Protection against type 1 diabetes upon Coxsackievirus B4 infection and iNKT cell stimulation: role of suppressive macrophages

Liana Ghazarian¹,², Julien Diana¹,², Lucie Beaudoin¹,², Pär G. Larsson⁵, Raj K. Puri³, Nico Van Rooijen⁴, Malin Flodstrom-Tullberg⁵, and Agnès Lehuen¹,²

¹INSERM, U1016, Hospital Cochin/St Vincent de Paul, Paris, France; ²Université Paris Descartes, Paris, France; Laboratoire d’Excellence INFLAMEX, Sorbonne Paris Cité, ³Tumor Vaccines and Biotechnology Branch, Division of Cellular and Gene Therapies, Center for Biologies Evaluation and Research, Food and Drug Administration, Bethesda, Maryland; ⁴Department of Molecular Cell Biology and Immunology, VU University Medical Center, Amsterdam, The Netherlands, ⁵Center for Infectious Medicine, Department of Medicine, The Karolinska Institute, Stockholm, Sweden

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Correspondence should be addressed to Agnès Lehuen, INSERM U1016, Hôpital Saint-Vincent de Paul/Cochin, 84 Avenue Denfert-Rochereau, 75014 Paris, France

Phone: 33-1-43217384 Fax: 33-1-40488352

E-mail: agnes.lehuen@inserm.fr
Abstract

iNKT cells belong to the innate immune system and exercise a dual role as potent regulators of autoimmunity and also participants in responses against different pathogens. They have been shown to prevent type 1 diabetes development and promote antiviral responses. Many studies on the implication of environmental factors on the etiology of type 1 diabetes have suggested a link between enteroviral infections and the development of this disease. Our study using the pancreatropic enterovirus Coxsackievirus B4 (CVB4) shows that while infection accelerated type 1 diabetes development in a subset of pro-insulin 2 deficient (Pro-ins2⁻/⁻) NOD mice, the activation of iNKT cells by a specific agonist, α-Galactosylceramide (αGalCer), at the time of infection, inhibited the disease. Diabetes development was associated with the infiltration of pancreatic islets by inflammatory macrophages producing high levels of IL-1β, IL-6 and TNF-α and activation of anti-islet T cells. On the contrary, macrophages infiltrating the islets after CVB4 infection and iNKT cell stimulation expressed a number of suppressive enzymes, among which IDO was sufficient to inhibit anti-islet T cell response and prevent diabetes. Our study highlights the critical interaction between virus and the immune system in the acceleration or prevention of type 1 diabetes.

Abbreviations used in this paper: 1MT, 1-Methyltryptophan; αGalCer or αGC αGalactosylceramide; DC, dendritic cell; CVB4, coxsackievirus B4; IDO, indoleamine 2,3-dioxygenase; iNKT, invariant natural killer T cell; iNOS, inducible nitric oxide synthase; pDC, plasmacytoid dendritic cell; PLN, pancreatic lymph node.
Introduction

Type 1 diabetes is characterized by the destruction of pancreatic islet beta cells by autoreactive CD4 and CD8 T cells leading to low insulin production and incapacity to regulate blood glucose levels (1). Despite numerous studies, the etiology of type 1 diabetes stays elusive. Besides genetics (2-4), environmental factors such as viral infections have been suggested as triggers of type 1 diabetes (5-7). Most striking of these infections concern type B Coxsackieviruses (CVB) belonging to the enterovirus genus whose genome and anti-CVB antibodies were detected more frequently in the blood of recently diagnosed patients compared to healthy controls (8; 9). Besides, enteroviral RNA or enteroviral particles were directly detected in pancreas of type 1 diabetes patients while they were undetectable in pancreas of healthy donors (9; 10). In mouse model of type 1 diabetes, Serreze et al. have shown that diabetes can develop rapidly after Coxsackieviruses B4 (CVB4) infection if mice had an advanced age and sufficient insulitis (11). Others have reported that inefficient islet beta cell response, viral dose and replication rate, as well as the lack of islet neogenesis could also promote accelerated diabetes development after CVB4 infection (12-14).

Natural Killer T (NKT) cells are CD1d restricted non-conventional T cells recognizing self and exogenous glycolipids. Most NKT express an invariant TCR-α chain Vα14-Jα18 (Vα14) in mice and Vα24-Jα18 in humans and are named invariant (i)NKT cells. They can promptly secrete copious amounts of IFN-γ and IL-4 and provide maturation signals to dendritic cells (DC) and lymphocytes, thereby contributing to both innate and acquired immunity (15; 16). iNKT cells are potent regulatory cells that can inhibit autoimmunity and promote immune
responses against pathogens (1; 17). Diabetes can be prevented in NOD mice by increasing iNKT cell numbers and by iNKT cell stimulation with exogenous ligands such as αGalCer (15; 18; 19). NOD mice protected from diabetes by iNKT cells have weak Th1 anti-islet beta cell responses (20). Indeed, iNKT cells can impair the differentiation of anti-islet CD4 and CD8 T cells, which become hypo-responsive or anergic (21). Contrary to their suppressive role in type 1 diabetes, iNKT cells can enhance immune responses to pathogens such as parasites, bacteria and viruses (22; 23).

Our previous studies conducted in a murine model of type 1 diabetes with lymphocytic choriomeningitis virus infection revealed that iNKT cells could promote systemic anti-viral CD8 T cell responses while inhibiting the deleterious anti-islet T cell responses thereby preventing type 1 diabetes (24; 25). In this study we investigated the role of iNKT cells after CVB4 infection. Our study reveals that diabetes development following CVB4 infection is associated with the infiltration of inflammatory macrophages into the pancreatic islets with subsequent activation of anti-islet T cells. However, the activation of iNKT cells during CVB4 infection results in the infiltration of suppressive macrophages into pancreatic islets. IDO expressed by these macrophages was critical for the inhibition of diabetes development.
Research design and methods

Mice

Female Pro-ins2/−/− NOD (Pro-ins2/−/) mice, Vα14 transgenic NOD mice expressing the Vα14-Jα18 TCRα chain and BDC2.5 Cα/−/− mice were previously described (15; 21; 25; 26). NOD Vα14 were crossed to Pro-ins2/−/− NOD mice to generate Vα14 Pro-ins2/−/− NOD. Mice were bred and housed in specific pathogen-free conditions. This study was approved by the local ethics committee on animal experimentation (P2.AL.171.10).

