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

Liana Ghazarian

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Université Paris 5 Rene Descartes

Ecole doctorale GC2ID
Spécialité : Immunologie

THESE
Pour obtenir le grade de
DOCTEUR DE L'UNIVERSITE PARIS 5 RENE DESCARTES

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

Présentée et soutenue publiquement le 10 Octobre 2013 par

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Scientific summary

iNKT cells are non-conventional T lymphocytes that are restricted to glycolipid presenting CD1d molecule. iNKT cells express an invariant TCR α chain (V α 14-J α 18 in mice and V α 28-J α 18 in humans). Their particularity is to rapidly produce copious amounts of cytokines (IFN- γ and IL-4) after activation and to activate other cells of the immune system such as dendritic cells, NK cells and T lymphocytes. iNKT cells, therefore, form a bridge between innate and adaptive immune responses.

Type 1 diabetes is an autoimmune disease characterized by the destruction of pancreatic β cells whose role is to produce insulin. While diabetes development can clearly be associated with genetic polymorphisms, environmental factors were also implicated in the etiology of the disease. Numerous studies suggest that viral infections, particularly infections with Coxsackievirus B4 (CVB4), could be implicated in the development of type 1 diabetes.

Our study was performed with NOD mice that develop type 1 diabetes around 15 weeks of age and with proinsulin 2 knockout NOD mice (Pro-ins2^{-/-}) which become diabetic around 8 weeks of age. Our results show that CVB4 infection induces accelerated diabetes in around half of NOD and Pro-ins2^{-/-} mice compared to uninfected mice. However, the activation of iNKT cells with their agonist, α Galactosylceramide (α GalCer), at the time of infection greatly decreases diabetes incidence. CVB4 infection induces a strong recruitment of macrophages into the pancreas. Interestingly, iNKT cell activation modifies the function of these macrophages. Indeed, pancreatic macrophages of CVB4 infected mice strongly express IL-1, IL-6 and TNF- α , indicating their pro-inflammatory character. On the contrary, macrophages of mice infected with CVB4 and treated with α GalCer express low levels of these cytokines, but strong levels of suppressive enzymes iNOS (inducible NO synthase), IDO (Indoleamine 2,3-dioxygenase) and arginase I. The use of inhibitors of these enzymes showed that diabetes prevention is induced by IDO. We have also observed that autoreactive T cells strongly infiltrate the pancreatic islets after CVB4 infection. It is interesting to note that the high diabetes incidence of CVB4 infected mice is associated with an increased frequency of IFN- γ producing autoreactive T cells in pancreatic islets. On the contrary, the frequency of these cells is very low in infected mice treated with α GalCer. The inhibition of IFN- γ production is dependent on IDO enzyme, since the use of its inhibitor strongly increases IFN- γ production by anti-islet T cells and diabetes incidence.

To summarize, our results show that iNKT cell activation during the infection with CVB4 induces immunosuppressive macrophages in the pancreas. These cells inhibit the function of autoreactive T cells and prevent diabetes development.

Résumé scientifique

Les cellules NKT invariantes (iNKT) sont des lymphocytes T non conventionnels restreints par la molécule CD1d qui présente des glycolipides. Les cellules iNKT expriment un TCR avec une chaîne α invariante, V α 14-J α 18 chez la souris et V α 28-J α 18 chez l'homme. Elles ont la particularité de produire de grande quantité de cytokines (IFN- γ et IL-4) rapidement après leur activation et peuvent à leur tour stimuler d'autres cellules du système immunitaire comme les cellules dendritiques, les cellules NK et les lymphocytes T. Elles représentent ainsi un pont entre les réponses immunitaires innées et adaptatives.

Le diabète de type 1 est une maladie auto-immune caractérisée par la destruction des cellules β pancréatiques productrices d'insuline. Bien que l'apparition de diabète de type 1 soit associée à des polymorphismes génétiques, les facteurs environnementaux ont également été impliqués dans l'étiologie de cette maladie. De nombreuses études suggèrent que les infections virales, en particulier les infections par le virus de coxsackie B4 (CVB4), pourraient être impliquées dans le développement de cette maladie.

Notre étude a été réalisée avec des souris NOD qui développent un diabète de type 1 vers 15 semaines d'âge et des souris NOD déficientes pour la proinsuline 2 (Pro-ins $2^{-/-}$) développant un diabète vers 8 semaines d'âge. Nos résultats montrent qu'après infection par CVB4, la moitié des souris NOD et Pro-ins $2^{-/-}$ développent un diabète accéléré par rapport à des souris non infectées. Toutefois, une injection de l'agoniste des cellules iNKT, la molécule α Galactosylceramide (α GalCer), au moment de l'infection des souris, diminue fortement l'incidence de diabète. L'infection par CVB4 induit un fort recrutement de macrophages dans le pancréas et l'activation des cellules iNKT modifie la fonction de ces macrophages. En effet, les macrophages pancréatiques des souris infectées par CVB4 expriment fortement les cytokines IL-1 β , IL-6 et TNF- α , révélant leur caractère pro-inflammatoire alors que les macrophages des souris infectées et traitées par α GalCer expriment faiblement ces cytokines inflammatoires et fortement des enzymes immunosuppressives iNOS (inducible NO synthase), IDO (Indoleamine 2,3-dioxygénase) et arginase I. L'utilisation d'inhibiteurs de ces enzymes montre que la protection contre le diabète est induite par IDO. Nous avons également observé une forte infiltration de lymphocytes T autoréactifs dans les îlots pancréatiques des souris infectées. De façon intéressante, l'incidence accrue de diabète du groupe CVB4 est associée à une fréquence élevée de cellules T autoréactives produisant de l'IFN- γ dans le pancréas, alors que la production d'IFN- γ par les cellules T autoréactives est très faible dans les souris du groupe CVB4+ α GalCer. Cette inhibition de la production d'IFN- γ est dépendante de l'enzyme IDO, car l'utilisation d'un inhibiteur d'IDO augmente fortement la production d'IFN- γ par les lymphocytes T anti-îlots et l'incidence de diabète.

Dans l'ensemble nos résultats montrent, que l'activation des cellules iNKT lors de l'infection par CVB4 induit des macrophages immunosuppresseurs dans le pancréas, ces cellules inhibant la fonction des lymphocytes T autoréactifs et ainsi le développement du diabète.

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List of Abbreviations

AAMφ	Alternatively activated macrophages	mAb	Monoclonal antibody
ACS	ACS transporter	MAIT	Mucosal-associated invariant T
AhR	Aryl hydrocarbon receptor	MCP-1	Monocyte chemoattractant protein-1
APC	Antigen presenting cells	MDA5	Melanoma differentiation-associated protein 5
BB	BioBreeding	MDSC	Myeloid derived suppressor cells
CAMφ	Classically activated or type 1 macrophages	Met	Methionine
CAR	Coxsackie and adenovirus receptor	MHC	Major histocompatibility complex
CMV	Cytomegalovirus	MHV	Mouse hepatitis virus
CTLA-4	Cytotoxic T lymphocyte antigen 4	MS	Multiple sclerosis
CVB3	Coxsackievirus group B3	Mφ	Macrophages
CVB4	Coxsackievirus group B4	NF-κB	Nuclear factor kappa beta
Cys	Cysteine	NKT	Natural killer T
Cys2	Cystine	NO	Nitric oxide
DC	Dendritic cells	NOD	Non Obese Diabetic
DN	Double negative	pDCs	Plasmacytoid DCs
EAE	Experimental autoimmune encephalomyelitis	PD-L1	Programmed cell death 1 ligand 1
EIF2	Eukaryotic initiation factor 2	PFU	Plaque forming unit
EMC-D	Encephalomyocarditis D virus	PKR	dsRNA-dependent protein kinase
GAD	Glutamic acid decarboxylase	PLN	Pancreatic lymph node
HCG	Human chorionic gonadotropin	Pro-ins2 ^{-/-}	Proinsulin 2 knockout NOD mice
HLA	Human leukocyte antigen	qPCR	Quantitative RT-PCR
IDDM1	Insulin-dependent diabetes mellitus locus	RA	Rheumatoid arthritis
IDO	Indoleamine -2,3-deoxygenase	ROS	Reactive oxygen species
IFIH1	IFN induced with helicase C domain 1	SEA	<i>S. mansoni</i> egg soluble antigen
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit related protein	SLE	Systemic lupus erythematosus
IL-	Interleukin-	T1D	Type 1 diabetes
iNKT	Invariant NKT cells	T3A	Type 3 Abney
iNKT17	INKT cells producing IL-17	TGF-β	Tumor growth factor β
iNOS	Inducible nitric oxide synthase	Th	T helper
IP-10	IFN-γ-induced protein-10	Thi	Thioredoxin
ISH	In situ hybridization	TLR	Toll-like receptor
KRV	Kilham rat virus	TNF-α	Tumor necrosis factor α
L. major	Leishmania major	Tregs	Regulatory T cells
LCMV	Lymphocytic choriomeningitis virus	Wt	Wild type
		αGalCer	αGalactosylceramide

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Introduction

I - Type 1 diabetes

Type 1 diabetes (T1D) is an organ-specific autoimmune disease characterized by the destruction of β -cells within the islets of Langerhans in the pancreas. Pancreatic islet β -cells produce and secrete insulin and once 80–90% of the β -cells have been destroyed, insulin production becomes insufficient, resulting in hyperglycemia. T1D is considered a childhood disease because most patients develop T1D by 20 years of age and accounts for 1–5% of all diabetes cases. The typical symptoms are constant thirst, weight loss, polyuria, and polydipsia. Studying diabetes is often difficult because its development is very heterogeneous among patients. In some diabetic patients, it can develop rapidly without clear signs of autoimmunity, such as the presence of autoantibodies (1). Other patients can have a subclinical phase of various durations, characterized by the presence of autoantibodies and autoreactive T cells recognizing islet antigens before the onset of overt diabetes (2). Furthermore some patients can harbor islet autoantibodies for many years without ever progressing to T1D (3). Still, even though autoantibodies are not considered to directly cause T1D, they are a useful tool for predicting diabetes development since the number of autoantibodies found in the serum of patients is predictive of T1D. The circulating autoantibodies and autoreactive T cells mostly target β -cell antigens such as proinsulin, glutamic acid decarboxylase (GAD), tyrosine phosphatase IA-2, islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) and chromogranin A (4). It is not yet clear what initiates the breakdown of tolerance towards β -cells, but genetic and environmental factors have been implicated, both alone and in synergy.

T1D is suggested to begin with the death of some islet β -cells that can result from a natural process of tissue remodeling, from a metabolic stress due to change in a diet or from viral infections. Pancreatic antigen presenting cells (APC), particularly dendritic cells (DC) and macrophages, then clear apoptotic bodies by engulfing them. While apoptosis by itself is not immunogenic, factors such as the inefficiency of immune cells to clear apoptotic bodies or a viral infection can induce a strong inflammatory environment in pancreatic islets that will activate APC and render them immunogenic. After migration to the pancreatic lymph node (PLN) and the spleen, immunogenic APCs would present the islet antigens to autoreactive T cells, activate them and induce their proliferation. Autoreactive T cells would then infiltrate the pancreas and destroy islet β -cells thereby inducing T1D.

Much knowledge about T1D comes from animal models because human pancreatic tissues are not easily available. Among rodents, Non Obese Diabetic (NOD) mice have particularly been used as they spontaneously develop T1D bearing similarities with human T1D. The analysis of pancreas from diabetic patients has revealed that CD8 cells formed the most prominent cell type in pancreatic lesions, followed by B lymphocytes and macrophages and CD4 T cells (5). Similarly, immune cells, such as DCs, macrophages, NK cells, CD4 and CD8 T cells infiltrate the pancreas of NOD mice. The infiltration can be observed already at three weeks of age even though diabetes will develop after twelve weeks of age suggesting that a similar preclinical phase persists in NOD mice. Besides, a number of diabetes susceptibility loci have been found to be common among humans and mice which concern the Major histocompatibility complex (MHC), the interleukin-2 (IL-2) and the suppressor receptor cytotoxic T lymphocyte antigen 4 (CTLA-4) (6). Another advantage of NOD mice is the possibility of generation of different genetically modified mice.

The role of T lymphocytes in T1D

The immune mechanisms leading to T1D development are very complex. Both cells of the innate and adaptive immune systems have been shown to participate in T1D pathogenesis. However, the absence of diabetes development in NOD scid mice, devoid of lymphocytes, showed the critical role of lymphocytes as effector cells.

B lymphocytes were shown to participate in T1D pathogenesis in both humans and NOD mice. A recent phase two clinical trial showed that B lymphocytes depletion using rituximab resulted in better preservation of islet β -cells compared to untreated subjects (7). NOD mice, devoid of B lymphocytes, were shown to have a complete inhibition or delayed T1D development in several but not in all studies (8; 9). While autoantibodies generated by B lymphocytes are used in clinic to evaluate T1D development, they are not actually believed to cause β -cell death. Rather, the role of B lymphocytes in human T1D pathogenesis is attributed to their capacity to present autoantigens to autoreactive T cells. Recently, however, the works performed in our laboratory showed that by B-1a lymphocytes are part of complex immune interplay involving IgGs secreted by B-1a cells, neutrophils and IFN- α secreting pDCs that are all critical for the initiation of the diabetogenic T cell response and type 1 diabetes development (10).

Studies showing evidence of the role of T lymphocytes in T1D development are numerous. T1D diabetes can be transferred into NOD neonates by T cells from diabetic NOD donors (11). Thymectomy could also strongly decrease T1D of neonatal BB/W rats (12). Treatment of newly born NOD mice with a depleting anti-CD3 antibody strongly reduces T1D incidence (13). Similarly, anti- α/β TCR mAb was shown to effectively inhibit T1D development (14). Moreover, T cell modulation in already diabetic NOD mice by both anti-CD3 and anti- α/β TCR mAb induces long term remission (14; 15). Based on the results in NOD mice several clinical trials with anti-CD3 mAbs were performed. In a phase II clinical trial performed by Herold et al. T1D patients were given two courses of anti-CD3 mAb (Teplizumab), one year apart, soon after the onset of diabetes. The results showed that this treatment strongly reduced the loss of islet β -cells in a subset of patients compared to untreated patients (16).

1.1 The role of CD4 and CD8 T cells in T1D

The susceptibility or resistance to the development of T1D is strongly determined by MHC class II alleles suggesting that T1D was caused mainly by autoreactive CD4 T cells. Indeed, the depletion of CD4 T cells prevents T1D development in NOD mice (17). Additionally, β -cell chromogranin A restricted BDC2.5 CD4 T cells, that were isolated from diabetic NOD mice, can effectively transfer diabetes to NOD scid recipients in the absence of CD8 T cells (18; 19). In humans CD4 T cells directed against islet antigens such as insulin, IGRP and IA-2 have been identified. Even in fully competent NOD mice, the absence of certain anti-islet CD4 T cells can completely abolish diabetes development (20). However, later studies defined that not only CD4, but CD8 T cells as well were important for promoting T1D development. Thus, NOD mice develop neither insulinitis nor diabetes if they lack the expression of MHC class I molecule and therefore CD8 T cells or when they only lack CD8 T cells (21). Moreover, NOD mice lacking the expression of MHC class I molecules on either islet β -cells or APCs do not develop T1D highlighting the necessity of interactions of CD8 T cells with islet β -cells as well as with APCs presenting autoantigens (22). Interestingly, the potential to induce diabetes when transferred into recipient mice is not the same for CD4 and CD8 T cells. Using adoptive transfer experiments, Bendelac et al. showed that diabetes development in neonatal NOD mice requires the transfer of both CD4 and CD8 T cells from

diabetic NOD donors (11). Christianson et al. have shown T1D could be induced in NOD scid mice by transferring CD4 T cells isolated from diabetic NOD mice. This T1D incidence was increased when CD8 T cells were co-transferred as well. Contrary to CD4 T cells, CD8 T lymphocytes alone rarely transfer diabetes. They can however induce T1D in recipient mice in which CD4 T cells were already present (23). In a different study Yagi et al. transferred either CD8, or CD4, or both CD8 and CD4 T cells from nondiabetic mice into NOD scid mice and treated the recipient mice with cyclophosphamide to promote the onset of overt diabetes (24). While CD8 or CD4 T cells transferred alone did not induce diabetes, the co-transfer of both CD8 and CD4 T cells resulted in high diabetes incidence. Thus, an effective T1D development requires the presence of both of these populations.

Exactly which of these two populations, CD4 or CD8 T cells, plays a dominant role in the actual killing of islet β -cells has also been studied. Both of these populations have a cytotoxic potential by expressing cell surface death molecules and releasing soluble mediators. Inflammatory cytokines such as IFN- γ and TNF- α released by CD4 T cells and granzyme/perforin released by CD8 T can kill islet β -cells (25). Regarding interactions with HLA molecules, human islet β -cells constitutively express low level of HLA class I molecules. In T1D patients there is a hyperexpression of HLA class I making them fully capable of presenting autoantigens to CD8 T cells. While human islet β -cells do not express HLA class II molecules under steady state conditions, they were shown to upregulate this molecule in the presence of inflammatory cytokines (26). Therefore, islet β -cells can also present autoantigens to CD4 T cells. The histological analysis of pancreata from recent T1D patients allowed shedding some light on this point. It has revealed that CD8 T cells form the most predominant population in the pancreatic islets of T1D patients (Fig. 1) (27; 28). The second most abundant cell type is macrophages, while CD4 T cells and B lymphocytes are less prevalent. These histological data let to suggestion that the actual killing of β -cells is mediated by CD8 T. Given that diabetes can be prevented by CD4 T cell depletion, CD4 T cells were suggested to be required for their function as helper cells in the activation of effector CD8 T cells. Additionally, when found in insulitic lesions, they are thought to promote inflammation. This notion comes from several publications that described the immune environment in pancreas as being Th1 rather than Th2. Rothe et al. compared the immune response to cyclophosphamide treatment between diabetes prone NOD mice and several diabetes resistant strains (29). The results showed an upregulation of Th1 type cytokines IL-12, IL-18 and IFN- γ in NOD mice compared to diabetes resistant strains. IFN- γ upregulation was seen only in one diabetes resistant strain, but interestingly, IL-4 was

upregulated as well suggesting that elevated Th2 responses could counterbalance the pathogenic effect of IFN- γ in mice that do not develop T1D. In line with this observation, IFN- γ was shown to be expressed, while IL-4 expression was shown to be reduced in new-onset T1D patients compared to healthy controls (30; 31).

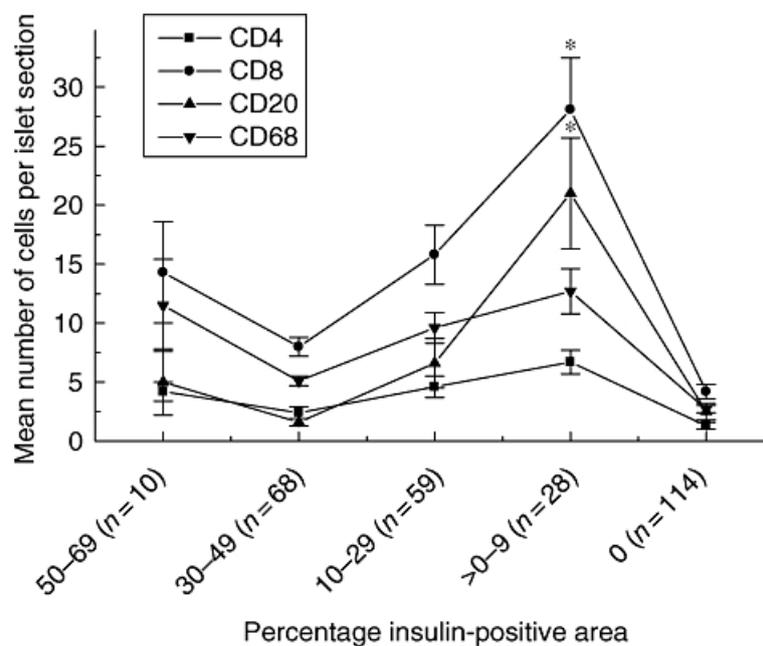


Figure 1. Relationship between insulin immunopositivity and different immune subsets in the islets of patients with recent-onset type 1 diabetes) (Figure source: (28)).

Identification of islet autoantigens targeted by T cells is of great interest since they can allow the development of therapies for preventing or arresting T1D development. Just before the onset of T1D, a large fraction of pancreatic CD8 T cells of NOD mice was shown to be composed of T cells that share TCR α chain bearing the Va17-Ja42 rearrangement (32). These T cells produce IFN- γ when stimulated with their high affinity NRP-V7 mimotope. The identification of NRP-V7 has allowed developing MHC class I tetramers loaded with this mimotope and identifying IGRP specific T cells by flow cytometry analysis. This tool also allowed determining that IGRP specific T cells can account for up to 40% of pancreatic CD8 T cells. Besides, the frequency of these T cells in the peripheral blood of NOD mice could predict whether the onset of T1D is imminent or not. A representative T cell clone from this population was used to generate transgenic NOD mice (8.3 NOD) expressing the NRP-V7

reactive TCR. These 8.3 NOD mice develop accelerated diabetes compared to NOD mice, but only when CD4 T cells are present (33). In this case CD4 T cells induced the migration of diabetogenic CD8 T cells into the pancreas. These observations further highlight the importance of CD4 T lymphocytes as helper cells. Further studies discovered that the natural target for these T cells was IGRP. 8.3 CD8 T cells recognized particularly the peptide ranging from residues 206-214 of IGRP (VYLKTNVFL peptide sequence) (34). IGRP specific CD8 T cells can be found in the pancreatic islets of NOD mice as early as three weeks of age. At this stage however their avidity for IGRP₂₀₆₋₂₁₄ is low. These CD8 T cells undergo avidity maturation and increase in frequency both in the pancreas and peripheral blood of NOD mice as T1D progresses (35). Importantly, autoreactive T cells directed against several other IGRP peptides were also detected among peripheral blood mononuclear cells as well as in pancreatic lesion of diabetic patients but not in healthy controls (36; 37).

Given the diabetogenic potential of anti-IGRP T cells, Krishnamurthy et al. studied the implication of these cells in T1D initiation in NOD mice. They developed transgenic NOD mice that overexpressed IGRP under the MHC class II promoter thereby increasing IGRP presentation by APCs (38). As a result, the frequency of anti-IGRP CD8 T cells was strongly decreased in these mice due to increased IGRP presentation in the thymus and an effective negative selection. Interestingly, diabetes developed at a similar rate between IGRP transgenic and non-transgenic NOD mice. Therefore, reactivity to IGRP is not required for T1D initiation. Moreover, the normal progression to T1D in transgenic mice showed that decreased numbers of anti-IGRP CD8 T cells can be compensated by anti-islet T cells directed against other antigens.

NOD mice and T1D patients share islet β -cell antigens such as insulin, IGRP or GAD. While the majority of autoreactive CD8 T cells recognize IGRP₂₀₆₋₂₁₄ in prediabetic and diabetic NOD mice, in T1D patients CD8 T cell response is dominated by pre- (pro-) insulin antigen (39; 40). The autoantigen driving T1D initiation in NOD mice is suggested to be insulin as well (41). In NOD mice the 9–23 amino acid region of the insulin B-chain (B:9-23) is an immunodominant T-cell target. Nakayama et al. have shown that NOD mice which express a single proinsulin gene with a mutated immunodominant B:9-23 epitope are completely protected from diabetes (42). Additionally, by contrast to IGRP, the elimination of anti-insulin T cells, by overexpressing insulin in APCs and enhancing the negative selection of anti-insulin autoreactive T cells, prevents insulinitis and diabetes development in NOD mice (43). The frequency of anti-IGRP CD8 T cells is significantly decreased in mice lacking anti-insulin T cells compared to non-transgenic NOD mice. This result suggests that perhaps anti-

insulin CD8 T cells promote T1D development by killing first islet β -cell and initiating the establishment of insulinitis. Consistent with this proposition, Coppieters et al. observed that single islets of new onset T1D patients contained only one specificity of islet-autoreactive CD8 T cells, whereas in patients with long lasting T1D islets were surrounded by CD8 T cells of multiple specificities, suggesting that T cells proliferate around the islets (37). Therefore, at least in young NOD mice anti-insulin CD8 T cells could infiltrate pancreas, proliferate and kill islet β -cells resulting in antigen spreading and activation of anti-IGRP CD8 T.

While insulin and IGRP antigens have been largely studied, numerous other autoantigen exist in T1D such as GAD65 (44-46), IA-2 and phogrin (47) and heat-shock protein 60 (48) chromogranin A (18; 19) and several others.

Until recently, it wasn't clear whether T cells infiltrated the pancreatic islets due to the expression of their cognate antigens in the pancreas or as a result of their non-specific recruitment due to the ongoing inflammation and expression of chemokines. However, several recent studies have demonstrated that T cells infiltrate the pancreas only if their antigen is expressed in the pancreas and is presented by MHC molecules. Lennon et al. have created transgenic mice expressing two CD4 T cell populations, only one of which expressed TCR specific for an islet autoantigen, while the second population expressed a TCR whose antigen was not expressed in the pancreas (49). In these mice, only T cells whose TCR recognized a β -cell antigen could accumulate in the pancreas while the numbers of nonspecific T cells remained extremely low. In a different study, the authors introduced two mutations into the dominant epitope of TCR contact residues of the IGRP gene. As a result anti-IGRP CD8 T cells could not recognize their antigen and neither activated nor naïve anti-IGRP CD8 T cells were able to enter the pancreas (50). These observations can explain why the accumulation of lymphocytes is only seen around islets that contain β -cells (28).

1.2 Th17 lymphocytes

Th17 cells were shown to be pro-inflammatory in different autoimmune pathologies such as multiple sclerosis and lupus, thus prompting investigations of the role of these cells in T1D. In a study by Arif et al. peripheral blood CD4 T lymphocytes of 54 T1D patients were found to secrete IL-17 when stimulated with islet antigens compared to only 10% of control subjects (51). Consistent with these results, monocytes from T1D patients were shown to

spontaneously secrete proinflammatory cytokines IL-1 β and IL-6, which are known to induce and expand Th17 cells (52). In vitro these monocytes induced IL-17 deviation in allogeneic memory T cells. An increase in Th17 cell numbers was also observed in the PLN of T1D patients in a study by Ferraro et al. even though not in the peripheral blood (53).

In NOD mice, the progression from insulinitis to overt diabetes is associated with the upregulation of IL-17 transcript in pancreatic islets (54). When comparing NOD mice with transgenic NOD mice in which the diabetes susceptibility locus *Idd3* was replaced with a protective *Idd3* locus from a non-diabetic strain (NOD.*Idd3*), Liu et al. observed that T cells from NOD mice differentiate into Th17 cells more effectively than T cells from NOD.*Idd3* congenic mice (55). This was associated with IL-21 cytokine that is present in *Idd3* locus and that promotes IL-17 production by Th17 cells. Similarly, the inhibition of Th17 function either by administration of an anti-IL-17 mAb or anti-IL-25 mAb, that favors Th17 differentiation, prevents T1D development in NOD mice (56). Diabetes prevention in these mice was associated with reduced peri-islet T cell infiltration and an increased numbers of regulatory T cells (Treg) cells in PLN compared to untreated NOD mice suggesting that Th17 cells controlled the activation of Treg cells. In the course of T1D progression, Th17 cells are suggested to interfere only at the later stages of the disease since inhibition of Th17 cells in young NOD (5 week old) mice has no effect on T1D progression. However, in older mice (10 week old), Th17 cell inhibition results in the prevention of T1D (56). The pathogenic role of Th17 cells was also studied in transfer experiments in which in vitro differentiated BDC2.5 Th17 cells could rapidly induce T1D when transferred into NOD scid recipients (57). However, diabetes development in this setting was rather due to in vivo differentiation of these Th17 cells into Th1 T cells (57; 58). The reason for this conversion is suggested to be the unusual upregulation of IL-12 receptor on in vitro differentiated Th17 cells (59). Natural Th17 cells do not express the IL-12 receptor. Therefore the upregulation of this receptor can favor Th1 phenotype after the in vivo transfer.

One of the mechanisms of Th17 pathogenicity was proposed in a study using mouse insulinoma cell line (MIN6) and pancreatic islets (60). In combination with other cytokines, IL-17 treatment of MIN6 cells and murine pancreatic islets was shown to induce the upregulation of iNOS synthase and NO which can be cytotoxic for islet β -cells. In another study, IL-17 was shown to enhance β -cell death by proinflammatory cytokines such as IL-1 β , IFN- γ and TNF- α (51). Finally, Th17 lymphocytes are suggested to disrupt the balance between effector T cells and Treg cells in favor of pathogenic effector T cells.

Besides Th17 cells, studies performed in our laboratory have shown that NKT 17 cells exacerbated T1D induced by diabetogenic BDC2.5 CD4 T cell transfer in IL-17 dependent manner since the inhibition of IL-17 prevented T1D exacerbation blocked by anti-th17 treatment (61).

