Internship report

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Study of antimicrobial peptides in the development of auto-immune type 1 diabetes

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ABSTRACTS
Type 1 diabetes (T1D) is an autoimmune disease characterized by the destruction of insulin-secreting pancreatic β cells by autoreactive T lymphocytes. Yet, the exact role of innate immune cells in the development of this disorder is insufficiently understood. An emerging theme in the field of autoimmunity is the role of antimicrobial peptides in the development of autoimmune disorders such as psoriasis and lupus erythematosus. A study published earlier this year showed that when secreted by neutrophils, the murine cathelicidin-related antimicrobial peptide (CRAMP) has a role in the initiation of T1D in non-obese diabetic (NOD) mice. However, its role in the later phase (pre-diabetic) of the disease has never been investigated before. In this work, we show that exogenous CRAMP induces an immunoregulatory environment in the pancreatic islets of pre-diabetic NOD mice. Indeed, injection of CRAMP induces the recruitment of regulatory macrophages, T cells and dendritic cells in pancreatic islets, thus possibly reducing the onset of the disease. We also observed that CRAMP can be directly produced by pancreatic β cells, and that this production is partly defective in NOD mice compared to other non-autoimmune strains. These data suggest that pancreatic β cells may use the antimicrobial peptide CRAMP as a defense against autoimmune cells and a defect in the production of this peptide might favor the development of T1D in NOD mice.

**Key words:** Type 1 diabetes, autoimmunity, antimicrobial peptides, cathelicidin-related antimicrobial peptide (CRAMP), macrophages, dendritic cells, insulin, β cells, aldehyde dehydrogenase (ALDH)

Le diabète auto-immun de type 1 se déclare lorsque des lymphocytes T autoréactifs détruisent les cellules β sécrétrice d’insuline dans les îlots pancréatiques (ou îlots de Langerhans). Le rôle exact des cellules du système immunitaire inné dans le développement de cette maladie reste encore à découvrir. Ces dernières années, le domaine de l’auto-immunité a vu naître un intérêt particulier pour les peptides antimiobiens : en effet, il a été prouvé qu’une mauvaise expression de ces derniers est responsable de l’apparition de maladies auto-immunes, tels le psoriasis ou le lupus érythermal. Cette année, le rôle du peptide antimicrobien CRAMP a été montré pour la première fois dans l’initiation du diabète de type 1 chez la souris NOD (non-obese diabetic), lorsqu’il est sécrété par les neutrophiles. Cependant, son implication dans la phase prédébétique n’a cependant encore jamais été décrite. Ici, nous montrons que l’injection de CRAMP exogène est capable de diminuer l’inflammation des îlots pancréatiques en induisant des macrophages, des lymphocytes T et des cellules dendritiques régulateurs, ce qui pourrait repousser l’apparition de la maladie. Nous montrons également que CRAMP est produit directement par les cellules β du pancréas, et que sa production chez la souris NOD est moins importante que chez d’autres espèces murines non auto-
immunes. Ces résultats semblent montrer que les cellules β seraient capable d’utiliser le peptide antimicrobien CRAMP pour se défendre contre les réactions auto-immunes et qu’un défaut de production favoriserait l’apparition du diabète de type 1 chez la souris NOD.

**Mots-clés** : diabète de type 1, auto-immunité, peptides antimicrobiens, cathelicidin-related antimicrobial peptide (CRAMP), macrophages, cellules dendritiques, insuline, cellules β, aldehyde dehydrogenase (ALDH)
ABBREVIATIONS
ALDH: Aldehyde dehydrogenase

AMPS: Antimicrobial peptides

BMDCs: Bone marrow dendritic cells

CRAMP: Cathelicidin-related antimicrobial peptide

cDCs: Conventional dendritic cells

DCs: Dendritic cells

EGF-R: Epidermal growth factor receptor

EGF-R a: EGF-R antagonist

FBS: Fetal bovine serum

GM-CSF: Granulocyte macrophage colony-stimulating factor

HLA: Human leukocyte antigen

IL: Interleukin

LAP: Latency associated peptide

LPS: Lipopolysaccharide

M1: Inflammatory macrophages

M2: Regulatory/suppressive macrophages

mAb: Monoclonal antibodies

MCSF: Macrophage colony-stimulating factor

MFI: Mean fluorescent intensity

NOD: Non-obese diabetic

NOD SCID: Non-obese diabetic, severe combined immuno-deficiency

NOR: Non-obese resistant

P2RX7: Purinergic receptor P2X, ligand-gated ion channel, 7
P2RX7 a: P2RX7 antagonist

PBA: Phenylbutyrate

PBS: Phosphate buffered saline

pDCs: Plasmacytoid dendritic cells

RBC: Red blood cells

SCFA: Short chain fatty acids

T1D: Type 1 diabetes

T2D: Type 2 diabetes

TGF: Transforming growth factor

TLR: Toll-like receptor

TNF: Tumor necrosis factor

T\textsubscript{reg}: regulatory T cells

Tr1: IL-10-producing subpopulation of T\textsubscript{reg} (IL-10 is a regulatory cytokine)
INTRODUCTION
1) Diabetes

1.1) The main types of diabetes: type 1 and type 2

Type 1 diabetes (T1D), or formerly called insulin-dependent diabetes, is the direct result of the insulin-producing pancreatic β cells destruction by auto-immune cells. This disorder is generally diagnosed in childhood or adolescence with three principal symptoms: polydypsia (high need to drink), polyphagia (insatiable hunger) and polyuria (frequent need to urinate). A hyperglycemia is observed at the same time, forcing the patient to inject himself or herself exogenous insulin as a lifetime replacement treatment [1].

