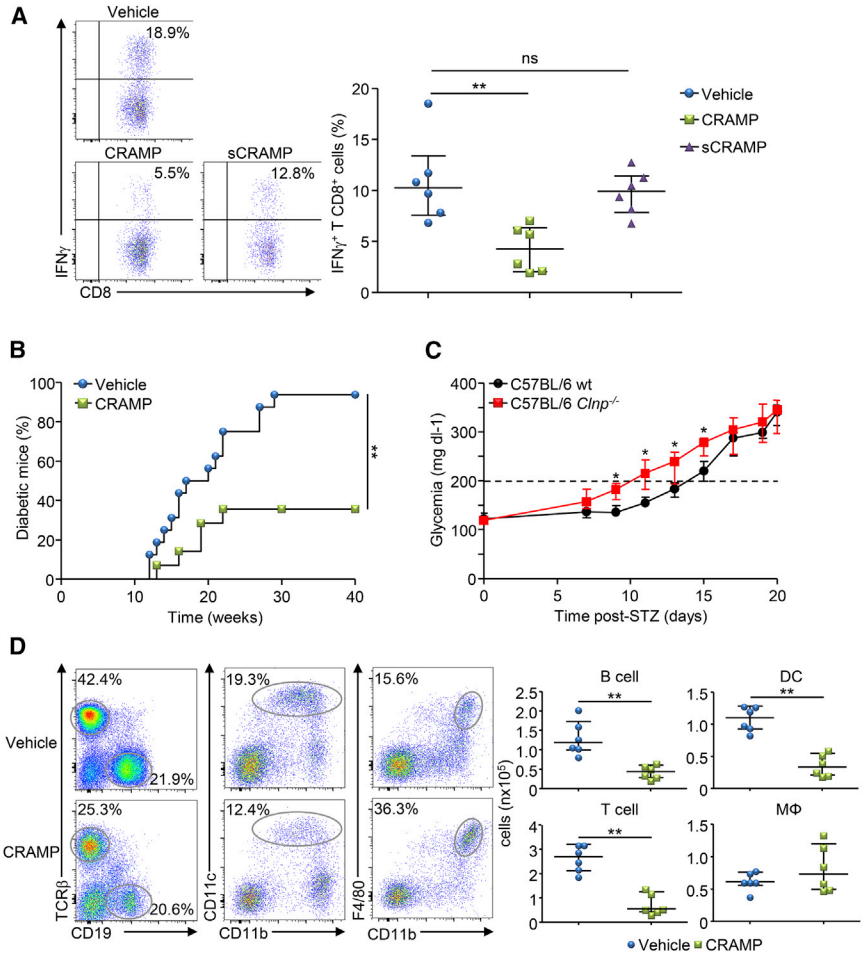

Figure 2. CRAMP Is Protective against Autoimmune Diabetes in Adult NOD Mice

(A) Pancreatic islets were isolated from female NOD mice treated with CRAMP, scrambled CRAMP (sCRAMP), or vehicle (day 10). Cells were stimulated with $IGRP_{206–214}$-loaded DCs, before staining for TCR-β, CD8, and intracellular IFN-γ. Data are the frequency of IFN-γ^+ cells among $CD8^+$ T cell population and are representative or the median ± interquartile range from four independent experiments with two independent mice per group in each experiment.

(B) Female NOD mice were treated twice a week with CRAMP and incidence of diabetes was followed, n = 12 mice per group.

(C) C57BL/6 WT or *Cnlp*$^{-/-}$ littermate mice were treated with multiple low-dose streptozotocin and blood glucose level was followed (day 0: day of the first injection). Data are the median ± interquartile range with 8 independent mice per group. Diabetes was diagnosed in mice with blood glucose level > 200 mg dl^{-1}.

(D) Immune cell populations in the pancreatic infiltrate from female NOD mice treated with CRAMP or vehicle (day 2) were determined by flow cytometry. Data are the frequency of gated cells ($CD19^+$ B cell, $TCR\beta^+$ T cell, $F4/80^-$ $CD11b^+$ $CD11c^+$ DC, and $F4/80^+$ $CD11b^+$ macrophage) among the $CD45^+$ population or the absolute number of cells per mouse. Data are representative or are the median ± interquartile range from six independent experiments with two pooled mice per group in each experiment. *p < 0.05, **p < 0.01, ***p < 0.001.

produced by β-cells might have different roles on the pancreatic immune environment than CRAMP from neutrophils. Collectively, these data showed that endocrine cells produced CRAMP in the islets of adult mice, but this production was faulty in female NOD mice. These data prompted us to further investigate the immunomodulatory role of CRAMP in adult NOD mice.

CRAMP Is Protective against Autoimmune Diabetes in Adult NOD Mice

As CRAMP production was defective in the pancreatic islets of female adult NOD mice, we investigated the effect of CRAMP injection on diabetes development in these mice. Intraperitoneal administration of CRAMP but not of scrambled CRAMP (sCRAMP) reduced the frequency of pancreatic IFN-γ^+CD8^+ T cells specific of the β-cell antigen islet-specific glucose-6-phosphatase catalytic subunit related protein $(IGRP)_{206–214}$ and dampened the incidence of autoimmune diabetes (Figures 2A and 2B). To determine the impact of CRAMP-deficiency on autoimmune diabetes development, we followed the development of multiple low-dose streptozotocin- (MLD-STZ)-induced diabetes in the CRAMP-deficient *Cnlp*$^{-/-}$ and WT C57BL/6 mice (Nizet et al., 2001). We observed an accelerated incidence of diabetes in the CRAMP-deficient mice compared to WT mice (Figure 2C). CRAMP expression was not detected in the infiltrating immune cells from MLD-STZ-treated C57BL/6 mice excluding a role of CRAMP from these cells in MLD-STZ-induced diabetes (Figure S1B). To decipher whether CRAMP regulates the diabetogenic immune response, we analyzed the pancreatic immune infiltrate in CRAMP-treated female NOD mice. Two days after treatment, we observed a reduction in the total number of infiltrating immune cells and particularly a decreased number of B cells, T cells, and conventional dendritic cells (cDCs) in CRAMP-treated but not sCRAMP-treated NOD mice (Figures 2D and S2A). In contrast, the frequency of macrophages increased, while their total number remained unchanged. No difference was observed in the pancreatic lymph nodes (PLN) or the spleen except for an increased frequency and number of medullary macrophages in the PLN (Figures S2B and S2C), supporting a pancreas-specific effect of CRAMP treatment. Together these data supported a protective effect of CRAMP treatment against autoimmune diabetes by altering the pancreatic immune infiltrate in adult NOD mice.