1.3 Regulatory T cells

Treg cells are potent suppressor cells that maintain peripheral tolerance to autoantigens. Both humans and mice lacking FoxP3 cells develop T1D, along with several other autoimmune diseases suggesting the role of FoxP3 cells in the control of T1D. Studies have shown that peripheral blood Treg cells of T1D patients suppress T cell proliferation less effectively than Treg cells of healthy controls (62; 63). Besides blood, functional defects in Tregs in PLN of T1D patients compared to healthy controls were also documented (53). By contrast, Putnam et al. did not observe any differences in Treg cell numbers or suppressive function between chronic T1D patients and healthy controls (64). In humans, these contradictory results can reflect the differences between T1D patients as being newly diabetic vs. chronic diabetic. Studies with NOD mice have also yielded somewhat controversial results. The deficiency in Foxp3 cell numbers were reported in peripheral lymphoid organs in some studies (65; 66), but not confirmed in others (67; 68). Also, when comparing lymphoid organ Treg cells of NOD mice with Treg cells of diabetes resistant C57Bl/6 mice equal suppressive capacities were reported (69). In mice, these differences they can arise because of genetic drifts among various NOD colonies or environmental conditions in animal housing facilities that can influence the Treg cell population. However, these differences might not be important since Treg cell numbers and function in the pancreas seems to be a much more important factor for the control of T1D development than in peripheral organs. For example, NOD mice develop T1D despite having more Treg cells in the thymus compared to C57BL/6 mice (70). By contrast, the transgenic overexpression of chemokine CCL22 that allows increasing the infiltration of Treg cells into pancreas strongly decreases diabetes development (71). Treg cell function and survival depends on the signaling of IL-2 cytokine via CD25 on Treg cells. In NOD mice, pancreatic islet Treg cells were shown to express low levels of CD25, the IL-2 receptor, and BCL-2 rendering them prone to apoptosis and failed to control T1D development (72). The importance of CD25 signaling in Tregs in T1D was further demonstrated in experiments where the administration of IL-2 could reverse T1D in already

diabetic mice (73). This IL-2 administration was shown to increase Treg cell numbers in pancreas as well as the expression of several molecules associated with Treg cell functions such as CTLA-4, GITR and the receptor for IL-2, CD25. Consistent with these results, IL-2/CD25 and CTLA-4 gene polymorphisms are genetic predisposing factors to T1D development in humans and NOD mice (74). Interestingly, defect in IL-2R signaling was shown to decrease the maintenance of FoxP3 expression in Treg cells of type 1 diabetic subjects (75). Given that FoxP3 controls Th17 cells by antagonizing ROR γ and ROR α transcription factors required for Th17 differentiation, it is therefore not surprising that the imbalance of Th17 cells and Treg cells has been suggested to promote T1D pathogenesis. Besides Foxp3, Treg cells have been shown to control Th17 cells through Stat3 as the invalidation of Stat3 in Treg cells led to increase of Th17 cells (76). Consistent with these data, Ferraro et al. observed that functional defects in Treg cells of PLN in T1D patient correlated with the increase of Th17 cells (53). It is worth mentioning that the protection from T1D after the inhibition of IL-17 cytokine was associated with increased Treg cell numbers in pancreas.

Besides the localization of Treg cells, the age at which these cells are studied in NOD mice can account for discordance among different studies. Using transgenic BDC2.5 NOD model, Tritt et al. observed that Treg cell numbers in peripheral lymphoid organs remain unchanged in young (4 week-old) and old (8 week-old) NOD mice compared to diabetes resistant strains (68). However, using transfer experiments, they observed that Treg cells of older mice prevented T1D less effectively than Treg cells of younger mice. This observation was suggested to result from a reduced proliferative capacity of Treg cells from older mice in pancreas, thereby contributing to the shift of Treg/T effector balance towards T effector cells.

II. Relationship between genetics, environment and autoimmune diabetes

The frequency of autoimmune diseases has increased in recent decades. This rise includes both allergic diseases such as asthma, whose incidence has more than doubled since 1980 in the United States, rhinitis and atopic dermatitis (77; 78), and autoimmune diseases such as T1D (79; 80), multiple sclerosis (81) and Crohn's disease (82). The annual increase in the frequency of T1D is estimated to be 3% (83), although its global distribution is not homogenous. In fact, the distribution of many autoimmune diseases forms a gradient with the highest frequency in the north that decreases towards the south of the northern hemisphere and from the south to the north in the southern hemisphere (84). Such gradients have been observed for multiple sclerosis in the United States and Australia (85; 86) and for T1D, with the Canadian province of Newfoundland and Labrador and European countries having the highest rates (Fig. 2) (87). In Europe, the highest incidence was observed in Nordic countries (88), with Finland being on top of the list (40.9/100,000/year) followed by Sweden (30/100,000/year) and Norway (20.8/100,000/year) while in most Asian countries the incidence was lower than 1/100,000/year (89). Genetic and environmental factors have been proposed to explain these differences.

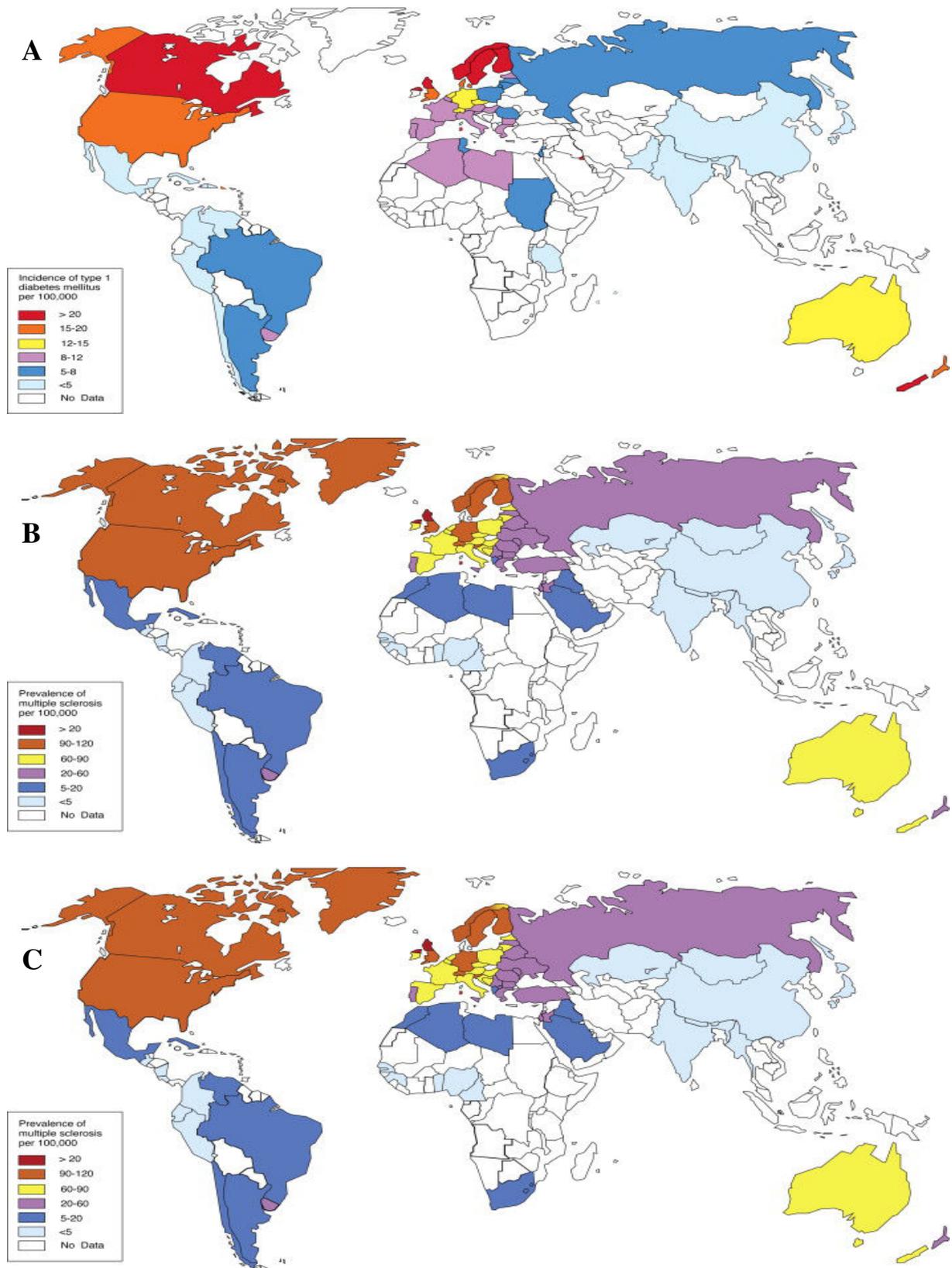


Figure 2. Worldwide distribution of autoimmune diseases: (A) Type 1 diabetes, (B) multiple sclerosis, (C) ulcerative colitis (Figure source : Shapira et al. Journal of Autoimmunity 2010 (90)).

1.1 Genetics

The development of T1D is under genetic control. T1D is particularly common in families with one or more diabetic siblings or first-degree relatives (91) and there is a high (40–60%) concordance of diabetes in identical twins (92; 93). More than 40 disease susceptibility genetic loci have been identified in T1D, which include the genes coding insulin, CTLA-4, IL-2 receptor α , the tyrosine phosphatase PTPN22 and the intracellular viral RNA sensor IFIH1 (94). However, the strongest risk for T1D is associated with human leukocyte antigen (HLA) loci, also known as insulin-dependent diabetes mellitus locus (IDDM1), particularly the HLA class II DR and DQ alleles, which is found in around 40% of cases, although these loci can also confer protection against T1D. Interestingly, European regions with the highest incidence of T1D, such as Finland or Sardinia, also have the highest frequency of T1D-predisposing MHC class II alleles, HLA DR3/4-DQ8 (95). However, not all individuals carrying a susceptibility allele develop T1D, and the susceptibility alleles can have different effects in different nations. For example, while DR3/4-DQ8 alleles confer increased risk in Bahraini Arabs, they actually play a neutral role in the Lebanese population (96). Meanwhile, Japanese individuals carry both susceptibility and protective alleles, and it is the balance between these alleles that contributes to the low incidence of T1D in Asia. Thus, the distribution of different HLA alleles at least partly accounts for the differences in T1D incidence worldwide; however, other factors are also involved. For example, the frequency of susceptibility and protective HLA-DQ alleles is 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 (97). Similarly, while the frequency of various alleles does not differ between Baltic States and Nordic countries, the incidence of T1D is higher in Nordic countries (98). There is also a difference in the incidence of T1D between eastern and western Germany, even though both populations share the same genetic background. Another striking observation concerns immigrant families. First generation Pakistani children born in the United Kingdom have a similar rate of T1D as the local population (11.7/100,000/year), but this is 10 times higher than in the incidence in Pakistan (1/100,000/year) (99). Another recent study showed that children with non-Swedish parents but living in Sweden have an increased risk of T1D compared with children in their native countries (100). All of these observations support the role of non-genetic factors in the etiology of T1D. Nevertheless, the genetic background is important because the incidence of T1D in Sardinian families migrating to a country with a lower incidence of T1D remains high, similar to that in their native Sardinia (101; 102). Clearly, genetic susceptibility is a very strong factor underlying the development

of T1D, but external factors might play a decisive role in either inducing or protecting against T1D.

1.2 Environment

It is clear, that genetics is a risk factor that accounts for at least some of the pattern of T1D distribution; however, it cannot explain the rapid worldwide increase estimated to be 3% per year (103). If this rise was solely dependent on genes, one would expect an increase in the frequency of predisposing HLA alleles among newly diabetic patients. However, this is not the case. In fact, the frequency of susceptibility alleles has actually decreased while that of protective HLA alleles has increased among newly diagnosed children (104; 105). Therefore, the role of changing environmental factors has been proposed and examined in epidemiological and animal studies. As a result, epidemiological studies have suggested an association between the incidence of T1D and socio-economic status, which reflects the exposure to microbial agents and dietary habits. Sun exposure and vitamin D intake have also been proposed to influence T1D onset.

1.2.1 Socio-economic status and the role of infectious diseases

The European north–south gradient not only correlated with T1D distribution but also with the degree of development and national growth income of the countries in Europe. The richest and most developed countries have the highest incidence of T1D (Fig. 3) (106).

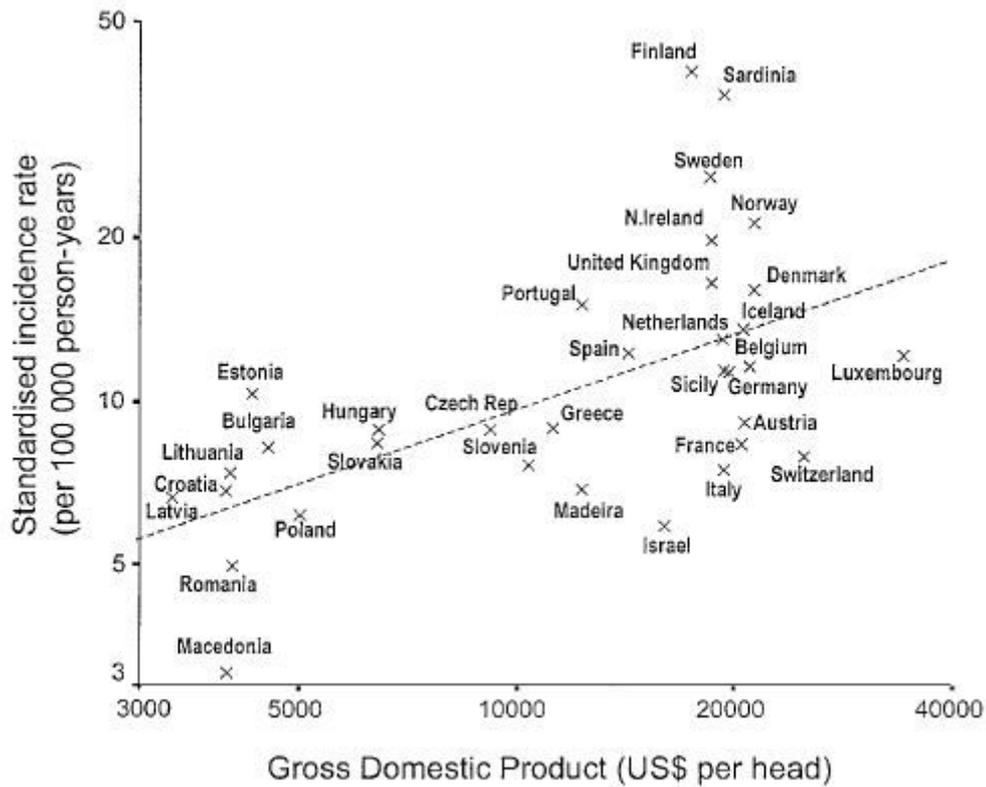


Figure 3. Association between type 1 diabetes prevalence during 1989-1994 and gross domestic product (Figure source : Patterson et al. Diabetologia 2001 (106)).

There are numerous differences between poor and rich countries, and one particularly interesting finding relevant for T1D is the increase in hygiene and decrease in infection because both phenomena are very recent. Developed countries have better hygiene because they invest in general cleanliness of cities, in education, and in medical care such as vaccination. These approaches have eliminated the favorable niches where pathogens used to proliferate, such as sewage, thus limiting the numbers of pathogens. In addition, if an infection occurs, its spread is often better controlled through greater accessibility to drugs and campaigns aimed at instilling people to follow basic rules of hygiene, such as frequent hand washing. Consequently, people are no longer exposed to the wide variety of pathogens that they used to be, and the age at which children encounter such pathogens has increased. Consistent with these observations, the increasing frequency of immune-mediated diseases such as T1D, allergy and asthma has been correlated with the decreasing rate of infections with mumps, tuberculosis, and hepatitis B or C viruses (Fig. 4).

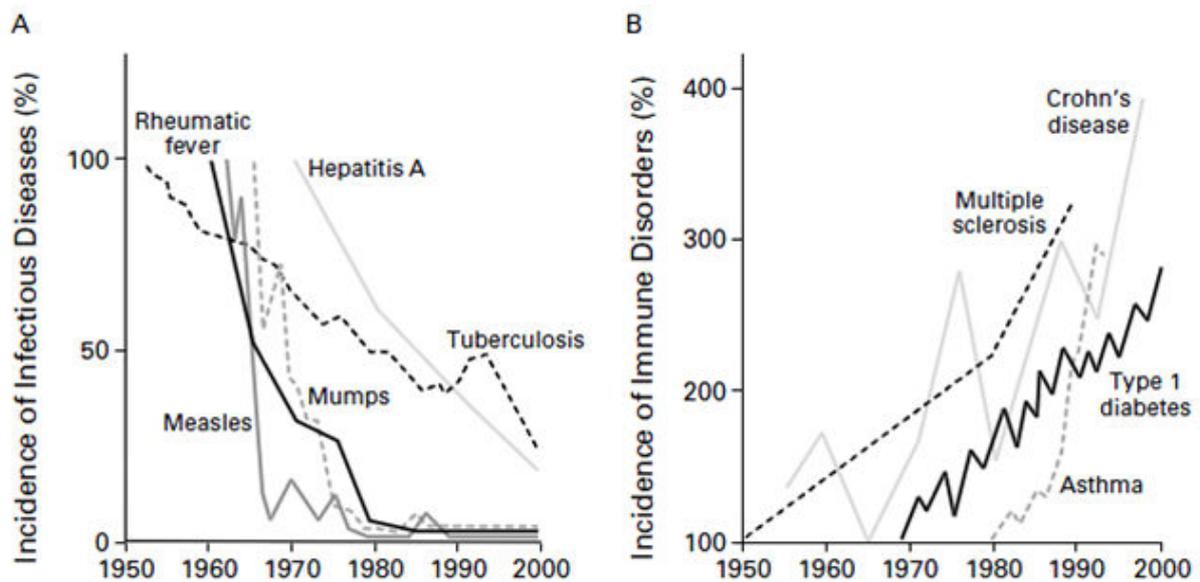


Figure 4. Correlation between (A) the incidence of infectious diseases and (B) autoimmune diseases (Figure source : Bach et al. N Engl J Med. 2002 (84)).

In addition to the time pattern, the geographical distribution of some infections inversely correlates with the distribution of autoimmune diseases. Thus the incidence of hepatitis A, schistosomiasis, tuberculosis and other infections has a low prevalence in European countries and USA (Fig. 5). On the contrary, diabetes incidence is the highest in these countries. This observation further supports the protective role of infection in preventing some autoimmune diseases.

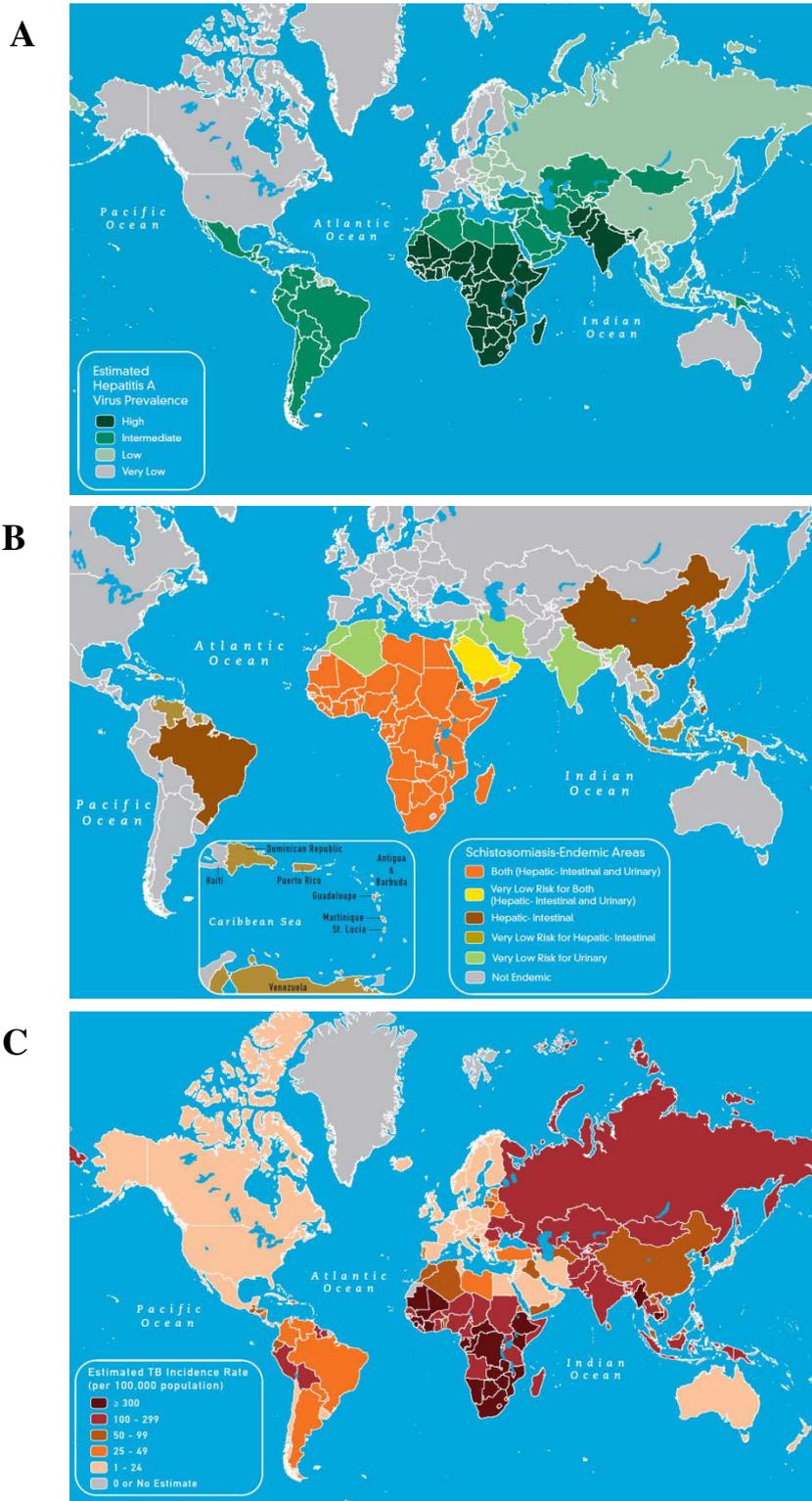


Figure 5. World distribution of infections: (A) hepatitis A infection (Center for disease control and prevention), (B) schistosomiasis (Center for disease control and prevention), (C) tuberculosis (Figure source : World Health Organization report, 2010)

In 1989, David Strachan, a scientist studying the relationship between autoimmune hay fever, hygiene and household factors, proposed that frequent encounters with parasites, bacteria and viruses in early childhood favor the development of a balanced immune system. Otherwise, the untrained immune system may develop inappropriate immune reactions to the self, thus provoking autoimmune diseases. His proposal was later coined as “Hygiene Hypothesis”, and has since been applied to numerous autoimmune and inflammatory diseases including T1D, multiple sclerosis, Crohn’s disease, inflammatory bowel disease, allergy and asthma. This hypothesis is supported by the observation that, in large families, the incidence of T1D is lower among the youngest children than in their oldest siblings, possibly because the youngest children are more exposed to pathogens brought home by their siblings. Most importantly, this happens from a very young age. In a similar way, children who attend daycare less frequently develop autoimmune diseases compared with children who are kept at home and who do not socialize with other children as much.

1.2.2 Protective role of parasitic and bacterial infections in mouse models

Parasites and bacteria can inhibit the development of T1D in animal models (107). For example, infection with *Schistosoma mansoni* or injection of *S. mansoni* egg soluble antigen (SEA) prevents T1D in NOD mice, the mouse model of T1D (108). Gastrointestinal parasites, such as *Trichinella spiralis* or *Heligmosomoides polygyrus*, can also inhibit the development of diabetes in NOD mice by diminishing insulinitis, inducing the secretion of cytokines such as IL-4, IL-10 and IL-13, and skewing T lymphocyte responses towards a T helper (Th)2 profile (109; 110). Infection with the nematode Filarial activates Th2 T cells and Foxp3⁺ Tregs and protects NOD mice from T1D (111). These mice are also protected from T1D by Salmonella infection, which upregulates the inhibitory programmed cell death 1 ligand 1 (PD-L1) receptor (112). Infection with a laboratory strain of *Mycobacterium avium* induced the expression of the death receptor Fas on autoreactive T lymphocytes, which enhanced their killing by other immune cells (113; 114). Interestingly, the *M. avium* subspecies paratuberculosis is currently a focus of research as a possible trigger of T1D in humans (115-117).

1.2.3 Cow's milk

Early introduction of cow's milk into the diet of children has been proposed to trigger T1D. Enhanced expression of antibodies to cow's milk was observed in children who later develop T1D (118). Elliott et al. compared the consumption of milk proteins in 14 different countries and found that the incidence of diabetes increased with increasing consumption of milk protein β -casein A1 and the B variants, with Nordic countries having the highest intake (119). Iceland, where the consumption of these two proteins is lower than in Nordic countries, has a lower incidence of T1D despite similar environmental conditions (Fig. 6) (120). Other milk proteins such as lactoferrin, bovine serum albumin and immunoglobulin against bovine insulin are considered harmless. However, other studies found no such associations and the causative role of cow's milk remains unconfirmed. It is possible that the increased consumption of cow's milk in young infants simply reflects the decreased tendency towards breastfeeding in wealthy Western countries.

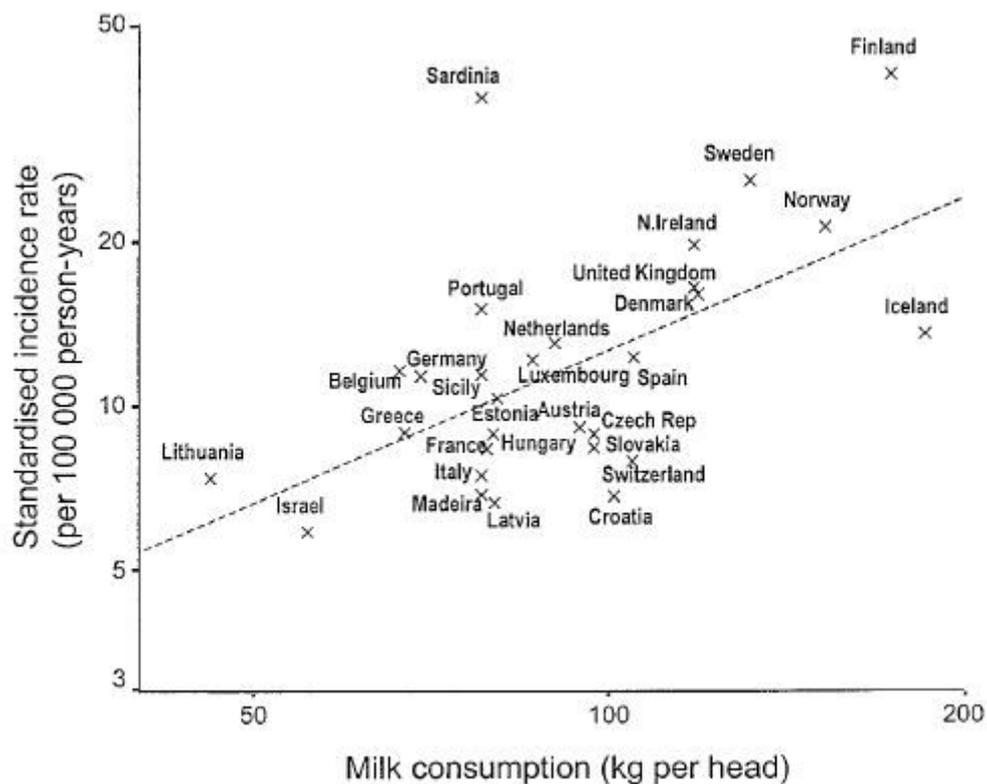


Figure 6. Association between type 1 diabetes prevalence during 1989-1994 and national milk consumption (Figure source : Patterson et al. Diabetologia 2001 (106)).

1.2.4 Wheat and gluten

T1D and celiac disease, an immune disorder characterized by intolerance to gluten present in some cereals, share several common susceptibility HLA alleles and non-HLA alleles, such as CTLA-4 and C-C chemokine receptor type 5 (121). Celiac disease is more frequent among T1D patients than in control subjects. Therefore, dietary gluten was proposed as a link between the gut, immune activation in gut-associated lymphoid tissues and the development of T1D. The German BABYDIAB study of more than 1600 children with T1D parents showed that the introduction of gluten before 3 months of age increased islet autoantibody risk (122). The BABYDIAB investigators are currently investigating whether eliminating gluten in genetically susceptible newborns during the first year of life can delay or decrease T1D incidence.

1.2.5 Sun and vitamin D

The distribution of T1D along the north–south gradient is correlated with exposure to sunlight and, consequently, the amount of vitamin D produced in the presence of solar ultraviolet B rays. Vitamin D was suggested to play a protective role because of its immunosuppressive capacity, and countries with greater solar exposure and enhanced vitamin D synthesis generally show a reduced incidence of T1D (Fig. 7) (123; 124). Mohr et al. reported that each time the daily recommended dose of vitamin D was lowered in Finland (from 4500 IU to 2000 IU in 1960s, to 1000 IU in 1975 and to 400 IU in 1992), the incidence of T1D increased sharply (125). However, other researchers have presented contradictory results.

Dietary supplementation of vitamin D in pregnancy or in the first year of life in children with little sun exposure was reported to reduce the risk of T1D in some studies but not all (126-128). Importantly, sunlight exposure cannot explain the differences in T1D in adjacent Finland and Karelia or in eastern and western Germany, where sunlight exposure is the same. Moreover, the incidence of T1D in Sardinia remains high, despite high sunlight exposure. Therefore, the contribution of this factor to the development of T1D remains under investigation.