Type 2 diabetes (T2D) is a trouble of storage, utilization and metabolism of food sugar because of insulin resistance and a relative dysfunctional secretion of insulin. The intensity of the symptoms is usually lower than in T1D; that is why the hyperglycemia is usually diagnosed by chance, very often in overweighted adults [2]. Nevertheless the frequency of T2D in young patients is becoming more and more important, because of the lack of exercise and richer diets observed in Western countries [3],[4].

1.2) Epidemiology of type 1 diabetes

According to the World Health Organization (WHO), 347 million people are diabetic and 5-10% of them have T1D.

European (EURODIAB) and international (DIAMOND) programs aim at determining the incidence of type 1 diabetes. They report that the incidences around the world are completely different from one country to another, although there is a clear North-South gradient (Figure 1) [1],[2],[5]. For example, in Europe, the incidence in Macedonia is 3.6 per 100 000 per year against 43.9 per 100 000 per year in Finland.

The world incidence (apparition of new cases for a certain period of time) of T1D is constantly increasing every year (about 3.7% in 2013) and is becoming one of the most common diseases in children and adolescents, according to WHO.
1.3) Genetics of type 1 diabetes

About 50 loci in the Human genome are known to influence the development of T1D [6]. Even though the Human Leukocyte Antigen (HLA) complex plays a major part in its onset with an approximate 60% contribution, the disease cannot be predicted thanks to the presence or the absence of one particular gene [6,7]. Other loci are thought to enhance the genetic susceptibility to the disease, including the genes coding cytotoxic T lymphocytes antigen 4 (CTLA-4), interleukin (IL)-2 receptor A, tyrosine phosphatase PTPN22 and insulin [6].

Studies on families and twins have shown that both genetic and environment play a role in the development of the disease [8,9]: people having a first-degree relative have a 0.05% risk to develop T1D, whereas in the whole population of the United States of America the average risk is about 0.003%. Monozygotic and dizygotic twins are known to have a disease concordance rate of around 40% and 8% respectively [9]. Interestingly, it is important to note that depending on which parent has T1D, the child does not have the same risk to develop the disease: 2% if the mother is diabetic, 7% if the father is [9]. In summary, despite the important role of genetics in its development, T1D is nowadays considered as a multifactorial disease not only based on inheritance [10].

1.4) Environmental factors

The fact that monozygotic twins have only 40% of concordance rate and the variance of incidence in populations sharing the same genetic background shows that environment plays a considerable role in the development of the disease. For example, the frequency of susceptibility and
protective HLA alleles are similar among children from Finland and Karelia (a neighboring region in Russia); however, the incidence of T1D in Finland is six times higher than in Karelia \[11\]. In the same line, first-generation immigrant Pakistani children born in the United Kingdom have a similar rate of T1D to the local population but, this rate is ten times higher than the incidence in Pakistan \[12\].

Some studies support the fact that environmental stresses, in particular infantile obesity, increase the insulin need hence the over-stimulation and damages of islet cells \[1\],\[13\]. Other works attribute the incidence variations of T1D to the lack of immune-system stimulations and claim that infections could prevent autoimmune diseases (and T1D) \[14\].

Finally, depending on the pathogen involved, studies show that bacterial or viral infections can whether accelerate or retard the onset of T1D \[15\].

1.5) The immunopathology of type 1 diabetes

As described before, type 1 diabetes is caused by the progressive selective destruction of the pancreatic β cells by auto-immune cells recruited in the inflamed tissue (called insulitis). Nevertheless, the destruction of β cells within the pancreas represents the last step of T1D development started many years before and requires both innate and adaptive immune systems. Importantly, among these cell types, some cells promote and other regulate the disease, offering various therapeutic targets.

1.5.1) Adaptive immune cells: T lymphocytes

There are many evidences supporting the critical role of T cells in diabetes pathogenesis, as the disease can be transferred by T cell populations or auto-reactive T cell clones. Indeed, diabetes can be transferred from diabetic NOD mice (Non-Obese Diabetic; see section 1.6) to NOD SCID mice (devoid of B and T lymphocytes) by the transfer of splenic CD4+ and CD8+ T cells \[16\],\[17\]. It is important to emphasize that both CD4+ and CD8+ T cells are necessary for the destruction of pancreatic β cells (Figure 2). Further studies have evidenced the prevention of autoimmune type 1 diabetes by inducing T cell tolerance using anti-CD3 antibodies (CD3 is a specific T lymphocytes marker that leads to T cell tolerance in absence of costimulation) (Figure 2) \[18\].

1.5.2) Innate immune cells: macrophages and dendritic cells

Innate cells are able to produce pro-inflammatory or suppressive cytokines, conditioning the environment for specific T lymphocytes to differentiate into effector T cells or regulatory T cells (Figure 2).
The deleterious role of macrophages in type 1 diabetes is supported by the fact that the depletion (reduction) of this cell type by toxic drugs or the prevention of macrophage recruitment in the pancreatic islets prevent the development of the disorder \cite{16,19,20}.