CRAMP Induces a Phenotypic Switch in Pancreatic Macrophages

As the number of pancreatic macrophages remained similar in CRAMP-treated NOD mice, we evaluated how CRAMP might alter their phenotype. Macrophages play a crucial role in

diabetes development through the induction of pancreatic inflammation (Calderon et al., 2008), and the transfer of regulatory macrophages prevents diabetes in female NOD mice (Parsa et al., 2012). We observed that CRAMP treatment in female NOD mice rapidly decreased the frequency and number of pancreatic inflammatory macrophages ($F4/80^+CD11c^+CD206^-$), while the frequency and number of regulatory macrophages ($F4/80^+CD11c^-CD206^+$) were enhanced as shown by a reduced ratio of inflammatory/regulatory macrophages (Figure 3A). This effect was dose-dependent (Figure S3A), remained observed 7 days after treatment (Figure S3B), and also observed with the human cathelicidin LL-37, but not with the scrambled mouse or human cathelicidins (Figure S3C). In order to compare the phenotype of pancreatic macrophages between autoimmune and non-autoimmune mice, we injected STZ that induces a rapid recruitment of macrophages into the islets of both mouse strains (Diana et al., 2013). While STZ maintained a high ratio of inflammatory/regulatory macrophages in female NOD mice, it resulted in the recruitment of regulatory macrophages into the islets of non-autoimmune strains (Figure 3A). Conversely, STZ-injection in CRAMP-deficient C57BL/6 mice resulted in the recruitment of inflammatory macrophages, confirming a role of CRAMP in governing the phenotype of pancreatic macrophages (Figure 3B). Functionally, CRAMP-induced pancreatic macrophages harbored a low expression of the inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-12 (IL-12), while a high expression of the regulatory cytokines IL-4 and active transforming growth factor-β (TGF-β) (LAP) was evident (Figures 3C and S3D). A similar phenotype was observed in the PLN, but not in the spleen, supporting a pancreas-specific effect of CRAMP (Figure S4). In addition, as described for tolerogenic gut-associated myeloid cells, CRAMP-induced pancreatic macrophages exhibited a high aldehyde dehydrogenase (ALDH) activity reflecting retinoic acid (RA) production (Guilliams et al., 2010) (Figure 3D).

CRAMP Regulates the Pi3K Pathway in Pancreatic Macrophages

Next, we attempted to determine through which receptor CRAMP exerted its regulatory effect on pancreatic macrophages. In adult NOD mice, nearly all the pancreatic macrophages expressed the epidermal growth factor receptor (EGFR) contrary to DCs or T cells (Figure 4A). By utilizing specific inhibitors (AG1478, A438079, and PB10) against known surface-receptors for cathelicidin (EGFR, P2X7R, and FPR2, respectively), we determined that EGFR was required for the immunoregulatory effect of CRAMP on pancreatic macrophages (Figure 4B). However, as we did not observe a complete reversion of the effect of CRAMP after EGFR blockade, we cannot exclude the involvement of other receptors. Next we determined the molecular pathway regulated by CRAMP in pancreatic macrophages, by analyzing the expression of SHIP1, a SH2-containing inositol-5′-phosphatase critical in macrophage programming via the regulation of the phosphoinositide 3-kinase (Pi3K) pathway (Parsa et al., 2006). Twelve hours after CRAMP treatment, SHIP1 expression in pancreatic macrophages increased (Figure 4C), and the blockade of SHIP-1 with 3 α-aminocholestane (3AC) (Brooks et al., 2010) prevented the induction of regulatory macrophages by CRAMP (Figure 4D); both data supporting that CRAMP induced regulatory macrophages via SHIP1 induction. Accordingly, the phosphorylation of Akt and NFκBp65, two downstream key molecules of the Pi3K pathway inducing pro-inflammatory cytokine gene expression in macrophages (Zhou et al., 2014), was reduced in CRAMP-induced pancreatic macrophages compared with macrophages from untreated NOD mice (Figure 4E). Together, these results supported that CRAMP regulated pancreatic inflammatory macrophages via SHIP-1 induction regulating the Pi3K pathway.

CRAMP Induces Pancreatic Regulatory Dendritic Cells

CDCs are key players in autoimmune diabetes pathogenesis, transporting β-cell antigens from the pancreatic islets to the draining lymph nodes and presenting these self-antigens to autoreactive T cells (Ganguly et al., 2013). We observed that CRAMP treatment of adult NOD mice induces pancreatic regulatory cDCs characterized by low expression of costimulatory markers (Figure 5A) and a regulatory cytokine profile with a low level of IL-12, a high level of active TGF-β (Figure 5B), and a high ALDH activity (Figure 3E). CDCs with a regulatory cytokine profile were also observed in the PLN, but not in the spleen, of CRAMP-treated NOD mice (Figure S4). However, increased ALDH activity in cDCs and macrophages was observed only in the islets of CRAMP-treated NOD mice (Figure S4). Together, these data supported that CRAMP induced regulatory cDCs in the pancreatic islets of adult prediabetic NOD mice.

CRAMP Generates Pancreatic Regulatory T (Treg) Cells

Both regulatory macrophages and cDCs have the potential to induce Treg cells necessary for long-term protection against autoimmune diabetes (Tang and Bluestone, 2008). Eight days after CRAMP treatment, we observed an increased frequency and number of both $Foxp3^+$ Treg cells and IL-10-producing Tr1 cells in the islets, but not in the PLN or the spleen, of CRAMP-treated female NOD mice (Figures 5C and S5). CRAMP also stimulated the expression of regulatory molecules (TGF-β and CD103) by Treg cells in the islets, but not in the PLN (Figures 5D and S6A). CRAMP-induced pancreatic Treg cells expressed low amounts of neuropilin compared with Treg from vehicle- or sCRAMP-treated NOD mice supporting that these Treg cells originated from the conversion of naive T cells into inducible Treg cells (Yadav et al., 2012) (Figure S6B). This result was confirmed in vitro using purified pancreatic myeloid cells ($CD11b^+$ cells) cultured with monoclonal naive autoreactive T cells devoid of Treg cells ($CD62L^+CD25^-CD4^+$ T cells) isolated from the spleen of BDC2.5 TCR transgenic NOD mice. We observed that myeloid cells from CRAMP-treated NOD mice induced a higher number of Treg cells compared to their counterparts from vehicle-treated NOD mice (Figure 5E). Accordingly with the high ALDH activity of CRAMP-induced myeloid cells (Figures 3D and 3E), this induction of Treg cells was dependent on RA as revealed by the use of the RA-receptor inhibitor LE135 (Figure 5E). Likely due to their inability to produce RA, myeloid cells from the PLN or the spleen of CRAMP-treated NOD mice failed to induce Treg cells (Figure S6C). Finally, using neutralizing anti-CD25 antibody, we observed that CRAMP treatment in prediabetic NOD mice was no more protective against diabetes in absence of functional Treg cells (Figure S6D). Together, our data demonstrated that