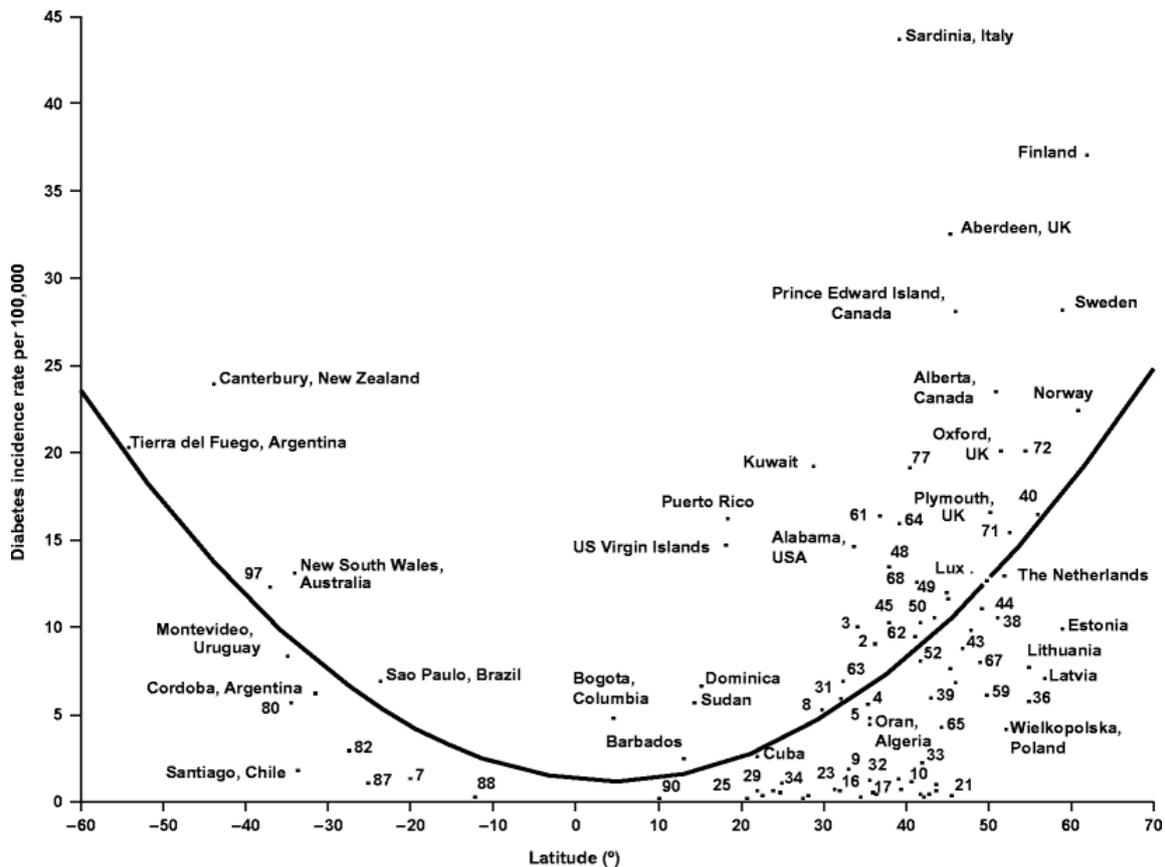


Figure 7. Age-standardized incidence rates of type 1 diabetes per 100,000 boys <14 years of age, by latitude, in 51 regions worldwide, 2002 (Figure source : Mohr et al. Diabetologia 2008 (123)).

2. Role of viruses in T1D

Viruses have been documented as possible causative agents of T1D in humans, and can act via numerous mechanisms. One of these mechanisms is molecular mimicry in which a pathogen-derived peptide shows sequence homology with a self-peptide and the host's T cells mistakenly attack self-tissue. Another mechanism is bystander activation of T cells. Viral infection can provoke significant inflammation and destruction of its target tissue with subsequent release of autoantigens that can activate autoreactive T cells. Inflammation can also induce stress in the endoplasmic reticulum, causing misfolding of proteins and the creation of new autoantigens. Even if the initial amount of autoantigen released is minimal, the small pool of autoreactive T cells, by killing target cells, could provoke the release of normally sequestered autoantigens from β -cells, a process known as antigen spreading.

Viruses can also protect against T1D by several mechanisms. First, when an organism is subjected to repetitive infections, its resources can be used in priority for the expansion and action of antipathogenic immune cells, which limits resources available for autoreactive cells and may, therefore, control their numbers and activation (84; 129). Second, natural selection has made some pathogens able to modify or dampen the host's immune responses to promote their own survival. For example some viruses can alter the functions of macrophages and antigen-presenting cells (130; 131), while other viruses induce the proliferation and differentiation of Treg cells (132; 133). These mechanisms favor the maintenance of peripheral tolerance and prevent T1D onset. Moreover, not only pathogens can directly alter the immune system, but the human body can also promote immune regulatory mechanisms to avoid exacerbated responses that could damage host tissues.

2.1 Viruses and acceleration of T1D

Several murine and human viruses such as rubella, mumps, rotavirus and cytomegalovirus (CMV) can trigger the development of diabetes (Fig. 8). It is important to note that the induction of diabetes by these viruses involves various mechanisms, and the detection of anti-islet autoimmune responses remains elusive for rubella and mumps. The association with a particular HLA allele and/or the identification of antigenic epitopes shared by viruses and islet antigens suggests that specific anti-islet responses could occur.

Infection with **rubella** in the first trimester of pregnancy was associated with a 20% higher incidence of diabetes in children born with congenital rubella infection (134). A relationship between rubella and diabetes has been shown in rabbits and hamsters (134; 135). In New York, the incidence of diabetes increased after a rubella epidemic affecting mostly genetically predisposed children. In a cohort of over 200 infected patients, there was a decrease in the frequency of the HLA-DR2 allele and an increase in the HLA-DR3 allele among diabetic patients (136). Molecular mimicry between human GAD65 and rubella virus protein was suggested as a triggering factor (137). Despite the possible influence of HLA alleles and the described epitope mimicry, rubella-induced diabetes might differ from classical T1D as rubella infection affects many other organs as well.

Mumps virus infection was suggested to be associated with the presence of pancreatic autoantibodies (138). Interestingly, after virtually eliminating mumps infection through a

vaccination campaign in Finland, a plateau in the T1D incidence was observed (139). However, little is known about the autoimmune process induced by mumps virus.

Several studies by Honeyman and colleagues have pointed towards a role of **rotaviruses** in T1D induction. For example, the appearance of antiviral IgG was linked to the appearance of autoantibodies against GAD65, insulin and IA-2 in the Australian BabyDiab study involving at-risk children (140). The same authors also showed that rotavirus could infect pancreatic cells from NOD mice and macaque monkeys (141). Interestingly, the rotaviral VP7 protein shows high sequence homology with islet autoantigens, tyrosine phosphatase IA-2 and GAD65. T cells cross-reacting with VP7 peptides and epitopes of IA2 and GAD65 were recently characterized in T1D patients. These peptides bind strongly to HLA-DRB1*04, which confers susceptibility to T1D (142). All of these findings support the hypothesis that molecular mimicry between VP7 and islet autoantigens could facilitate the development or exacerbation of anti-islet autoimmunity and T1D onset. However, a Finnish study failed to find a link between rotavirus and T1D (143). Concerning mouse models, high replicative rate of rhesus monkey rotavirus strain RRV infection was shown to protect NOD mice (144).

In humans, initial reports found a correlation between **CMV** infection and the presence of anti-islet autoantibodies (145). Molecular mimicry between CMV peptide and GAD65 has been proposed (146). However, other studies failed to find a link between CMV infection and T1D in genetically predisposed children (147; 148). In rodents CMV can trigger T1D in susceptible LEW.1WR1 and BioBreeding (BB) rats (149; 150), and a recent study showed that CMV can infect human β -cells and induce the expression of inflammatory cytokines and chemokines (151).

Besides these viruses that can infect both humans and rodents, two other viruses whose natural host is a rodent are used in laboratory studies to decipher the mechanisms of viral pathogenesis. Even though **encephalomyocarditis D** (EMC-D) virus can directly infect β -cells, the mechanism by which it induced T1D is mostly indirect. After low-dose EMC-D virus infection, the infected macrophages were shown to be involved in the development of diabetes in DBA/2 mice through the production of mediators such as IL-1 β , tumor necrosis factor (TNF)- α and inducible nitric oxide synthase (iNOS). The depletion of macrophages or the inhibition of these three mediators decreased the incidence of T1D (152). IL-1 β and TNF- α can induce the expression of the death receptor Fas on β -cells, and subsequent binding of Fas to its ligand can lead to β -cell apoptosis. Notably, nitric oxide produced by iNOS can directly induce β -cell apoptosis.

Several laboratories have analyzed the pathogenic role of the **Kilham rat virus (KRV)** in T1D induction in diabetes-resistant BB rats (149; 153; 154). Even though the KRV does not infect β -cells, it skews the immune responses towards a deleterious Th1 profile (153). Subsequent studies showed that KRV induces the production of the proinflammatory cytokines, IL-12 and IL-6. Interestingly, IL-12 production was dependent on Toll-like receptor (TLR)9, and blocking of TLR9 by chloroquine prevented the development of T1D in BB rats (154).

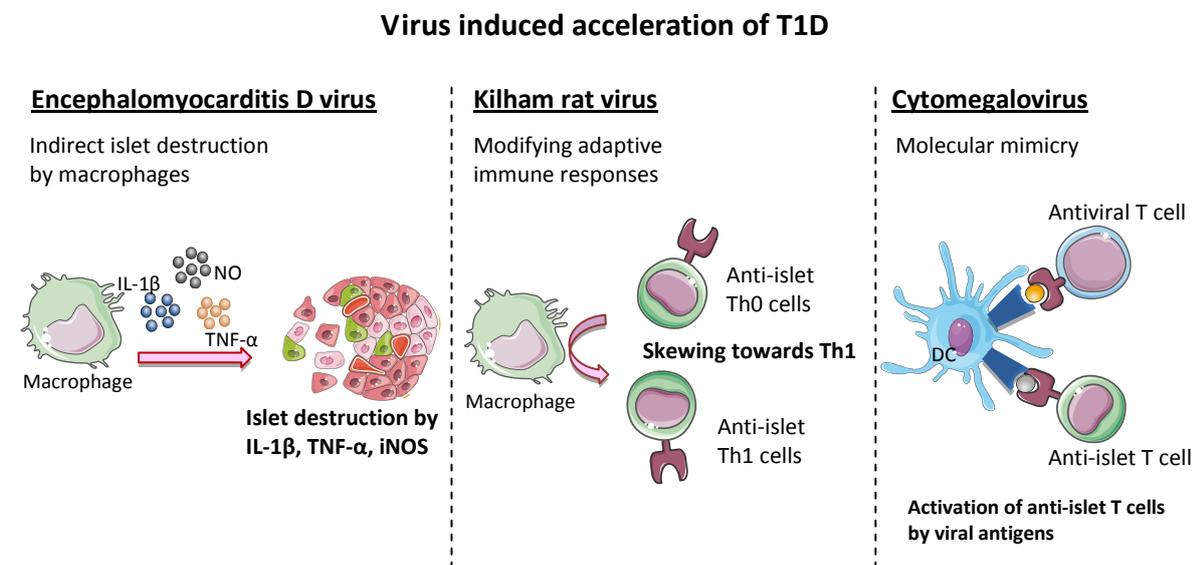


Figure 8. Virus induced acceleration of T1D. Viruses can accelerate T1D by inducing immune cells such as macrophages to produce proinflammatory cytokines that can kill pancreatic β -cells or by skewing immune responses towards generation of pathogenic anti-islet Th1 cells. Molecular mimicry can be another mechanism leading to the activation of autoreactive anti-islet T cells (Figure source : Ghazarian et al. Cell Mol Life Sci. 2013 (155)).

Fulminant type 1 diabetes

Fulminant T1D is a subtype of type 1 diabetes. The term fulminant characterizes the extreme rapidity of the disease development. Typically the duration from normoglycemia to hyperglycemia with almost complete destruction of islet β -cells rarely exceeds one week. Fulminant T1D is mostly observed in Japan where it accounts for 20% among rapid onset diabetes patents. By contrast, it is rarely reported in Western countries (156). Some similarities and differences were reported between classical T1D and fulminant T1D. Similar

to T1D, fulminant T1D development has been reported to be of similar frequency between both males and females. Genetic background has been linked to fulminant T1D. Thus, having HLA DR4-DQ4 confers susceptibility to both T1D and to fulminant T1D in Japanese population but is rare in Caucasian population suggesting its possible role in fulminant T1D development. Besides HLA, CTLA-4 has also been shown to confer susceptibility to fulminant T1D (157). In contrast to type 1 diabetes, no seasonal or regional variations have been reported, and the mean age of patients developing fulminant T1D is 40 years.

While the etiology of fulminant T1D stays elusive, viral infections are strongly associated with this disease. Patient with fulminant T1D present flu-like symptoms prior to developing the disease at significantly higher frequency compared to patients with classical T1D (158). Moreover, antibodies against a number of viruses such as human herpes virus 6, influenza B virus, mumps virus, coxsackievirus, hepatitis A virus, CMV and Epstein Barr virus were detected at the onset of fulminant T1D (159). Immune cells, particularly CD8 T cells and macrophages strongly infiltrate the pancreas of patients with fulminant T1D (159). However, whether the destruction of pancreatic islets is mediated by autoreactive T cells or whether, as suggested by Shimada et al., it is indirect and similar to EMC-D infection in mice, remains to be determined (160).

2.2 Protective effects of viruses against T1D

Because the protective role of viruses in T1D in humans can only be observed through the correlation between infection and reduced T1D incidence, the mechanisms by which viruses confer protection against T1D were mainly studied in rodent models. NOD mice have been widely used because they spontaneously develop diabetes with many characteristics of human T1D. Interestingly, these mice less frequently develop T1D in animal facilities that are not pathogen-free or after treatment with pathogen-derived molecules. For example, a single Bacillus Calmette-Guérin injection is sufficient to inhibit T1D when administered to young NOD mice (161). Similarly, treating NOD mice with complete Freund's adjuvant reduced the number of diabetogenic T cells, while incomplete Freund's adjuvant skewed the T cell responses towards a Th2 profile protecting against T1D (162; 163).

Protection against T1D was also observed after infection with **coxsackievirus group B3 (CVB3)**. Two mechanisms act in synergy to delay and reduce the onset of T1D (Fig. 9).

First, CVB3 infection upregulates the expression of the inhibitory receptor PD-L1 on lymphoid cells, which hinders the expansion of PD-1-expressing autoreactive CD8 T cells and delays T1D onset. Second, CVB3 infection enhances the proliferation of Tregs, which produce Tumor growth factor β (TGF- β) to confer long-term protection against diabetes (133). Studies of the **coxsackievirus group B4** (CVB4) strain, which is able to both induce and protect against diabetes, depending on the context, will be discussed later.

Infection of NOD mice with the murine **gammaherpesvirus-68** delays the onset of T1D by reducing the capacity of CD11c⁺ DCs to capture and process autoantigens. This results in the retention of autoreactive T cells in the spleen and pancreatic lymph node, limiting their homing to the pancreas and the killing of islet β -cells. The numbers of Tregs do not change during the course of infection (130).

Even though the reovirus strain **Type 3 Abney** (T3A) can infect β -cells and induce their apoptosis, the infection of newborn NOD mice with this virus reduces or delays the onset of T1D without preventing insulinitis (164). While the exact mechanism by which the T3A strain inhibits T1D has not been determined, the authors proposed two non-exclusive scenarios. The first one is based on the observation that the T3A strain infects various organs, including the thymus. Infection of the thymus of young animals could somehow eliminate autoreactive T cells in this tissue. In the second scenario, the infection and destruction of pancreatic islets could lead to the release of β -cell antigens and the establishment of an active tolerance through Treg cells.

Chronic **mouse hepatitis virus** (MHV) infection can prevent the development of T1D in NOD mice (165). Studies outside of the context of T1D have shown that MHV can, in some cases, suppress the activation of splenic T lymphocytes and reduce the functions of antigen-presenting cells, such as macrophages, DCs and B lymphocytes (166; 167).

The mechanism of protection of T1D by **lymphocytic choriomeningitis virus** (LCMV) has extensively been studied by our group. Since iNKT cells were shown to be key regulators of diabetes prevention by LCMV, this virus will be discussed in the section dedicated to iNKT cells and the role that they play in T1D.

Finally, lactate dehydrogenase virus infection also suppresses T1D onset in NOD mice by reducing the numbers of inflammatory macrophages in the peritoneum (131).

Virus induced protection against T1D

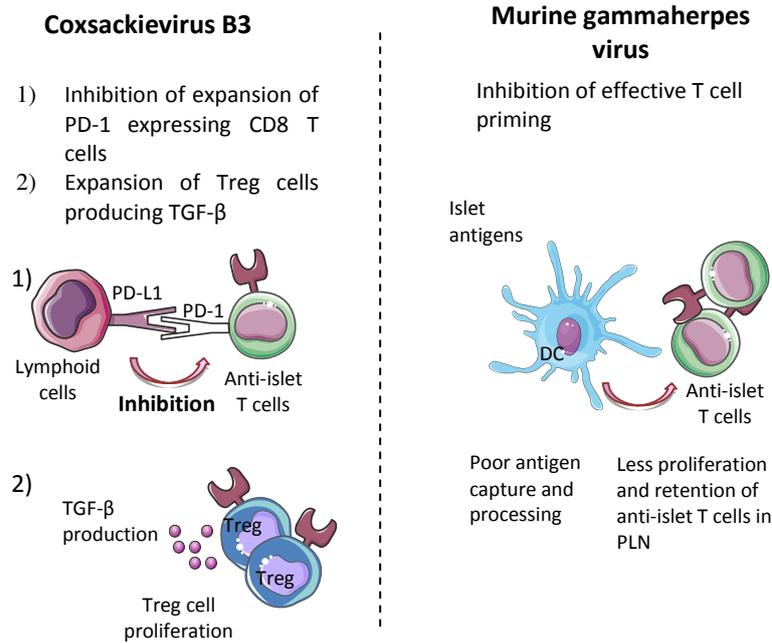


Figure 9. Virus induced protection against T1D. Viruses can induce protection from T1D by eliciting immune mechanisms such as induction of Treg cells, inhibition of anti-islet T cells through suppressive receptors like PD-1 and suppressive cytokines like TGF- β or by blocking the efficient antigen capture and processing by antigen presenting cells (Figure source : Ghazarian et al. Cell Mol Life Sci. 2013 (155)).

3. Dual role of enteroviruses in T1D

The role of enteroviruses in T1D is of great interest. They seem to represent a perfect illustration of the hygiene hypothesis. In times when enterovirus infections were frequent, the incidence of diabetes was low (168). Currently, however, the rarity of enterovirus infection is thought to make them more aggressive in susceptible individuals and can favor the onset of T1D.

The enterovirus genus belongs to the Picornaviridae family. Their genome is composed of a single positive RNA molecule encapsulated in capsid without an envelope. Human enteroviruses include polioviruses, echoviruses, rhinoviruses, enterovirus 71 and coxsackieviruses, which is the most prevalent group after the introduction of poliovirus vaccination. Coxsackieviruses are divided in two groups, A and B, with 24 and six serotypes, respectively. Coxsackievirus group B (CVB), particularly CVB4, is widely implicated in

T1D. Infections with enteroviruses are mostly asymptomatic and only rarely cause complications such as hand, foot and mouth disease, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, and severe neonatal sepsis-like disease. They are predominantly transmitted via the oral–fecal route (i.e. consumption of food or water containing contaminated feces).

3.1 Epidemiological perspectives of enteroviruses

Studies examining the relationship between enteroviruses and T1D probably started with two articles published by Gamble and Taylor in 1969. In their first article, they reported that patients with recent onset of T1D (less than 3 months) had higher antibody titers against CVB4 compared with control individuals and patients whose T1D was not recent (169). In their second article, they reported a seasonal onset of T1D with peaks in late fall/early winter, which followed the seasonal outbreak of CVB infection in late summer/early fall (170). Since then, many studies have analyzed the role of CVB in T1D. The major difficulty in providing direct proof for the pathogenic role of enteroviruses is the interval between enteroviral infection and onset of T1D, meaning the enteroviral infection may be undetectable. Enteroviral infection is mostly identified by the detection of viral RNA in the serum. However, in healthy individuals, enteroviral RNA is detectable for only a few days after infection and most studies analyzed serum samples at intervals of several months. Nevertheless, with the exception of a few studies (171; 172), more than 24 retrospective and prospective studies have detected a link between enteroviruses and T1D (173).

3.1.1 Prospective studies

In 1995, the Finnish DiMe study group showed that enterovirus infections, identified by the presence of antibodies against enteroviral antigens, were almost two times more frequent in siblings who developed clinical T1D compared with siblings who remained diabetes-free (174). Similarly, in the Finnish Diabetes Prediction and Prevention (DIPP) study, anti-enteroviral antibodies were detected in 26% of children who developed diabetes compared with 18% of children in the control group. They reported a temporal relationship

between enteroviral infection and the onset of T1D as 57% of cases had autoantibodies present within 6 months after infection (175). Similar results were observed in the Finnish Trial to Reduce IDDM in Genetically at Risk (TRIGR) project, which revealed that enteroviral RNA was more frequently detected in children developing autoantibodies than in children who did not have autoantibodies (176). The Diabetes and Autoimmunity Study in the Young (DAISY) conducted in Denver, CO, USA, followed 2,365 at-risk children, of which 140 subsequently developed autoantibodies. Among them, 61% of children with enteroviral RNA developed T1D as compared with 28% of children who did not have enteroviral RNA (177). Even in Cuba where the incidence of T1D is low, the presence of enteroviral RNA was reported to be associated with T1D (178). Thus, enterovirus infection is often considered a pivotal event that shifts the balance from chronic subclinical autoimmunity towards destructive autoimmunity.

3.1.2 Retrospective studies

In the United Kingdom, the study by Nairn et al. found that 27% of newly diagnosed patients harbored enteroviral RNA in serum samples taken within 1 week after confirmation of diagnosis, as compared with only 4.9% of healthy controls (179). A recent study by Schulte et al. also detected enteroviral RNA at the onset of T1D whereas no enteroviral RNA was detected in healthy controls (180). This study also underlined the importance of assessing peripheral blood mononuclear cells because 4/10 patients with T1D were positive for enteroviral RNA in peripheral blood mononuclear cells as compared with 2/10 in serum (180). In a Swedish study, anti-CVB4 neutralizing antibodies were more frequently detected in patients with newly diagnosed T1D as compared with healthy controls (181).

Because detecting enteroviral presence in the pancreas requires biopsies or pancreatic samples from postmortem donors, such analyses have been limited. However, in 1979, Yoon et al. isolated a virus from the pancreas of a deceased patient diagnosed with diabetic ketoacidosis. This virus was later identified as being related to the diabetogenic CVB4 strain. When injected into mice, this virus had infected pancreatic islets and caused β -cell loss (182). Similarly, Dotta et al., using immunohistochemistry, detected enteroviral VP1 capsid protein in islets from patients with T1D, whereas islets from healthy donors were virus-free (183). They sequenced the viral genome present in the pancreatic islets of 1 out of 3 VP1 positive diabetic donors and identified this virus as CVB4. The isolated CVB4 could infect human

islets from healthy donors and reduce insulin secretion (183). Richardson et al. detected enteroviral capsid VP1 protein in the pancreatic islets of 44/72 (66%) patients with recently diagnosed T1D, while this protein was not detected in healthy controls (184). In this study, VP1 was also detected in 42% of T2D patients. Interestingly, the authors propose that enteroviral infection could also play a role in T2D, since enteroviral infection of β -cells in vitro decreases glucose-induced insulin secretion. Thus, in individuals with increased insulin resistance and higher insulin requirements, enteroviral infection could contribute to the development of T2D. Enteroviral RNA was also detected by *in situ* hybridization of pancreatic islets from two deceased donors with fulminant CVB3 infection and in some CVB3-infected patients with T1D (185). Indirectly, the presence of a virus has also been suggested by the detection of the antiviral cytokine IFN- α in the pancreas and β -cells of patients with T1D (186; 187).

Finally, echoviruses 4, 6, 9, 14 and 30, other members of the enterovirus family, have been implicated in the etiology of T1D and were shown to impair or kill human β -cells in vitro (188-194). However, studies focusing on echoviruses remain very scarce.

3.2 Understanding the relationship between enteroviral infections and T1D: studies in NOD mice

Because the sequence of P2-C protein of CVB4 is very similar to that of the highly immunogenic GAD65 expressed in β -cells, molecular mimicry was proposed to explain the pathogenic effects of CVB4 in T1D in humans (195). This hypothesis implies that anti-CVB4 T lymphocytes cross-react with GAD65, destroy β -cells, and induce T1D. However, molecular mimicry was ruled out in later studies (196).

CVB4 infection in NOD mice seems to yield contradictory results. The discrepancy might reflect the complexity of the interactions among CVB4, the immune system and pancreatic β -cells, which could lead to the induction or prevention of T1D. Indeed, several parameters, including timing of infection, type of mice, viral dose and viral strain with its particular virulence, play important roles in the outcome of CVB infection in animal models (Fig. 10). All of these factors, in combination, might explain why CVB4 could accelerate T1D in some individuals while remaining asymptomatic in others.

Factors involved in enteroviral induced T1D in mouse models

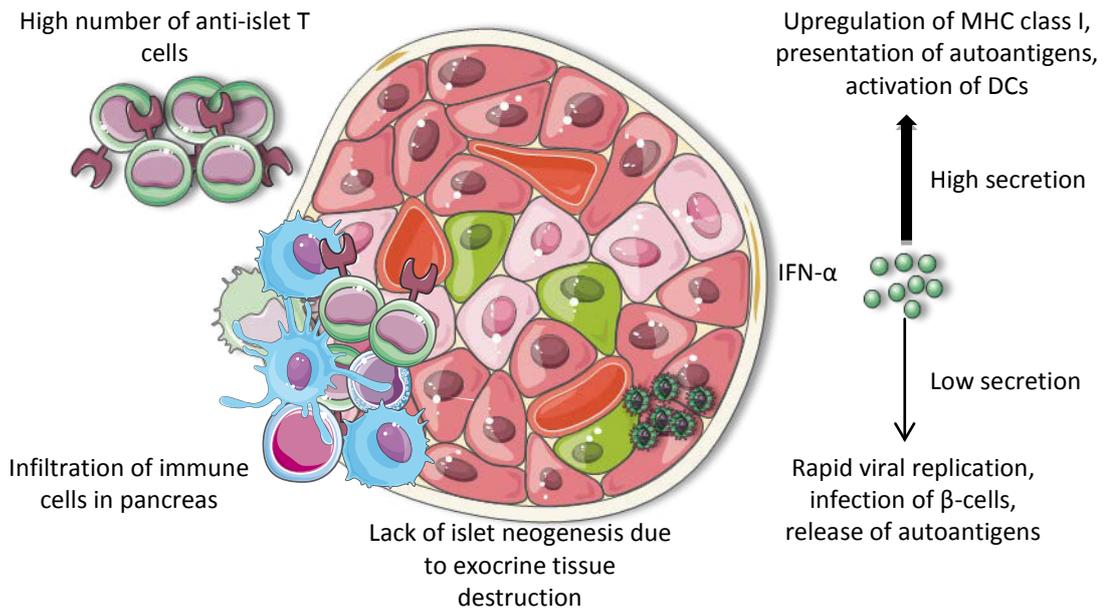


Figure 10. Factors involved in enteroviral induced T1D. Studies in mouse models have revealed that in order for enteroviruses to accelerate T1D, a high number of autoreactive anti-islet T cells are necessary. In addition, pancreas must be infiltrated by cells of the immune system. As for the IFN- α , its high secretion can result in a strong activation of the immune system favoring the presentation of islet autoantigens. On the other hand, if IFN- α secretion is too low viruses will replicate freely, infect islet β -cells and provoke the release of autoantigens leading to the activation of anti-islet T cells and provoking diabetes (Figure source : Ghazarian et al. Cell Mol Life Sci. 2013 (155)).

3.2.1 Age and associated numbers of autoreactive T cells

Using BDC2.5 transgenic NOD mice, Horwitz et al. showed that CVB4 infection of prediabetic mice, harboring a greater number of anti-islet BDC2.5 CD4 T cells compared with younger mice, induces the acceleration of T1D through bystander activation of T cells (197). This study suggested that the number of anti-islet T cells at the time of infection is a critical factor. Two subsequent studies strengthened this hypothesis by infecting non-transgenic NOD mice at different ages. CVB4 infection of young NOD mice, aged 4–8 weeks, did not accelerate or induce T1D (198; 199). However, CVB4 infection of older mice accelerated T1D onset in 61% of mice (199). Interestingly, it seems that both the number and location of autoreactive T cells are important factors. F1 mice, obtained by crossing BDC2.5 NOD mice with BALB/c or C57BL/6 mice had the same numbers of peripheral autoreactive T cells. However, these T cells only infiltrated the pancreas in BDC2.5NOD×BALB/c mice. Upon CVB4 infection, BDC2.5NOD×BALB/c mice but not BDC2.5NOD×C57BL/6 mice,

developed diabetes, indicating that infiltration of pancreas by diabetogenic T cells before infection is an important factor that promotes the onset of diabetes (200).