It has also been observed that within the infiltrate, macrophages are able to produce inflammatory cytokines such as IL-12, TNF and IL-1β in NOD mice (see section 1.6) or patients, allowing the recruitment and activation of diabeticogenic CD8+ T cells and the emergence of T1D \cite{20,21}. Interestingly, NOD mice peritoneal macrophages secrete more inflammatory cytokines (especially IL-12) than the same macrophages in NOR mice (Non-Obese Resistant; the genetically closest strain to NOD mice, that do not develop T1D) when stimulated with lipopolysaccharide (LPS) \cite{22}.

The harmful activity of antigen-presenting cells like conventional dendritic cells (cDCs) present in the islets was observed with a better survival of islet-grafted mice in which DCs were depleted (reduced) \cite{23}. Other studies stated that β cell-derived self antigens are captured by cDCs and presented to islet antigen-specific CD4+ T cells in the pancreatic lymph nodes to start the disease (Figure 2) \cite{24}. cDCs are also able to activate diabetogenic T cells by secreting inflammatory cytokines in the pancreatic islets (such as IL-12), which induces type 1 diabetes \cite{25,26}.

Yet, patients born without DCs are known to develop autoimmune disorders \cite{26}; which means that these cells are able to encourage effector T cells lowering, T-cell anergy (unresponsiveness state) or activation of regulatory T-cell (T_{reg}) by secreting TGF-β \cite{27,28}. Indeed, cDCs are responsible for the inhibition of self-reactive effector cells like T cells or the secretion of self-antibodies by B lymphocytes; so called peripheral tolerance \cite{16,26}.

![Figure 2: Type 1 diabetes onset](image)

**Figure 2: Type 1 diabetes onset** \cite{29}
1.6) A murine model for type 1 diabetes: the Non-Obese Diabetic (NOD) mice

Twenty years ago, as researchers were trying to obtain a cataract-prone strain, Makino et al. generated the NOD mice strain\(^\text{[30]-[32]}\). These mice spontaneously develop type 1 diabetes as they grow old: the infiltration begins around 3 to 6 weeks after birth, where only innate immune cells are involved. Then, from 6 to 12 weeks old, begins the pre-diabetic phase with the infiltration of T cells in the pancreatic islets. Finally the diabetic phase starts from 12 to 30 weeks old with a noticeable hyperglycemia caused by the destruction of $\beta$ cells. However females show signs of T1D sooner than males\(^\text{[30]}\). The incidence of the disease in NOD mice is 60 to 80% in females and 20 to 30% in males\(^\text{[30],[32],[33]}\).

As all animal models, the NOD mouse-model presents some flaws (high homogeneity concerning HLA and the development of T1D). However it recapitulates the major steps of the autoimmune diabetes and is a crucial tool for the development of therapeutic strategies against T1D.

1.7) Treatments

Patients with type 1 diabetes cannot be cured yet. However, a few leads are being or have been investigated: so far, the most known and effective substitution therapy for T1D is therapeutic insulin. Since its discovery and isolation in 1922 by Frederick Grant Banting\(^\text{[34]}\), insulin was first isolated from cattle and pork, but is nowadays produced by bioengineered organisms. A very common way for insulin administration is the use of syringes multiple times a day before eating, but this device can be replaced by insulin pumps for a continuous subcutaneous delivery\(^\text{[29],[35]}\). Self-blood monitoring also allows patients to effectively control their glycemia.

Islets transplantations have also been performed, but the lack of donor, the limited survival of grafted-islets (less than 5 years) and the strong immuno-suppressive treatments with important side-effects like renal failure in 7 to 21% of the cases limited their success\(^\text{[29],[36],[37]}\).

Many treatments based on the modulation of the immune system are under further investigations, among them anti-CD3 antibodies to inhibit auto-reactive T cells (whether leading to anergy of diabetogenic T cells or regulatory T cells induction), anti-CD20 antibodies targeting B lymphocytes and other molecules inhibiting pro-inflammatory cytokines (IL-1\(\beta\), IL-6, IL-12, TNF-\(\alpha\))\(^\text{[29]}\).
2) Antimicrobial Peptides

In immunology, antimicrobial peptides (AMPs) occupy a growing place, not only due to their protective role against microbes, but also for their modulatory functions on immune system and cell physiology.

2.1) Biological properties of AMPs and their antimicrobial role

AMPs constitute a very large group of peptides expressed in many living organisms from all kingdoms [38]. One of the firsts AMPs, called gramadicin, was discovered by René Dubos in 1939 in the supernatant of the soil bacteria Bacillus brevis. Its antimicrobial activity was proved on Gram-positive bacteria-infected wounds in guinea-pig, pointing potential clinical use on humans [29].

Other works showed that AMPs are part of plant immune system: purotionin, a protein found in wheat flour, was shown to exhibit antimicrobial actions against fungi and pathogenic bacteria [40],[41].

In mammals, two main families have been enlightened and well-identified: the cathelicidins and the defensins. Various studies showed their antimicrobial effects on viruses, Gram-positive or Gram-negative bacteria, protozoa and fungi in vitro [42]. Depending on the peptide, the maximal effectiveness can be tissue-dependent: for example, α-defensins expressed in the gut and in granulocytes (innate immune cells, namely neutrophils, basophils, eosinophils, mast cells) can destroy some enveloped viruses and bacteria like Salmonella while β-defensins in the skin affect Staphylococcus aureus and Pseudomonas aeruginosa [43],[44]. However, some AMPs exhibit protective effects against several pathogens: it has been shown that a cathelicidin-knockout in mice enhanced their vulnerability to microbes like herpes and vaccinia viruses, group-A Streptococcus and E. coli.