In vivo treatments

Coxsackievirus B4 Edwards strain 2 was injected i.p. at the dose of 1x10^5 PFU/mouse. When indicated, mice were treated with a single i.p. injection of αGalCer (2 µg/mouse (Alexis) diluted in PBS/Tween 0.05%), at the time of CVB4 infection. For short-term blockade of IFN-γ and IL-4, mice were injected i.p. with 0.5 mg of purified anti-IFN-γ mAb (R46A2) or anti-IL-4 mAb (11B11) or corresponding isotype controls on days -1 and +1 of virus infection for PCR analysis and on days -1, +1, +3 for diabetes incidence. IL-13 was blocked with 10 µg of soluble extracellular domain of IL-13 receptor injected i.p. twice daily on days -1, 0 and +1 of infection. A selective iNOS inhibitor, 1400W (10 mg/kg/day; Calbiochem), and a selective arginase I inhibitor, N(omega)-hydroxy-nor-L-arginine (nor-NOHA 20 mg/kg/day; Calbiochem), were injected i.p. daily starting from the day of infection and up to day 8. To inhibit IDO, mice were given 1-methyl-tryptophan (1MT; Sigma) in drinking water (4 mg/mL) 2 days before the infection and for up to 8 days after. In some experiments 2x10^5 macrophages were isolated from the pancreas of Pro-ins2/−/− mice treated with CVB4 or CVB4+αGalCer two days earlier and transferred i.v. into recipient Pro-ins2/−/− mice that were infected with CVB4 one day earlier.
**Viral titration**

Pancreata were recovered and homogenized in liquid maintenance medium 199 (#11825-015; Gibco) complemented with distilled water (65%), sodium bicarbonate (2.7%), PBS (11.5%), penicillin/streptomycin (1.6%), L-glutamine (1.6%) and centrifuged at 2300 rpm for 20 min. Tenfold serial dilutions of the supernatant were overlaid on the HeLa cell monolayer and incubated for 2 h at 37°C. The monolayers were washed with PBS and overlaid with mixed equal portions of maintenance medium, containing FCS (PAA) instead of PBS, and 2.4% suspension of Avicel (RC581; BMC Biopolymer). Two days later, the overlay was removed; the monolayers were fixed with formaldehyde and colored with crystal violet oxalate solution.

**Diabetes diagnosis and histology**

Overt diabetes was defined as two positive urine glucose tests of glycaemia >200 mg/dl 48 h apart (Glukotest and Heamogokotest kits, Roche). For histology analysis, paraffin embedded sections were cut at three levels (200µm intervals) and stained with haematoxylin-eosin. Insulitis severity was scored in a blinded fashion by two examiners with following criteria: grade 0, no infiltration; grade 1, peri-islet lymphocytic infiltration; grade 2, <50% islet lymphocytic infiltration and 3, >50% islet lymphocytic infiltration. At least 40 islets from each mouse were analyzed.

**Preparation of single cell suspensions from pancreas**

Pancreata were perfused with 5 ml of Collagenase P solution (0.75 mg/ml, Roche), dissected free from surrounding tissues, digested for 10 min at 37°C and washed twice with RPMI–10% FCS. Islets were then purified on a Ficoll gradient and incubated with 1 ml of non-enzymatic cell
dissociation buffer (Invitrogen) for 10 min at 37°C and dissociated into a single cell suspension by pipetting.

**Flow cytometry**

Following mAbs were used: CD45 (30F11), CD11b (M1/70), Ly-6G (RB6-8C5), F4/80 (BM8), CD115 (AFS98), IL-4 (11B11), IL-13 (eBio13A) from eBiosciences and CD11c (HL3), 120G8, Ly-6C (AL21), IFN-γ (XMG1.2), CD62L (MelS14), CD4 (GK1.5), CD8 (53S6.7) anti-Shuman Ki67 (B56) from BD biosciences. Stainings were performed in PBS, 5% FCS for 20 min, at 4°C. Non-specific Fc binding was blocked using an anti-CD16/CD32 antibody (24G2). APC-conjugated αGalCer-loaded CD1d tetramer was prepared in our laboratory. For cytokine stainings, cells were stimulated with PMA (10 ng/ml) and ionomycine (1 µg/ml) in the presence of Brefeldin A (1 mg/ml) for 4 h at 37°C (all from Sigma). After the surface staining, cells were fixed, permeabilized during 30 min with cytofix-cytoperm kit (BD) and incubated with intracellular mAbs for 30 min. Cells were either analyzed using a BD Fortessa flow cytometer or sorted out using BD ARIA II sorter.

**Quantitative RT-PCR**

RNA was extracted using RNeasy mini kit (Qiagen) and reverse transcribed using Superscript III (Invitrogen). Quantitative-PCR was performed with SYBR Green (Roche) and analyzed with LightCycler 480 (Roche). Relative expression was calculated using the $2^{-ΔΔCt}$ method and normalized to the expression of the housekeeping gene GAPDH. The stability of GAPDH expression was confirmed by comparison to HPRT mRNA (Supplementary Fig. 1).

**In vitro T cell responses**
Single cell suspensions of pancreatic islets were cultured in the presence of IGRP<sub>206-214</sub> peptide (VYLKTNVFL; 10 µM) for 4 h 30min at 37°C in the presence of Brefeldin A (1 mg/ml). After surface staining cells were fixed, permeabilized and intracellular IFN-γ staining was performed. The proliferation was assessed in a thymidine incorporation assay. Sorted naïve BDC2.5<sup>+</sup> CD4 T cells (3x10<sup>4</sup>) were cultured with anti-CD3/CD28 beads (Invitrogen) and 3x10<sup>4</sup> macrophages, isolated either from the pancreas of mice infected with CVB4 alone or infected and treated with αGalCer. After 48 h culture, wells were pulsed with 1 µCi tritiated thymidine ([<sup>3</sup>H]-TdR) overnight. [<sup>3</sup>H]-TdR incorporation was measured using a TopCount counter (PerkinElmer) of Cochin cytometry and immunobiology facility. 1MT was prepared as a 20 mM stock solution in 0.1 M NaOH and added to T cell culture at final concentration of 200 µM.