Based on these experimental results, the pre-existence of a pool of anti-islet T cells and the infiltration of the pancreas by immune cells in children could determine whether or not T1D will develop following enteroviral infection.

3.2.2 Viral titer

A comparison of non-diabetogenic CVB3/GA and diabetogenic CVB3/28 strains prompted Tracy et al. to propose that viral dose and replication rate play an important role in the initiation of T1D (201). Infection of 12-week-old NOD mice with 5×10^5 TCID₅₀ (50% tissue culture infective dose) per mouse of the non-diabetogenic CVB3/GA strain did not induce diabetes, whereas a 100-fold higher dose induced diabetes in 30% of mice. On the other hand, the diabetogenic CVB3/28 strain induced T1D in up to 70% of NOD mice, although this rate decreased when a lower dose of CVB3/28 was administered. These results indicate that the pathogenicity of the strain could be dependent on its dose. Thus, children infected with a high viral dose or whose immune system would allow viruses to quickly reach high titers, could be at increased risk of developing T1D.

3.2.3 CVB4 infection of β -cells and the antiviral response

Some CVB strains have been reported to infect, proliferate, affect the metabolism and destroy human pancreatic islet cells in vitro. In human pancreas, CVB4 was detected in islets but not in the exocrine tissue, whereas it was mostly detected in pancreatic exocrine tissue in mice (202-204). The effects of infection of mouse β -cells with CVB4 are contradictory. It has been described that CVB4 primarily uses the coxsackie and adenovirus receptor (CAR) to enter cells (185; 205). Although CAR and CVB4 were not detected in some studies (206; 207), others have detected CVB4 in murine islets. Importantly, the permissiveness of islet β -cells was suggested to determine the outcomes of infections with different CVB strains. Using immunohistochemistry, Horwitz et al. demonstrated the presence of CVB4 in pancreatic islets at 7 days post-infection in BDC2.5 mice, and the pathogenic effect of the CVB4 strain was attributed to its capacity to infect β -cells whereas CVB3 could not (200; 208). Meanwhile in

old prediabetic NOD mice even the non-diabetogenic CVB3 could infect islets and accelerate diabetes (209). If infection of islet β -cells is an important factor, the efficacy of an individual's response to the virus could determine the progression to T1D.

IFN- α , IFN- β and IFN- γ are the major cytokines that are rapidly secreted after viral infection. The expression of IFN- γ in pancreatic islet β -cells of IFN- γ -deficient mice allows them to control viral replication and survive after CVB4 infection (210). Similarly in transgenic NOD mice in which IFN signaling is inhibited in pancreatic β -cells, CVB4 induces diabetes in 95% of mice while non-transgenic mice remain diabetes-free. This inability to respond to IFNs rendered β -cells permissive to CVB4 infection resulting in their killing by activated NK cells (211). It has been shown that during coxsackievirus infection IFN- α increases the expression of intracellular double-stranded RNA sensor 2-5AS, which activates the enzyme RNase L that cleaves viral RNA and, therefore, protects β -cells from infection. IFN- γ activates the enzyme PKR (dsRNA-dependent protein kinase), which can disturb protein synthesis and block CVB replication (212). Thus, the rapid islet response to viral infection through IFN secretion has a strong influence on diabetes outcome because it determines whether or not enteroviruses can infect islet β -cells. As described above, uncontrolled viral replication, which can yield high viral titers very quickly, can make an otherwise harmless viral strain highly pathogenic.

3.2.4 Islet neogenesis

Islet neogenesis has been proposed to be a factor determining the outcome of CVB infection in mice. Yap et al. used two CVB4 strains, the diabetogenic CVB4/E2 strain and the non-diabetogenic CVB4/JVB strain, and found that the severity of pancreatic acinar tissue damage caused by viral infection influences T1D development (207). While the E2 strain caused massive destruction of acinar tissue without islet destruction, the JVB strain caused only minor damage. This allowed regeneration of the acinar tissue and new pancreatic islets, which were not observed in the severely destroyed pancreas of CVB4/E2-infected NOD mice (207). Although it is unknown whether islet neogenesis occurs in the human exocrine tissue, a marker for cell proliferation, Ki-67, was expressed in human pancreatic islets positive for enteroviral VP1 protein (213). Therefore, the capacity of islet cells to proliferate after the infection might protect against T1D.

3.3 Dual roles of enteroviruses in T1D

3.3.1 Hygiene hypothesis

According to the hygiene hypothesis, the rarity of enteroviral infections could lead to decreased immunity against these viruses, thus increasing their invasiveness and pathogenicity (Fig. 11). To estimate the frequency of enteroviral infections, Viskari et al. studied the prevalence of enteroviral meningitis in Finnish children and concluded that the frequency of enteroviral infection in children aged ≥ 6 months had decreased (214). However, they found that the infection rate was actually increasing in younger children aged 0–6 months. One explanation for this rise in young infants could be the lack of protective anti-enteroviral antibodies of maternal origin that would pass to the child through the placenta and breastfeeding. Indeed, Sadeharju et al. found that children who were breastfed for > 2 weeks after the birth had a lower incidence of enteroviral infections than children breastfed for < 2 weeks (215). Moreover the percentage of women lacking antibodies against the CVB4 strain increased from 6% to 17% between 1983 and 1995 while the percentage of women lacking antibodies against enteroviral peptides increased from 13% to 42% during the same period (168; 216).

With fewer infections, pregnant women nowadays might lack anti-enteroviral antibodies or may have a repertoire that covers fewer serotypes compared with women at the beginning of the 20th century. Thus, young children would receive fewer or no protective antibodies from their mothers. In children with a weaker immune defense against enteroviruses, a single strong infection could then allow increased virus replication, promoting the development of T1D. Interestingly, this scenario proposing the increased pathogenic role of CVB in T1D is supported by observations of polioviral infections, another member of the enteroviral family. At the end of the 19th century, poliovirus infections became rare and the number of children developing a severe complication of polioviral infection, paralytic poliomyelitis, increased drastically. It is suggested that with frequent polioviral infections children encountered the virus at an early age when they still had protective antibodies transmitted by their mothers. These antibodies protected against paralysis by forming an immediate barrier and blocking the invasion of the central nervous system by poliovirus. Thus the infected individual had time to make his own protective antibodies to further eliminate the virus. However, because of improved sanitary conditions, the virus became rare and children were infected at an older age when the level of maternal antibodies had strongly decreased. Without any immediate protection, poliovirus attacked the central nervous system easier, thus increasing the chances to develop paralytic poliomyelitis (217).

The reduced frequency of enteroviral infection could favor delayed enteroviral encounters in genetically predisposed older children. The older the child is at the time of infection, the more likely the child is to have accumulated anti-islet T cells. It is important to remember that studies of NOD mice have shown that the number of anti-islet T cells and the degree of islet inflammation are critical factors and could explain why some children develop T1D while others do not.

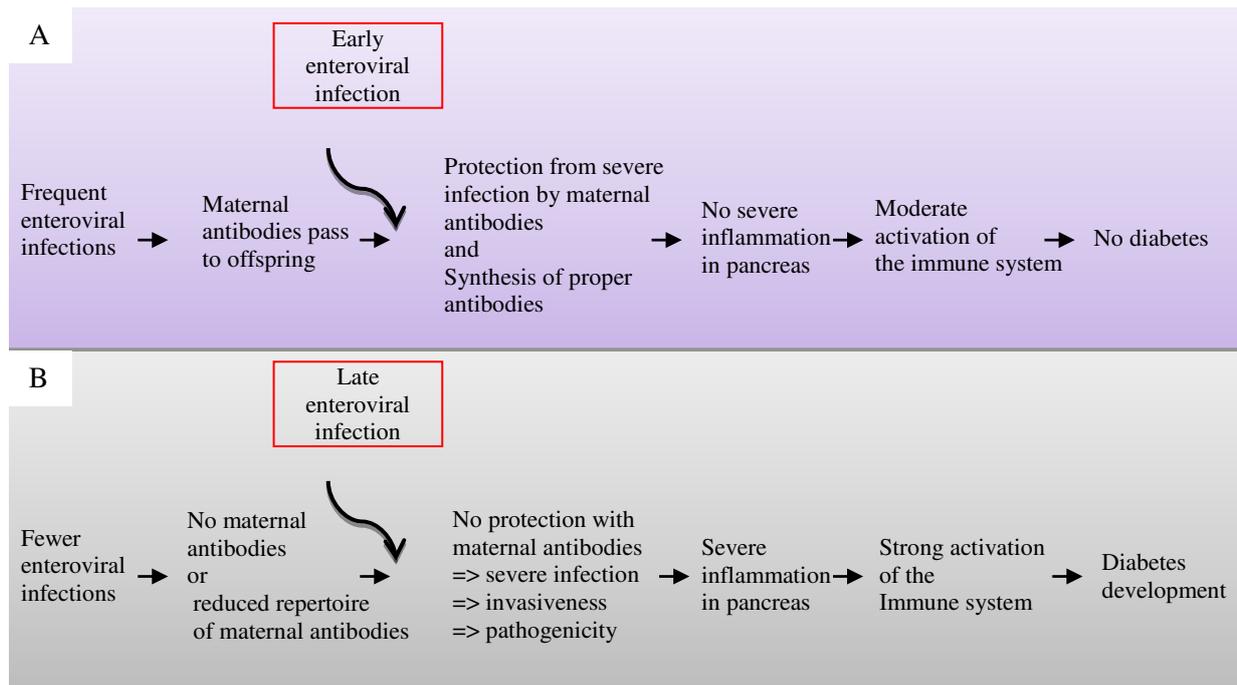


Figure 11. Hygiene hypothesis and enteroviral infections. (A) When enteroviral infections were frequent, maternal anti-enterovirus antibodies were passed to the offspring through placenta and breastfeeding. These antibodies would constitute the first barrier against the virus and prevent its spreading and severe inflammation. This is of particular importance in pancreas where inflammation can favor cell death and release of auto-antigens. With controlled viral infection, the activation of the immune system will remain moderate with reduced self-antigen presentation and activation of autoreactive T cells. Diabetes development, thus, will not be favored. **(B)** With fewer enteroviral infections, mothers might not have any anti-enteroviral antibodies or have a reduced repertoire to pass to their offspring. The virus will then cause severe widespread infection and high inflammation. The released auto-antigens will then be presented by APCs resulting in the activation of anti-islet T cells and diabetes development.

3.3.2 RNA sensors and T1D

To further support the pathogenic role of enteroviruses, the genomic region coding the viral RNA sensor IFN induced with helicase C domain 1 (IFIH1), otherwise known as melanoma differentiation-associated protein 5 (MDA5), has been identified as a T1D susceptibility locus. This is particularly important because MDA5 is critical for the recognition of picornaviruses to which the enterovirus genus belongs (218). This intracellular receptor recognizes double-stranded viral RNA that forms during viral replication, induces the expression of type I IFNs (IFN- α and IFN- β), and activates the immune system. Four rare mutations that reduce the expression of IFIH1 and type I IFNs were reported to have a protective role in T1D (219-221). These findings suggest that wild-type IFIH1 and effective recognition of viral infection actually predispose individuals to T1D. This role of MDA5 is supported by several previous studies. For example, IFN- α was detected in plasma samples of 70% of newly diagnosed T1D patients, of whom 50% carried enteroviral RNA in their blood samples; none of the IFN- α -negative patients had enteroviral RNA (222). The scenario proposes that after viral RNA binding to IFIH1, large amounts of type I IFNs are secreted, leading to the upregulation of MHC class I molecule expression on β -cells, increased presentation of autoantigens, and activation of DCs (223). Type I IFNs could also regulate cells of the innate and adaptive immune system, which could facilitate the killing of β -cells and destruction of pancreatic islets (224; 225).

Even though much less documented than MDA5, it has been proposed that another RNA sensor, TLR7, could be implicated in the etiology of T1D (226). TLR7, mainly expressed by plasmacytoid DCs (pDCs), can recognize coxsackieviruses and initiate an antiviral immune response by inducing the production of type I IFNs (227). Treatment with a TLR7 agonist, particularly in combination with other immune stimulatory molecules such as CD40, induced T1D in transgenic NOD mice bearing an increased number of autoreactive T cells (228). Interestingly, NOD mice deficient for Myd88, an adaptor molecule required for the signaling of most TLRs, including TLR7, do not develop diabetes in a specific pathogen-free mouse facility (229).

While an efficient MDA5-mediated response is associated with T1D susceptibility in humans, several studies have highlighted the critical role of IFN- α production in diabetes prevention in infected mice (211; 230-232). The amount and the environment in which IFN- α is secreted could perhaps account for this phenomenon. IFN- α is required to block viral replication, but excessive secretion in the context of an ongoing autoimmune response could render IFN- α pathogenic.

III - NKT cells

1. Characteristics of NKT cells

Natural killer T (NKT) cells are non-conventional T cells because they do not recognize MHC molecules but, instead, recognize the non-polymorphic non-classical MHC-like molecule CD1(233). Contrary to classical MHC molecules, CD1 presents hydrophobic molecules such as glycolipids. In humans, CD1 comprises CD1a, CD1b, CD1c, CD1d and the intracellular CD1e. In mice, however, only CD1d is expressed. NKT cells share some characteristics with NK cells such as the expression of NK1.1, NKG2D or Ly49 (234). They are considered to be innate cells due to their activatory/effector phenotype and are highly preserved in humans and in mice (235). There are different types of NKT cells: type I or invariant NKT cells, type II NKT cells and NKT-like cells.

1.1 Subpopulations of NKT cells

Type I NKT cells or invariant NKT cells (iNKT cells) express an invariant T cell receptor α chain (V α 24-J α 18 in humans and V α 14-J α 18 in mice) (Table 1). The human V α 24-J α 18 chain pairs with a single V β 11 chain. In mice, however, the V α 14-J α 18 chain can associate with V β 8.2, 7 or 2 chains. iNKT cells have an activated memory phenotype characterized by the expression of CD69 and CD44 and the lack of expression of CD62L. In humans iNKT cells can be CD4⁺, CD8⁺ or double negative (DN) while in mice only CD4⁺ and DN iNKT cells exist. iNKT cells are restricted to the non-classical MHC molecule CD1d that presents glycolipids. CD1d is expressed by DCs, monocytes, macrophages, B lymphocytes, epithelial cells and can be upregulated by some tumor cells. The activation of iNKT cells leads to rapid production of various Th1 and Th2 cytokines (IFN- γ , TNF- α , IL-2 and IL-4, IL-5, IL-13 respectively) (235-239). After this phase, iNKT cells act like classical T cells. More precisely, a phase of proliferation can be observed, followed by contraction and hyporesponsiveness to further stimulation that is maintained by interaction of PD1 on iNKT cells and inhibitory PD-L1 on other immune cells (240; 241). Besides cytokine production, iNKT cells can be cytotoxic by expressing Fas-L and releasing perforin (242).

Type II NKT cells, found in mice and humans, are also restricted to the CD1d molecule. However, they express a much wider array of TCR α chains. They recognize hydrophobic molecules such as a myelin-derived glycolipid sulfatide, lysophosphatidylcholine and small aromatic molecules. They express NK cell receptors and have an activated memory phenotype. Type II NKT cells were shown to play a suppressive role during infection and autoimmunity (243-245)

Finally, a third group is composed of NKT-like cells. Mucosal-associated invariant T (MAIT) cells, found in gut lamina propria and expressing an invariant TCR α chain (V α 33-J α 19 in mice and V α 7.2-J α 19 in humans), are an example of NKT-like cells. These cells are restricted to MHC class I-like non-polymorphic molecule MR1 and are considered NKT-like due to the expression of an invariant TCR α chain and NK cell receptors. MR1 knockout mice have a higher microbial burden suggesting their role in antimicrobial defense. Much research is focused on the identification of ligands of MAIT, which is suggested to be a well preserved microbial compound as MAIT can be activated by numerous microbes (246). However, a recent study has revealed that vitamin B metabolites can be presented by MR1 and stimulate MAIT cells (247).

	Type I - invariant NKT cell	Type II NKT cells	NKT-like cells (MAIT, ...)
CD1d restricted	+	+	- (can be restricted to MR1)
TCR α chain	V α 14-J α 18 in mice V α 28-J α 18 in humans	Diverse but enriched in V α 3 and V α 8 in mice	Diverse
TCR β chain	V β 8.2, 7, 2 in mice V β 11 in humans	Diverse but enriched in V β 8 in mice	Diverse
NK1.1	+/-	+/-	+
CD4, CD8	CD4, DN in mice CD4,CD8,DN in humans	CD4, DN in mice	Diverse

Table 1. Main phenotypic differences of NKT cells (adapted after Godfrey et al. Nat Rev Immunol. 2004 (248)).

1.2 Mouse strains and tools for studying NKT cells

1.1.1 NKT cell numbers

Over the time mice lacking or, on the contrary, having high numbers of NKT cells were developed to study NKT cells (Table 2). CD1d^{-/-} mice lack type I and type II NKT cells as the absence of glycolipid presentation by CD1d in the thymus makes it impossible to positively select CD1d restricted cells. Therefore, besides NKT cells, all other cells whose thymic selection requires the presentation of self-glycolipids by CD1d molecule, such as certain $\gamma\delta$ T cells, are also absent from these mice. J α 18^{-/-} mice lack iNKT cells since the J α 18 locus, that is necessary for iNKT V α 14-J α 18 TCR chain formation, is replaced with a neomycin selection cassette. The use of J α 18^{-/-} mice to study iNKT cells is more appropriate since other CD1d restricted cells are preserved in these mice. J α 18^{-/-} mice, however, have an important shortcoming. The deletion of the J α 18 locus somehow prevents the transcription of genes encoding J α chains upstream of J α 18 (from J α 1 to J α 18) and an estimated 60% of TCR α diversity is lacking in these mice (249). This shortcoming must be taken into account when using these mice. As opposed to these two strains, transgenic mice, with a tenfold increased iNKT cell frequency and numbers, have been generated in our laboratory by introducing an already rearranged V α 14-J α 18 locus. These mice can be used to study iNKT cells in organs where their frequency is very low. Additionally, these mice can be used as donors of iNKT cells for transfer experiments.

1.1.2 NKT cell activation

In experimental settings iNKT cells can be activated by a high affinity ligand α Galactosylceramide (α GalCer). α GalCer is a sphingolipid originally isolated from a marine sponge *Agelas Mauritanus* during pharmacological testing of anti-cancerous properties of natural compounds by Kirin pharmaceuticals. It is suggested that α GalCer actually derives from bacteria *Sphingomonas* that colonized the sponge *Agelas Mauritanus*. On the contrary, NKT cell activation can be temporarily blocked with an anti-CD1d antibody that will prevent antigen presentation to NKT cells.

1.1.3 NKT cell detection

The discovery of α GalCer greatly promoted studies of iNKT cells by allowing the development of a tool for their specific detection, which is made of CD1d tetramers loaded with α GalCer. Contrary to iNKT cells that can be studied with α GalCer loaded tetramers, no

single specific marker exists for the detection of type II NKT cells and NKT-like cells. Sulfatide loaded tetramers exist; however, they stain only a subpopulation of type II NKT cells (244). Rather, a combination of markers is used to detect these cells. Also, the comparison of CD1d^{-/-} mice with Jα18^{-/-} mice helps define specific roles of iNKT and type II NKT cells.

	iNKT	Type II NKT cells	Other cells
CD1d ^{-/-}	-	-	Present
Jα18 ^{-/-}	-	Present	Absence of T cells with Jα chains ranging from Jα1 to Jα18
Vα14-Jα18 transgenic	10 fold increased frequency and number	Present	Present
Anti-CD1d mAb	Temporal inhibition of antigen presentation by CD1d		
αGalCer reactivity	+	-	-

Table 2. Tools for studying NKT cells. CD1d^{-/-} mice lack iNKT, type II NKT cells, as well all other cell populations restricted to CD1d. Jα18^{-/-} mice lack iNKT cells and T cells with Jα chains ranging from Jα1 to Jα18. Vα14-Jα18 transgenic mice have a tenfold increased frequency and numbers of iNKT cells. The treatment with a blocking anti-CD1d antibody allows to temporarily block antigen presentation by CD1d.

2. Functional role of NKT cells

2.1 iNKT cell anti-microbial immune response

The important role of iNKT cells in anti-infectious immunity was shown in numerous studies. Their capacity to promptly secrete copious amounts of cytokines such as IFN-γ and IL-4 after activation places them in the first line of defense against the pathogen since these cytokines can promote either Th1 or Th2 immune responses. They also constitutively express IL-12 receptor and can be quickly activated by IL-12 secreting DCs. It has been shown that iNKT cells inhibit *Pseudomonas aeruginosa* as well as *Streptococcus pneumoniae* proliferation in the lung and prevent animal death. The mechanism by which iNKT cells control *Pseudomonas aeruginosa* is suggested to involve activation of macrophages and neutrophils since iNKT cell deficient mice had significantly fewer activated macrophages and

infiltrating neutrophils in the lung compared to wild type (wt) mice (Fig. 12A) (250). iNKT cell control of *Streptococcus pneumoniae* infection is suggested to involve a very similar mechanism (251). iNKT cells can also control infections with parasites such as *Leishmania major* (*L. major*) (Fig. 12B) (252). During *L. major* infection, macrophages express the 65 kDa heat shock protein (HSP65) only if iNKT cells are present. This protein prevents the apoptosis of infected macrophages and, thus, promotes parasite clearance. iNKT cells can also boost early immune responses against influenza infection and decrease animal mortality by mechanisms including the inhibition of suppressor cell numbers in the lung and activation of NK and CD8 T cells through IFN- γ secretion (253). Additionally, the activation of iNKT cells by α GalCer during vaccination with an inactivated influenza A virus, promotes the generation of long-term memory CD8 T cells and, therefore, better protection during the secondary challenge (254). The role of iNKT cells in anti-viral immune response is further confirmed by the ability of certain viruses to downregulate CD1d expression (255; 256).

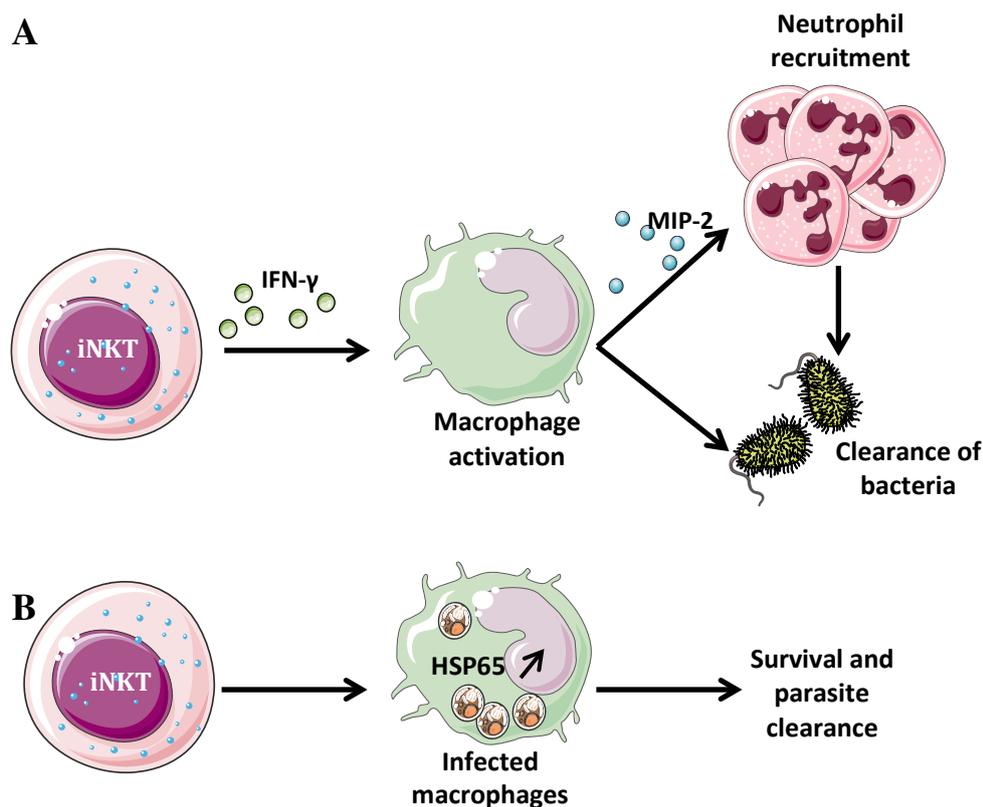


Figure 12. iNKT cell anti-microbial immune response. (A) iNKT cells induce activation of pulmonary macrophages during *Pseudomonas aeruginosa* infection possibly through IFN- γ production. Activated macrophages secrete MIP-2 (macrophage inflammatory protein 2) that allows the recruitment of neutrophils in lungs. Together, activated macrophages and neutrophils clear the infection through phagocytosis of bacteria. (B) During *L. major* infection iNKT cells induce upregulation of HSP65 in infected macrophages that promotes macrophage survival and parasite clearance.

One of the mechanisms of iNKT cell activation during infections is the recognition of an exogenous glycolipid presented by the CD1d molecule. Some of the exogenous ligands revealed are mycobacterial lipids, glycolipids derived from the bacteria *Borrelia burgdorferi*, sphingolipids of gram negative bacteria *Sphingomonas* and glycolipids from gram positive bacteria *Streptococcus pneumoniae* (257-260).

However, a strong recognition of exogenous ligands is not an absolute requirement for iNKT cell activation (Fig. 13). iNKT cells can be activated in the presence of pathogens lacking glycolipids such as viruses, parasite *Salmonella typhimurium*, bacteria *Staphylococcus aureus* and *Mycobacterium tuberculosis*. With such pathogens, the weak self-ligand presentation combined with inflammatory stimuli is suggested to replace the strong exogenous ligand presentation. The inflammatory stimuli can come from numerous other cells activated directly or indirectly by the pathogens. Among such cells are DCs that express numerous TLRs that sense the presence of pathogens. DCs then can activate NKT cells by secreting activatory cytokine IL-12, which can condition iNKT cells to produce IFN- γ rather than IL-4 and favor the pathogen clearance (261-263). Recently, the study by Brennan et al. demonstrated that a self-ligand, β -D-glucopyranosylceramide, accumulates in DCs after infections with *Escherichia coli* and *Streptococcus pneumoniae* or after TLR4 stimulation with LPS and directly activates iNKT cells (264). Another endogenous ligand, iGB3, activates iNKT cells when it is presented by DCs activated by LPS of gram negative bacteria *Salmonella typhimurium* (262). Additionally, stimulation of DCs through TLR4 and TLR9 induces the secretion of type I interferon and also de novo synthesis of self glycosphingolipids that together lead to iNKT cell activation (265).

The activation of iNKT cells by DCs does not always require CD1d presentation. In the study by Nagarajan et al. iNKT cell activation and secretion of copious amounts of IFN- γ after LPS stimulation is critically dependent on the presence of DCs during cell culture (266). In this case, LPS induces IL-12 and IL-18 synthesis by DCs that act synergistically to activate iNKT cells independently from CD1d since the addition of CD1d blocking antibody or the use of DCs from CD1d knockout mice does not change IFN- γ production by iNKT cells. Similarly, iNKT cell activation and IFN- γ secretion after murine CMV infection is dependent on the presence of cytokines IFN α/β and to a lesser degree on IL-12 but independent of CD1d (267). Additionally, the activation of iNKT cells after exposure to β -1,3 glucan, a major cell-wall polysaccharide present in numerous fungi, greatly depended on DC secretion of IL-12 (268). Interestingly, recently Brigl et al. showed that even in the presence of a strong exogenous ligand, DC derived cytokines can be the major activators of iNKT cells. In their

study they demonstrate that iNKT cell activation during *Sphingomonas yanoikuyae* or *Streptococcus pneumoniae* infections is independent of CD1d even though these bacteria express iNKT cell antigens (269). DC secreted IL-12, however, was critical for iNKT cell activation and IFN- γ secretion.

All these results demonstrate the existence of numerous pathways for iNKT cell activation that allow them to participate to the immune response against various types of pathogens.