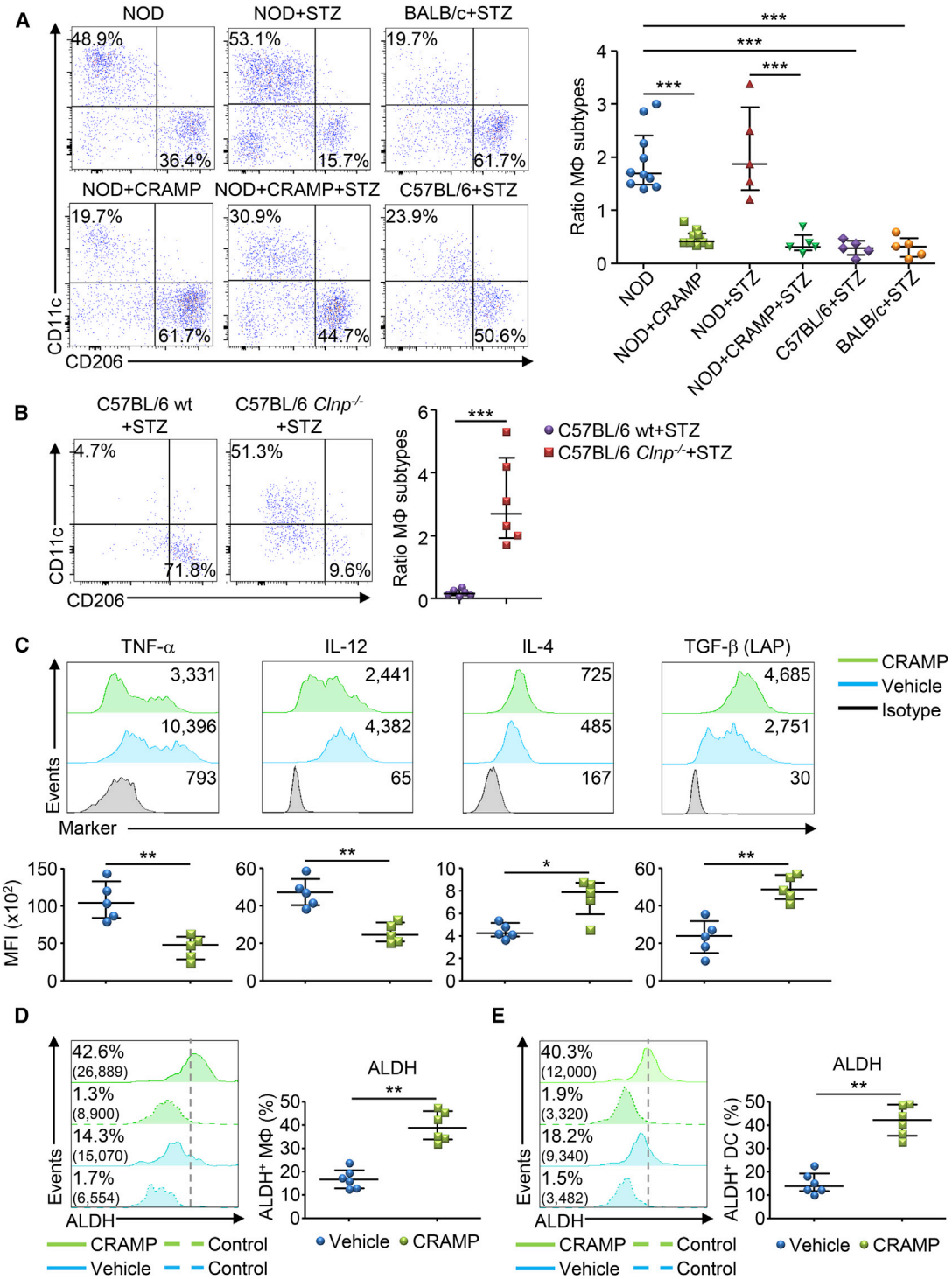

Figure 3. CRAMP Induces a Phenotypic Switch in Pancreatic Macrophages

(A and B) Macrophage sub-populations in the pancreatic infiltrate from female 8-week-old mice treated with CRAMP (day 2) and/or with streptozotocin (day 1). Data show the ratio between the frequency of inflammatory and regulatory macrophages. Data are representative or are the median ± interquartile range from five independent experiments with one or two independent mice per group in each experiment.

(C–E) Cytokine production and ALDH activity in pancreatic macrophages were analyzed in female NOD mice treated with CRAMP or vehicle (day 2). Islet cells were isolated, stimulated with LPS for 4 hr, and stained for CD45, CD11b, F4/80, and LAP; fixed and stained for TNF-α, IL-12, and IL-4 (C) or cells were isolated, stained for ALDH activity, and then stained for the surface markers (D and E). Data shown were obtained after gating on macrophages ($F4/80^+$ $CD11b^+$) in (D) or dendritic cells (($F4/80^-$ $CD11b^+$ $CD11c^+$) in (E). Data are representative or are the median ± interquartile range from five or six independent experiments with two pooled mice per group in each experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