As most AMPs are cationic peptides, they can bind and disrupt the membranes of microorganisms, eventually killing them. One of the reasons that could explain why AMPs do not affect eukaryotic cells is because their membranes contain cholesterol, unlike bacteria and viruses [41]. It also appears that the physiological eukaryotic cell concentration in salt (around 150mM of NaCl) protects them from the action of AMPs at low doses in vivo and in vitro, by increasing concentrations of serum proteins and divalent cations [42],[45].

2.2) Other roles in physiology?

In addition to their antimicrobial functions, several studies have shown the implication of AMPs in many physiological processes.
It appears that these peptides can act as chemoattractants (they can draw specific cells in a given locus) for neutrophils, immature dendritic cells, monocytes or T lymphocytes and even stimulate cytokine production by monocytes and epithelial cells \cite{46}. For example, the chemotactic activity of the human cathelicidin LL-37 recruits inflammatory cytokines-secreting cells such as monocytes, mast cells, T cells, neutrophils and activates dendritic cells (producing inflammatory cytokines and type-1 interferons) \cite{38,47,48}. On the other hand, AMPs can function as inhibitors of the immune response by preventing activation through toll-like receptors (TLRs), expressed on several immune cells and required for the recognition of microbe-derived antigens \cite{49}. Indeed, cathelicidins can block TLR-4 activation in dendritic cells \cite{50}. Moreover, cathelicidins are also able to inhibit pro-inflammatory cytokines: LL-37 is able to suppress the release of TNF-α by macrophages activated via by bacterial molecules such as lipopolysaccharide (LPS) \cite{51}.

Other studies have shown the involvement of some AMPs in wound healing when secreted by epithelial cells. Actually, it has been shown that LL-37 can participate to the re-epithelialization and the revascularization of harmed tissue by recruiting endothelial cells and promoting their proliferation and migration \cite{51}. Some works have demonstrated that cathelicidin-mediated processes are very important for neoangiogenesis (development of new blood vessels) in the skin \cite{38,52,53}.

2.3) Role in diseases

As they interfere with the adaptive and innate immune systems, it has been found that the alteration of antimicrobial peptides secretion can lead to various auto-inflammatory or auto-immune diseases.

For example, in atopic dermatitis, the cutaneous lesions often worsen because of \textit{Staphylococcus aureus} colonization. This colonization has been proved to be more likely with an insufficient expression of some AMPs \cite{54}. An overexpression of these peptides can lead to other skin disorders as well: in rosacea, an excess of LL-37 has been found to enhance the inflammation and aberrant blood vessels development \cite{55}. In autoimmune diseases like systemic lupus erythematosus and psoriasis, the oversecretion of LL-37 by neutrophils and keratinocytes allows the recognition of self-nucleic acids by dendritic cells, inducing the production of type I interferons and the establishment of a chronic inflammation \cite{38,56,57}.
3) Objectives

Understanding the exact role of AMPs in autoimmune disorders is crucial for the development of new treatments. As they occupy a growing place in the field of autoimmunity, it is fair to study their possible role in type 1 diabetes and make them possible tools or targets for new therapies.

Earlier this year, the role of the cathelicidin-related antimicrobial peptide (CRAMP), the murine analog of LL-37, was actually enlightened in the development of the T1D for the first time. This study demonstrates that during the first postnatal weeks in the NOD mice, neutrophils transiently infiltrate the pancreatic islets and secrete CRAMP. CRAMP binds extracellular self-DNA and forms a tripartite complex with anti-DNA and immunoglobulin G produced by pancreatic β cells. This complex is able to target plasmacytoid dendritic cells (pDCs, another subset of DCs) via TLR7/9 leading to the production of type I interferons in the pancreatic islets. These inflammatory cytokines likely activate the cDCs and trigger their migration to the pancreatic lymph nodes where they present pancreatic self-antigens and activate auto-reactive diabetogenic T lymphocytes, initiating the disease.

As the previous study described the role of neutrophil-secreted CRAMP during the initiation of the disease, the role of such AMP during the later phase of diabetes development remains to investigate. Indeed, as described above, AMP can be produced by other immune and non-immune cells and can harbor opposite roles on the immune systems, depending on various parameters. In this study, we focus on the pre-diabetic phase of T1D development in the NOD mice strain (around 8 weeks old) and we hypothesize that CRAMP may be produced by insulin-secreting β cells. We aim at determining the role of this antimicrobial peptide on the physiology of β cells and on the functions of the main immune infiltrating cells present at this age in the pancreatic isles: macrophages, dendritic cells and T lymphocytes.
MATERIAL AND METHODS
Animals

Female NOD mice (spontaneously develop autoimmune diabetes starting at 12 weeks of age) were used at 3 to 10 weeks of age. Female NOD SCID mice (diabetes-resistant, lacking T and B lymphocytes, absence of insulitis) were used at 12 weeks of age. Finally, female BALB/c mice (non-autoimmune-prone mice) were used between 8-12 weeks of age. All animals were maintained in specific pathogen–free conditions in the U1016 facility, Cochin Institute. All experiments were approved by the local ethic authorities.