**Statistical analysis**

Diabetes incidence was plotted according to the Kaplan-Meier method. Incidences between groups were compared with the log-rank test. For other experiments, comparison between means was performed using the nonparametric Mann-Whitney U test. P-values <0.05 were considered statistically significant. All data were analyzed using Prism version 5 software (GraphPad Software).
Results

iNKT cell activation inhibits diabetes development after CVB4 infection

As previously described by Serreze et al. (11), 50% of NOD mice developed diabetes after CVB4 infection at 10 weeks of age, while the remaining mice stayed diabetes free for up to 30 weeks of age (Fig. 1A). Analysis of diabetic mice showed that diabetes acceleration in a subset of mice after CVB4 infection was significant as compared to non-infected mice (Fig. 1B). Similar data were obtained with Pro-ins2/− mice (Fig. 1C and 1D), which were used since they develop accelerated spontaneous type 1 diabetes compared to wild type NOD mice, thus shortening the duration of experiments. Pro-ins2/− mice were used at 5-6 weeks of age when they already exhibit moderate insulitis. Interestingly, one single injection of the iNKT cell agonist, αGalCer, at the time of infection strongly decreased diabetes incidence in both NOD and Pro-ins2/− mice (13% and 25% respectively). Insulitis was also reduced in CVB4+αGalCer Pro-ins2/− mice compared to CVB4 infection only (Fig. 1E). Since islet beta cell infection could promote diabetes development (12; 27), we analyzed CVB4 pancreatic and islet beta cell infection. Viral titers determined by plaque forming unit were similar in the pancreas from infected mice treated or not with αGalCer (Fig. 1F and Supplementary Fig. 2A). While in situ hybridization showed CVB4 infection of exocrine tissue, it did not reveal islet beta cell infection on day 3 and 7 post-infection (data not shown). Altogether, our data show that iNKT cell activation decreased the incidence of diabetes following CVB4 infection in NOD and Pro-ins2/− mice.

iNKT cell activation dampens pancreatic inflammatory response and promotes the expression of suppressive enzymes

To study the mechanism of diabetes prevention by activated iNKT cells, the inflammatory response was analyzed in pancreatic islets of CVB4 infected mice. At day 2 post-infection, the
expression of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α was significantly decreased in Pro-ins2+/− and NOD mice from CVB4+αGalCer group as compared to untreated infected mice (Fig. 2 and Supplementary Fig. 2B). No differences were found in the mRNA level of the suppressive cytokines, IL-10 and TGF-β (data not shown). Strikingly, αGalCer treatment at the time of infection induced a strong upregulation of the suppressive enzymes iNOS, IDO1 and IDO2 (hereafter referred as IDO) and arginase I. While arginase I was induced by αGalCer alone, the strongest upregulation of iNOS and IDO required both αGalCer and CVB4 infection. Interestingly, Ym1/Ym2 mRNA was also upregulated in the islets from mice of CVB4+αGalCer group, suggesting the presence of alternatively activated macrophages (28; 29). The expression of these molecules was transitory with a peak on day 2 post-infection (Supplementary Fig. 3). Thus, the activation of iNKT cells during CVB4 infection favors the establishment of a less inflammatory and more immunosuppressive environment in pancreatic islets as compared to CVB4 infection only.

**CD11b+/Ly-6C+** macrophages are the main population expressing suppressive enzymes and Ym1/Ym2

We next investigated which cell population(s) expressed the immunosuppressive enzymes iNOS, IDO, arginase I, and Ym1/Ym2 molecules. On day 2 post-infection, the highest expression of these molecules were detected in myeloid cells isolated from pancreatic islets (islet myeloid cells) (CD11b+/CD11c−) of Pro-ins2+/− mice from CVB4+αGalCer group compared to CD45+ DCs (CD11c+), plasmacytoid DCs (CD11c\textsuperscript{low}/120G8+), remaining CD11b+/CD11c− and CD45− cells (Fig. 3A and B). Of note, these enzymes were not detected in sorted populations of
pancreatic lymph node (PLN) and spleen from same mice tested up to 10 days post-treatment (data not shown).

Further characterization of islet CD11b⁺/CD11c⁻ myeloid cells showed that they were predominantly F4/80⁺ and Ly-6G⁻ macrophages and that their frequency did not differ between CVB4 and CVB4+αGalCer treated mice (Fig. 4A). Interestingly, the kinetics of their infiltration into islets correlated with the pattern of expression of inflammatory and suppressive molecules in total islets (Supplementary Fig. 4A). Macrophages were further stained with Ly-6C and CD115 mAbs since these molecules can be upregulated on suppressive macrophages (30; 31). However, after infection Ly-6C and CD115 were upregulated to the same extent independently of αGalCer treatment. Similar data were obtained in NOD mice (data not shown). Analysis of sorted Ly-6C⁺/CD115⁺ and Ly-6C⁻/CD115⁻ pancreatic infiltrating cells revealed that the inflammatory cytokines and suppressive enzymes were mainly expressed by Ly-6C⁺/CD115⁺ macrophages (Fig. 4B and data not shown). Of note, Ly-6C⁺ macrophages from spleen and PLN did not express these suppressive enzymes (Supplementary Fig. 4B). Altogether, these results indicate that CVB4 infection induces the infiltration of Ly-6C⁺/CD115⁺ macrophages into pancreatic islets which resemble classically activated macrophages (CAMφ) strongly expressing inflammatory cytokines. However when iNKT cells are activated, macrophages express suppressive enzymes characteristic of myeloid derived suppressor cells (MDSC).