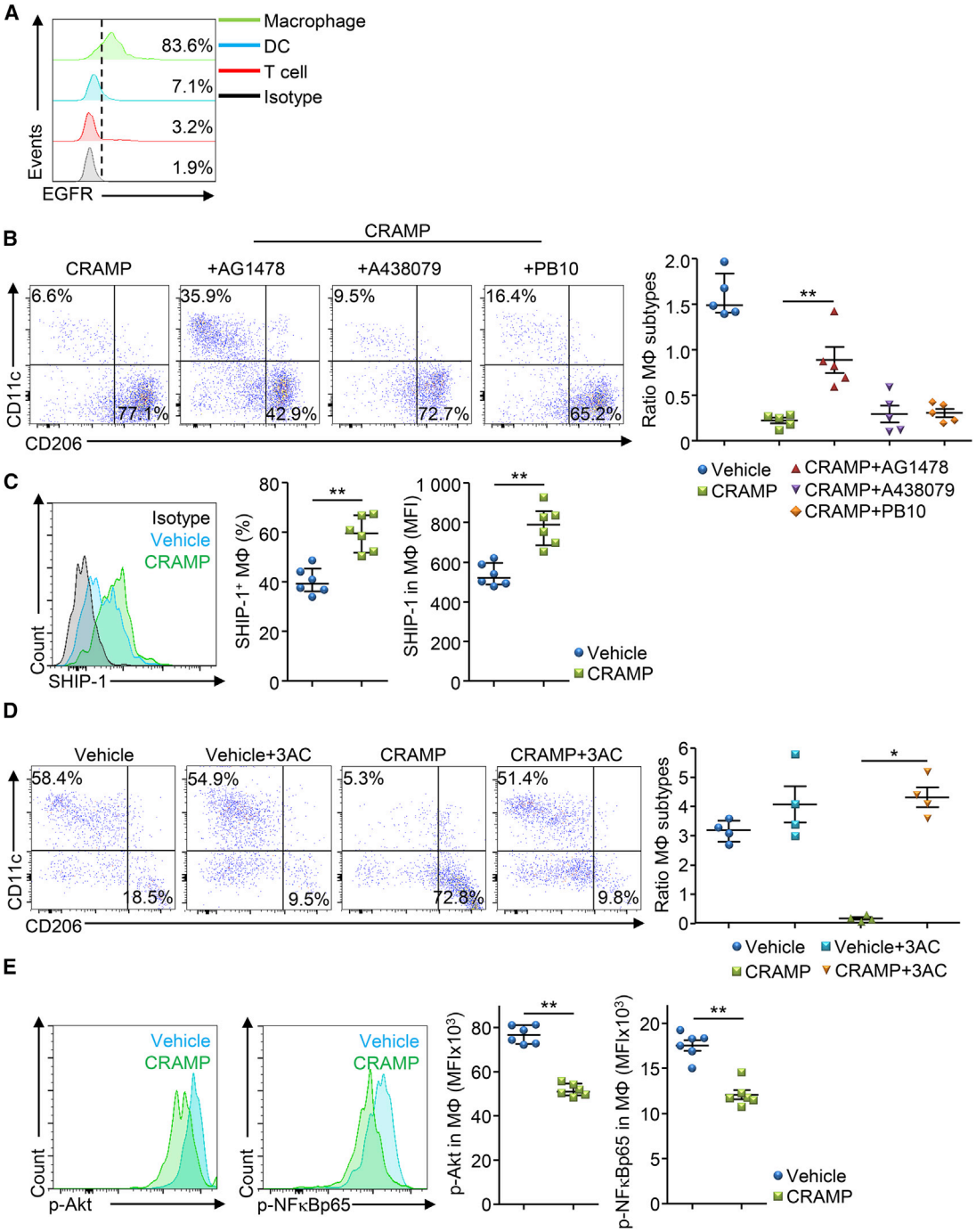

Figure 4. CRAMP Regulates the Pi3K Pathway in Pancreatic Macrophages

(A) Pancreatic macrophages expressed EGFR. Islets cells were isolated from 8-week-old female NOD mice and stained for CD45, CD11c, CD11b, F4/80, and EGFR. Data show the frequency of EGFR$^+$ cells in each population and are representative of three independent experiments with two pooled mice per group in each experiment.

(B) Macrophage sub-populations in the pancreatic infiltrate from female NOD mice treated with CRAMP and with various CRAMP-receptor inhibitors (AG1478, A438079, and PB10 for EGFR, P2X7R, and FPR2, respectively) (day 2). Data are representative or are the median ± interquartile range from five independent experiments with two pooled mice per group in each experiment.

(C and E) Expression of SHIP1, p-Akt, and p-NFκBp65 in the macrophage population from the islets of female NOD mice treated with CRAMP (day 1). Islet cells were isolated and stained for CD45, CD11b, F4/80, and then for SHIP1, p-Akt, and p-NFκBp65. Data are representative and the median ± interquartile range from three independent experiments with two independent mice per group in each experiment.

(D) Macrophage sub-populations in the pancreatic infiltrate from female NOD mice treated with CRAMP and with the SHIP-1 inhibitor 3AC (day 2). Data are representative or are the median ± interquartile range from four independent experiments. *$p < 0.05$, **$p < 0.01$.

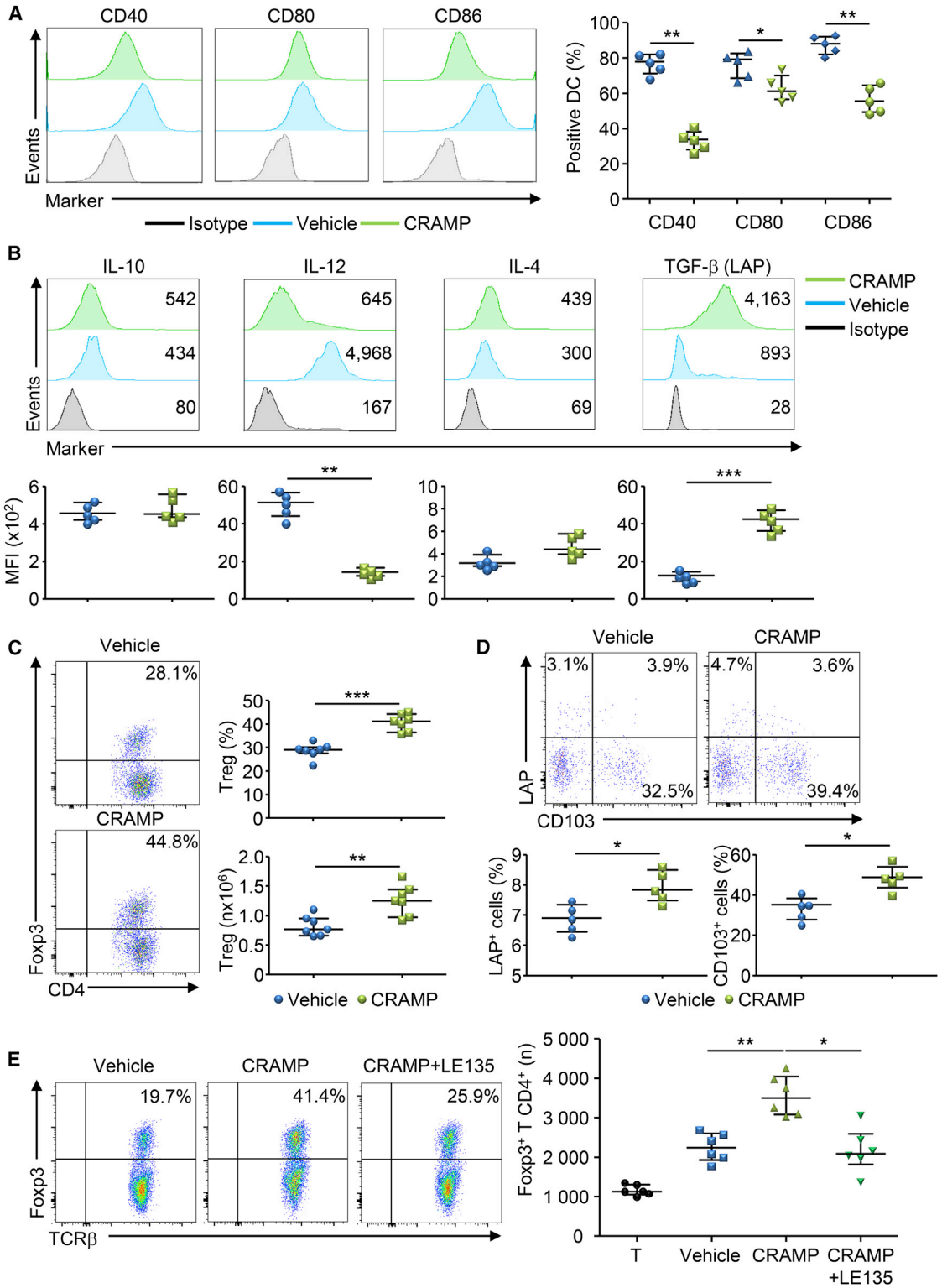

Figure 5. CRAMP Induces Regulatory Dendritic Cells and T Cells in the Pancreatic Islets

(A) Surface phenotype of cDCs from the pancreatic islets of female NOD mice treated with CRAMP or vehicle (day 2) was determined by flow cytometry. Data shown were obtained after gating on dendritic cells (F4/80$^-$ CD11b$^+$ CD11c$^+$). Data are representative and the median ± interquartile range from five independent experiments with two pooled mice per group in each experiment.

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CRAMP regulated the pancreatic immune environment in prediabetic NOD mice, inducing myeloid and lymphoid regulatory cells preventing autoimmune diabetes.