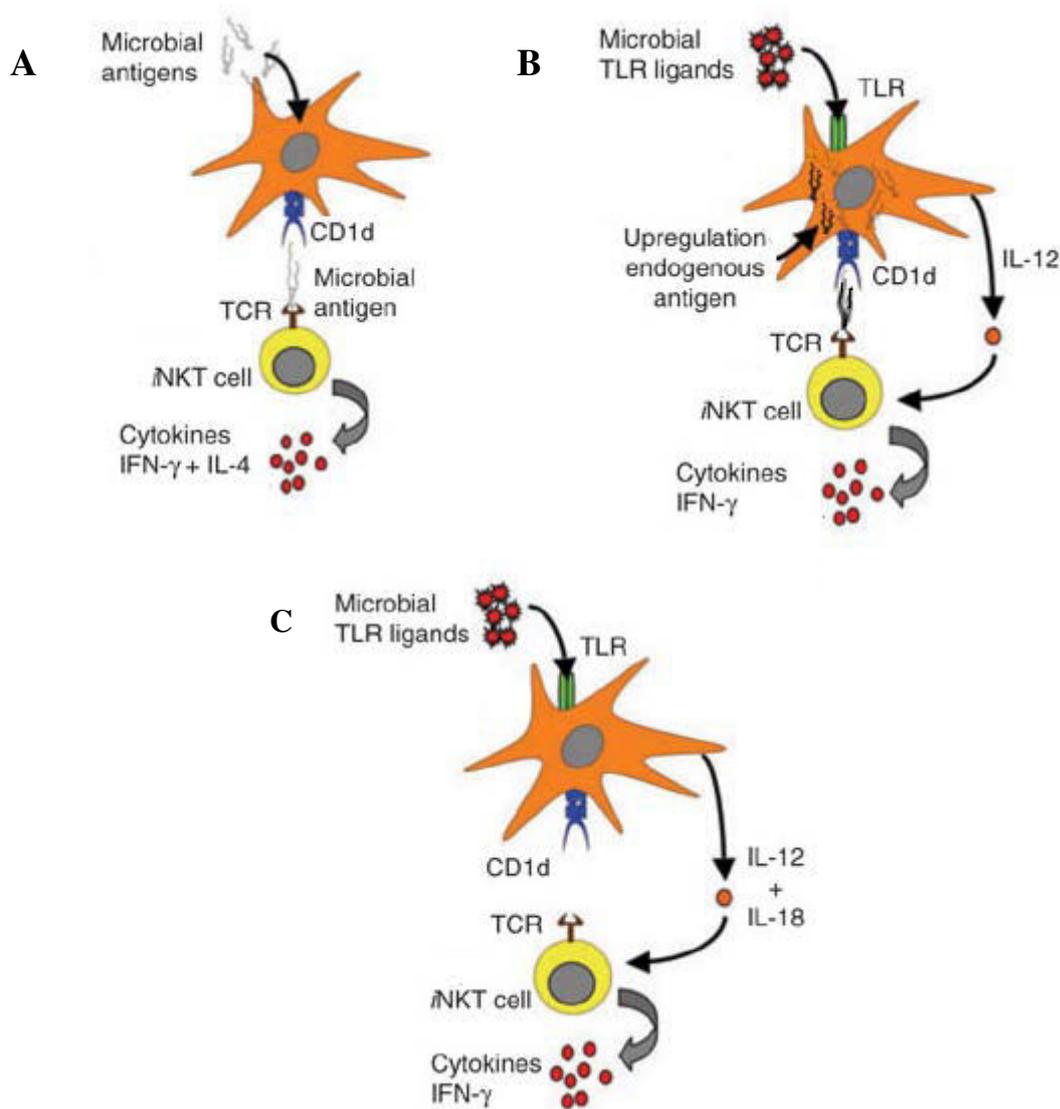


Figure 13. Mechanisms of activation of iNKT cells. (A) Direct exogenous activation: iNKT cells can be activated by ligands derived from pathogens presented by CD1d molecule. Depending on the ligand iNKT cells can either promote Th1 or Th2 type responses through IFN- γ or IL-4 secretion respectively. (B) Direct endogenous activation: TLR stimulation can promote production or upregulation of self-ligands. Self-ligand stimulation of iNKT cells is weak and needs additional stimulation by cytokines. (C) Indirect activation: iNKT cells can be activated by inflammatory cytokines independently from CD1d (Figure source : Modified after De Libero et al. Nat Immunol. 2007 (270)).

2.2 The role of iNKT cells in anti-tumor immune response

The role of iNKT cells in tumor immunity has been shown in numerous models and is supported by the observation that cancer patients frequently have lower numbers of iNKT cells compared to healthy controls, which correlates with poor clinical outcome (271-273). Most solid human tumors lack CD1d expression; however some tumors can upregulate the expression of CD1d, which can make them direct targets for NKT cell cytotoxicity. Thus, tumor cells from leukemia patients can express CD1d and are killed when cultured with iNKT cells (Fig. 14A) (272). iNKT cell mediated cytotoxicity depended on perforin/granzyme secretion and to a lesser degree on the expression of pro-apoptotic receptors such as TRAIL, Fas-L and TNF- α . Antigen presentation by CD1d was critical for cytotoxic effect of iNKT cells suggesting antigen depending recognition of tumor cells. On the contrary, methylcholanthrene (MCA) induced fibrosarcomas do not express MHC class I and CD1d but can still be directly killed by iNKT cells through perforin pathway (Fig. 14B) (271). α GalCer activated iNKT cells were shown to control tumor growth in MCA induced sarcomas, mammary carcinomas and spontaneous sarcomas in cancer prone p53^{-/-} mice by means of IFN- α production and the expression of pro-apoptotic TRAIL receptor (274). The metastasis of EL-4 mouse lymphoma cells into liver can also be controlled by α GalCer activated iNKT cells that produce IFN- γ , activate other immune cells and promote Th1 immune responses (Fig. 14C) (275).

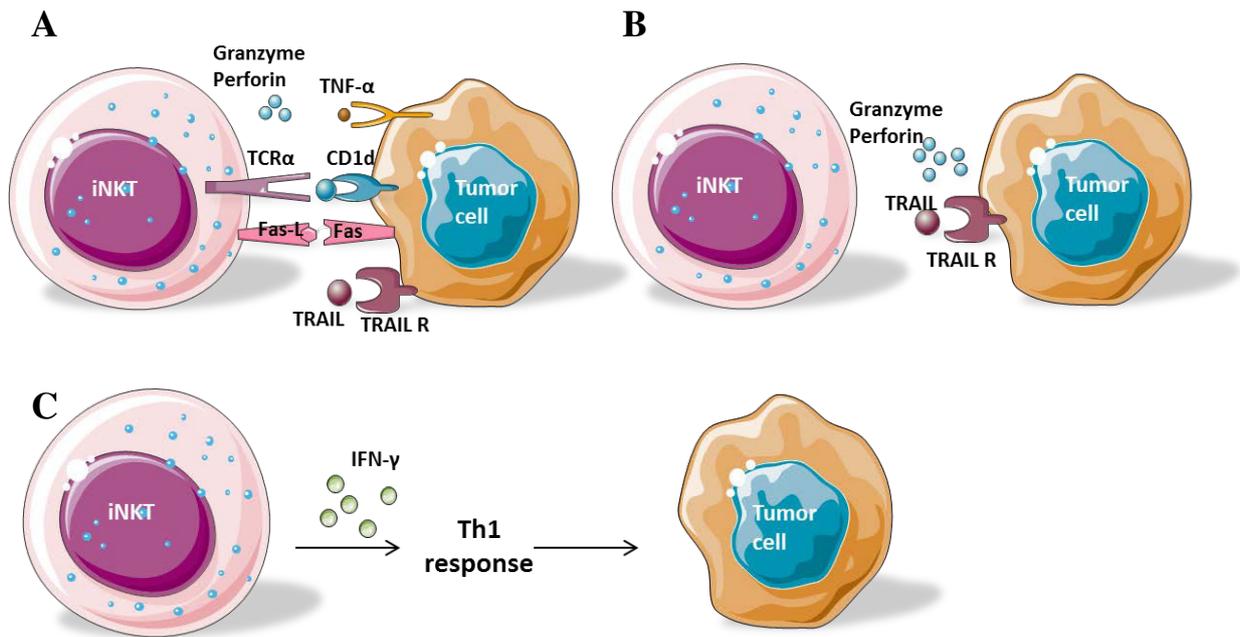


Figure 14. The role of iNKT cells in anti-tumor immune response. (A) iNKT can recognize and kill CD1d expressing tumor cells by releasing granzyme/perforin, TNF- α and TRAIL as well as by expressing pro-apoptotic receptor Fas-L. (B) iNKT cell induced apoptosis can be independent of CD1d recognition. (C) iNKT cells can induce apoptosis of tumor cells indirectly by secreting IFN- γ and promoting Th1 type immune responses.

2.3 iNKT cells and immune tolerance

iNKT cells can promote immune tolerance in various settings such as transplantation, burn injury, oral tolerance and autoimmunity.

Graft acceptance:

The acceptance of grafts by the host organism is critically dependent on immunosuppression that allows blocking the presentation of foreign antigens by APC and activation of the immune system. iNKT cells were shown to promote suppression and graft acceptance. Thus, rat islet xenographs are rejected in mice depleted of iNKT cells and corneal allografts are rejected significantly sooner in mice depleted of iNKT cells (276; 277).

Burn injury:

Injuries arising from trauma or burns induce a strong inflammation, which is later replaced by strong immunosuppression. This potent immunosuppression can be very

dangerous since it can result in sepsis and multiple organ failure due to autoimmunity. The main cell type responsible for this phenomenon is macrophages and their plasticity. Macrophages are hyperactivated by the injury, produce massive amounts of inflammatory mediators but several days later become suppressive by producing IL-10 and prostaglandin E2. Besides macrophages, iNKT cells were also shown to induce immune dysfunction through low IFN- γ and high IL-4 production since administration of an anti-IL-4 mAb after the injury or an anti-CD1d antibody prior to the injury reverses the immune suppression in mice (278; 279). Furthermore, reversing the IFN- γ to IL-4 ratio in favor of IFN- γ through activation of iNKT cells by α GalCer prevents immunosuppression (280).

Oral tolerance:

iNKT cells can promote oral tolerance to exogenous antigens by promoting tolerogenic DCs, de novo induction of Treg cells and by reducing the numbers of antigen specific T cells (281).

Autoimmunity:

The role of iNKT cells in controlling autoimmunity was suggested after several studies showed that iNKT numbers or functions were decreased in several autoimmune diseases. Patients with **multiple sclerosis** (MS) have lower numbers of iNKT cells compared to healthy controls (282). In experimental autoimmune encephalomyelitis (EAE), a murine model of MS, the numbers of iNKT cells was revealed to be a critical factor for the disease development. Thus, $J\alpha 18^{-/-}$ mice develop a more severe EAE compared to wt mice. On the contrary, $V\alpha 14$ transgenic NOD mice, which have an elevated numbers of iNKT cells, are protected from EAE (283). Similarly, restoration of iNKT cell function through α GalCer stimulation was shown to protect mice from EAE (284; 285). However, when mice were immunized by MOG and treated with α GalCer simultaneously, the protection from EAE was not observed any more suggesting that iNKT cell activation prior to the disease is protective, while their activation during the disease is, on the contrary, deleterious (286). The protective role of iNKT cells is attributed to their production of IL-4 and IL-10 and inhibition of Th1 and Th17 immune responses (287). Interestingly, iNKT cells from the blood of MS patients produce more IL-4 during the phase of remission compared to iNKT cells found in the relapse phase of the disease or in healthy controls thus supporting the protective role of iNKT cell

produced IL-4. However, other studies contradict the importance of IL-4 in the disease prevention (283; 288).

iNKT numbers are also reduced in patients with **systemic lupus erythematosus** (SLE) (289). There are different mouse models of SLE. In *lrp* mice, which are deficient for Fas, iNKT cell numbers are significantly reduced, which leads to the exacerbation of the disease and to inflammatory dermatitis (290; 291). Increasing the iNKT cell function through repetitive α GalCer stimulation can alleviate the disease (291). However, iNKT cells can also be deleterious for SLE depending on the stage of the disease development. Thus, in a different SLE model, (NZBxNZW)F1 mice iNKT cells were shown to be protective in young animals but deleterious in older ones. SLE is exacerbated in (NZBxNZW)F1 CD1d^{-/-} mice that are deficient in iNKT cells from birth showing the early protective role of NKT cells (292). On the contrary, the blocking of CD1d in 3 months old (NZBxNZW)F1 mice ameliorates the disease showing the deleterious role of NKT cells at an older age (293).

The role of NKT cells in **rheumatoid arthritis** (RA) has not been clearly defined yet. iNKT cell numbers are decreased in the blood and synovium of patients suffering from RA (294). Interestingly, the beneficial treatment of patients with an anti-CD20 monoclonal antibody (mAb) induces an increase in iNKT cell numbers (295). However, no such decrease was documented in the mouse model of RA (296). On the contrary, iNKT cell deficiency is protective in experimentally induced RA (297). Additional studies are, therefore, necessary to clarify the role of NKT cells in this pathogenesis between mice and humans.

Finally α GalCer activated iNKT cells were shown to prevent the development of experimental **myasthenia gravis** in mice through IL-2 secretion and the induction of Treg cells (298). In humans, however, the disease was associated with increased iNKT cell numbers in blood suggesting that altering the phenotype and numbers of iNKT cells can be beneficial for the disease treatment.

3. Role of NKT cells in type 1 diabetes

The role of NKT cells in T1D was extensively studied and demonstrated in genetically modified mice, lacking iNKT cells or, on the contrary, having an elevated frequency of these cells. The results of these studies strongly suggest that iNKT cells play a protective role in T1D and both their frequency and function are important for their regulatory role.

3.1 Frequency of iNKT cells in T1D

The first suggestion for the protective role of iNKT cells in T1D came from studies showing that iNKT cell numbers and frequency were lower in diabetes prone NOD mice compared to strains of mice that did not develop diabetes spontaneously (299; 300). Diabetes incidence is even more severe in CD1d^{-/-} NOD mice deficient for NKT cells compared to wt NOD mice (301; 302). Inversely, our group has shown that the increase in iNKT cell numbers, by the transgenic expression of an already rearranged V α 14-J α 18 TCR α chain, decreases the incidence of diabetes of NOD mice (303). The overexpression of the CD1d molecule by pancreatic islet β -cells has also been shown to induce iNKT cells accumulation in PLN and decrease diabetes incidence (304). These data strongly support the protective role of iNKT cells in T1D.

3.2 The functional role of iNKT cells in T1D

The development of diabetes is associated with the presence of autoreactive Th1 lymphocytes directed against pancreatic islet β -cells. On the contrary, the protection against diabetes is associated with Th2 lymphocytes. iNKT cells can produce both IFN- γ and IL-4, which can respectively drive Th1 and Th2 immune responses (Fig. 15). However, different studies have shown that one of the mechanisms by which iNKT cells prevent diabetes is through preferential IL-4 secretion and inhibition of Th1 immune responses. It is interesting to note that iNKT cells of NOD mice produce less IL-4 compared to other non-diabetic strains such as BALB/c. Furthermore, the administration of exogenous IL-4 or the expression of IL-4 in pancreatic islets prevents diabetes development of NOD mice (305). In addition to these data, our group has shown that the low diabetes incidence in V α 14 transgenic mice was associated with high level of IL-4 and low level of IFN- γ production locally in the pancreas (306). Similarly, another study showed that transferred thymic TCR α/β^+ /CD4⁻/CD8⁻ cell population that is highly enriched in iNKT cells prevents diabetes development through IL-4 and IL-10 production since the administration of IL-4 and IL-10 neutralizing antibodies reversed diabetes protection (307). The activation of iNKT cells through multiple injections of α GalCer prevents diabetes development in pre-diabetic NOD mice. This was associated with the accumulation of IL-4 producing iNKT cells in the pancreatic islets and PLN (308; 309).

The protective effect of this treatment is abrogated in IL-4 deficient mice, confirming the protective role of IL-4 in T1D (310).

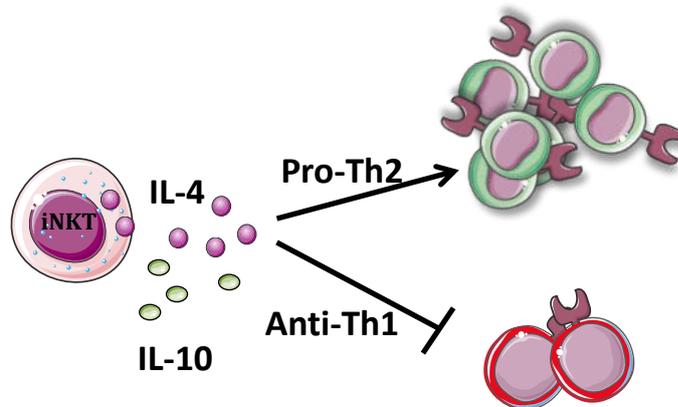


Figure 15. Role of iNKT cell cytokines in diabetes. Diabetes prevention by iNKT cells is associated with their preferential expression of IL-4 and IL-10 over IFN- γ . These cytokines promote protective Th2 immune responses and inhibit diabetogenic Th1 immune responses.

It is, however, important to stress that iNKT cells can prevent T1D by several other mechanisms other than IL-4 or IL-10. For example, iNKT cells were shown to inhibit the differentiation of both diabetogenic anti-islet CD4 and CD8 T cells into effector cells by making them anergic and non-functional (311; 312). The suppression of autoreactive CD4 T cell proliferation was shown to be independent of peripheral CD1d expression but still required cell contacts between T cells, NKT cells and APCs (Fig. 16) (313; 314). Thus, iNKT cell induced suppression can be indirect and involve tolerogenic APCs. Importantly, several groups have shown that diabetes prevention in mice treated with multiple α GalCer injections is associated with modification of APC function. Tolerogenic CD11c⁺/CD8 α ⁻ DCs were shown to accumulate in PLN of these mice. These APCs produced low levels of a pro-Th1 cytokine IL-12 and expressed low levels of costimulatory molecules CD80 and CD86 compared to DCs that had received a single α GalCer injection (that does not prevent diabetes) (315). Such DC modification can lead to the inhibition of T cell responses and at the same time induce regulatory T cells. Interestingly, iNKT cell induced T cell suppression seems to be specific to anti-islet T cells since the treatment of splenocytes by the non-specific T cell activator, Concanavaline A, induced the production of both IL-4 and IFN- γ . Furthermore, despite the very low production of cytokines by iNKT cells stimulated with an α GalCer

analog, C16 molecule, diabetes prevention is even more efficient than with α GalCer stimulation (316). Again, these results demonstrate that iNKT cell prevention of diabetes can be independent of cytokines.

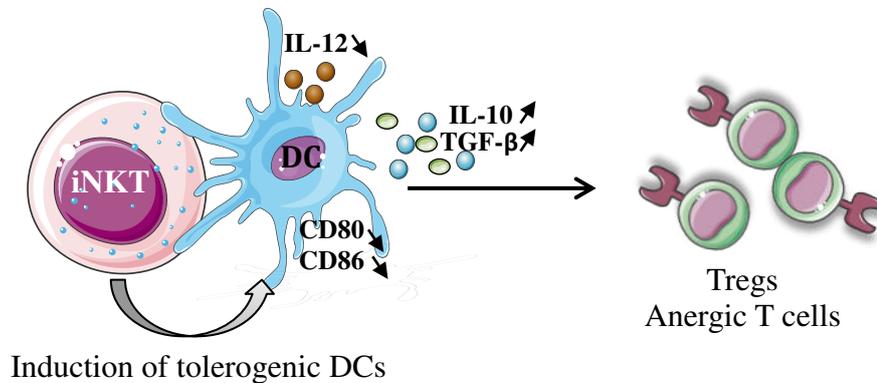


Figure 16. iNKT cells prevent diabetes by inducing tolerogenic DCs in PLN. Such DCs are characterized by low expression of activatory IL-12 cytokines, the high expression of suppressive IL-10 and TGF- β cytokines as well as the downregulation of costimulatory molecules CD80/CD86. The induction of tolerogenic DCs is independent of CD1d expression but still requires a physical contact between iNKT cells and DCs. The interactions of autoreactive T cells with tolerogenic DCs render them anergic and lead to prevention of diabetes.

Despite the well documented protective role of iNKT cells in T1D, our study showed that a new sub-population of iNKT cells producing IL-17 (iNKT17) plays a deleterious role in diabetes. iNKT17 cells are CD4⁻/NK1.1⁻ but can be stained with CD1d: α GalCer tetramer (317). We observed that NOD mice have a higher frequency of iNKT17 cells compared to non-autoimmune strains C57BL/6 and BALB/c (61). Moreover, when co-transferred with diabetogenic BDC2.5 CD4 T cells, iNKT17 cells exacerbated T1D. By contrast, the transferred CD4⁺/NK1.1⁺ iNKT cell population, depleted of iNKT17 cells, efficiently prevented diabetes.

3.3 Role of iNKT cells in human T1D

The first study showing that T1D patients had lower numbers of iNKT cells in their blood compared to healthy controls was published in 1998 in the journal Nature (318). Further

studies, however, yielded conflicting results. Oikawa et al. reported that iNKT cell numbers were increased in the blood of recent T1D patients compared to healthy controls, while others did not report any difference at all (319-321). In the mouse model of T1D, the secretion of IL-4 by iNKT cells was shown to be protective in T1D. Consistent with this data, the analysis of pancreatic lymph node iNKT cells revealed a defective IL-4 production in T1D patients compared to healthy controls (322). Besides, iNKT cells of T1D patients were shown to produce only IFN- γ upon stimulation while iNKT cells of healthy controls produced both IFN- γ and IL-4, yet again suggesting the protective role of IL-4 in T1D prevention (318). In contrast to these publications, Kukreja et al. found that iNKT cells of diabetic patients had a suppressed phenotype and produced very little quantities of cytokines compared to healthy controls (323). These controversial results do not eliminate the role of iNKT cells in T1D. They can on one hand reflect the great heterogeneity of iNKT cell frequency in humans. On the other hand the use of various techniques for iNKT cell detection such as a combination of antibodies versus CD1d: α GalCer tetramer staining and different protocols for iNKT cells stimulation could yield different results. Additionally, blood iNKT can have a different phenotype and function compared to pancreatic iNKT cells. Unfortunately, studies performed with pancreatic iNKT cells remain limited since they require pancreatic biopsies or samples from post-mortem donors.

3.4 The protective role of iNKT cells in diabetes during a viral infection

The ability of iNKT cells to be activated and to participate in both anti-infectious immune responses and autoimmunity motivated our group to study the regulatory role of iNKT cells in diabetes during a viral infection. The question asked was whether iNKT cells could be bi-functional i.e. maintain tolerance and promote anti-viral immune responses at the same time. To study this question, diabetes prone NOD mice were infected with LCMV. Twenty years ago, Oldstone and colleagues reported that LCMV infection in diabetes-prone rats and NOD mice decreased or prevented the development of autoimmune diabetes (324; 325). Subsequent studies have shown that the protection against diabetes is associated with an IFN- γ -induced protein-10 (IP-10) chemokine gradient, with the highest concentration in PLN. This results in the attraction of activated T lymphocytes in this tissue and subsequent apoptosis (326). The results of our group showed that after the infection iNKT cells activate

DCs in the spleen (230). DCs then produce IL-12 and promote strong anti-LCMV CD8 T cell responses that are crucial for the elimination of infected cells. Interestingly, in the pancreas activated iNKT cells do not interact with DCs but rather induce the recruitment of pDCs (Fig. 17). The recruited pDCs produce large quantities of IFN- α , which inhibits viral replication in the pancreas and limits pancreatic tissue destruction. In the second step, pDCs migrate to pancreatic lymph node where they produce TGF- β and induce the conversion of naïve T lymphocytes into Treg cells (Foxp3⁺). These newly induced Treg cells migrate to the pancreas where they produce TGF- β , which locally inhibits anti-islet T cell responses (132). Thus this study showed that iNKT cells can boost anti-viral immune responses while promoting tolerance and protecting mice from developing diabetes.

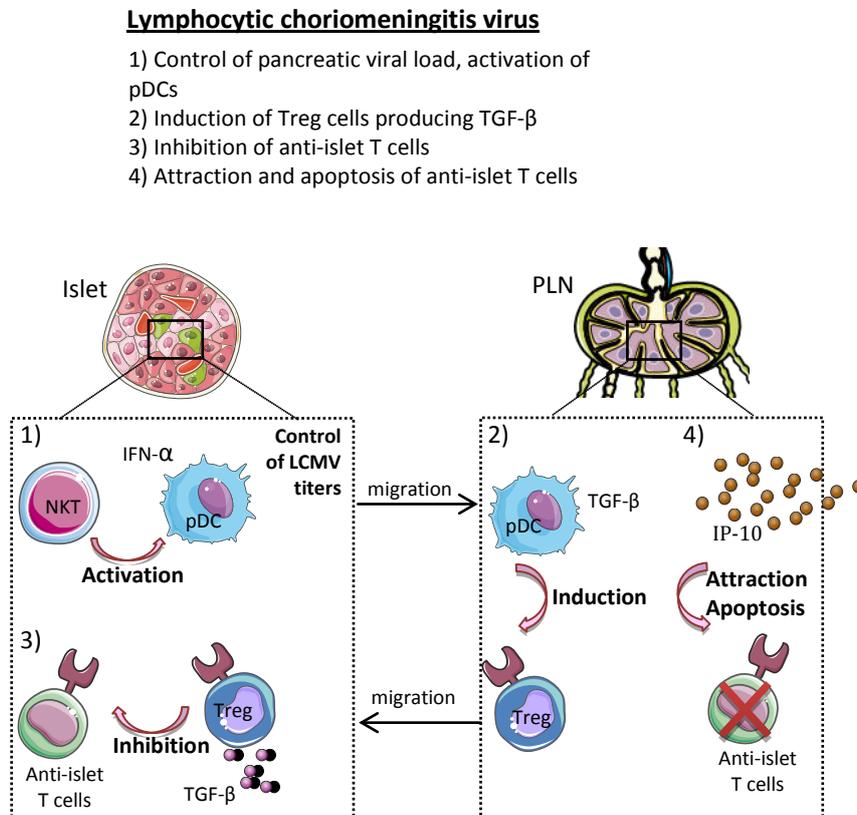


Figure 17. LCMV induced protection against T1D. During LCMV infection iNKT cell activation of pDCs lead to production of IFN- α that controls LCMV replication in the islets. pDCs then migrate to PLN where they produce TGF- β and induce Treg cells. Treg cells then leave PLN and migrate to pancreas where they produce TGF- β and suppress anti-islet T cell responses. Besides, IP-10 chemokine gradient induces T cell migration to PLN where they are eliminated through apoptosis. These two mechanisms allow an efficient prevention of diabetes after LCMV infection (Figure source : Ghazarian et al. Cell Mol Life Sci. 2013 (155)).

3.5 Type II NKT cells and T1D

Besides iNKT, several studies have demonstrated that type II NKT cells can also play a protective role in T1D. Duarte et al. generated transgenic NOD mice that are overexpressing the CD1d specific $V\alpha 3.2^+/V\beta 9$ TCR and are thus enriched in type II NKT cells. While insulinitis developed normally in these transgenic mice, they never progressed to overt diabetes (245). Diabetes prevention could be abolished through blocking of ICOS or PD1/PD-L1 pathways (327).

IV - Macrophages

1. Characteristics of macrophages

Macrophages belong to the innate immune system and play an important role in the regulation of immune responses. They constitutively express numerous scavenger receptors that allow them to effectively phagocytize pathogens and apoptotic and necrotic bodies. They express various pathogen and danger recognition receptors that enable them to be activated by engulfed self and pathogen derived molecules. Once activated, they mount an immune response such as killing of pathogens and clearance of apoptotic and necrotic cells thus participating in immune responses and tissue homeostasis. They can secrete both pro and anti-inflammatory cytokines and regulate T cell responses. Macrophages have a high plasticity and whether they will enhance or dampen the inflammation depends on the immune microenvironment. Three major types of macrophages have been described: classically activated or type 1 macrophages (CAM ϕ), alternatively activated or type 2 macrophages (AAM ϕ) and regulatory macrophages referred as myeloid derived suppressor cells (MDSC).

1.1 Types of macrophages

1.1.1 Classically activated macrophages

CAM ϕ are induced by Th1 type cytokines such as IFN- γ , TNF- α and/or by microbial products such as LPS (Fig. 18). CAM ϕ can express the enzyme iNOS that catalyzes the conversion of the amino acid L-arginine into nitric oxide (NO) and reactive oxygen species (ROS). These molecules induce DNA damage in pathogens and thus have a powerful microbicidal activity. However, these toxic mediators can also cause tissue damage of the host organism. Additionally, CAM ϕ secrete various pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α and IL-12 that favor Th1 T cell differentiation.

1.1.2 Alternatively activated macrophages

AAM ϕ on the contrary can be anti-inflammatory. They are induced by Th2 type cytokines IL-4 and IL-13 and are found during Th2 type immune responses such as

helminthes infections. They can downregulate Th1 immune responses and promote Th2 type immune responses important for the elimination of the pathogen (Fig. 18) (328; 329). However, during asthma, AAM ϕ are suggested to be pathogenic as they exacerbate the inflammation in the lung (330). AAM ϕ secrete low levels of pro-inflammatory cytokines but high levels of regulatory cytokines IL-10 and TGF- β . By contrast to CAM ϕ , AAM ϕ express the enzyme arginase I that also catalyzes the amino acid L-arginine but generates polyamines important for cell proliferation and the amino acid proline that is essential for building collagen fibers and subsequent tissue repair and wound healing. They can also express PD-L1 and PD-L2 and suppress the proliferation of activated T cells (331). AAM ϕ can be distinguished by the expression of several markers including mannose receptor (CD206), Fizz1, Ym1/Ym2, Dectine 1 and IL-4R.