Reagents

CRAMP (CRAMP_{1-39}, Innovagen®) was diluted in water/PBS (vol/vol) and used at a 10 µg/mL in vitro and 100 µg were intraperitoneally injected per one mouse for in vivo experiments. Phenyl butyrate (PBA, Sigma®) was diluted in PBS and used in vitro at 200 µg/mL. EGF-R antagonist (AG 1478 hydrochloride) or the P2X7R antagonist (A438079 hydrochloride) (both from Tocris used at 500µg/mouse in 300µL PBS) was injected day-2,-1 before CRAMP injection.

Generation of Bone Marrow derived Dendritic Cells (BMDCs)

BMDCs were grown from bone marrow progenitors. The bone marrow was flushed out of mice femur and tibia with HBSS, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S). After centrifugation (350 xg/min for 5 min at 4°C), the pellet was resuspended in 1mL of lysis Buffer for 30 seconds to eliminate the red blood cells. After wash, 1.5x10^6 cells/mL were put in well of 24-well plate (corning®) containing 1mL of BMDCs medium (RPMI, 10% FBS plus GM-CSF (R&D, 10 ng/mL). CRAMP (50µg/mL) was added 8 days after putting the cells in culture and LPS (100ng/mL) 24 hours later.

BMDCs were prepared from progenitor cells isolated from the femurs and tibias of female mice between 6 and 8 wk of age. Briefly, after lysis of the red blood cells, BM cells (1.5x10^6/mL) were plated on 24-well low-cluster plates in RPMI 1640 medium containing 10% FCS (fetal calf serum) and 1% penicillin/streptomycin and supplemented with 10 ng/ml murine GM-CSF (R&D Systems) for 8 days. Half-medium changes took place on day 4 of culture. CRAMP (10 µg/mL) was added on day 6 in
all experiments. In some experiments, LPS (100 ng/mL, Sigma®) were added 12 h before DC analysis on day 8.

**Isolation of pancreatic islets**

Mice were killed and pancreases were perfused with 3 mL of a solution of collagenase P (Roche Applied Science®, 0.75 mg/mL) in HBSS 1% Hepes, then dissected with no surrounding tissue. Pancreases were then digested at 37 °C for 8 min. Digestion was stopped by adding cold HBSS followed by several washes. Islets were then purified on a discontinuous Ficoll® gradient and then disrupted adding 1 mL of non-enzymatic cell dissociation buffer (Sigma®) for 8 min at 37 °C. After another wash, the cells were resuspended and ready for flow cytometry analysis.

**Flow cytometry**

Cell suspensions were prepared from various tissues (spleen, pancreatic lymph nodes and pancreas) and were stained at 4 °C in PBS containing 2% FCS and 0.5% EDTA after blocking FcR with 2.4G2 mAb (eBioscience). Surface staining was performed with antibodies all from BD Biosciences or eBioscience (anti-CD11c (clone N418), anti-CD11b (clone M1/70), anti-CD45 (clone 30-F11), anti-LAP (clone TW7-16B4), anti-F4/80 (clone BM8), anti-CD206 (clone 19.2), anti-CD49b (clone DX5), anti-LAG3 (clone C9B7W), anti-CD40 (clone 1C10), anti-CD80 (clone 16-10A1) and anti-CD86 (clone GL1) mAbs). For cytokine detection by intracellular staining, the cell suspension was incubated 4 h at 37 °C with LPS (100 ng/mL) in presence of protein transport inhibitory cocktail (eBioscience). After fixation and permeabilization (BD Fix&Perm), cells were first stained with anti–IL-4, anti–IL-10, anti–IL-12, and anti-TNF-α antibodies. For CRAMP intracellular staining, cells were left unstimulated and stained sequentially with rabbit anti-CRAMP pAb (Innovagen) and guinea pig anti-insulin pAb (Abcam) and then with anti-rabbit-PE and anti-guninea pig-FITC antibodies (Invitrogen). Treg were detected using the anti-mouse/rat Foxp3 staining set (FJK-16s; eBioscience). ALDH activity was measured using the ALDEFLUOR™ kit (STEMCELL™) according to the instructions of the manufacturer. Cell analysis was performed with the BD Biosciences® cell sorter BD FACSARia™ III. All the data were acquired with the BD FACSDiva™ software. Then, all the data were analyzed with the FlowJo® software.
Pancreatic islet culture for CRAMP secretion measurement

After digestion of the pancreas by collagenase P treatment, islets were isolated by handpicking to avoid any contamination by exocrine tissue. Then, islets (100 islets/well/200µL) were cultured overnight in DMEM, 10%FBS, 1% penicillin/streptomycin. The next day LPS (100 ng/mL) or PBA (200 µg/mL) were added for additional 24h. The supernatants were analyzed with an ELISA-CRAMP kit from MyBioSource®.

Statistical analyses

For all experiments, comparison between groups was performed using the non-parametric Mann-Whitney U-test. Reported values are mean +/- SEM. P values < 0.05 were considered statistically significant. All data were analyzed using GraphPad Prism v5 software.
1) CRAMP and β cells

1.1) Pancreatic islet cells secrete CRAMP constitutively and after in vitro stimulation

To test the hypothesis that pancreatic β cells can produce the antimicrobial peptide CRAMP, we isolated by handpicking pancreatic islets from NOD SCID mice and BALB/c mice and we cultured them in vitro overnight. The islets were incubated alone or in presence of phenylbutyrate (PBA) or lipopolysaccharide (LPS), two bacterial-derived molecules known to enhance the production of cathelicidins by several cell types [58]-[60]. The concentrations of CRAMP in the supernatants were measured by ELISA tests (Figure 3). Interestingly, islets from both strains secreted CRAMP constitutively and their secretion was increased in presence of LPS and PBA. Importantly we observed that CRAMP secretion in all condition was higher with BALB/c islets compared to their NOD SCID counterparts (Figure 3). This suggests that the secretion of CRAMP in pancreatic islets is partly defective in the autoimmune NOD mice.