CRAMP Production by β-cells Is Controlled by Short-Chain Fatty Acids

Finally, we aimed to determine why CRAMP production by endocrine cells was defective in female NOD mice. In mammals, short-chain fatty acids (SCFAs), produced by specific gut bacteria, was demonstrated to induce cathelicidin expression in colonocytes, lung epithelial cells, and gastrointestinal cells (Kida et al., 2006; Schauber et al., 2004; Schauber et al., 2003). Consequently, we hypothesized that the reduced pancreatic CRAMP production in female NOD mice might be due to a lack of endogenous stimulation of endocrine cells by SCFAs. By GC-MS, we observed that the amount of SCFAs, and particularly of butyrate, in the feces and the blood of female NOD mice were lower than in the male NOD mice or female BALB/c or C57BL/6 mice (Figure 6A and S7A). While butyrate was not detectable in the islets from untreated mice, after intraperitoneal (i.p.) injection of butyrate, butyrate concentration increased in the islets and the blood but remained lower in the female NOD mice than in the other mice (Figure S7A). Injection of butyrate to female NOD mice increased pancreatic CRAMP production in a dose-dependent manner (Figures 6B and S7B) and addition of butyrate or other SCFAs to islet culture also increased CRAMP production, supporting a direct effect of SCFAs on endocrine cells (Figures 6C and 6D). The G protein-coupled receptors (GPR)43 and GPR41, two receptors for SCFAs (Brown et al., 2003), have been described to be expressed by β-cells (Regard et al., 2007). Adding pertussis toxin prior to the addition of SCFAs to islet culture, we confirmed that the induction of CRAMP by SCFAs required functional GPRs (Figure 6D). According to its stimulatory effect on CRAMP production, we showed that butyrate mimicked the immunoregulatory effects of CRAMP in the islets, decreasing the ratio of inflammatory/regulatory macrophages and increasing the frequency of ALDH$^+$ DCs and Treg cells (Figures 6E–6G). The injection of butyrate to SZT-treated CRAMP-deficient C57BL/6 mice failed to increase the frequency of pancreatic regulatory macrophages, supporting that the effect of butyrate on pancreatic macrophages was mediated via CRAMP induction (Figure S7C). Finally, butyrate treatment of prediabetic NOD mice dampened the frequency of pancreatic diabetogenic IFN-γ^+CD8$^+$ T cells and reduced the incidence of diabetes (Figures 6H and 6I). These data supported that CRAMP production by β-cells is controlled by SCFAs via GPRs and such pathway is defective in the female NOD mice.

Gut Microbiota Modulates CRAMP Production in the Pancreatic Islets

Because SCFAs are produced by specific gut bacteria during dietary fibers fermentation, we addressed the role of the gut microbiota in the control of pancreatic CRAMP production. We treated male NOD mice per os with broad-spectrum antibiotics (ABX) to deplete these mice of their gut microbiota, and 10 days after treatment, we observed that pancreatic CRAMP production decreased (Figure 7A). Next, we transferred gut microbiota from male NOD mice or from female BALB/c or C57BL/6 mice to female NOD mice, and 10 days after fecal transfer, we observed that pancreatic CRAMP production increased in grafted female NOD mice (Figure 7A). Accordingly with the immunoregulatory role of CRAMP, ABX treatment of male NOD mice increased the inflammatory/regulatory macrophage ratio in the islets, while gut microbiota transfer from male to female NOD mice decreased this ratio in female NOD mice (Figure 7B). The pro-inflammatory effect of ABX treatment in male NOD mice was reversed by subsequent CRAMP treatment, supporting that the gut microbiota controlled the phenotype of pancreatic macrophages via CRAMP induction (Figure 7C). As expected, ABX treatment increased the incidence of diabetes in male NOD mice, while gut microbiota transfer from male to female NOD mice reduced the incidence of diabetes (Figures 7D and 7E). Together, our results supported that gut microbiota modulated the pancreatic immune environment via SCFAs and CRAMP induction and thus controlled the development of autoimmune diabetes.

DISCUSSION

Our study demonstrates that the gut microbiota via SCFAs controls the production of the antimicrobial peptide CRAMP by the pancreatic endocrine cells in adult mice. CRAMP harbors positive immuno-regulatory effect on pancreatic macrophages and cDCs maintaining immune homeostasis in this tissue via Treg cell induction. Thus, in female NOD mice, a defective production of SCFAs resulted in low amounts of pancreatic CRAMP, an unopposed pancreatic inflammation, and the development of autoimmune diabetes.

The role of AMPs, and more specifically of cathelicidins, in immunity appears to be complex with both anti-inflammatory and pro-inflammatory properties (Hilchie et al., 2013). Cathelicidins are directly chemotactic for various innate immune cells such as neutrophils, macrophages, or cDCs and also promote the secretion of chemokines by epithelial cells (Oppenheim and Yang, 2005). Cathelicidins have been shown to suppress the pro-inflammatory activity of TLR ligands in macrophages via various mechanisms: ligand sequestration by binding to LPS

(B) Cytokine by pancreatic cDCs were analyzed in female NOD mice treated with CRAMP or vehicle (day 2). Islet cells were isolated, stimulated with LPS, and stained. Data shown were obtained after gating on dendritic cell (F4/80$^-$ CD11b$^+$ CD11c$^+$). Data are representative or are the median ± interquartile range from five or six independent experiments with two pooled mice per group in each experiment.

(C–E) Regulatory T cells were analyzed in the islets from female NOD mice treated with CRAMP or vehicle (day 8). Cells were stained for CD45, TCRβ, CD4, CD103, and LAP, fixed, and then stained for Foxp3. Results show the frequency and absolute number of Treg cells (C, Foxp3$^+$) among the CD4 T cell population or results are the frequency of the gated cells among the CD45$^+$ Foxp3$^+$ CD4$^+$ T cell population (D). Data are representative or are the median ± interquartile range from three independent experiments with two or three independent mice per group in each experiment. (E) Pancreatic myeloid cells isolated from CRAMP-treated NOD mice induce Treg cells in vitro. Myeloid cells (CD11b$^+$) were isolated from the islets of female NOD mice treated with CRAMP or vehicle and cultured for 3 days with naive BDC2.5 T cells with the presence of TGF-β and with the presence or not of the RA inhibitor LE135. Then cells were stained for TCR-β and CD4, fixed, and then stained for Foxp3. Data show the number and frequency of Treg cells (Foxp3$^+$) among the CD4 T cell population. Data are the median ± interquartile range from six independent experiments with four pooled mice per group in each experiment. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