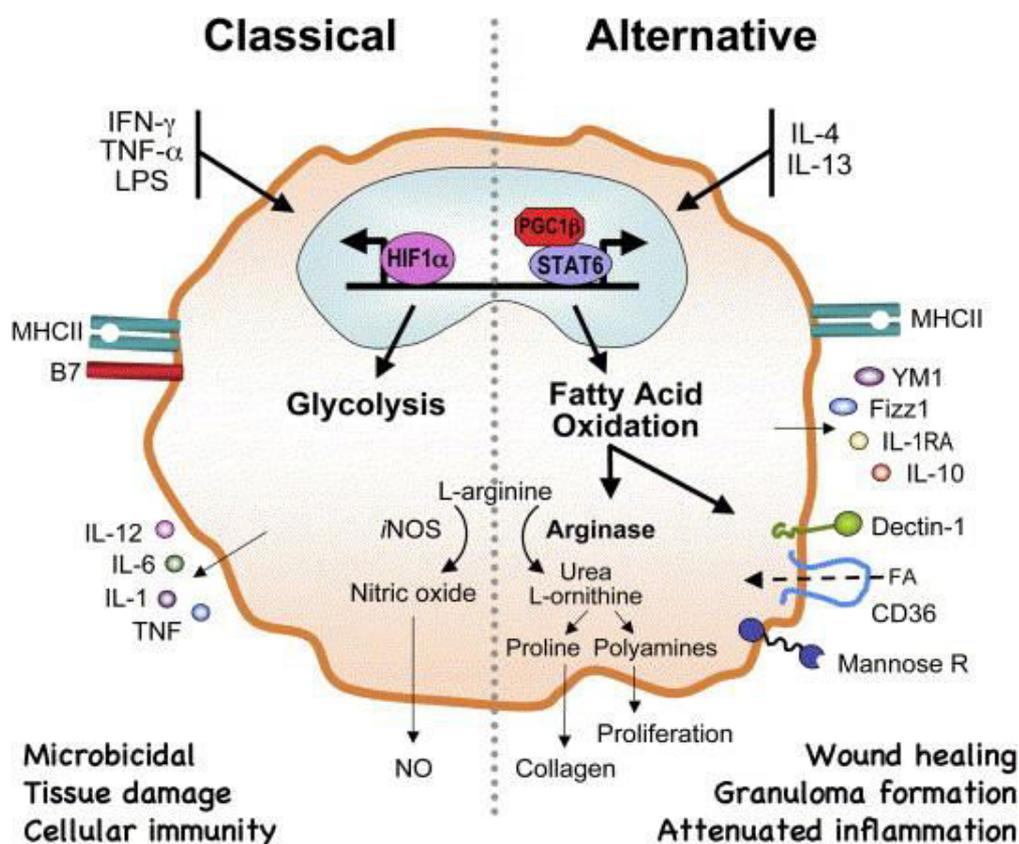


Figure 18. Macrophage subtypes. Classically activated macrophages are induced by inflammatory stimuli such as IFN- γ , TNF- α or microbial molecules. They are characterized by the expression of proinflammatory cytokines and the upregulation of the enzyme iNOS that produces NO. CAM ϕ are microbicidal but can, however, cause tissue damage. Alternatively activated macrophages are induced by cytokines IL-4 and/or IL-13. They express the enzyme arginase I and molecules such as CD206, Fizz1, Ym1/Ym2, Dectine 1 and IL-4R. They promote wound healing, granuloma formation and suppress inflammation (Source : Lacy-Hulbert et al. Cell Metabolism, 2006).

1.1.3 Myeloid Derived Suppressor Cells

MDSC form a highly heterogeneous population of myeloid cells that can comprise granulocytes, macrophages and DCs. The role of MDSC is to suppress the function of immune cells such as T lymphocytes or NK cells and prevent excessive immune responses that can lead to tissue damage (332; 333). Initially, in mice, MDSC were described to express myeloid cell marker CD11b, and GR1 (334). The anti-GR1 antibody was later found to recognize Ly-6G and Ly-6C molecules and two subsets of MDSC were described based on these markers: monocytic MDSC, which have CD11b⁺/Ly-6G⁻/Ly-6C^{high} phenotype, and granulocytic MDSC, which have CD11b⁺/Ly-6G⁺/Ly-6C⁻ phenotype (Table 3) (335). Other markers associated with MDSC are CD80, CD115 and IL4 Receptor alpha (336-338). In humans MDSC are defined as CD14⁻/CD11b⁺ cells that express the common myeloid marker CD33 but lack the expression of markers of mature myeloid and lymphoid cells, and of the MHC class II molecule HLA-DR (339). However, the phenotype of MDSC is not fixed and can vary depending on the pathological context in which these cells are found.

MDSC were often described in cancer patients (339; 340). However, they can also accumulate during infections where they are thought to prevent hyperactivation of immune cells and prevent exacerbated immune responses (341-343). In line with these findings, an increase in the numbers of MDSC was observed after different immunization protocols such as staphylococcal enterotoxin A or vaccinia virus. MDSC can also accumulate during autoimmune diseases such as EAE (344), experimental autoimmune uveoretinitis (345), experimentally induced eczema (346), and in inflammatory bowel diseases (347).

Mouse MDSC		Human MDSC	
Obligatory marker	Additional markers	Obligatory marker	Additional markers
CD11b	Ly-6C	CD11b	CD14 ⁻
	Ly-6G	CD33	
	CD115	HLA-DR ^{-low}	
	IL-4R α		

Table 3. Main characteristics of mouse and human MDSC.

1.2 Suppressive mechanisms of MDSC

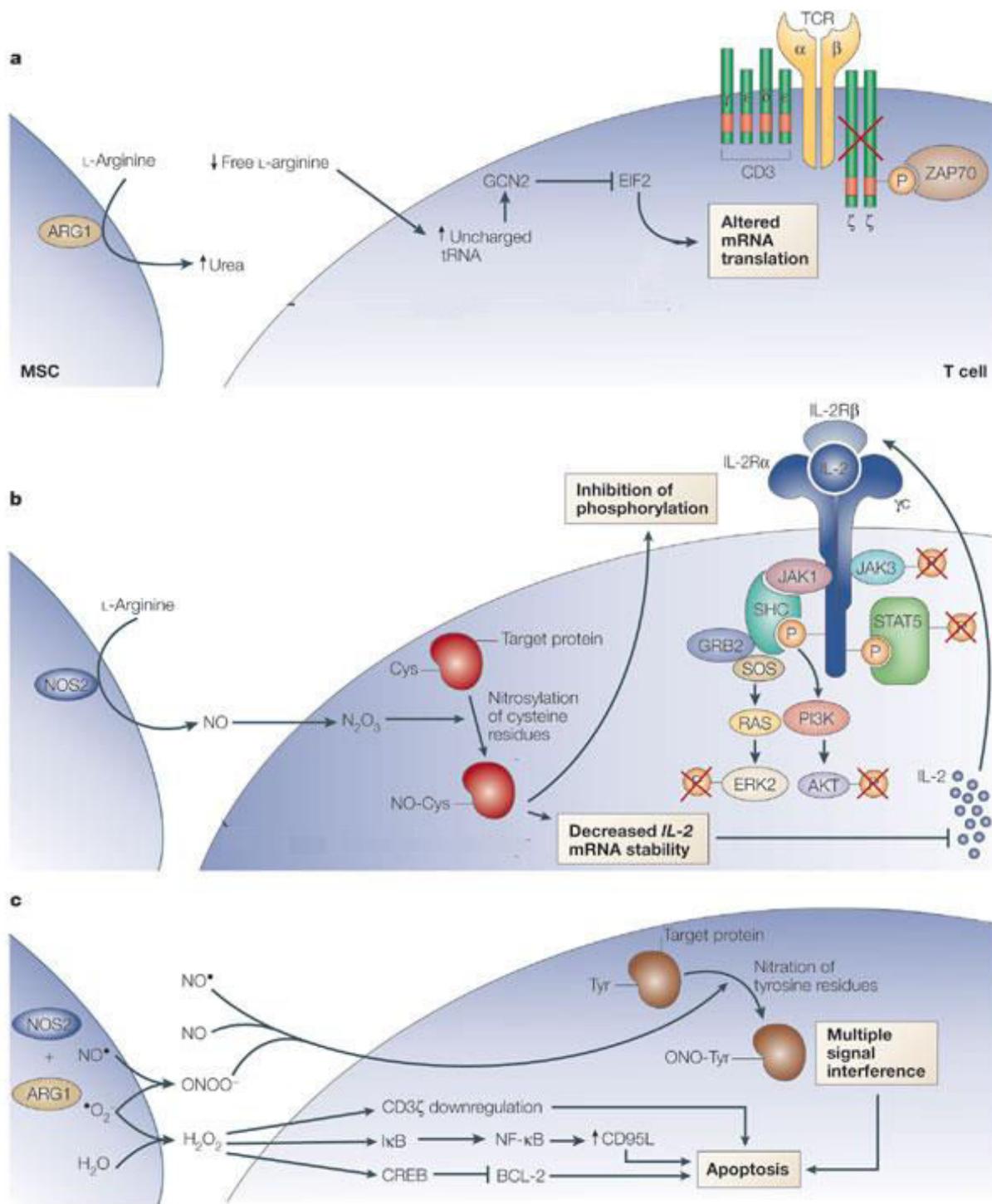
MDSC can mediate suppression by various mechanisms specific to the pathology. Some of the mechanisms are suppression of T cell proliferation and function through the depletion of the milieu in essential amino acids and induction of regulatory T cells.

1.2.1 Arginase and iNOS

The suppressive function of MDSC was linked to the upregulation of enzymes arginase I and iNOS, which degrade the amino acid L-arginine (348; 349). Both L-arginine depletion as well as the metabolites generated by iNOS and arginase I, as a result of L-arginine catabolism, can suppress T cells.

When an amino acid is withdrawn, its uncharged transfer RNA accumulates in cells and is sensed by a stress-response kinase GCN2 (Fig. 19A) (350). GCN2 then phosphorylates eukaryotic initiation factor 2 (EIF2) that, in its turn, blocks the ribosomal translation of most mRNA leading to cell cycle arrest and anergy of CD8 T cells. GCN2 kinase can also lead to the downregulation of the CD3 ζ chain of CD8 T cells and impair T cell signaling (351).

The catabolism of L-arginine by iNOS generates NO, which can inhibit IL-2 signaling on T cells and decrease the stability of IL-2 mRNA resulting in inhibition of T cell activation (Fig. 19B) (352; 353). High concentrations of NO can also directly induce apoptosis of T cells (354). When arginase I and iNOS are expressed simultaneously, their cooperation generates superoxide O₂⁻ that allows the formation of reactive nitrogen and oxygen species such as peroxynitrites or H₂O₂ (Fig. 19C) (355). Peroxynitrites can induce post-translational protein modifications by the nitration of tyrosine amino acid (356). As a result, proteins can lose or gain new functions. Thus, Nagaraj et al. showed that MDSC can induce nitration of TCR/CD8 complex making it impossible for T cells to recognize their antigens (357). Peroxynitrites and H₂O₂ were also shown to sensitize T cells to apoptosis by inducing the upregulation of proapoptotic Fas-L and downregulation of anti-apoptotic BCL-2 (358; 359). Finally, H₂O₂ can suppress T cell responses through the downregulation of the CD3 ζ molecule (360-362).



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Figure 19. Mechanisms of suppression by iNOS and arginase I. (A) L-Arginine depletion by arginase is sensed by GCN2 kinase that inhibits EIF2 and blocks mRNA translation inducing the downregulation of CD3ζ chain and cell cycle arrest. (B) NO generated by iNOS can decrease IL-2 mRNA stability as well as prevent IL-2 signaling in T cells, which can block T cell proliferation. (C) When both arginase and iNOS are active, generated H₂O₂ can induce CD3ζ downregulation and T cell apoptosis while peroxynitrites can induce protein modifications that can interfere in numerous pathways (Source : Bronte et al. Nature Reviews Immunology 2005 (348)).

1.2.2 Indoleamine-2,3-deoxygenase (IDO)

IDO is a suppressive enzyme that catalyzes the rate-limiting step of the amino acid tryptophan leading to tryptophan depletion and generation of different metabolites such as kynurenicins (363; 364). Both tryptophan depletion and metabolites can induce potent immunosuppression. Besides MDSC, IDO can be expressed by pDCs, B lymphocytes but also by non-immune cells including some tumor cells, epithelial and endothelial cells. IDO enzyme exists in two forms transcribed from two genes, IDO1 and IDO2; however not much is known about their functional differences, and the two isoforms are rarely differentiated in scientific publications.

The strongest inducer of IDO is IFN- γ due to the presence of an interferon response element in the promoter of IDO. However, IDO can be induced in DCs of IFN- γ ^{-/-} mice after CTLA-4/B7 engagement showing that the presence of IFN- γ is not an absolute pre-requisite. In fact, IFN α/β , TGF- β , TLR9 ligand CpG combined with CTLA-4 and PD-1/PD-L1 pathways were all described to induce IDO (365-367). Interestingly, low dose of CpG induces a robust classic Th1 immune response while high CpG dose induces strong upregulation of IDO suggesting that the intensity of the inflammation can dictate whether IDO will be upregulated or not.

In non-inflammatory conditions, IDO maintains fetal, oral and gut tolerance (368; 369). In inflammatory settings it can participate in the immune response against the pathogen. IDO upregulation during the immune response against a pathogen can be both good and bad for the host. The good consequence of IDO expression is the capacity of IDO to directly kill pathogens that are auxotroph for tryptophan. For example, the replication of *Toxoplasma gondii* was reduced in human fibroblasts by IDO upregulation and tryptophan degradation (370). Similarly, IFN- γ treated human macrophages were shown to upregulate IDO that inhibited the replication of *Chlamydia psittaci* (371). Also, the replication of herpes simplex virus type 2 in HeLa cell line can be inhibited by synergistic action of IFN- γ and TNF- α that induce a strong expression of IDO (372). Importantly, the viral replication could be restored with the addition of tryptophan showing that anti-viral response was mediated directly by IDO. IDO upregulation was also shown to be protective of human CMV, measles virus and vaccinia virus infections (373-375). Besides fighting infections, IDO, expressed during inflammation, can also suppress excessive immune responses and protect the tissue from destruction. However, if the suppression by IDO is too strong or if it arrives too early after the pathogen attack, it can dampen the protective host immunity and favor the escape of the pathogen. Such pathogenic expression of IDO was observed with *L. major* infection, in mice

infected with murine leukemia virus and during respiratory infections such as tuberculosis (376-378).

Mechanisms of suppression by IDO

Depletion of tryptophan by IDO has numerous consequences on CD4 and CD8 T cells. Similar to L-arginine depletion, when tryptophan is withdrawn, its uncharged transfer RNA accumulates in cells and is sensed by GCN2 kinase that activates EIF2 and provokes cell cycle arrest and anergy of CD8 T cells (Fig. 20) (379). CD3 ζ chain downregulation on CD8 T cells and impaired T cell signaling were also observed after tryptophan depletion (380). In CD4 T cells the activation of GCN2 kinase was shown to inhibit Th17 differentiation (381) and induce Treg cell differentiation (380; 382)

Another mechanism of suppression by IDO is the formation of kynurenins, the metabolites of tryptophan catabolism. Kynurenins were shown to suppress both CD4 and CD8 T cells, as well as NK cells (383; 384). Kynurenins are also natural ligands of the transcription factor Aryl hydrocarbon receptor (AhR). Activation of AhR by kynurenins induces the differentiation of CD4 T cells into regulatory Foxp3 cells and reduces the immunogenicity of DCs (385). Besides kynurenins, other tryptophan metabolites can induce T cell suppression. For example, 3-hydroxyanthranilic acid enhances Treg numbers and inhibits Th1 and Th17 responses in EAE (386).

Recently it has been shown that IDO can also induce suppression independently from its enzymatic activity. The study by Pallotta et al. showed that TGF- β can induce the expression of IDO in pDCs (366). The ITIM domains of IDO enzyme are then phosphorylated leading to the activation of the non-canonical nuclear factor kappa beta (NF- κ B) pathway that confers long term suppressive ability to pDCs.

Finally, IDO was shown to induce the upregulation of the suppressive HLA-G expression on DCs and also to increase the shedding of HLA-G molecule (387).

These different mechanisms of T cell suppression are not mutually exclusive and can act synergistically to induce a very potent suppression (306).

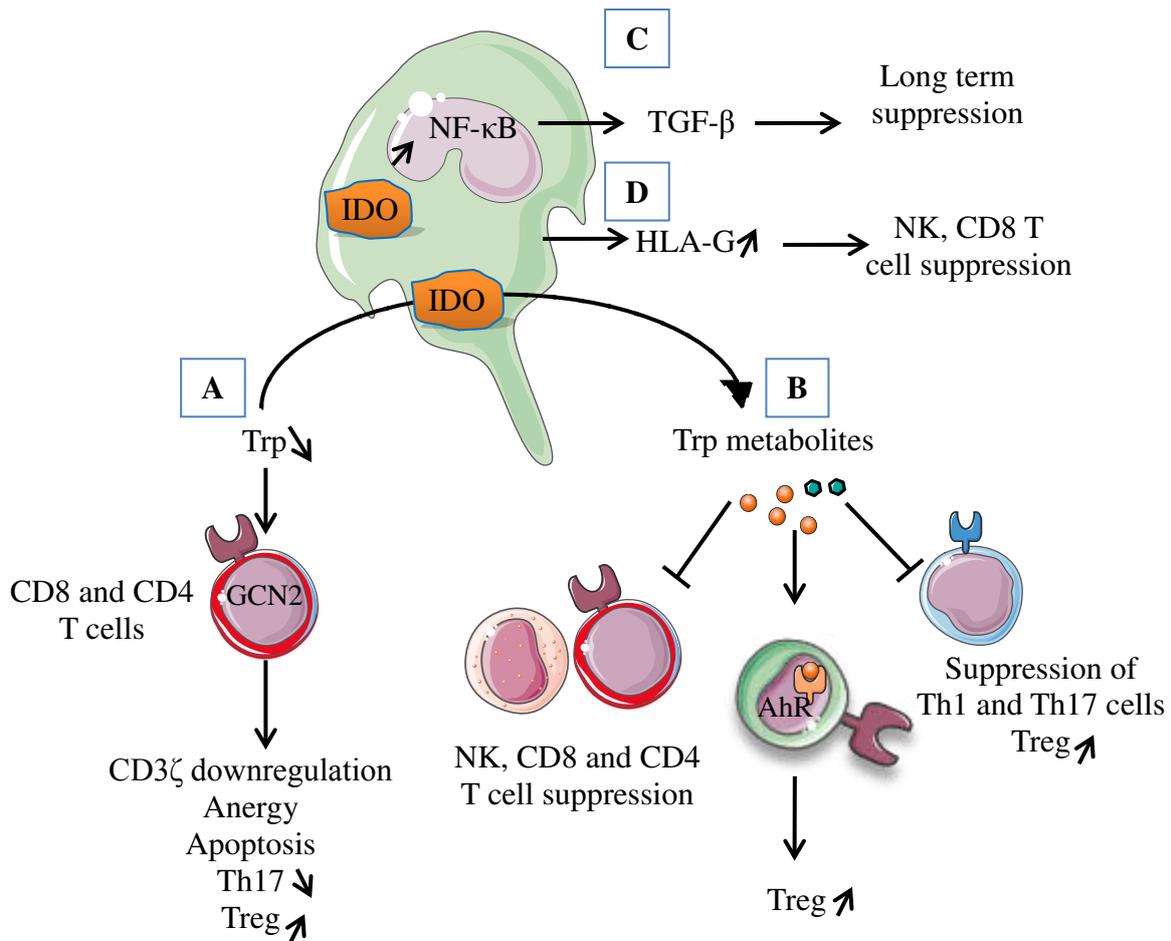


Figure 20. Mechanisms of suppression by IDO. (A) Tryptophan depletion by IDO is sensed by GCN2 kinase that can induce downregulation of CD3 ζ expression, energy and apoptosis of CD8 and CD4 T cells. GCN2 activation can also inhibit differentiation of CD4 T cells into Th17 cells and, on the contrary, promote Treg cell induction. (B) Tryptophan catabolism generates metabolites that can suppress NK, CD4 and CD8 T cells. Among these metabolites, kynurenin, were shown to bind the transcription factor Aryl hydrocarbon receptor and induce Treg cells. Another metabolite, 3-hydroxyanthranilic, can suppress Th1 and Th17 T cell responses and also promote Treg cells. (C) IDO can mediate suppression independent of tryptophan catabolism by acting as a signaling molecule and activating the non-canonical NF- κ B. This mechanism was shown to induce a long term suppressive capacities to IDO expressing pDCs. (D) IDO can induce the upregulation of the HLA-G molecule that can suppress T and NK cells.

1.2.3 Cysteine depletion

Cysteine is yet another amino acid that is suggested to be depleted by MDSC (388). T lymphocytes are dependent on cysteine for activation and proliferation. Cysteine can either be converted inside the cell from imported cystine or from the intracellularly available methionine (Fig. 21). However, T cells neither express the cystine transporter, nor have the

necessary enzyme to convert methionine. They typically depend on DCs and/or macrophages for cysteine production. Once produced inside APCs, cysteine is exported through ASC neutral amino acid transporter and can enter T cells through this same transporter. Additionally, DCs and macrophages secrete the protein thioredoxin that converts extracellular cystine to cysteine, which then can enter T cells through the amino acid transporter. While, MDSC can perfectly uptake cystine and convert it to cysteine, they lack the expression of ASC neutral amino acid transporter and retain the produced cysteine instead of releasing it. Besides, MDSC sequester cystine thus reducing its availability for the extracellular thioredoxin. As a result of cysteine depletion, the activation of T cells is hindered.

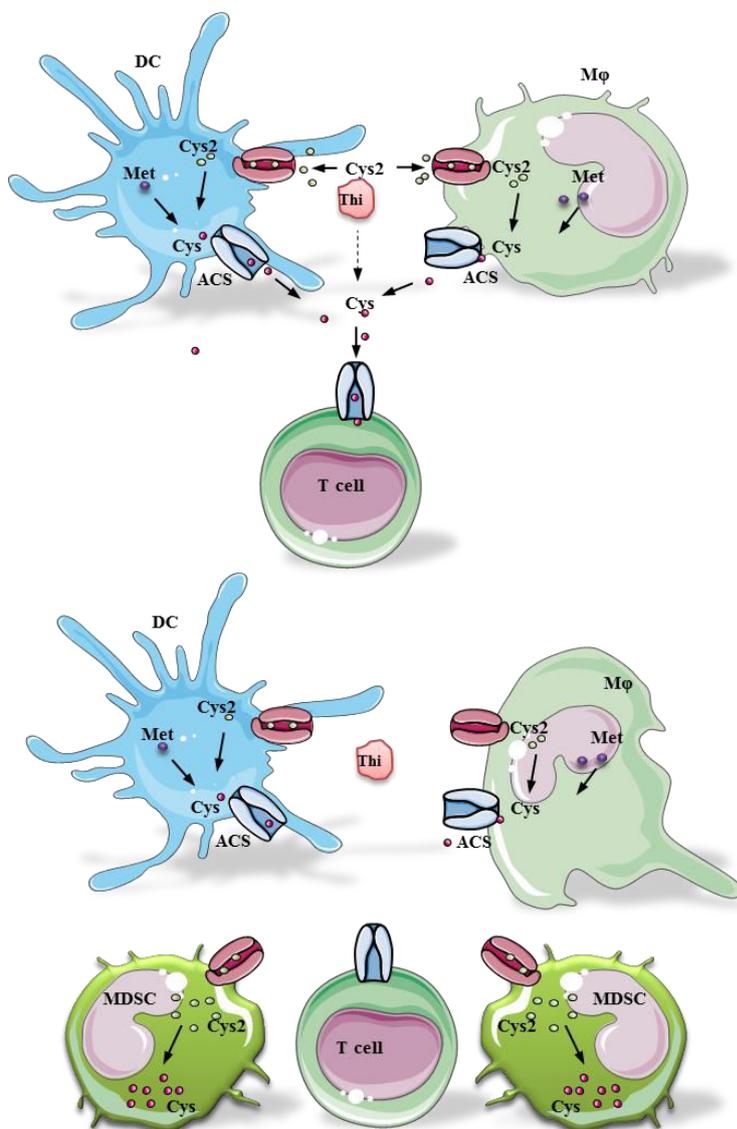


Figure 21. Suppression of T cells by cysteine depletion by MDSC. (A) DCs and macrophages produce cysteine (Cys) by converting the amino acid methionine (Met) and cystine (Cys2) that they uptake from the environment. Cysteine is then released through ACS transporter (ACS). Additionally, the enzyme thioredoxin (Thi) converts extracellular cystine into cysteine. T cells then uptake cysteine from the extracellular environment. (B) While MDSC uptake cystine and convert it to cysteine, they do not release it because they lack the ACS transporter. This results in the depletion of cysteine (Modified after Srivastava et al. *Cancer Res* 2010 (388)).

1.2.4 TGF- β production

The suppressive cytokine TGF- β is strongly associated with tumor growth (389). One such source of TGF- β was found to be tumor infiltrating MDSC that effectively suppressed cytotoxic T cells (390). Similarly, Valenti et al. showed that monocytes cultured with microvesicles released by tumor cells differentiated into MDSC that produced TGF- β and induced T cell suppression (391). Thus, these experiments show the direct suppression of T cells by TGF- β producing MDSC. Besides, it is possible that T cells can also be suppressed indirectly by TGF- β induction of Treg cells.

2. Role of macrophages in T1D

2.1 The pathogenic role of CAM ϕ in T1D

The pathogenic role of macrophages in T1D has been suggested in several publications. In prediabetic NOD mice a short term macrophage depletion (total of 2 injections 2 days apart at 8 weeks of age) greatly delayed diabetes incidence (392). Interestingly, pancreatic insulinitis resolved completely with the disappearance of macrophages and slowly reappeared once macrophages returned into pancreas. This study, therefore, revealed the important role of macrophages in the retention of lymphocytic infiltrate in pancreas probably by inducing the upregulation of adhesion molecules on pancreatic endothelium (393). In transgenic models with increased numbers of anti-islet CD4 and CD8 T cells macrophage depletion completely prevented diabetes development and was associated with decreased production of a pro-Th1 cytokine IL-12 and lower levels of T cell activation (394; 395). Macrophage depletion also allows better survival of islet grafts in diabetic NOD mice (396). Interestingly, the inhibition of macrophage migration into the islets alone is enough to prevent diabetes development by diabetogenic T cell transfer (397).

2.1.1 Initiation of T1D

In rodents, a massive developmentally programmed wave of apoptosis of pancreatic islet β cells occurs between days 17 and 20 of age. Apoptotic and necrotic bodies, as well as cellular debris generated during this process are normally cleared by macrophages. In NOD

mice, however, macrophages were found to have reduced capacity of phagocytosis compared to strains that do not develop diabetes such as BALB/c (398; 399). The accumulation of apoptotic and necrotic bodies in combination with cytokines is suggested to activate APCs, that uptake and present self-antigens to autoreactive T cells in PLN, thus initiating diabetes (Fig. 22) (400). Surprisingly, macrophage depletion prevents spontaneous diabetes development in diabetes prone rodents despite the increased accumulation of apoptotic and necrotic bodies (401-403). These results suggest that the inefficient clearance of apoptotic bodies is not the only mechanisms by which macrophages favor in type 1 diabetes development.

2.1.2 Cytokines and T1D

In recently diagnosed type 1 diabetes patients islet macrophages and DCs were found to secrete IL-1 β and TNF- α while these cytokines were not found in macrophages from control patients (404). Similarly, islet macrophages from NOD mice strongly express TNF- α (405). Islet macrophages of T1D patients are, therefore, suggested to have a CAM ϕ phenotype.

Cytokines can be deleterious for pancreatic islet β -cells (406). For example, TNF- α and IL-1 β can activate the NF- κ B signaling pathway, which can increase the expression of Fas and promote β cell apoptosis (407). Additionally, TNF- α synergized with IFN- γ can induce iNOS expression, generation of NO leading to β -cell death (406; 408). Yang et al. further confirmed the pathogenic role of TNF- α by demonstrating that the administration of TNF- α to newborn NOD mice during three weeks accelerated diabetes development while the administration of an anti-TNF- α antibody completely inhibited it (409). The treatment with TNF- α induced DC maturation while anti-TNF- α treatment prevented it (410). These experiments suggest that TNF- α plays a role in the initiation of T1D perhaps by favoring the induction of immunogenic DCs that would present auto-antigens in PLN. However, TNF- α also plays an important role in the effector phase of diabetes since even though TNF-R1^{-/-} mice develop insulinitis similar to wt NOD mice, they do not progress to overt diabetes (411). As for IL-1 β , Thomas et al. showed that IL-1R knockout mice have a slower diabetes progression compared to wt NOD mice (412). Besides, the administration of IL-1R antagonist after syngeneic pancreatic islet transplantation prevents hyperglycemia during the period of the treatment (413).

Another pro-Th1 cytokine, IL-12, produced by macrophages is suggested to favor diabetes development in NOD mice. It was shown that upon activation macrophages of NOD mice produce higher levels of IL-12 compared to diabetes resistant strains (414). Interestingly, in macrophage depleted mice diabetes prevention correlates with decreased IL-12 production (403) but can be restored by IL-12 administration. The low IL-12 production is suggested to drive Th2 differentiation of splenic T cells since splenocytes of macrophage depleted mice fail to induce diabetes when transferred into NOD Scid recipients compared to untreated mice.

It is interesting to note, that macrophages can induce diabetes without the involvement of the adaptive immune system. C57Bl/6 transgenic mice, expressing CCL2 (also known as monocyte chemoattractant protein-1 (MCP-1)) specifically in islets, have an increased macrophage accumulation in pancreas, which is associated with high diabetes incidence (415). However, this is suggested to be related to the mouse strain used in this article, since transgenic overexpression of CCL2 in the islets of NOD mice gives a completely different result. Indeed, while the insulinitis score is higher in transgenic NOD mice, diabetes incidence is actually lower compared to wt NOD mice and is associated with the presence of CD11b⁺/CD11c⁺ suppressive cells in PLN (416). This last study doesn't disprove the role of CAM ϕ in the pathogenesis of diabetes in NOD mice but perhaps reflects the negative control loop. Thus, in these transgenic NOD mice, which have a persistent inflammation in islets, the recruited macrophages would rather differentiate into suppressive cells in order to dampen the inflammation and protect pancreas.