Figure 3: CRAMP secreted by NOD SCID and BALB/c strains islets in vitro. 100 islets from 8 weeks old NOD SCID and BALB/c mice were incubated overnight in DMEM, 10% FBS and 1% penicillin/streptomycin. Then the islets were put in presence of lipopolysaccharide (LPS ; 100 ng/mL) and phenylbutyrate (PBA ; 200 µg/mL) for additional 24 hours. Concentrations of CRAMP were measured in the supernatants by ELISA (*: p<0.05).
1.2) CRAMP expression by β cells is lower in NOD SCID mice than in BALB/c mice in vivo

Once the secretion of CRAMP by islets cells was observed, we evaluated if β cells were responsible for this production in vivo. Intracellular production of CRAMP by pancreatic β cells was measured by flow cytometry, using anti-CRAMP and anti-insulin antibodies (Figure 4). First we observed that insulin⁺ β cells were the main producers of CRAMP among the islet cells (Figure 4A). Secondly, confirming our ex vivo data, we observed that the expression of CRAMP by the β cells was reduced in NOD SCID mice compared to BALB/c mice (Figure 4B). Together these data suggest that β cells produce CRAMP in the pancreatic islets and that this production is reduced in the NOD mice.

Figure 4: CRAMP-expression by β cells in NOD SCID and BALB/c mice in vivo. (A) Intracellular secretion of CRAMP was measured in β cells with flow cytometry using anti-CRAMP and anti-insulin antibodies. NOD SCID and BALB/c mice >12 weeks old were used. BALB/c mice naturally produce more CRAMP than NOD SCID mice in vivo. The frequency of CRAMP⁺ cells among insulin⁺ cells is represented in (B).
2) Role of CRAMP in the modulation of the immune diabetogenic response

The effect of the antimicrobial peptide CRAMP as a modulator of the innate or adaptive immune diabetogenic response had never been investigated; however the immunomodulatory functions of CRAMP and other antimicrobial peptides have been demonstrated in other contexts \[48\]. Thus, we investigated the potential effect of exogenous CRAMP on the various immune cells infiltrating the pancreatic islets during diabetes development.

2.1) Effects of CRAMP on macrophages

2.1.1) Phenotype of pancreatic macrophages in NOD mice islets at various ages.

Macrophages are one of the first cell types to infiltrate the islets during diabetes development in both NOD mice and T1D patients. As NOD mice spontaneously develop T1D growing old, we first evaluated the phenotype of the macrophages in the islets at various ages. In 3 weeks old mice, pancreatic macrophages belonged to the M2 regulatory subtype (CD206+/CD11c-) with a frequency of 60.8% among the macrophage population (Figure 5). However, with age, an increased frequency of the inflammatory M1 subtype was detected: from 17.7% in 3 weeks old mice to 46.1% of the total macrophage population in 9 week old mice. The frequency of regulatory macrophages (M2) progressively declines to reach 29.6% in 9 weeks old NOD mice (Figure 5). These data suggest that the phenotype of the pancreatic macrophages evolves according to the step of development of the disease and that the pre-diabetic phase is characterized by the accumulation of inflammatory macrophages.
2.1.2) The injection of exogenous CRAMP induces regulatory macrophages

As a direct effect of CRAMP on macrophages has been already described in vitro\(^{60}\), we investigated if exogenous CRAMP may influence the phenotype of the pancreatic macrophages in the pre-diabetic NOD mice. Interestingly, we observed that the injection of CRAMP in 10 week-old NOD mice modified the relative frequency of each macrophage subtypes. Indeed, CRAMP injection reduced the frequency of the M1 inflammatory macrophages (from 60.4% in untreated mice to 25.4% in treated mice) and increased the frequency of the M2 regulatory subtype (from 19.0% in untreated mice to 46.8% in treated mice) (Figure 6A). Importantly the effect of CRAMP on macrophage phenotype was dose dependent since growing dose of CRAMP increased the frequency of the macrophages in the islets and increased the frequency of the M2 subtype among the pancreatic population (Figure 6B).

![Figure 6: Injection of CRAMP induces the recruitment of regulatory macrophages and this effect is dose-dependent.](image)

10 week-old mice were treated or not with 100μg/mouse (A) or various doses of exogenous CRAMP (B) 3 days before analysis. The frequency of each subtype among the macrophage population (F4/80\(^+/\)CD11b\(^+\)) is represented in A and the frequency of macrophages among immune cells (CD45\(^+\)) and M1/M2 ratio are represented in (B)( M1 are inflammatory macrophages and M2 regulatory macrophages).
2.1.3) Action of exogenous CRAMP on macrophage-secreted cytokines