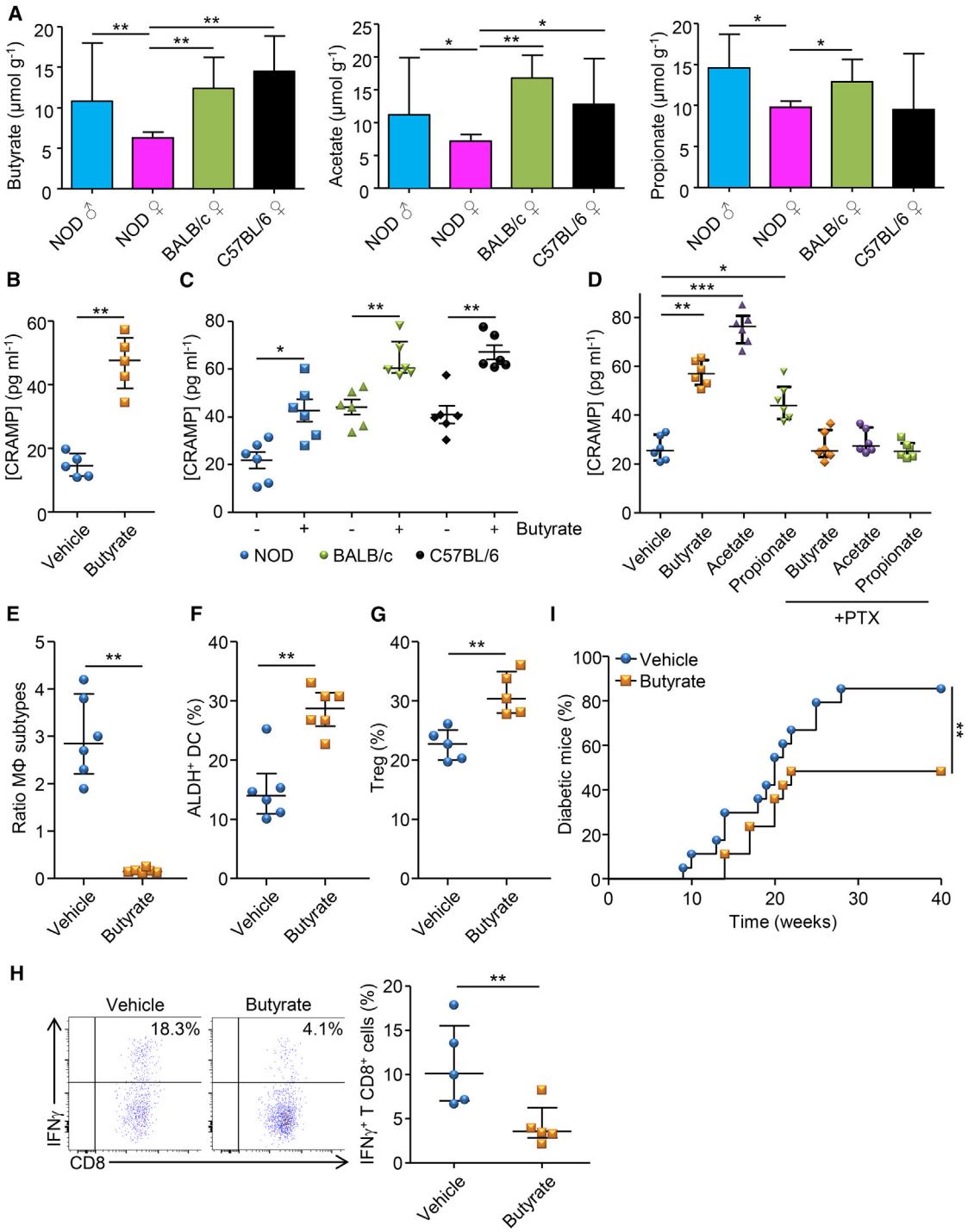

Figure 6. CRAMP Production by β-Cells Is Controlled by Short-Chain Fatty Acids

(A) Concentration of SCFAs in the feces of 8-week-old female and male NOD, BALB/c, and C57BL/6 mice was performed by GC-MS. Data are the median ± interquartile from six independent mice per group.

(B, E–G) Eight-week-old female NOD mice were treated with butyrate and 3 days later CRAMP production was analyzed in the short-term islet culture (B), the subtypes of pancreatic macrophage (E), or the ALDH activity in pancreatic DCs (F); pancreatic Treg cell frequency was analyzed 8 days after butyrate treatment (G). Data are representative or are the median ± interquartile range from five or six independent experiments with two pooled mice per group in each experiment.

(C and D) Islets were isolated from 8-week-old female mice and treated in vitro with SCFAs and/or pertussis toxin (PTX), and CRAMP production was evaluated by ELISA. In (D), islets from female NOD mice were used. Data are the median ± interquartile range from six independent experiments with two pooled mice per group in each experiment.

(legend continued on next page)

(Larrick et al., 1995); inhibition of the translocation of NF-κB subunits (Mookherjee et al., 2006) or perturbation of the MAPK and MyD88 pathways (Pinheiro da Silva et al., 2009). In line with our present results, the human cathelicidin has been shown to reduce pro-inflammatory activity of inflammatory macrophages, while preserving anti-inflammatory activity of regulatory macrophages in the lungs (Brown et al., 2011). Similar anti-inflammatory effects have been described for cDCs in a mouse model of allergic contact dermatitis (Di Nardo et al., 2007). Together, these results suggest that in case of infection, cathelicidins would first promote the innate defense by recruiting innate immune cells and then dampen the activity of these cells to avoid immunopathologic tissue damage. The pro-inflammatory activity of cathelicidins has been particularly described in autoinflammatory and autoimmune contexts such as atherosclerosis, psoriasis, systemic lupus erythematous, and initiation of autoimmune diabetes (Kahlenberg and Kaplan, 2013). In these contexts, the proinflammatory activity of cathelicidins is linked to their ability to bind extracellular self-nucleic acids and to potentiate the activation of type I IFN secreting-pDCs via TLR7/9. In these diseases, cathelicidins are produced by activated neutrophils producing neutrophil extracellular traps (NETs) with major consequences for the function of cathelicidins (Pinegin et al., 2015). First, their local concentration in the tissue would be high since immature neutrophils store high amounts of cathelicidins in their granules and release them after activation. It has been estimated that one hundred thousand activated neutrophils secrete around 10 ng ml^{-1} of cathelicidin in 15 min culture (Jann et al., 2009), whereas we observed in the present study that similar number of islet cells secrete around 80 pg ml^{-1} of cathelicidin in overnight culture. Second, we suggest that, as many proteins of the NETs (Wang et al., 2009), cathelicidins produced by activated neutrophils might be citrullinated, while cathelicidins expressed by endocrine cells were not and it has been shown that this post-translation modification suppresses the immunoregulatory properties of cathelicidins (Kilsgård et al., 2012; Koziel et al., 2014). Collectively, these data support that the diverse cellular source and associated post-translational modifications of cathelicidins might explain their opposite roles in inflammation. This hypothesis might reconcile our previous (Diana et al., 2013) and present studies, demonstrating two opposing roles of cathelicidins in different stages of development of autoimmune diabetes. Citrullinated cathelicidin produced by NETotic neutrophils in young NOD mice would initiate the disease via the activation of pDCs, while non-modified cathelicidin produced by endocrine cells in adult mice would regulate infiltrating immune cells and prevents the development of the disease.

In the present study, we show that the production of CRAMP by endocrine cells is dependent of SCFAs and their associated receptors GPR41 and GPR43. These receptors have been shown to be expressed by β-cells (Bahar Halpern et al., 2012) but their functions on these cells remained unclear. In other inflammatory context, stimulation of GPR43 by SCFAs was required for the normal resolution of inflammation, because GPR43-deficient mice showed exacerbated or unresolving inflammation in models of colitis, arthritis, and asthma (Maslowski et al., 2009). The anti-inflammatory role of SCFAs has been also described in allergic inflammation in the lung (Trompette et al., 2014) and in colitis (Smith et al., 2013). Additionally, butyrate is also anti-inflammatory via its ability to promote differentiation of colonic and peripheral Treg cells (Arpaia et al., 2013; Furusawa et al., 2013).