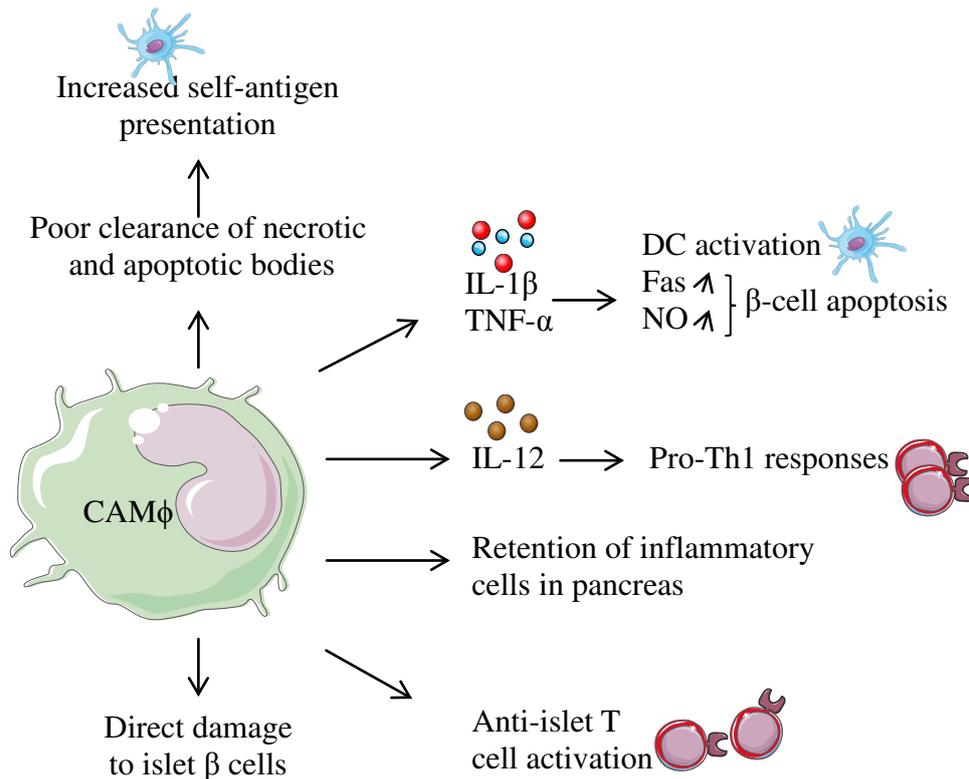


Figure 22. Pathogenic role of macrophages in T1D. In diabetes prone mice poor clearance of necrotic and apoptotic bodies by macrophages is suggested to initiate T1D. In these mice islet macrophages also produce high amounts of IL-1 β , TNF- α and IL-12 that can promote DC activation, upregulation of pro-apoptotic Fas receptor and NO and Th1 immune responses.

2.2 Role of alternatively activated macrophages in T1D

Contrary to CAM ϕ , AAM ϕ are suggested to be protective from T1D. This notion is based on the correlation between infections that favor AAM ϕ phenotype and decreased diabetes incidence. Infection with *Schistosoma mansoni* or the treatment with *Schistosoma mansoni* eggs induces a decrease of diabetes incidence in NOD mice. The infection also induces suppressive DCs that produce less pro-inflammatory IL-12 and more suppressive TGF- β . Moreover, *Schistosoma mansoni* promotes the differentiation of AAM ϕ and the skewing of T lymphocytes towards the production of IL-4 and IL-10 (417). Similarly, the diabetes prevention in NOD mice infected with gastrointestinal nematode, *Heligmosomoides polygyrus* is associated with the induction of AAM ϕ in numerous organs such as the colon and the spleen (109; 418). Another nematode, *Taenia crassiceps*, was shown to decrease chemically induced diabetes in NOD mice. Infected mice had an increased IL-4 and reduced

TNF- α production as well as an increase in AAM ϕ numbers compared to uninfected mice (419). Finally, in two studies with adoptive transfer of in vitro differentiated AAM ϕ , a marked reduction in pancreatic islet injury and in diabetes incidence was observed (420; 421). Together, these data strongly support the protective role of AAM ϕ in T1D.

2.3 MDSC and T1D

The list of factors suggested to favor the development of diabetes in NOD mice is long and includes, among others, failed central tolerance, defects in inhibitory CTLA-4 and PD-1 signaling, defect in macrophage phagocytosis, deficiencies in iNKT and Foxp3 cell numbers and others. Recently, MDSC and particularly IDO expressing MDCS were included into this list. A study by Grohmann et al. showed that CD8 regulatory DCs of NOD mice failed to upregulate IDO after stimulation with IFN- γ (422). This defect was proper to DCs of young mice (4 weeks old) since DCs of older mice (8 weeks old) could upregulate IDO. In old mice IFN- γ induced IDO upregulation was dependent on the phosphorylation of Stat1 transcription factor. In young NOD mice, however, IFN- γ induced the production of peroxynitrites that inhibited Stat1 phosphorylation and IDO induction. Furthermore, the use of peroxynitrite inhibitor greatly reduced diabetes incidence of NOD mice (423). The inability of IFN- γ to upregulate IDO is thought to be the result of inflammation in islets that develops after the wave of β -cell apoptosis at 3 weeks of age. Thus the inflammation favors immunogenic DCs that can prime and activate autoreactive T cells leading to T1D development.

Interestingly, autoimmune disorders, such as rheumatoid arthritis and multiple sclerosis, can have a temporal remission during pregnancy. Munn et al. have shown the role of IDO in maintaining fetal tolerance (368; 369). Importantly, the treatment with human chorionic gonadotropin (HCG) hormone, that is one of the earliest synthesized hormones during pregnancy, was shown to prevent diabetes in NOD mice by upregulating IDO in splenic DCs in IFN- γ independent manner (424). Splenocytes of HCG treated mice induced diabetes only when DCs were depleted prior to transfer suggesting that the suppression was mediated only by DCs and not by increased Treg numbers. It would, therefore, be interesting to test the role of IDO in the remission phase of different autoimmune diseases. The transgenic induction of IDO expression by syngeneic fibroblasts can also prolong the survival of islet allograft (425). The graft survival correlated with effector T cell suppression,

induction of Th2 immune responses, anti-inflammatory cytokine expression and generation of Treg cells in transplant draining lymph node. Finally, MDSC from tumor bearing mice prevented diabetes when co-transferred with diabetogenic T cells into NOD Scid mice. However, the mechanisms of T cell suppression was not elucidated in this publication (426).

V - Interactions of NKT cells with MDSC

There exists a relationship between NKT cells and MDSC. Interestingly, type I and type II NKT cells seem to have opposing roles: type I NKT cells inhibiting them and type II NKT cells promoting them.

Cancer

Besides direct killing of tumor cells and activation of DCs, iNKT cells can play an anti-tumor role by regulating MDSC. Thus, α GalCer activated iNKT cell were shown to convert CD11b⁺/GR1⁺ suppressor cells into immunogenic APCs, which upregulated CD86 and induced the activation rather than the suppression of T cell responses (427). Similarly, iNKT cells could also render cytotoxic T cells resistant to MDSC mediated suppression in a breast cancer model (428). Not only tumor cells can be targets for iNKT cells cytotoxicity. The study by Song et al. suggests that iNKT cells can also kill monocytes infiltrating neuroblastoma xenografts and, by this, prevent the tumor growth. In vitro, iNKT cells effectively killed PBMC that were pulsed with neuroblastoma lysate, suggesting that tumor antigens must be presented by CD1d for cytotoxicity (429). Similarly, Renukaradhya et al. reported that in B cell lymphoma model, iNKT cell deficient BALB/c mice had higher numbers of CD11b⁺/GR1⁺ cells both in the tumor and the spleen compared to wt BALB/c mice, suggesting iNKT cell mediated control of MDSC numbers and the tumor growth (430). Interestingly, type II NKT cells were on the contrary promoting the tumor of B cell lymphoma.

Type II NKT cells were found to promote the metastasis of colon cancer cells into lungs by secreting IL-13 and inducing TGF- β producing CD11b⁺/GR1⁺ suppressor cells (390; 431). In the 4T1 breast cancer tumor model, NKT cells suppress tumor immunity by producing IL-13 and polarizing macrophages towards AAM ϕ phenotype rather than CAM ϕ that are otherwise tumoricidal through NO production (432).

Autoimmunity

In animal model of multiple sclerosis α GalCer activated iNKT cells induce the expansion and recruitment of MDSC to the central nervous system that protects against EAE.

Activated MDSC express immunosuppressive enzymes iNOS and arginase I and secrete IL-10 and their depletion completely abrogates the protective effect of α GalCer (433).

Infections

Furthermore De Santo et al. reported that both murine and human iNKT cells can control influenza A infection by inhibiting the expansion of MDSC in the lung (253). Without iNKT cells, MDSC dampen influenza-specific CD8 T cell responses, which eventually leads to increased viral titers and mortality (253). However, iNKT cell stimulation with α GalCer during vaccination with inactivated influenza A virus, on the contrary, induces IDO expressing MDSC in the draining lymph node that suppresses acute CD8 T cell responses and promote memory CD8 T cell responses (254).

Aim of the study

iNKT cells are potent regulators of autoimmunity, and their manipulation was shown to prevent T1D development in numerous publications. iNKT cells also participate in immune responses against pathogens. Our previous studies conducted in a murine model of T1D with LCMV 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 T1D. The aim of this study was to investigate whether the dual role of iNKT cells in infections and autoimmunity was specific to LCMV, or whether it could be related to other viral infections. In humans, T1D development is suggested to be favored by enteroviral infections. We, therefore, chose to study the T1D development after coxsackievirus infection. Importantly, iNKT cells can be used in therapy in humans since these cells can be activated in humans by α GalCer injection. Therefore, the manipulation of α GalCer activated iNKT cells and coxsackievirus could be a promising therapy.

It has previously been published that CVB4 infection accelerated T1D development in a subset of mice, but prevent it in others. However, the molecular mechanisms, particularly the role of iNKT cells after the infection, have not been described yet. In our study we tried to determine the inflammatory response after coxsackievirus infection and whether or not α GalCer activated iNKT could shape it to prevent T1D development similar to LCMV infection. α GalCer activated iNKT cells secrete various cytokines such as INF- γ and IL-4, IL-13. INF- γ secreted by iNKT cells could promote viral clearance. However, it could potentially accelerate T1D development by promoting Th1 type immune responses. By contrast, IL-4 and IL-13 could prevent T1D by skewing T cell responses towards Th2 type, but this could also result in viral escape. We also studied the recruitment of immune cells into pancreas and whether the manipulation of iNKT cells favored the infiltration of suppressive cells into pancreas such as Treg cells and MDSC. We searched to determine whether they modified the phenotype of APCs by making them diabetogenic or, on the contrary, tolerogenic. Finally we investigated the role of α GalCer activated iNKT cells in anti-islet T cell response.

In summary, we aimed at a better understanding of the biological role of iNKT cells in this pathological condition. Determining the interactions between iNKT cells and the virus in mouse models is the first step towards designing of novel therapies with iNKT cell agonists.

Experimental design and methods

Mice

Female Pro-ins2^{-/-} NOD mice, V α 14 transgenic NOD mice expressing the V α 14-J α 18 TCR α chain, BDC2.5 C α ^{-/-} and J α 18^{-/-} mice were previously described (41; 132; 303; 311; 434). NOD V α 14 were crossed to Pro-ins2^{-/-} NOD mice to generate V α 14 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 a single dose of 1x10⁵ PFU/mouse. When indicated, mice were treated with a single i.p. injection of α GalCer (2 μ g/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 μ g of soluble extracellular domain of IL-13 receptor injected 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. 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. To deplete macrophages, mice were injected i.v. with 200 μ l of clodronate or PBS containing liposomes on days -1, +1 of virus infection for PCR analysis and -1, +1 and +3 for diabetes incidences. Clodronate was a gift from Roche Diagnostics (435).

Viral titration by plaque forming unit essay (PFU)

Pancreata were recovered and homogenized in liquid maintenance medium 199 (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 20min. 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. In situ hybridizations were performed by our collaborators Dr. Malin Flodström et al.

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 (200 µm 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) and dissected free from surrounding tissues. Pancreata were 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. Islets were then 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), CD86 (137-2), Foxp3 (150D/E4), PD-L1 (MIH5), CD103 (2E 7), OX-40 (OX86), GITR (DTA-1) from eBiosciences and CD11c (HL3), Ly-6C (AL21), IFN- γ (XMG1.2), CD62L (Mel-14), CD4 (GK1.5), CD8 (53-6.7), anti-human Ki-67 (B56), IL-10 (JES5-16E3), IL-12 (C15.6), CTLA-4 (UC10-4F10-11), IA-k (10-3.6) from BD biosciences. Stainings were performed in PBS, 5% FCS for 20min, at 4°C. Non-specific Fc binding was blocked using an anti-CD16/CD32 antibody (24G2). APC-conjugated α GalCer-loaded CD1d tetramer was prepared in our laboratory. For intracellular cytokine stainings, cells were stimulated with PMA (10 ng/ml) and ionomycin (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 30min. Figure 1. For IGRP tetramer staining, pancreatic islets were recovered, dissociated into a single cell suspension and stained with NRP-V7 (IGRP₂₀₆₋₂₁₄ mimotope; KYNKANVFL) loaded Kd tetramer and unrelated TUM peptide (KYQAVTTTL) loaded Kd tetramer both synthesized by the National Institute of Health Tetramer Core Facility. Tetramer staining was performed at room temperature for 1 hour followed by 15 minute surface staining with mAbs directed against CD45, TCR α , CD8 and CD4. Cells were either analyzed using a BD Fortessa flow cytometer or sorted out using BD FACS ARIA II sorter.

Quantitative RT-PCR

RNA was extracted using RNeasy mini kit (Qiagen) and reverse transcribed using Superscript III (Invitrogen). Quantitative RT-PCR (qPCR) was performed with SYBR Green (Roche) and analyzed with LightCycler 480 (Roche). Relative expression was calculated using the $2^{-\Delta\Delta C_t}$ method and normalized to the expression of the housekeeping gene GAPDH. The stability of GAPDH expression was confirmed by comparison to HPRT mRNA (Appendix 1). The primers used for qPCR analysis are listed in table 1 (Appendix 2, all from Eurofins). They were all designed and tested in our laboratory prior to use.

In vitro T cell responses

Single cell suspensions of pancreatic islets were cultured in the presence of IGRP₂₀₆₋₂₁₄ peptide (VYLKTNVFL; 10 μ M) for 4 h 30 min 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⁺ CD4 T cells (3×10^4) were cultured with anti-CD3/CD28 beads (Invitrogen) and 3×10^4 macrophages, isolated either from the pancreas of mice infected with CVB4 alone or infected and treated with α GalCer. After 48 h culture, wells were pulsed with 1 μ Ci tritiated thymidine ($[^3\text{H}]\text{-TdR}$) overnight. $[^3\text{H}]\text{-TdR}$ 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 μ M.

Adoptive transfer of pancreatic islet macrophages

CD11b⁺/F4/80⁺/Ly-6C⁺ macrophages were sorted out from pancreatic islets of 5-6 weeks old female Pro-ins2^{-/-} mice from CVB4 and CVB4+αGalCer groups on the second day of infection using FACS Aria II cell sorter. A total of 2x10⁵ macrophages were injected i.v. into each recipient 5-6 weeks old female Pro-ins2^{-/-} mouse infected with 1x10⁵ PFU of CVB4 one day earlier. Diabetes development was monitored twice weekly using urine glucose tests.

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 stimulation with α GalCer inhibits diabetes development upon CVB4 infection

The infection of female NOD mice by CVB4 can lead to either development of diabetes or long term protection from diabetes depending on the age at which mice are infected (199). The infection of NOD mice, that are younger than 8 weeks of age and that do not have sufficient insulinitis yet, leads to long term protection against diabetes. However, the infection of mice older than 8 weeks of age that have an established insulinitis leads to accelerated diabetes development in 60% of mice compared to uninfected NOD mice. Similar to results obtained in previous publications, we observed that diabetes development of CVB4 infected 10 week old female NOD mice (CVB4 group) was accelerated compared to uninfected mice (Fig. 23A and B). To evaluate the role of iNKT cells, we treated NOD mice with a single dose of iNKT cell agonist, α GalCer, at the time of infection (CVB4+ α GalCer group). Interestingly, while this single α GalCer treatment had no effect on diabetes development in uninfected mice, it strongly decreased diabetes incidence of CVB4 infected NOD mice since only around 20% of mice became diabetic.

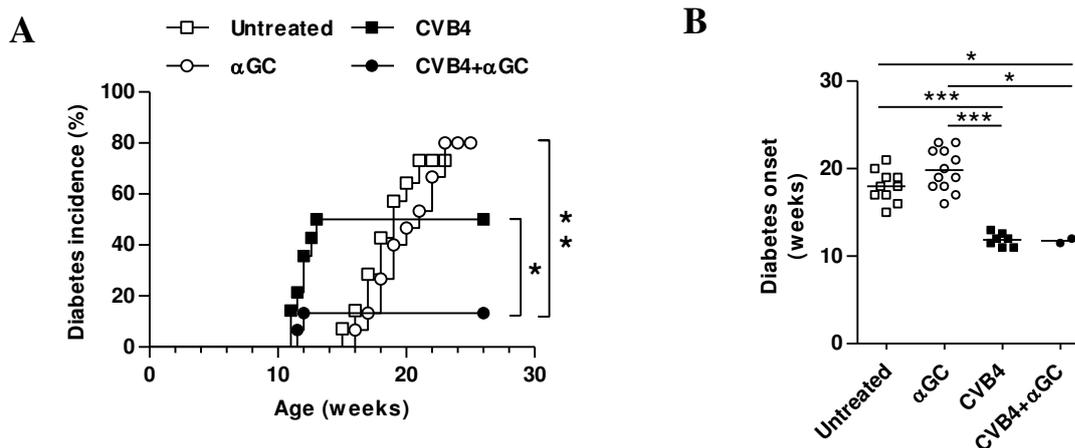


Figure 23. NKT cell activation prevents diabetes development of NOD mice upon CVB4 infection. (A) Diabetes incidence of female NOD mice (10 weeks old) inoculated i.p. with CVB4 or PBS and treated with α GalCer (α GC) or control vehicle at (untreated n=14, α GalCer n=15, CVB4 n=14, CVB4+ α GalCer n=15). *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.

Working with 10 weeks old NOD mice that require an additional follow-up of 10-20 weeks is very time consuming. To shorten the duration of experiments, we tested the same experimental protocol in Pro-ins2^{-/-} mice generated by the group of Christian Boitard (41). These mice have accelerated diabetes compared to wt NOD mice as almost 100% of mice become diabetic between 10 to 15 weeks of age compared to NOD mice whose diabetes onset only starts around this age.

In mice two isoforms of proinsulin exist (proinsulin 1 and proinsulin 2) that are coded by two genes located on chromosomes 7 and 19. These two isoforms differ only by several amino acids and are very similar in their protein primary structure. However, they are not expressed similarly. While proinsulin 2 is expressed both in the thymus and in pancreatic islet β -cells, proinsulin 1 is expressed only in pancreatic β -cells. Thus, only proinsulin 1 is expressed in islets of Pro-ins2^{-/-} mice. Importantly, normoglycemia is preserved in these mice due to increased proinsulin 1 expression in islets that compensates for the lack of proinsulin 2. Rather, the absence of proinsulin 2 expression in the thymus prevents the negative selection of autoreactive T cells directed against proinsulin 2. However, since the structure of proinsulin 1 and 2 are very similar, these T cells can also recognize proinsulin 1 expressed in pancreatic islets. The high frequency of such T cells is suggested to be responsible for the accelerated diabetes observed in these mice. Moreover, diabetes does not develop in NOD Scid Pro-ins2^{-/-} mice confirming the role of the adaptive immune system in T1D development in these mice (data not shown).

Similar to NOD mice, Pro-ins2^{-/-} female mice infected with CVB4 at 4 weeks of age were completely protected from diabetes (data not shown). We, therefore, chose to infect them between 5-6 weeks of age when they have sufficient insulinitis for diabetes development. In line with this, 60% of Pro-ins2^{-/-} female mice developed diabetes between 1 and 3 weeks post CVB4 infection (Fig. 24A). Even though less apparent compared to NOD mice, diabetes development of CVB4 infected Pro-ins2^{-/-} was significantly accelerated compared to untreated mice (Fig. 24B). Similarly, the treatment of Pro-ins2^{-/-} mice with α GalCer at the time of infection decreased the diabetes incidence to 25%. Since Pro-ins2^{-/-} mice gave similar results compared to NOD mice, we chose to perform our study mainly with Pro-ins2^{-/-} mice while confirming the most important data in NOD mice.

Overall, our results showed that iNKT cell activation decreases diabetes incidence in both NOD and Pro-ins2^{-/-} mice infected with CVB4.

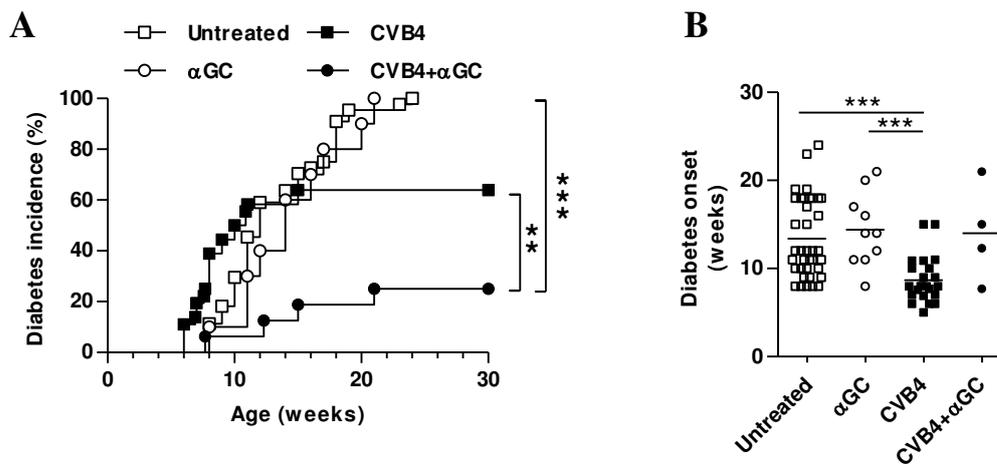


Figure 24. NKT cell activation prevents diabetes development of Pro-ins2^{-/-} mice upon CVB4 infection. (A) Diabetes incidence of female Pro-ins2^{-/-} mice inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle at 5-6 weeks of age (untreated n=44, α GalCer n=10, CVB4 n=36, CVB4+ α GalCer n=17). **p<0.005, ***p<0.0005 using log-rank test analysis. Data represent 2-4 pooled independent experiments. (B) Age of diabetes onset of Pro-ins2^{-/-} mice that became diabetic. ***p<0.0005, Mann Whitney.

α GalCer stimulated iNKT cells induced a potent protection from diabetes after CVB4 infection. However, even without α GalCer stimulation, diabetes was prevented in 40% of CVB4 infected mice. We, therefore, wondered if unstimulated iNKT cells were responsible for diabetes prevention in this subset of CVB4 infected mice. To answer this question, we studied the diabetes incidence in CVB4 infected Ja18^{-/-} Pro-ins2^{-/-} mice, which completely lack iNKT cells. CVB4 infection induced accelerated diabetes in around 38% of Ja18^{-/-} Pro-ins2^{-/-} mice compared to uninfected mice and the overall diabetes incidence was 67% (Fig. 25). These results are similar to those obtained with wt Pro-ins2^{-/-} mice and suggest that diabetes prevention after CVB4 infection is not mediated by unstimulated iNKT cells.

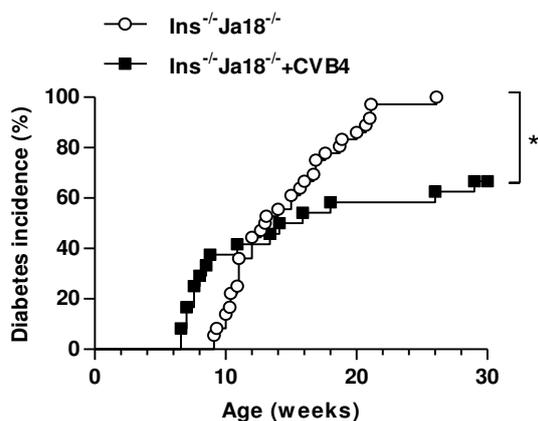


Figure 25. Diabetes incidence of Ja18^{-/-} Pro-ins2^{-/-} mice after CVB4 infection. Diabetes incidence of female Ja18^{-/-} Pro-ins2^{-/-} mice inoculated i.p. with CVB4 or PBS at 6 weeks of age (untreated n=36, CVB4 n=24). *p<0.05 using log-rank test analysis. Data represent 3-5 pooled independent experiments.

The histological analysis of pancreata of Pro-ins2^{-/-} mice one week after the infection showed that there were more islets with mononuclear cells infiltration in mice of CVB4 group compared to mice from CVB4+ α GalCer group (Fig. 26). In this last group, mononuclear cells accumulated around the islets rather than infiltrated them. These results are in line with the protective role of α GalCer in diabetes development after CVB4 infection.

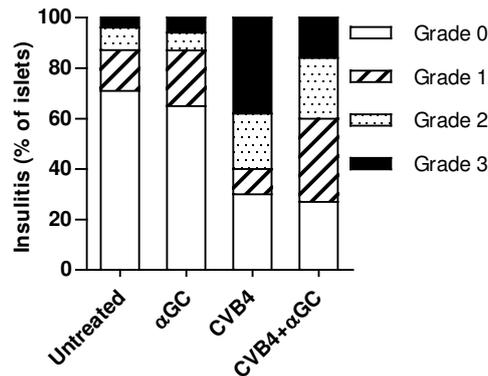


Figure 26. More islets of CVB4 infected mice are infiltrated by immune cells compared to in CVB4+ α GalCer group. Histological scoring of insulinitis was performed on pancreatic sections of Pro-ins2^{-/-} mice from days 7-10 post-infection stained with haematoxylin/eosin (n=6 mice per group). Grade 0, no infiltration; grade 1, peri-islet lymphocytic infiltration; grade 2, <50% islet lymphocytic infiltration and 3, >50% islet lymphocytic infiltration.

The study by Kanno et al. has previously demonstrated that the replication rate and the dose of CVB can directly influence diabetes development (201). Thus, diabetes incidence decreased from 70% to 30% when the viral dose was reduced 5 times. In line with this data, we hypothesized that the activation of iNKT cells by α GalCer during CVB4 infection could result in lower viral titers. As a consequence, the inflammatory response in the islets would not be strong, which would favor the preservation of pancreatic islets and decrease self-antigen presentation by APCs, thereby preventing diabetes. We, therefore, compared viral titers in total pancreas of infected Pro-ins2^{-/-} and NOD mice treated or not with α GalCer by PFU assay. Pancreatic viral titers peaked on the second day of infection and became undetectable by the 8th day of infection without any significant differences between the both groups of mice (Fig. 27A and B). Because PFU technique detects viral replication in total pancreas, we asked the group of our collaborator, Dr. Malin Flodström, to perform in situ hybridization (ISH) to determine if CVB4 specifically infected pancreatic islets and, if yes, whether there were differences in the level of infection between mice treated or not with

α GalCer. CVB4 infection of pancreatic islet β -cells has yielded conflicting results in previous publications (200; 209). We found that CVB4 infected the pancreatic exocrine tissue causing its destruction to a similar degree in both CVB4 and CVB4+ α GalCer groups. However, we did not detect CVB4 in pancreatic islets of CVB4 and CVB4+ α GalCer treated mice tested 3 days post-infection and 7 days post-infection, while CVB4 was still present in the exocrine tissue (Fig. 28) Altogether, these results strongly suggest that differences in diabetes incidence between CVB4 and CVB4+ α GalCer treated mice were not due to altered viral replication or clearance.

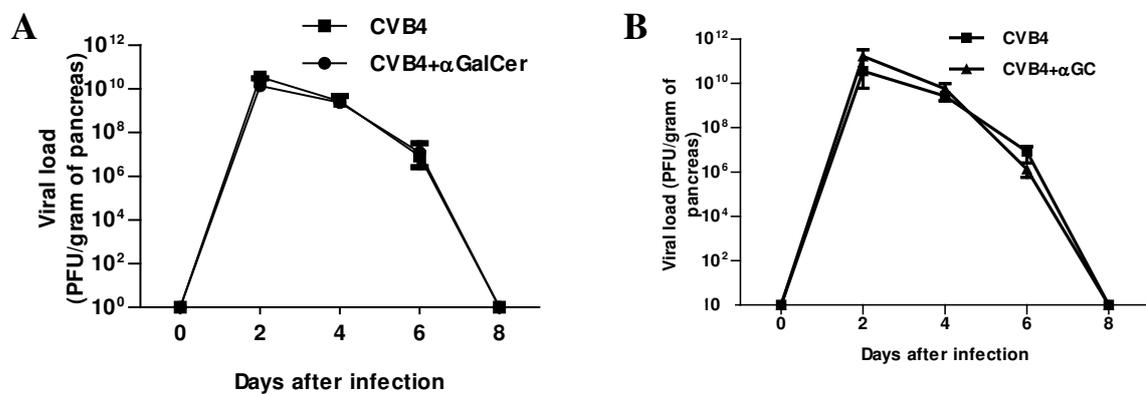


Figure 27. CVB4 replication is not different between mice from CVB4 and CVB4+ α GalCer groups. Pancreata were isolated from 5-6 weeks old female Pro-ins2^{-/-} mice (A) and 10 weeks old female NOD (B) mice on different days post-CVB4 infection, weighed and viral titers were determined on HeLa cell monolayers by PFU technique. Mean viral titers are expressed as PFU/gram of pancreas \pm SD (n=3-6 mice/group for each day).