**iNKT cell activation and critical role of IFN-γ and IL-13 in the expression of suppressive enzymes**

Since iNKT cell manipulation leads to infiltration of suppressive macrophages and has a major impact on the development of diabetes after CVB4 infection, iNKT cells were analyzed in
pancreatic islets (Fig. 5). Vα14 transgenic Pro-ins2−/− mice were used for this study, since they exhibit a tenfold increased frequency and number of iNKT cells, making them easier to detect. CVB4 infection induced the activation of pancreatic iNKT cells that upregulated CD69 without increasing their proliferation (no Ki-67 upregulation) and their cytokine production. In contrast, αGalCer injection led to a strong iNKT cell proliferation and massive production of IFN-γ, IL-4 and IL-13. We next investigated the role of these cytokines in the induction of MDSC and alternatively activated macrophages in mice from CVB4+αGalCer group, since IFN-γ can induce the expression of iNOS and IDO, while IL-4 and IL-13 can induce arginase I and Ym1/Ym2 expression. The blockade of IL-4 by specific mAb did not alter the expression of any of these enzymes at day 2 post-infection (Fig. 6). In contrast, blocking IFN-γ by a specific mAb significantly reduced the expression of iNOS, IDO, and Ym1/Ym2, while blocking of IL-13 significantly decreased the expression of arginase I. Thus, cytokines produced by iNKT cells might be the key mediators in the induction of pancreatic suppressive macrophages after CVB4 infection.

**IDO is required for the inhibition of diabetes onset**

To determine the role of suppressive enzymes in the protection against diabetes, Pro-ins2−/− mice from CVB4+αGalCer group were treated with specific inhibitors of iNOS, IDO and arginase I or control vehicles. Treatment with the iNOS inhibitor, 1400W, or the arginase I inhibitor, nor-NOHA, did not abrogate the protection against diabetes (Fig. 7A). However, while the IDO inhibitor, 1MT, did not affect diabetes incidence of control Pro-ins2−/− mice (Fig. 7B), it abolished the protection of mice from CVB4+αGalCer group since 86% of mice became diabetic compared to only 27% of mice from CVB4+αGalCer group treated with vehicle. Importantly,
combined treatment with the three inhibitors resulted in a similar incidence of diabetes as with the IDO inhibitor only, suggesting that iNOS and arginase I did not play any significant role in the protection against diabetes in this setting. Consistent with the incidence of diabetes, 1MT treatment increased the severity of insulitis in mice from CVB4+αGalCer group (Fig. 7C). Since IDO expression in islets was induced by IFN-γ, we next tested the role of IFN-γ in the protection against diabetes. Short-term IFN-γ blockade, during the period when IDO is detected in islets, increased diabetes incidence of CVB4+αGalCer treated mice (Fig. 7D). Of note, both 1MT treatment and IFN-γ blockade induced a moderate increase of diabetes incidence of mice from CVB4 group (Supplementary Fig. 5). Together these results show that IDO plays a critical role in the inhibition of diabetes development during CVB4 infection and iNKT cell activation.

The suppressive capacity of macrophages was then assessed in vitro in a T cell proliferation assay (Fig. 7E). Macrophages were isolated from the pancreas of Pro-ins2<sup>−/−</sup> mice of CVB4 and CVB4+αGalCer groups on day two post-infection and cultured with naïve BDC2.5 CD4 T cells stimulated with anti-CD3/CD28 beads. Macrophages from CVB4 infected mice enhanced BDC2.5 CD4 T cell proliferation even though this increase did not reach statistical significance. On the contrary, macrophages from mice of CVB4+αGalCer group significantly suppressed T cell proliferation and the suppression was reversed when 1MT was added to the culture. To demonstrate the role of pancreatic islet infiltrating macrophages in vivo, macrophages were sorted out from pancreatic islets of either CVB4 or CVB4+αGalCer treated Pro-ins2<sup>−/−</sup> mice on day two post-infection and transferred into CVB4 recipients infected the day before. The transfer of macrophages from CVB4 group significantly increased diabetes incidence of CVB4 infected recipient mice whereas the transfer of macrophages from CVB4+αGalCer
group significantly reduced diabetes incidence (Fig. 7F). Altogether these results highlight the dual role of pancreatic macrophages after CVB4 infection.

**Strong pancreatic anti-islet T cell response is associated to diabetes induction by CVB4**

To further decipher the immune mechanisms involved in the development of diabetes after CVB4 infection and its inhibition by iNKT cell activation, we evaluated specific anti-islet T cell responses. CVB4 infected Pro-ins2^{-/-} and NOD mice were analyzed from one to three weeks post-infection when diabetes was clearly established. Untreated, αGalCer treated or CVB4 infected non-diabetic mice were tested in parallel. CVB4 infection dramatically increased the total numbers of both CD4 and CD8 T cells infiltrating the pancreas of Pro-ins2^{-/-} and NOD mice as compared to untreated and mice treated only with αGalCer (Fig. 8A and B and Supplementary Fig. 6A). IGRP specific IFN-γ producing CD8 T cells were detected in all Pro-ins2^{-/-} and NOD mice that became diabetic after CVB4 infection regardless of the treatment received. In contrast, the frequency of IGRP specific IFN-γ producing CD8 T cells remained low in untreated or non-diabetic infected mice (Fig. 8C and D and Supplementary Fig. 6B). Treatment with IDO inhibitor, that abolished diabetes protection, induced a strong anti-IGRP effector CD8 T cell response only in diabetic mice. Altogether, our results strongly suggest that diabetes development after CVB4 infection is caused by anti-islet T cell responses.
Discussion

The present study shows that while CVB4 infection can rapidly induce diabetes in a subset of Pro-ins2−/− and NOD mice, the concomitant activation of iNKT cells inhibited diabetes development after CVB4 infection very efficiently. This prevention was associated with the reduction of inflammatory cytokine expression and the induction of suppressive enzymes in pancreatic infiltrating macrophages. Pancreatic iNKT cells produced IFN-γ, which was required for the induction of high levels of IDO that prevented diabetes onset. The development of diabetes was associated with an increased anti-islet T cell response, while the frequency of these IFN-γ producing autoreactive T cells remained very low in non-diabetic mice.