Here we also demonstrates that the gut microbiota via SCFAs directly shape the immune environment in the pancreas regulating autoimmune diabetes. A role for the gut microbiota has been proposed in various autoimmune diseases including T1D via the modulation of both the function of the colonic epithelium and the gut immune system (Mathis and Benoist, 2012). The gut microbiota of T1D animal models and T1D patients is altered with a lower proportion of butyrate-producing bacteria species (i.e., Firmicutes Clostridium) than controls (de Goffau et al., 2013; Giongo et al., 2011; Roesch et al., 2009). Furthermore, the partial protection against autoimmune diabetes observed in the male NOD mice compared to the female counterpart has been associated with difference in the composition of their gut microbiota (Markle et al., 2013; Yurkovetskiy et al., 2013). In these studies, the transfer of gut microbiota from male to female NOD mice confers protection against diabetes that is attributed to hormonal-dependent mechanism. However, male microbiota transferred to female also results in an increased proportion of butyrate-producing bacteria in the gut of female NOD mice. Altogether, these data support that the gut microbiota might regulate the development of autoimmune diabetes via hormonal-dependent and SCFA-dependent complementary pathways.

Diet plays a crucial role in the production of SCFAs in the gut by shaping the composition of the microbiota and by being the fuel for the production of SCFAs from dietary fibers (Thorburn et al., 2014). T1D is a complex autoimmune disease under polygenic control, but its development also depends on various environmental factors such as microbes, pollutants, and diet (Bach and Chatenoud, 2012). Thus, it is tempting to speculate that the rising incidence of T1D and other autoimmune diseases observed in western countries may be associated with nutritional changes that have appeared during the last century. More importantly is the reduced consumption of fruit and vegetables rich in fibers necessary for the maintenance of a "healthy" microbiota. In conclusion, our study suggests that the simple manipulation of our gut microbiota via the use of specific diet might directly impact on the production of AMPs in peripheral tissues via SCFAs, thus maintaining the local immune homeostasis and prevent the development of autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice and Treatments

Female and male BALB/c, C57BL/6J, C57BL/6J *Cnlp*$^{-/-}$, NOD, and NOD scid mice between 8 and 10 weeks of age were used, bred, and housed

(H) Islets were isolated from female NOD mice treated with butyrate, or vehicle (day 10). After 8-day culture, cells were stimulated with $IGRP_{206-214}$-loaded DCs, before staining for TCR-β and CD8 and IFN-γ. Data are the frequency of IFN-γ^+ cells among $CD8^+$ T cell population and are representative or the median ± interquartile range from four independent experiments with two independent mice per group in each experiment.

(I) Female 8-week-old NOD mice were treated twice a week with butyrate and incidence of diabetes was followed, n = 12 mice per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

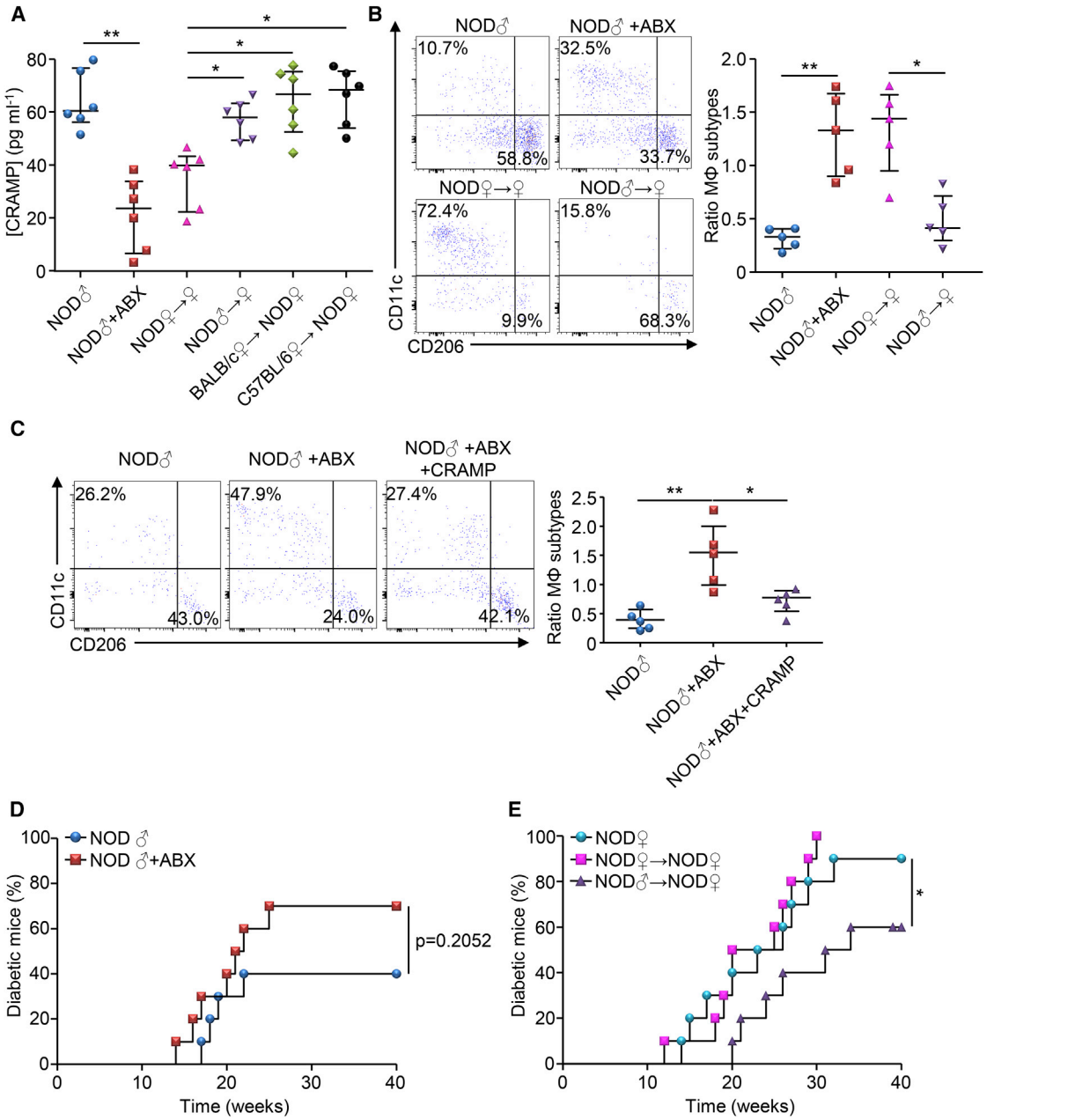

Figure 7. Gut Microbiota Modulates CRAMP-Production in the Pancreatic Islets

(A and B) Six-week-old male NOD mice were treated for 7 days with antibiotic cocktail (ABX) or 6-week-old female NOD mice were transferred with female or male gut microbiota from various mouse strains (→). Islets were isolated and cultured overnight to measure CRAMP secretion by ELISA (A) or macrophage sub-populations in the islets was directly analyzed by flow cytometry (B). Data are representative or are the median ± interquartile range from 5 or 6 independent experiments with 2 pooled mice per group in each experiment.

(C) Six-week-old male NOD mice were treated for 7 days with antibiotic cocktail (ABX) and then with CRAMP (100 μg/mouse). Islets were isolated and macrophage sub-populations in the islets was directly analyzed by flow cytometry. Data are representative or are the median ± interquartile range from five independent experiments with two pooled mice per group in each experiment.

(D) Six-week-old male NOD mice were treated for 7 days with antibiotic cocktail (ABX) and incidence of diabetes was followed, n = 10 mice per group.