To functionally characterize the phenotype of pancreatic macrophages after CRAMP injection, we evaluated their expressions of various cytokines. The cytokine-production of the total macrophage population in the islets was studied, in addition to the pancreatic lymph nodes; where the local antigen presentation to T cells takes place) and the spleen. According to their surface markers, we observed that CRAMP injection strongly decreased the expression of inflammatory cytokines while increased the expression of regulatory molecules. Indeed, we observed a significant decreased expression of TNF-α and IL-12 associated with a significant increased expression of LAP (Latency Associated Peptide) reflecting the secretion of active TGF-β (Figure 7). Indeed, TGF-β is stored in an inactive proform in the cell. The activation is possible when the proform is cleaved into LAP and the active form of TGF-β. We also observed a slight increase in IL-4 expression while the expression of IL-10 remained unchanged (Figure 7). IL-12 and TNF-α production, both inflammatory cytokines, dropped. Interestingly, the same results were obtained with the macrophages from the pancreatic lymph nodes (data not shown) suggesting that CRAMP injection may impact on the activation of the diabetogenic T cell response. Of note, the effect of CRAMP was not systemic since the cytokine profile of splenic macrophages remained unchanged after CRAMP injection (data not shown).

All together these results support that the injection of exogenous CRAMP to pre-diabetic NOD mice leads to the recruitment of regulatory macrophages in the pancreatic islets and lymph nodes.
Figure 7: Cytokine production by macrophages in NOD mice islets after CRAMP injection. Cells from pancreatic islets were stimulated during 4h in presence of LPS and protein transport inhibitor cocktail. Then, cytokine production was evaluated after surface staining for CD11b, F4/80 and LAP and intracellular staining for IL-4, IL-10, TNF-α and IL-12. Data are the mean fluorescence index (MFI) of each molecules for the macrophage population (CD11b⁺ F4/80⁺) (*: p<0.05).

2.1.4) CRAMP acts on macrophages via the EGF receptor

Some studies have shown that CRAMP binds to the epidermal growth factor receptor (EGFR) and P2X7 receptor (P2RX7) at the surface of the epithelial cells and neutrophils respectively, but this had never been described in macrophages. Antagonists for each receptor (EGF-R-a and P2RX7-a) have been used to determine the surface receptor for CRAMP responsible for the phenotype modification of the pancreatic macrophages (Figure 8). We observed that the blocking of EGFR and not of P2RX7 partly abrogated the decreased frequency of M1 and the increased frequency of M2 macrophages observed in the pancreatic islets after CRAMP injection (Figure 8). These data suggest that the effect of CRAMP on pancreatic macrophages may be mediated via EGFR and not by P2RX7.

Figure 8: Exogenous CRAMP binds to EGF receptor on macrophages. Mice were treated with EGFR or P2X7R antagonists 2h before injection of CRAMP. Three days later the phenotype of pancreatic macrophages were determined by flow cytometry. Data are the frequency of inflammatory M1 (CD11c⁺ CD206⁻) or regulatory M2 (CD11c⁻ CD206⁺) macrophages among the macrophage population (CD11b⁺ F4/80⁺) (*: p<0.05).
2.2) Effects of CRAMP on dendritic cells

Dendritic cells (DCs) are professional antigen presenting cells that are crucial for T cell activation in the lymph nodes and are required for the presentation of β-cell antigens to the autoreactive T cells in the pancreatic lymph nodes and their activation.

2.2.1) CRAMP prevents the activation of dendritic cells in vitro

In order to study the effect of CRAMP on DCs, bone marrow derived dendritic cells (BMDCs) from NOD mice were generated in vitro. When activated via TLR ligands, DCs express a high quantity of specific markers on their surface such as co-stimulation markers required for efficient T cell activation. When LPS (TLR4 ligand) was added in the medium, BMDCs expressed more the co-stimulation markers (here CD40, CD80 and CD86), with a clear shift of the spike compared to the control (Figure 9). However, BMDCs treated with CRAMP kept a minimum expression of co-stimulation markers, similar to the control. Interestingly, this treatment of the BMDCs with CRAMP rendered them refractory to the activation by LPS (Figure 9). This result shows that in vitro an early exposition to CRAMP prevents the activation of BMDCs by activating factor such as LPS.

![Co-stimulation markers](image)

**Figure 9: CRAMP prevents the activation of BMDCs in presence of LPS.** DCs were grown in vitro from NOD mice bone marrow progenitors. CRAMP was added 8 days after the cell culture started, and LPS 24h after that. The surface expression of each co-stimulation markers was evaluated by flow cytometry.
2.2.2) Effect of CRAMP on pancreatic dendritic cells in vivo

Since we observed that CRAMP prevents the activation of DCs in vitro we next wanted to determine the cytokine production by pancreatic DCs after CRAMP injection in the NOD mice. We observed that CRAMP injection decreased the expression by the DCs of the inflammatory cytokine IL-12 while it increased the expression of LAP (reflecting the active TGFβ) (Figure 10). No difference was observed for IL-4 and IL-10. In the gut, tolerogenic DCs are well-characterized. Several studies have shown the ability of certain DCs to secrete retinoic acid inducing regulatory T cells ($T_{reg}$), especially $T_{reg}$ Foxp3+ [63]. The production of retinoic acid is controlled by a specific enzyme, the aldehyde dehydrogenase (ALDH); so the presence of ALDH on DCs was considered a marker of a tolerogenic population. After CRAMP injection we observed an increased frequency of ALDH+ DCs in the pancreatic islets (Figure 10). Similar results were obtained in the pancreatic lymph nodes, but not in the spleen (data not shown). These data support that CRAMP injection induces tolerogenic DCs in the pancreatic islets and the pancreatic lymph nodes.