Numerous studies have shown the pathogenic role of macrophages in type 1 diabetes (32-35). CD11b+/F4/80+/Ly-6C+/CD115+ inflammatory macrophages (36) highly infiltrated pancreatic islets after CVB4 infection in both Pro-ins2−/− and NOD mice but differed in their regulatory functions between mice treated or not with αGalCer. Pancreas of mice that developed diabetes after CVB4 infection harbored macrophages with classically activated phenotype expressing high levels of IL-1β, IL-6 and TNF-α, which can favor diabetes development (37-39). According to these previous articles, it is not surprising that as a consequence of macrophage depletion and low expression of inflammatory cytokines, fewer mice developed diabetes after CVB4 infection (Supplementary Fig. 7).

In contrast to the production of proinflammatory cytokines by the pancreatic macrophages from CVB4 infected mice, in CVB4+αGalCer group macrophages produced suppressive enzymes including IDO that was required for the decrease of diabetes incidence in these mice. The high IDO expression was due to IFN-γ, a cytokine highly produced by αGalCer activated iNKT cells. IFN-γ alone was not sufficient for diabetes protection since αGalCer
treated mice had similar diabetes incidence as untreated mice despite high levels of IFN-γ. Importantly, while CVB4 infection did induce some IDO, αGalCer treatment alone did not induce any, suggesting that viral infection might be the initiator of IDO induction. Interestingly previous studies have suggested the role of IDO in the control of diabetes development. The poor induction of IDO in DCs from prediabetic NOD mice was shown to favor diabetes development (40), whereas transplanted pancreatic islets are protected by IDO expressing neighboring fibroblasts (41).

CVB4 induced diabetes depends on lymphocytes since NOD Scid, NOD Igµ<sup>S/S</sup> (11) and NOD Ca<sup>C</sup> (data not shown) mice do not develop diabetes after CVB4 infection. Similarly, CVB4 infected Pro-ins2<sup>S/S</sup> Scid mice did not become diabetic (data not shown). Diabetes development only in a subset of CVB4 infected Pro-ins2<sup>–/–</sup> mice could result from differences in insulitis level before infection as suggested in NOD mice (11). While CD8 T cell numbers highly increased in islets of all infected mice, IFN-γ producing anti-SIGRP CD8 T cells were only detected in the pancreas of diabetic mice, therefore strengthening the role of T cells in diabetes induced by CVB4, as previously suggested (11; 42). The inhibition of IDO in CVB4+αGalCer treated mice resulted in a strong anti-SIGRP CD8 T cell effector response and a high incidence of diabetes showing the protective role of IDO in diabetes. Most often IDO induces tolerance by suppressing T cell proliferation through tryptophan depletion or by inducing apoptosis through by-products of tryptophan degradation, such as kynurenins (43; 44). However the numbers of T cells did not differ in mice expressing low or high level of IDO, suggesting that IDO does not affect the proliferation of T cells but rather induces their local inhibition in the pancreas. Our data showing that IDO inhibits the function of CD8 T cells is reminiscent of a previous study in
Moreover local regulation by IDO has been previously observed in other infectious context as well as in cancer (46).

Interestingly, our study suggests that IFN-γ can play a protective or deleterious role in diabetes development depending of the setting (Fig. 8E). We hypothesize that a strong burst of IFN-γ early after viral infection upregulates IDO expression, which is initially induced by CVB4 infection, in order to down-regulate the inflammation after viral infection. In contrast, in the absence of iNKT cell activation, the production of inflammatory cytokines could promote the recruitment and activation of pathogenic T cells which would accumulate and produce IFN-γ in a more chronic way. At this later point, IDO is no longer expressed in pancreas and IFN-γ production would lead to islet beta cell death rather than IDO upregulation. Of note, we did not detect any difference of viral load in the pancreas of infected mice treated or not with αGalCer. However, we cannot rule out that IFN-γ, produced by αGalCer treatment, might contribute to the prevention of diabetes by dampening CVB4 infection specifically in islet beta cells. The infection of islet beta cells could lead to T1D development since it would favor the engulfment of infected islet beta cells by antigen presenting cells, presentation of islet beta cell autoantigens to autoreactive T cells and initiation of diabetes (27; 47; 48). Indeed, studies in mouse models and human samples have shown that IFNs, including IFN-γ, are important in regulating islet beta cell permissiveness to infection and replication in islet beta cells (12; 49; 50). Interestingly, some reports described islet beta cell infection by enterovirus in recent type 1 diabetic patients (51; 52). Therefore, IFN-γ production in the islets of CVB4+αGalCer treated mice could not only prevent diabetes by inducing IDO but also by limiting islet beta cell infection.

IDO mediated suppression could involve several mechanisms including regulatory FoxP3+ T cell induction in pancreatic islets (53). However in our study the percentages of
FoxP3+ CD4 T cells, their expression of CTLA-4, CD103, GITR and OX40, as well as their production of IL-10 and TGF-β were similar in the islets, PLN and spleen of infected mice with or without αGalCer treatment both in Pro-ins2−/− and NOD mice (Supplementary Fig. 8 and data not shown). Tryptophan metabolites can also render DCs tolerogenic with intermediate expression of stimulatory MHC-II, CD86 and upregulation of the suppressive PD-L1, PD-L2 (54). These molecules were highly upregulated by DCs in the pancreas, PLN and spleen after the infection. However the expression of these molecules was similar between CVB4 infected mice, treated or not with αGalCer. Similarly the production of IL-10 and IL-12 by DCs did not differ in any of the organs studied (data not shown).

In conclusion, this study shows how the manipulation of iNKT cells can lead to the induction of suppressive macrophages in the pancreas of CVB4 infected mice and highlights the role of macrophages in the development or prevention of diabetes after CVB4 infection. The outcome of this study can help design novel therapeutic approaches consisting of manipulation of iNKT cells and suppressive macrophages.

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L.G. researched data, performed experiments and wrote the manuscript. J.D. researched data and reviewed the manuscript. L.B. and P.G.L. performed experiments. R.K.P. provided IL-13 inhibitor and reviewed and edited the manuscript. M.F.T. provided CVB4 and reviewed and edited the manuscript. N.V.R provided Clodronate liposomes and reviewed and edited the manuscript. A.L. designed and supervised the study, and wrote the manuscript. L.G. and A.L. are the guarantors of this work and as such had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

Figure 1. NKT cell activation prevents diabetes development after CVB4 infection.