(E) Six-week-old female NOD mice were transferred with female or male gut microbiota from 8-week-old NOD mice and incidence of diabetes was followed, n = 10 mice per group. *$p < 0.05$, **$p < 0.01$.

in specific pathogen-free conditions. C57BL/6J *Cnlp*$^{-/-}$ mice were purchased from the Jackson Laboratory before housing and breeding in our laboratory. The C57BL/6J *Cnlp*$^{-/-}$ strain was maintained as heterozygote animals to provide genetically matched WT and KO littermate mice. $CRAMP_{1-39}$, scrambled (s) $CRAMP_{1-39}$, LL-37, and sLL-37 (all from Innovagen) were administrated i.p. at the dose of 100 μg diluted in 200 μl of vehicle (PBS-1% H_2O). See Supplemental Experimental Procedures for complete description of the treatments used. All animal experimental protocols

were approved by the ethic committee for animal experimentation (CEEA34.JD.046.12).

Spontaneous Diabetes Incidence

Eight-week-old NOD female mice received two injections of CRAMP (2 × 100 μg) during one week or two injections of sodium butyrate (2 × 1 mg) or two injections of vehicle (PBS-1% H_2O). Overt diabetes was defined as two positive urine glucose tests, confirmed by a glycemia > 200 mg dL^{-1}. Glukotest kit was purchased from Roche. Glucose tests and measure of glycemia were performed in a blind fashion.

Preparation of Pancreatic Islets

Pancreata were perfused with a solution of collagenase P in HBSS-1%HEPES (0.75 mg mL^{-1}, Roche), then dissected free from surrounding tissues. Pancreata were digested at 37°C for 8 min. Digestion was stopped by adding HBSS-10% FCS-1% EDTA followed by extensive washes. For flow cytometry analysis, islets were purified on a discontinuous Ficoll® gradient (Sigma-Aldrich) and then cells were released from the islets by incubation at 37°C for 6 min in non-enzymatic cell dissociation solution (Sigma-Aldrich). For islet cultures, to avoid potential contamination by exocrine tissue, islets were purified by handpicking in three consecutive baths of HBSS-10% FCS supplemented with 1% DNase 1.

Flow Cytometry

Single cell suspensions were prepared from various tissues and were stained for 30min at 4°C after FcγRII/III blocking with anti-CD16/CD32 monoclonal antibody (mAb). Staining buffer was PBS containing 2% FCS, 0.5% EDTA, and 0.1% sodium azide. See Supplemental Experimental Procedures for complete description of the antibodies used. For cytokine expression by macrophages and DCs, cell suspension was incubated 5 hr at 37°C with LPS (1 mg ml^{-1}, Sigma-Aldrich) in the presence of a protein transport inhibitor cocktail (eBioscience). For ALDH activity measurement, cells were stained using the ALDEFLUOR kit according to the manufacturer's protocol (Stemcell). For measurement of diabetogenic $CD8^+$ T cell response, isolated cells were stimulated for 4 hr at 37°C with bone-marrow derived DCs loaded with $IGRP_{206–214}$ peptide in presence of protein transport inhibitor cocktail. For regulatory T cell detection, cells were surface stained with anti-TCRβ, -CD4 (BD, RM4-5), fixed and then stained for Foxp3 expression, utilizing the Foxp3 staining kit (eBioscience). In all experiments dead cells were excluded using Fixable Viability Dye (eBioscience). Stained cells were analyzed on a Becton Dickinson Fortessa flow cytometer.

Western Blot Analysis of CRAMP

After overnight culture islet-free supernatant and islets were recovered. Islets were lysed with 60% acetonitrile in 1% trifluoroacetic acid (TFA) overnight at 4°C. On the second day, islet lysates in acetonitrile were lyophilized and the dried cellular components were dissolved in H_2O with 0.1% TFA. Polypeptides were then enriched from acidified islet supernatants or islet lysates using reverse-phase chromatography (OASIS™ cartridge, Waters®). Western blot analysis of CRAMP was performed in the islet supernatant (secreted) and in the islet lysates (cellular), synthesized CRAMP peptide was used as control. See Supplemental Experimental Procedures for complete description of the method used.

CRAMP Measurement by ELISA

One hundred handpicked pancreatic islets were cultured in 200 μl of a solution of DMEM, 10% FBS, 1% penicillin/streptomycin overnight, and CRAMP concentration were analyzed using the mouse CRAMP ELISA kit (Mybiosource). In some experiments, sodium butyrate (500 μg ml^{-1}) and/or pertussis toxin (100 ng ml^{-1}, Sigma-Aldrich) were added to the islet culture.

Cytology

Handpicked pancreatic islets were seeded on SuperFrost Gold Plus microscope slide by cytospin. Then, islets were fixed, permeabilized, and after stained with anti-insulin pAb (Abcam), anti-glucagon mAb (Abcam) and anti-CRAMP pAb or anti-LL-37 pAb (Innovagen), overnight at 4°C. After washing, second-step reagents were applied: anti-guinea pig-AlexaFluor488 (insulin), anti-mouse-Alexa647 (glucagon), and anti-rabbit-AlexaFluor555 pAbs (CRAMP and LL-37) (Invitrogen). Nucleuses were stained with DAPI. Image acquisition was performed on Necker Institute Imaging Facility using a Leica SP8 confocal microscope.

In Vitro Treg Cell Induction

All cells were magnetically isolated using MACS cell separation system (Miltenyi). $CD62L^+$ $CD4^+$ $CD25^-$ BDC2.5 T cells (4 × 10^4 cells per well) from splenocytes of BDC2.5 TCR transgenic NOD mice were incubated with 2 × 10^3 myeloid cells ($CD11b^+$) obtained from pancreatic islets of NOD mice treated with CRAMP or vehicle. Culture were performed for 4 days in complete IMDM with 5 ng ml^{-1} recombinant human IL-2 (R&D), 1 ng ml^{-1} recombinant mouse TGF-β (R&D) and 20 ng ml^{-1} of peptide 1040-51, a mimotope of BDC2.5 T cells. In some conditions retinoic acid inhibitor LE135 (1 μM, Santa Cruz) was added to the culture.

SCFA Measurement by Gas Chromatography–Mass Spectrometry

The short-chain fatty acids (SCFAs)—acetate, propionate and butyrate—present in the fecal content of male and female NOD mice were analyzed by GC/MS. See Supplemental Experimental Procedures for complete description of the method used.

Statistical Analysis

Diabetes incidence was plotted according to the Kaplan-Meier method. Incidences between each group were compared with the log-rank test. Reported values are median ± interquartile range as indicated. Comparison between each group was performed using the non-parametric Mann-Whitney U-test. p values < 0.05 were considered statistically significant. All data were analyzed using GraphPad Prism v5 software.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.07.013.

AUTHOR CONTRIBUTIONS

J.S. and J.D. performed experiments and analyzed data, with general assistance from L.F. for Western blot analysis, A.M.v.D. and E.L. for experiments with *Cnlp*$^{-/-}$ C57BL/6 mice, and L.S. for confocal microscopy. Y.C. and P.v.E. provided intellectual inputs. B.A. and J.D. designed and interpreted experiments and wrote the paper.

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