Figure 10: CRAMP induces tolerogenic dendritic cells in NOD mice islets in vivo. 8 week-old NOD mice were analyzed 2 days after injection of CRAMP. The presence of ALDH and cytokines was revealed with surface and intracellular staining respectively (*: $p<0.05$).
2.3) Effects of CRAMP on T lymphocytes

CRAMP favored the regulatory behavior of the macrophages and dendritic cells, part of the innate immune cells. T cells are directly responsible for β cell destruction, thus it was interesting to examine the action of CRAMP on these adaptive immune cells. Injected exogenous CRAMP increased the frequency of T\textsubscript{reg} in NOD mice islets (Figure 11). Indeed the frequency of both T\textsubscript{reg} Foxp3\textsuperscript{+} and Tr1 LAG3\textsuperscript{+} CD49\textsuperscript{+} lymphocytes, two different subpopulations of T\textsubscript{reg}, increased with the presence of CRAMP. In untreated NOD mice, T\textsubscript{reg} Foxp3\textsuperscript{+} and Tr1 LAG3\textsuperscript{+} CD49\textsuperscript{+} frequencies were approximately 30% and 8% respectively, whereas in presence of CRAMP they raised 40% and 16% respectively (Figure 11). This data support that the injection of exogenous CRAMP induces regulatory T cells in NOD mice.

Figure 11: Percentages of Treg Foxp3\textsuperscript{+} and Tr1 in NOD mice islets enhanced with the presence of CRAMP. 8 week-old female NOD mice were injected with CRAMP (100µg/200µL PBS/mouse) 8 and 4 days before analysis. The percentage of positive cells is among the CD4\textsuperscript{+} T cell population (*: p< 0.05).
CONCLUSIONS AND PERSPECTIVES
The cathelicidin CRAMP acts as a relatively good immunomodulator in the pre-diabetic phase. Indeed, this antimicrobial peptide is able to induce regulatory cell phenotypes in both innate (macrophages and DCs) and adaptive (T lymphocytes) immune cells in NOD mice. With time, the population of macrophages in the pancreatic infiltrate in NOD mice increases, especially inflammatory macrophages. The injection of exogenous CRAMP, which most likely binds to the EGF receptor on macrophages, allows the arrival of regulatory macrophages within the insulitis, possibly blocking the onset of T1D in NOD mice. On the same line, CRAMP can prevent the activation of dendritic cells in vitro and induce regulatory ALDH+ dendritic cells in vivo. Finally, CRAMP is able to recruit regulatory T cells, which could protect NOD mice from the disease. Its low secretion by pancreatic β cells in autoimmune mice strains might be one of the reasons the disease develops: in NOD mice, the poor secretion of CRAMP by β cells cannot stop or delay the pancreatic inflammation by inducing tolerogenic or regulatory immune cells, macrophages and dendritic cells in particular, on the front line of the whole process. An ELISA test has shown that CRAMP stimulates the secretion of insulin by β cells, even in NOD mice (data not shown). Altogether, these results show that CRAMP, and maybe other AMPs (cathelicidins or defensins) that have to be studied, could protect from the development of T1D.

The role of the gut microbiome as a stimulator of AMP-secretion is described very well. Indeed, short-chain fatty acids (SCFA), butyrate in particular, are studied for their important role as inflammatory-response and cytokine-release modulators and their ability to prevent immune cell infiltration [64]. Many studies have shown that the gut of NOD mice and patients with T1D are colonized with different strains than non-autoimmune strains or healthy people, which could explain why they develop the disease [65]-[67].

In other words, and regarding this project, the lack of insulin secretion and AMP expressions by β cells to prevent insulitis and the infiltration of immune cells in the pancreas could be the consequence of an insufficient secretion of SCFA by the gut microbiome. That is why transfers of microbiome are under investigation in human and NOD mice [68], treatments involving butyrate and AMP-targeting could be interesting new leads to treat T1D, as previous methods (like islets grafts) failed.
REFERENCES


7. Pociot et al. 2010. Genetics of Type 1 Diabetest: What’s Next? *Diabetes*


40. Balls AK et al. 1942. A crystalline protein obtained from a lipoprotein of wheat flour. *Cereal Chemistry*


46. De Y. et al. 2000. LL-37, the neutrophil granule and epithelial cell-derived cathelicidin, utilizes formyl-peptide receptor-like 1 (FPRL1) as a receptor to chemoattract human peripheral blood neutrophils, monocytes, and T cells. Journal of Experimental Medicine 192:1069–74


ABOUT THE AUTHOR
I am a third-year student of the Frontières du Vivant (FDV) bachelor. I have always wanted to work in the medicine (my wish was to become a surgeon) or the research field. Before entering this program, I first tried to pass the exam for medical studies. The second time I took the exam, I failed very close to the numerous clauses, and felt bad about it. I decided to enter this bachelor because students bathe in research within the first year, with lab internships, new ways to study, etc.

My main interests in Science are neurobiology, stem cells and immunology. During the first two year of the FDV bachelor, we had the opportunity to do one-week long (at least) internships; I was already able to do internships in the neurobiology (in the Ecole Supérieure de Physique et de Chimie Industrielle (ESPCI) in Zolt LENKEI’s lab) and the stem cells fields (in Institut Pasteur, Cellules souches et Développement department, in Shahragim TAJBAKHSH’s lab). That is why for my third-year internship I chose to explore autoimmune disorders, as this is what interests me the most in immunology.