(A) Diabetes incidence of female NOD mice (10 weeks old) inoculated i.p. with CVB4 (1x10^5 PFU/mouse) or PBS and treated with αGalCer (αGC) or control vehicle (untreated n=14, αGalCer n=15, CVB4 n=14, CVB4+αGalCer n=15). *p<0.05, **p<0.005 using log-rank test analysis. Data represent 2 pooled independent experiments. (B) Age of diabetes onset of NOD mice that became diabetic. *p<0.05, ***p<0.0005, Mann Whitney. (C) Diabetes incidence of female ProSins2^−^ mice inoculated i.p. with CVB4 (1x10^5 PFU/mouse) or PBS and treated with αGalCer or control vehicle at 5-6 weeks of age (untreated n=44, αGalCer n=10, CVB4 n=36, CVB4+αGalCer n=17). **p<0.005, ***p<0.0005 using log-rank test analysis. Data represent 2-4 pooled independent experiments. (D) Age of diabetes onset of Pro-ins2^−/−^ mice that became diabetic. ***p<0.0005, Mann Whitney. (E) Histological scoring of insulitis was performed on pancreatic sections of Pro-ins2^−/−^ mice from days 7-10 post-infection stained with haematoxylin/eosin (n=6 mice per group). Grade 0, no infiltration; grade 1, peri-islet lymphocytic infiltration; grade 2, <50% islet lymphocytic infiltration and 3, >50% islet lymphocytic infiltration. (F) Pancreata were isolated from Pro-ins2^−/−^ mice on different days post-infection, weighed and viral titers were determined on HeLa cell monolayers using a plaque assay technique. Mean viremia titers are expressed as PFU/gram of pancreas ± SD (n=6 mice/group for each day).

Figure 2. iNKT cell activation dampens pancreatic inflammatory response and promotes the expression of suppressive enzymes.
Female Pro-ins2/− mice at 5-6 weeks of age were either inoculated i.p. with CVB4 (1x10^5 PFU/mouse) or PBS and treated with αGalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by quantitative RT-PCR. Data are presented as specific gene expression relative to GAPDH. Each symbol represents pooled islets of an individual mouse (untreated n=6, αGalCer n=12, CVB4 n=16, CVB4+αGalCer n=16). *p<0.05, **p<0.005, ***p<0.0005, Mann Whitney.

Figure 3. CD11b+/CD11c− cells are the main population expressing the suppressive enzymes and Ym1/Ym2.

Female Pro-ins2/− mice at 5-6 weeks of age were either inoculated i.p. with a single dose of CVB4 or PBS and treated with αGalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, dissociated into a single cell suspension and cells were stained with surface antibodies directed against CD45, CD11c, 120G8 and CD11b. Cells were then sorted out using BD ARIA II sorter. (A) Dot plots correspond to a representative staining in pancreatic islets with gates used for sorting. (B) Total RNA was isolated from sorted populations from CVB4 and CVB4+αGalCer treated mice and mRNA levels were measured by quantitative RT-PCR. Data are presented as the mean of specific gene expression relative to GAPDH ± SD. Data were obtained from 3 independent experiments with 3 mice in each group. *p<0.05, Mann Whitney.

Figure 4. Inflammatory and suppressive molecules are expressed by CD11b+/F4/80+/Ly-6C+/CD115+ cells.

Female Pro-ins2/− mice at 5-6 weeks of age were either inoculated i.p. with CVB4 (1x10^5 PFU/mouse) or PBS and treated with αGalCer or control vehicle. On day 2 post-infection,
pancreatic islets were harvested, dissociated, stained with different surface antibodies and analyzed by flow cytometry. (A) Dot plots correspond to a representative staining in pancreatic islets. On the right, summary of data obtained from 3 independent experiments with 3 mice in each group ± SD. (B) At day 2 post-infection, pancreatic islet CD45⁺/CD11b⁺/F4/80⁺/Ly-6C⁺/CD115⁺ cells were sorted out from CVB4 infected mice treated or not with αGalCer. Total RNA was extracted and mRNA levels were measured by quantitative RT-PCR. Data are presented as the mean of specific gene expression relative to GAPDH ± SD. Data were obtained from 3 independent experiments with 3 mice in each group. *p<0.05, **p<0.005, ***p<0.0005, Mann Whitney.

**Figure 5. iNKT cell activation and cytokine production.**

iNKT cells were analyzed in Vα14 transgenic Pro-ins2⁺/⁻ female mice inoculated with CVB4 or PBS and treated with αGalCer or control vehicle. Pancreatic islets were harvested on day 2 of the treatment and dissociated into a single cell suspension. Cells were stimulated with PMA/ionomycin in the presence of Brefeldin A for 4 h then intracellular staining was performed. Dot plots correspond to a representative staining in pancreatic islets. On the right, summary of data obtained from 3 independent experiments with 3 mice in each group ± SD. *p<0.05 Mann Whitney.

**Figure 6. Critical role of IFN-γ and IL-13 in the induction of suppressive enzymes.**

CVB4 infected and αGalCer treated female Pro-ins2⁺/⁻ mice were injected with blocking anti-IL-4 mAb (11B11) or anti-IFN-γ mAb (R46A2) or respective isotype control antibodies on days -1 and +1 of infection. To block IL-13, mice were injected with IL-13 inhibitor twice a day on days -1, 0 and +1 of infection. On day 2 of infection, pancreatic islets were isolated and quantitative
RT-PCR was performed with total islet RNA. Data are presented as the mean of specific gene expression relative to GAPDH ± SD. Data represent 2 independent experiments with 3 mice per group. *p<0.05, **p<0.005, Mann Whitney.

**Figure 7. Critical role of IDO in diabetes prevention**

Pro-ins2−/− females, infected with CVB4 and injected with αGalCer, received treatments as indicated; 1400W, 1MT and nor-NOHA to inhibit iNOS, IDO and arginase I respectively or control vehicles. (A) Incidence of diabetes following different treatments. (CVB4+αGC n=15, CVB4+αGC+1400W n=12, CVB4+αGC+nor-NOHA n=12, CVB4+αGC+1MT n=15, CVB4+αGC+1400W+nor-NOHA+1MT n=12). **p<0.005 using log-rank test analysis. Data represent 2 pooled independent experiments. (B) Incidence of diabetes of control Pro-ins2−/− females treated or not with 1MT (n=9 for each group). (C) Histological scoring of insulitis was performed on pancreatic sections of Pro-ins2−/− females from days 7-10 post-infection stained with haematoxylin/eosin (n=6 mice). (D) Incidence of diabetes of female Pro-ins2−/− mice infected with CVB4 and injected with αGalCer and treated with an anti-IFN-γ mAb (R46A2) (n=9) or control isotype mAb (n=15). (E and F) CD11b+/F4/80+/Ly-6C+ macrophages were sorted out from pancreatic islets of Pro-ins2−/− females from CVB4 or CVB4+αGalCer groups on the second day of infection. (E) Macrophages were cultured (3x10⁴/well) with sorted CD62L+ BDC2.5 CD4 T cells (3x10⁴/well) in the presence of anti-CD3/CD28 beads ± 1MT (200µM). After 48 h of culture, tritiated thymidine was added to wells overnight. Results are expressed as percentage of [³H]thymidine uptake compared to control BDC2.5 CD4 T cells cultured with anti-CD3/CD28 beads only, which was considered as 100%. Data represent means ± SD for 3 independent experiments. **p<0.005, ***p<0.005, Mann Whitney. (F) Sorted macrophages
were injected i.v. (2x10^5 per mouse) into Pro-ins2−/− females infected with a single dose of CVB4 (1x10^5 PFU/mouse) one day earlier. Data represent the incidence of diabetes of the three groups of mice (n=10 for each group) from two independent experiments. *p<0.05, using log-rank test analysis.

Figure 8. Anti-islet T cell response in the development of diabetes.

(A and B) Representative CD8 versus CD4 dot plots of T cells from islets of female Pro-ins2−/− mice two weeks after the treatments as indicated on the figure (left panel) and summary of CD8 and CD4 T cell counts in the islets of Pro-ins2−/− females from day 7 to 3 weeks post CVB4 infection of several independent experiments (right panel). (C) Representative plots of intracellular IFN-γ staining among islet CD8 T cells from CVB4 infected diabetic and non-diabetic Pro-ins2−/− females two weeks after the infection after 4 h 30 min stimulation with IGRP_{206–214} peptide. (D) Graph showing the percentage of islet CD8 T cells secreting IFN-γ after IGRP_{206–214} peptide stimulation of diabetic and non-diabetic mice receiving different treatments as indicated on the figure. The horizontal line shows the limit between diabetic and non-diabetic mice. Each symbol represents a single mouse. (E) Schematic view of the immune cell interplay in the islets after infection. After CVB4 infection macrophages strongly infiltrate pancreatic islets and resemble classically activated macrophages (CAMφ) with low IDO expression and high secretion of proinflammatory cytokines IL-1β, IL-6 and TNF-α. In this setting, anti-islet T cells strongly infiltrate the islets and produce IFN-γ, which can kill islet beta cells. However, when iNKT cells are activated by αGalCer at the time of infection, they produce large amount of IFN-γ inducing a strong upregulation of IDO expression in islet infiltrating suppressive
macrophages (MDSC). IDO expressing MDSC suppress IFN-γ production by anti-islet T cells in the pancreas, thereby preventing type 1 diabetes onset.
Figure 1

A

Diabetes incidence [%]

Age (weeks)

B

Diabetes onset (weeks)

C

Diabetes incidence [%]

Age (weeks)

D

Diabetes onset (weeks)

E

Insulitis (% of islets)

Unretrated  αGC  CVB4  CVB4+αGC

Grade 0  Grade 1  Grade 2  Grade 3

F

Viral load (PFU/ml of plasma)

Days after infection

194x215mm (600 x 600 DPI)
Figure 2

104x64mm (600 x 600 DPI)
Figure 3

A

Gated among live cells

- Untreated
- oGC
- CVB4
- CVB4+oGC

CD45^+ cells
CD45^- cells

Gated among CD45^+ cells

CD11b^-/CD11c^- myeloid cells
CD11c^- DCs
CD11b^-/CD11c^- cells

Gated among CD11c^- cells

CD11c^-/120G8^- pDCs

B

Gene expression levels

- MyD88
- Apoptosis-1
- IκBα
- TLR3
- ICOS

219x311mm (300 x 300 DPI)
Figure 6

Diabetes
Figure 7

A

Diabetes incidence (%)

Age (weeks)

CVB40+GC
CVB40+GC+1406W
CVB40+GC+nor-NOHA
CVB40+GC+1MT
CVB40+GC+3 inhibitors

B

Diabetes incidence (%)

Age (weeks)

Pro-ins2
Pro-ins2+1MT

C

Insulitis % of Islets

CVB4
CVB4+GC
CVB4+GC+1MT

Grade 0
Grade 1
Grade 2
Grade 3

D

Diabetes Incidence (%)

Age (weeks)

CVB40+GC
CVB40+GC + anti-IFN-γ

E

% Thymidine Incorporation (% of control)

BDC2.5
CD3/CD28
CVB4 Mø
CVB4+GC Mø
1MT

F

Diabetes Incidence (%)

Age (weeks)

Mø CVB4
Non transferred
Mø CVB4+GC
Figure 8

A

B

C

D

E

219x288mm (600 x 600 DPI)
Supplementary figure 1.

Female Pro-ins2\textsuperscript{−/−} mice at 5-6 weeks of age were either inoculated i.p. with a single dose of CVB4 (1x10\textsuperscript{5} PFU/mouse) or PBS and treated with \(\alpha\)GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by quantitative RT-PCR. Data are presented as specific gene expression relative to HPRT and GAPDH. Each symbol represents pooled islets of an individual mouse.

Supplementary figure 2.

(A) Female NOD mice at 10 weeks of age were inoculated i.p. with a single dose of CVB4 (1x10\textsuperscript{5} PFU/mouse) or PBS and treated with \(\alpha\)GalCer or control vehicle. Pancreata were isolated on different days post-infection, weighed and viral titers were determined on HeLa cell monolayers using a plaque assay technique. Mean viral titers are expressed as PFU/gram of pancreas ± SD (n=3 mice/group for each day). (B) Female NOD mice at 10 weeks of age were either inoculated i.p. with a single dose of CVB4 (1x10\textsuperscript{5} PFU/mouse) or PBS and treated with \(\alpha\)GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by quantitative RT-PCR. Data are presented as specific gene expression relative to GAPDH. Bars correspond to data obtained from 3 pooled mice.

Supplementary figure 3.

Pro-ins2\textsuperscript{−/−} females at 5-6 weeks of age were inoculated i.p. with a single dose of CVB4 (1x10\textsuperscript{5} PFU/mouse) or PBS and treated with \(\alpha\)GalCer or control vehicle. On days 1, 2, 4 and 8 post-
infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by quantitative RT-PCR. Untreated mice are represented as day 0. Data are presented as specific gene expression relative to GAPDH. Data represents means of 3 pooled mice from two independent experiments ± SEM.

Supplementary figure 4.

(A) Kinetics of macrophage infiltration in pancreatic islets. Pro-ins2−/− mice at 5-6 weeks of age were either inoculated i.p. with a single dose of CVB4 (1×10^5 PFU/mouse) or PBS and treated with αGalCer or control vehicle. On days 1, 2, 4 and 8 post-infection, pancreatic islets were harvested, dissociated into a single cell suspension, stained with different surface antibodies and analyzed by flow cytometry.Untreated mice are represented as day 0. Data represent means ± SD of 2 pooled mice from three independent experiments. (B) Female Pro-ins2−/− mice at 5-6 weeks of age were infected with CVB4 (1×10^5 PFU/mouse i.p.) and treated with αGalCer. On days 2, 4, 6, 8, 10 post-infection, pancreatic islets, PLN and spleen were harvested, dissociated, stained with different surface antibodies and CD45^+/CD11b^+/F4/80^+/Ly-6C^+/CD115^+ cells were sorted out. Untreated mice are represented as day 0. Total RNA was extracted from sorted cells and mRNA levels were measured by quantitative RT-PCR. Data are presented as specific gene expression relative to GAPDH ± SD. Data were obtained from 3 independent experiments with 3-6 mice in each group.

Supplementary figure 5.
Incidence of diabetes of Pro-ins2^-/- females infected with CVB4 (1x10^5 PFU/mouse) at 5-6 weeks of age and treated with (A) 1MT (4 mg/mL in drinking water) or control vehicle 2 days before the infection and for up to 8 days after (CVB4=9, CVB4+1MT n=7) or (B) an anti-IFN-γ mAb (R46A2; 0.5mg) or control isotype mAb (0.5mg) injected on days -1, +1, +3 of virus infection (CVB4+isotype control n=10, CVB4+anti-IFN-γ n=10).

**Supplementary figure 6.**

(A) Summary of CD8 and CD4 T cell counts in pancreas of female NOD mice (infected at 10 weeks of age) from day 7 to 3 weeks post CVB4 infection (1x10^5 PFU/mouse) of two independent experiments after the treatments as indicated on the figure. (B) Graph showing the percentage of islet CD8 T cells secreting IFN-γ after IGRP_{206-214} peptide stimulation of diabetic and non-diabetic NOD females receiving different treatments as indicated on the figure. The horizontal line shows the limit between diabetic and non-diabetic mice. Each symbol represents a single mouse.

**Supplementary figure 7.**

(A, B and C) Pro-ins2^-/- females at 5-6 weeks of age were inoculated i.p. with a single dose of CVB4 (1x10^5 PFU/mouse) and treated with αGalCer or control vehicle. To deplete macrophages, mice were injected i.v. with 200 µl of clodronate or control PBS loaded liposomes on days -1, +1 of virus infection (Van Rooijen N. et al., J Immunol Methods 1994). (A) On day 2 post-infection, pancreatic islets were harvested, dissociated and stained with surface antibodies. Data represent means ± SD of 2 pooled independent experiments. (CVB4 n=9, CVB4+clodronate n=8,
CVB4+αGalCer n=9, CVB4+αGalCer+clodronate n=8) **p<0.005 Mann Whitney. (B) On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by quantitative RT-PCR. Data are presented as specific gene expression relative to GAPDH. Each symbol represents pooled islets of an individual mouse. (C) Diabetes incidence of 5-6 weeks old Pro-ins2−/− females inoculated i.p. with CVB4 (1x10^5 PFU/mouse) and treated with αGalCer or control vehicle. Mice were also injected i.v. with either clodronate or control PBS containing liposomes on days -1, +1 and +3 of infection (CVB4+PBS n=11, CVB4+clodronate n=12, CVB4+αGalCer+PBS n=11, CVB4+αGalCer+clodronate n=12). Data represent 2 pooled independent experiments.

**Supplementary figure 8.**
Female Pro-ins2−/− mice at 5-6 weeks of age were inoculated i.p. with a single dose of CVB4 (1x10^5 PFU/mouse) or PBS and treated with αGalCer or control vehicle. 7 days to three weeks after the infection, pancreatic islets were isolated, dissociated into single cell suspension and stained with different antibodies as indicated on the figure. (A) Dot plots correspond to a representative staining of islet CD4+FoxP3+ cells stained with anti-IL-10 and anti-TGF-β mAbs after stimulation with PMA and ionomycin for 6 h in the presence of Brefeldin A. (B) Summary of data obtained from 3 independent experiments with 3 mice in each group ± SD.
Supplementary online figure 1

Relative to GAPDH

Relative to HPRT

IDO1 expression

TNF-α expression

HPRT expression

GAPDH expression

Untreated  αGC  CVB4  CVB4+αGC

227x323mm (600 x 600 DPI)
Supplementary figure 3

119x84mm (600 x 600 DPI)
Supplementary online figure 4

A

B

211x262mm (600 x 600 DPI)
Supplementary online figure 5

A

B

Diabetes incidence (%)

Age (weeks)

Diabetes incidence (%)

Age (weeks)

40x18mm (600 x 600 DPI)
Supplementary online figure 6

A

CNP absolute numbers (n psi)

B

IFN-γ among CD8+ cells (%)
Supplementary online figure 8

A

Gated among FoxP3+ cells

B

250x369mm (600 x 600 DPI)