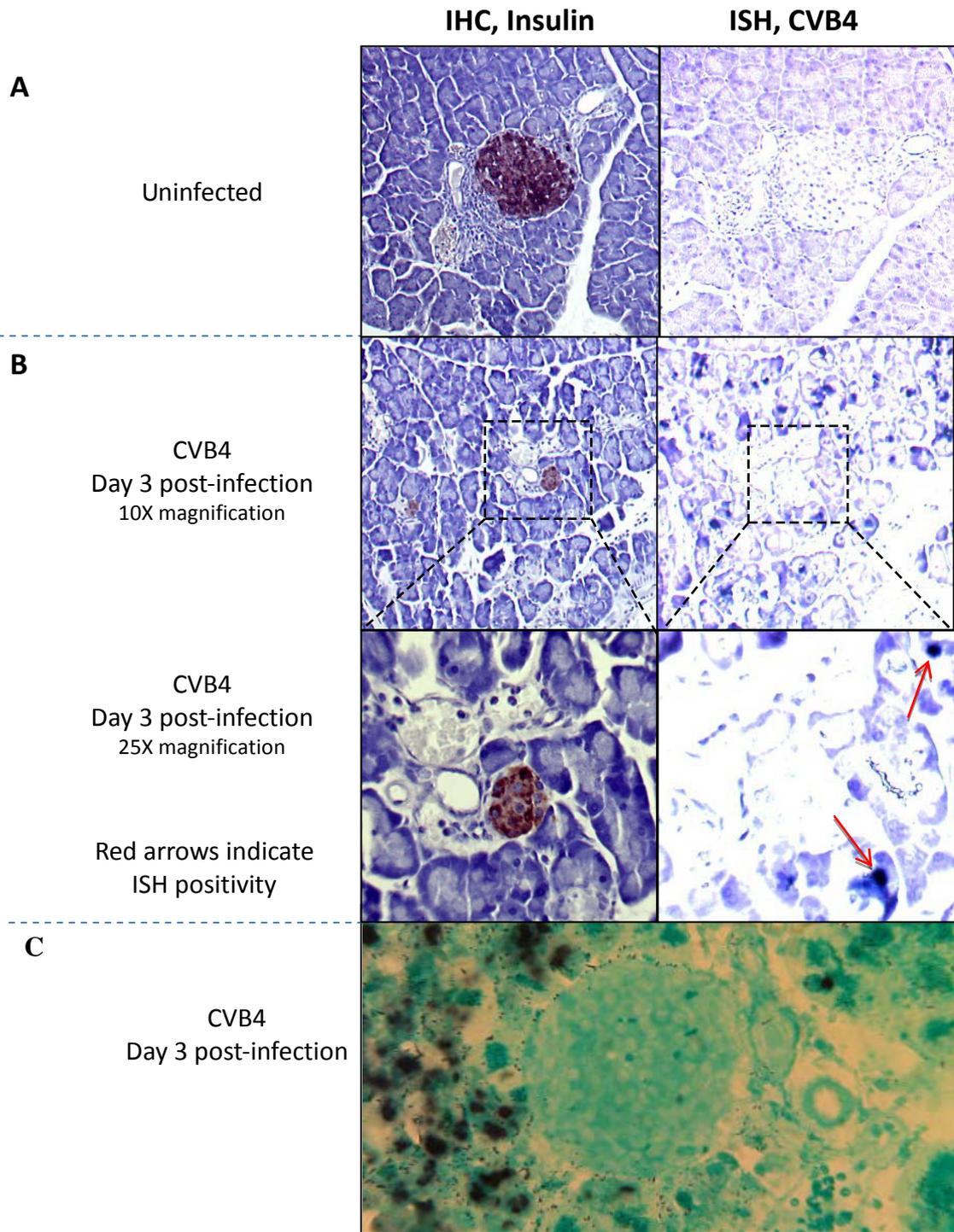


Figure 28. CVB4 does not infect islet β -cells. (A, B and C) Representative anti-insulin and anti-CVB4 staining in pancreas. Two consecutive sections from pancreas of day 3 CVB4 infected Pro-ins2^{-/-} mice were used; one to detect islets by immunochemistry using an anti-insulin mAb (left panel), the other for CVB4 detection by in situ hybridization (right panel). Sections are counterstained with haematoxylin/eosin stains since haematoxylin stains the nuclei rather than cytoplasm, which improves the visibility and makes it easier to detect islets. (A) Two consecutive sections of pancreas from an untreated mouse at 10x magnification. (B) Two consecutive sections of pancreas from CVB4 infected mouse at day 3 post-infection at 10x magnification (top row) and 25x magnification (bottom row). Red arrows indicate CVB4 positivity. (C) Section of pancreas from day 3 CVB4 infected NOD mouse at 10x magnification. The section is counterstained with Light green.

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

To investigate the mechanism by which α GalCer stimulated iNKT cells prevent diabetes after CVB4 infection, we analyzed the immune environment of pancreatic islets of Pro-ins2^{-/-} mice receiving different treatments. For this, pancreatic islets were harvested, total RNA was extracted and mRNA levels of different molecules were determined by qPCR. The data represented in the figure 28 were obtained from islets harvested on the second day of infection since the expression of different molecules was still very low on the first day post-infection and rapidly decreases after the second day (Appendix 3).

CVB4 infection induced the upregulation of numerous pro-inflammatory cytokines such as IL-1 β , IL-6, TNF- α , IFN- α or IL-15 (Fig. 29). Interestingly, the expression of these cytokines was significantly decreased when iNKT cells were stimulated by α GalCer. Moreover, while the expression of suppressive cytokines IL-10 and TGF- β did not differ between mice from CVB4 and CVB4+ α GalCer groups, iNKT cell stimulation induced the high upregulation of suppressive enzymes iNOS, IDO1 and IDO2 (further collectively referred as IDO) and arginase I in pancreatic islets. The strong upregulation of these enzymes suggested the presence of MDSC. We also detected the high upregulation of Ym1/Ym2 mRNA expression in islets of CVB4+ α GalCer group. Ym1 and Ym2 belong to the family of chitinases, act as eosinophil chemotactic factor and are strongly expressed by AAM ϕ suggesting the infiltration of AAM ϕ in pancreas of CVB4+ α GalCer treated mice (436). Similar results were obtained with NOD mice (Appendix 4). Thus our results indicated that iNKT cell activation by α GalCer during CVB4 infection induced a less pro-inflammatory and more immunosuppressive environment in pancreatic islets compared to CVB4 infection only.

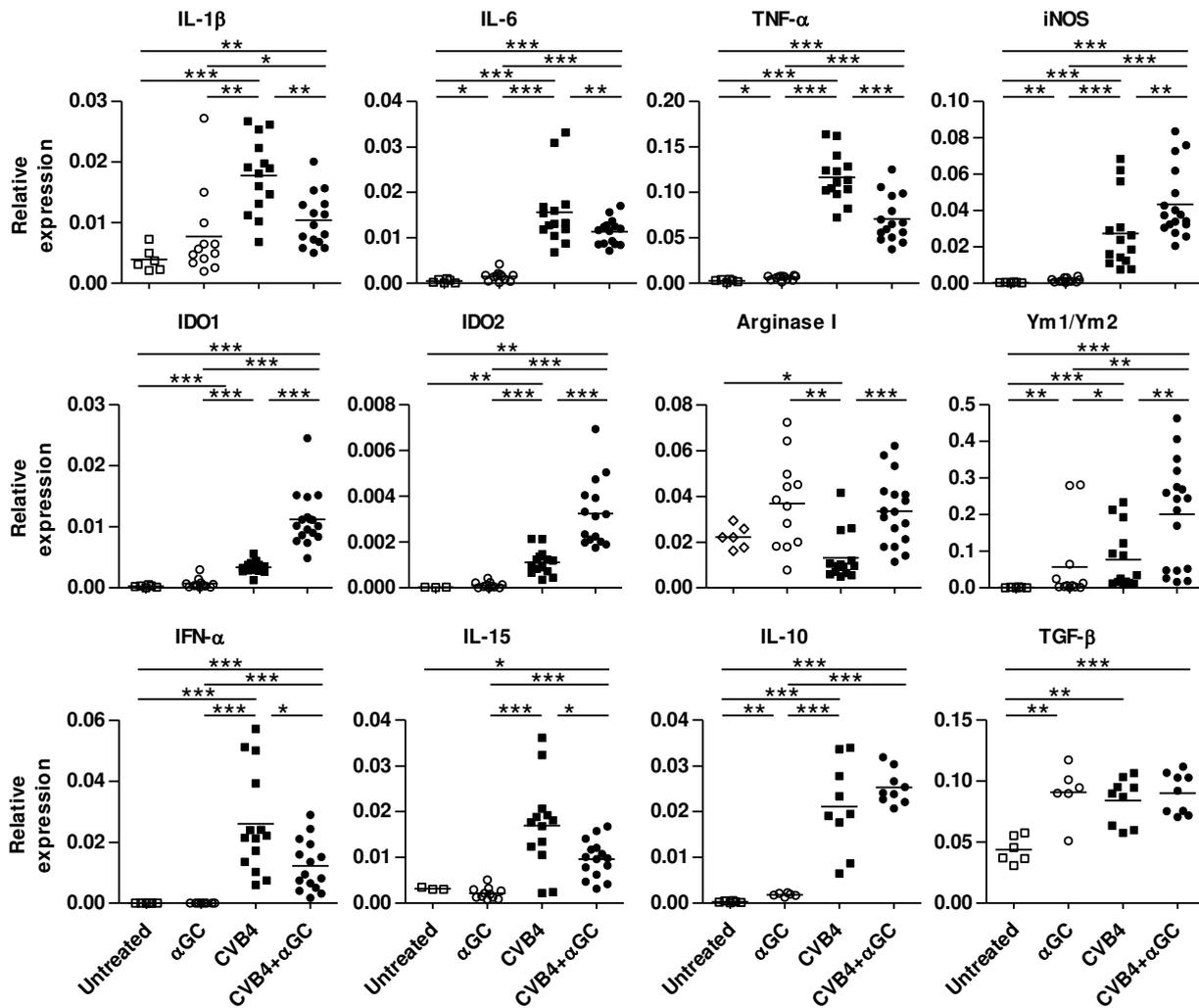


Figure 29. iNKT cell activation dampens pancreatic inflammatory response and promotes the expression of suppressive enzymes after CVB4 infection. Female Pro-ins^{2-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by qPCR. Data are presented as specific gene expression relative to GAPDH. Each symbol represents pooled islets of an individual mouse (untreated n=6, α GalCer n=6-12, CVB4 n=9-16, CVB4+ α GalCer n=9-16). *p<0.05, **p<0.005, ***p<0.0005, Mann Whitney.

The high upregulation of suppressive enzymes does not depend on iNKT cell numbers but depends on α GalCer treatment

α GalCer treatment can induce both the proliferation and production of cytokines by iNKT cells. To determine if the induction of suppressive enzymes in islets of CVB4+ α GalCer infected mice was a result of iNKT cell proliferation, we compared the level of expression of

iNOS, IDO and arginase I between CVB4 infected Pro-ins2^{-/-} mice that were treated with αGalCer and CVB4 infected Vα14 Pro-ins2^{-/-} that have an increased frequency of iNKT cells in islets compared to wt Pro-ins2^{-/-} mice (Fig. 30). Vα14 transgenic Pro-ins2^{-/-} mice were generated in our laboratory by crossing Pro-ins2^{-/-} mice with Vα14 transgenic NOD mice expressing the already rearranged Vα14-Jα18 transgene (303). The results of qPCR analysis performed on the second day of infection showed that the level of expression of suppressive enzymes was significantly lower in CVB4 infected Vα14 Pro-ins2^{-/-} mice compared to infected Pro-ins2^{-/-} mice that were treated with αGalCer (Fig. 31). In fact, the expression of iNOS, IDO and arginase I was similar between CVB4 infected Pro-ins2^{-/-} and Vα14 Pro-ins2^{-/-} mice. These results show that the upregulation of suppressive enzymes in CVB4+αGalCer treated mice strongly depends on stimulation of iNKT cells by αGalCer and cannot be compensated by increasing iNKT cell numbers.

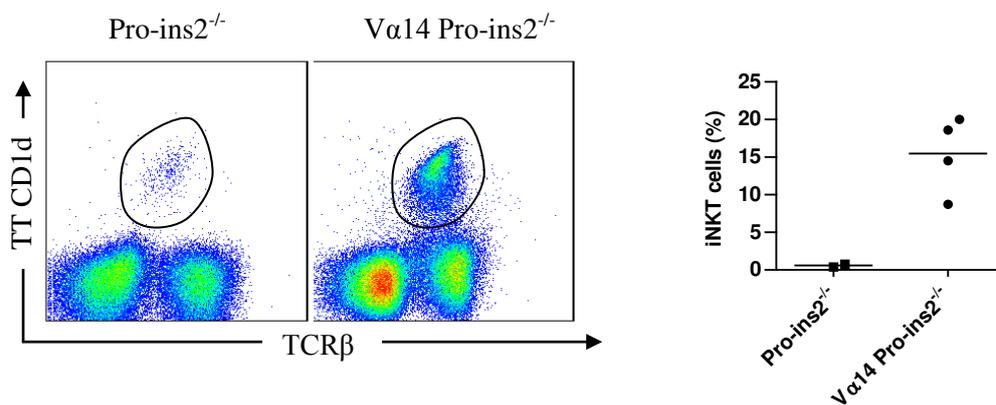


Figure 30. Vα14 Pro-ins2^{-/-} mice have an increased frequency of iNKT cells in pancreatic islets compared to wt Pro-ins2^{-/-} mice. Pancreatic islets of female Pro-ins2^{-/-} and Vα14 Pro-ins2^{-/-} mice 5-6 weeks old were harvested, dissociated and stained with αGalCer loaded CD1d tetramers and mAbs to CD45 and TCRβ for the detection of iNKT cells. Dot plots correspond to a representative staining of TT CD1d⁺/TCRβ⁺ cells in pancreatic islets gated among CD45⁺ cells. On the right, summary of iNKT cells frequencies in wt Pro-ins2^{-/-} and Vα14 Pro-ins2^{-/-} mice (Pro-ins2^{-/-} n=2, Vα14 Pro-ins2^{-/-} n=4)

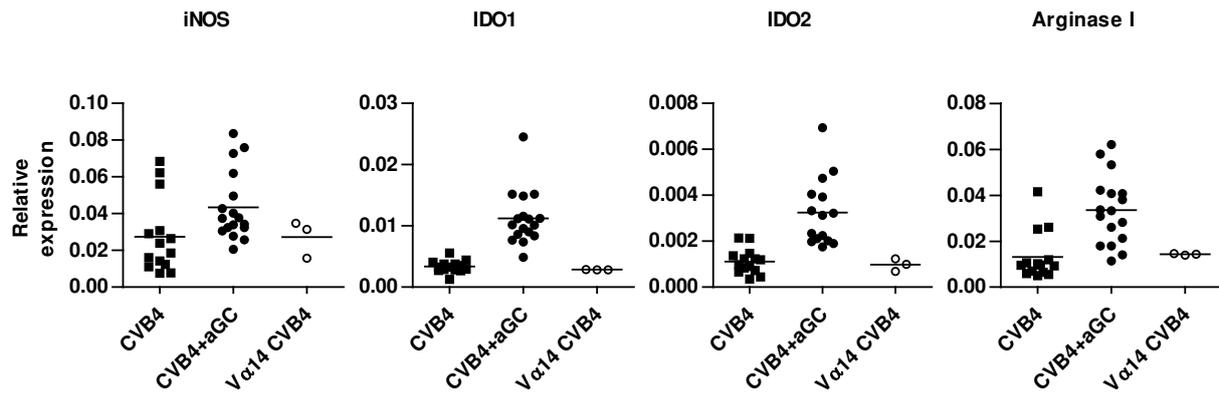


Figure 31. The upregulation of suppressive enzymes does not depend on iNKT cell numbers but depends on α GalCer treatment. Female Pro-ins2^{-/-} and V α 14 Pro-ins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested and total RNA was extracted from islets for qPCR analysis. Data are presented as specific gene expression relative to GAPDH. Each symbol represents pooled islets of an individual mouse (CVB4 n=16, CVB4+ α GalCer n=16, V α 14 CVB4 n=3).

iNKT cells are dispensable for the induction of iNOS and IDO in untreated CVB4 infected mice

While α GalCer activated iNKT cells are critical for the strong upregulation of iNOS and IDO in CVB4+ α GalCer treated mice, a low level of these enzymes is also detected in CVB4 infected mice. We, therefore, wanted to determine if the induction of these suppressive enzymes in the islets of CVB4 group was dependent on iNKT cells as well. To answer this question, we compared the level of expression of iNOS and IDO between infected Pro-ins2^{-/-} and J α 18^{-/-} Pro-ins2^{-/-} mice lacking iNKT cells. Our qPCR analysis performed with total islets extracted on day 2 post-infection showed that these molecules were expressed at the same level between CVB4 infected Pro-ins2^{-/-} and J α 18^{-/-} Pro-ins2^{-/-} mice (Fig. 32). As expected, α GalCer treatment of infected J α 18^{-/-} Pro-ins2^{-/-} mice did not increase the expression of iNOS and IDO contrary to infected Pro-ins2^{-/-} mice treated with α GalCer. Altogether these results show that iNKT cells are not necessary for the induction of iNOS and IDO expression after CVB4 infection. In line with these findings, we found iNOS and IDO to be expressed in NOD SCID and C α ^{-/-} mice, which lack T cells (data not shown). However, the strong upregulation of these enzymes directly depends on iNKT cells stimulated by α GalCer.

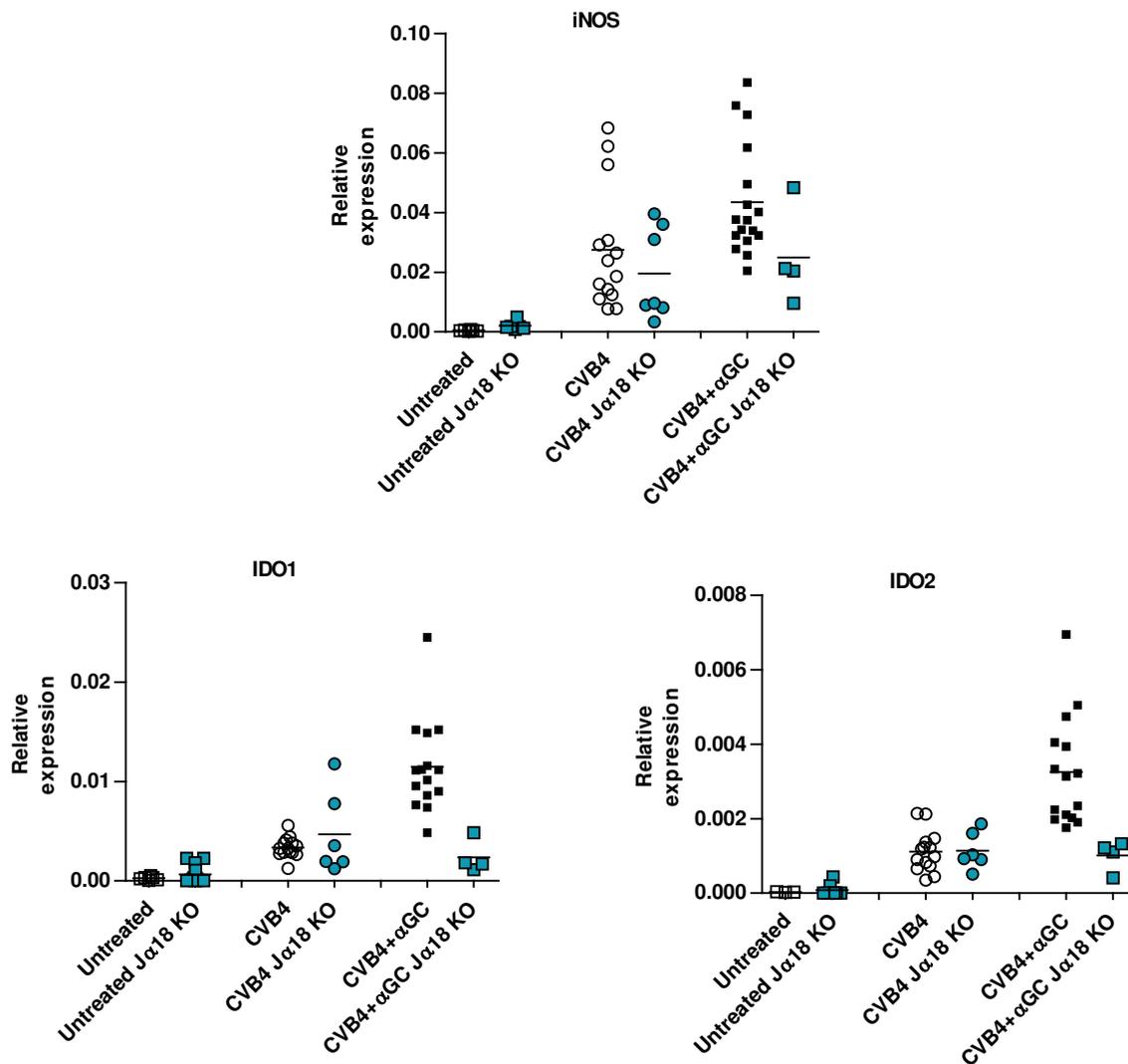


Figure 32. iNKT cells are not necessary for the induction of iNOS and IDO in CVB4 infected mice. Female $J\alpha 18^{-/-}$ Pro-ins2 $^{-/-}$ mice (blue symbols) at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by qPCR. Data are presented as specific gene expression relative to GAPDH. The data of Pro-ins2 $^{-/-}$ mice are the same as in figure 28 and are presented for comparison. Each symbol represents pooled islets of an individual mouse (Untreated n=8, CVB4 n=6, CVB4+ α GalCer n=4 for $J\alpha 18^{-/-}$ Pro-ins2 $^{-/-}$ mice).

iNKT cell phenotype in pancreatic islets

To understand the mechanisms of diabetes prevention after CVB4 infection by α GalCer stimulated iNKT cells, we studied their phenotype in pancreatic islets. In 6 weeks old Pro-ins2 $^{-/-}$ mice the frequency of pancreatic iNKT cells is less than 1% among all CD45 $^{+}$

cells (Fig. 30). While having a low frequency does not prevent these cells from exercising a potent regulatory role, it does, however, make their characterization difficult. Thus, pancreatic islets of numerous mice must be pooled for a single accurate staining. We, therefore, used V α 14 Pro-ins2^{-/-} for iNKT cell characterization since their increased frequency of iNKT cells facilitates the detection of these cells.

CVB4 infection induced the activation of iNKT cells that upregulated the early activation marker CD69 in CVB4 and CVB4+ α GalCer treated mice compared to untreated or α GalCer treated animals (Fig. 33). Interestingly, despite being activated by CVB4 infection, iNKT cells did not proliferate (upregulation of Ki-67) nor produced typical iNKT cell cytokines IFN- γ , IL-4 or IL-13. On the contrary iNKT cells proliferated and produced these cytokines in mice treated with α GalCer independently from CVB4 infection. Thus iNKT cells clearly have different phenotypes in CVB4 and CVB4+ α GalCer treated mice.

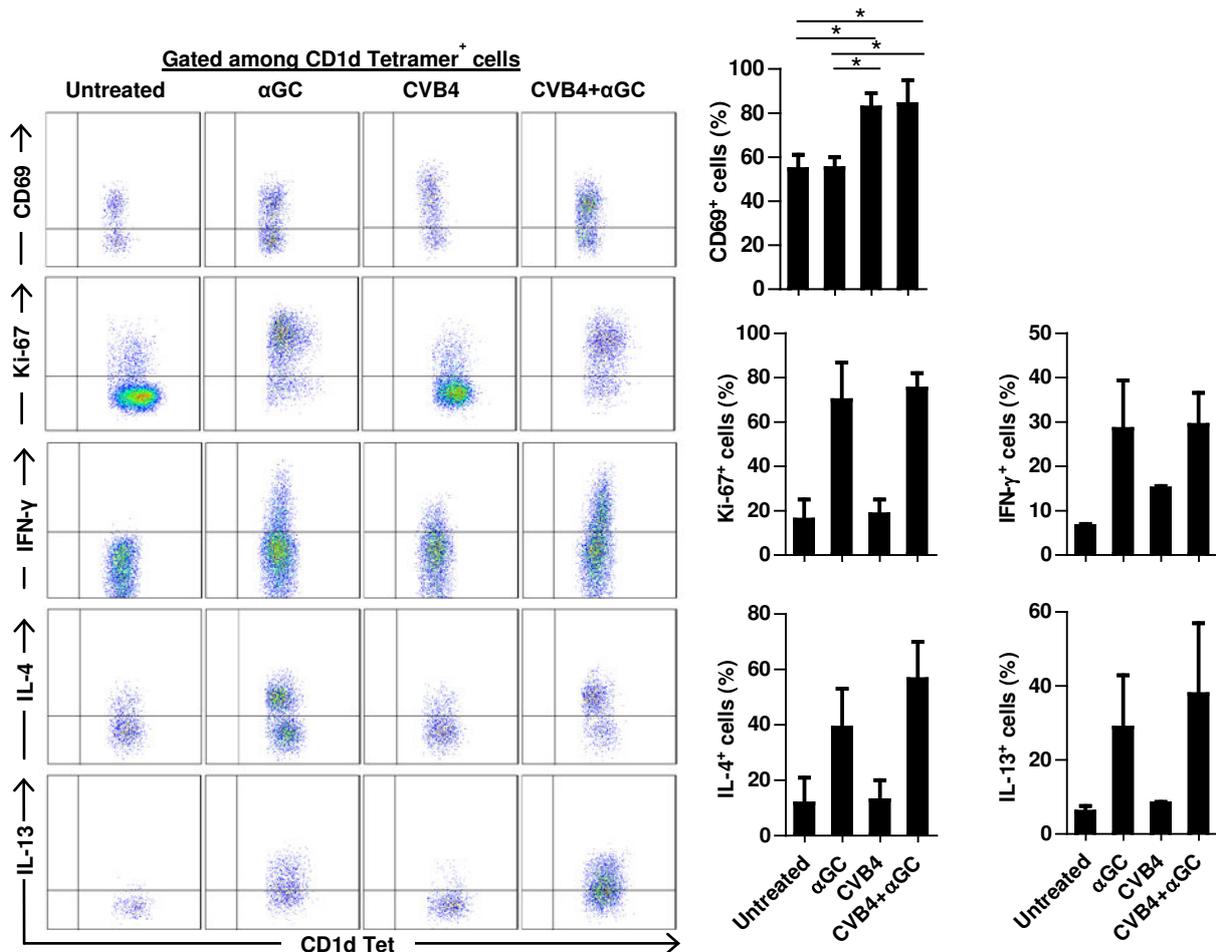


Figure 33. iNKT cells are activated but produce cytokines in CVB4+αGalCer group only. iNKT cells were analyzed in *Vα14 Pro-ins2^{-/-}* female mice inoculated with CVB4 or PBS and treated with αGalCer or control vehicle. Pancreatic islets were harvested on day 2 of the treatment and dissociated into a single cell suspension. Cells were stimulated with PMA/ionomycin in the presence of Brefeldin A for 4 h, then intracellular staining was performed. On the left, representative staining of islet iNKT cells (CD1d Tet⁺/TCRβ^{int}) gated among pancreatic islet CD45⁺ cells and expressing different markers. On the right, summary of the frequency of iNKT cells expressing different markers among CD45⁺ cells obtained from 3 independent experiments with 3 mice in each group ± SD. *p<0.05 Mann Whitney.

Critical role of IFN-γ and IL-13 in the expression of suppressive enzymes

Cytokines produced by iNKT cells are strong candidates for the induction of suppressive enzymes since IFN-γ was shown to induce the expression of iNOS and IDO, and IL-4 and IL-13 were shown to induce the expression of arginase I and Ym1/Ym2 molecules.

We, therefore, investigated the role of these cytokines in the expression of these suppressive enzymes. For this, we blocked IFN- γ and IL-4 with monoclonal antibodies directed against them and we blocked IL-13 with the administration of the extracellular domain of IL-13 receptor, which captures this cytokine. We then performed qPCR analysis with total pancreatic islets extracted on the second day of infection. Our results revealed that, while the inhibition of IL-4 did not affect the expression of any of these enzymes, the inhibition of IFN- γ strongly decreased the expression of iNOS, IDO and Ym1/Ym2, and the inhibition of IL-13 decreased the expression of arginase I (Fig. 34). Thus these results strongly suggest that cytokines produced by α GalCer activated iNKT cells might play an important role in the enhancing of the expression of suppressive enzymes.

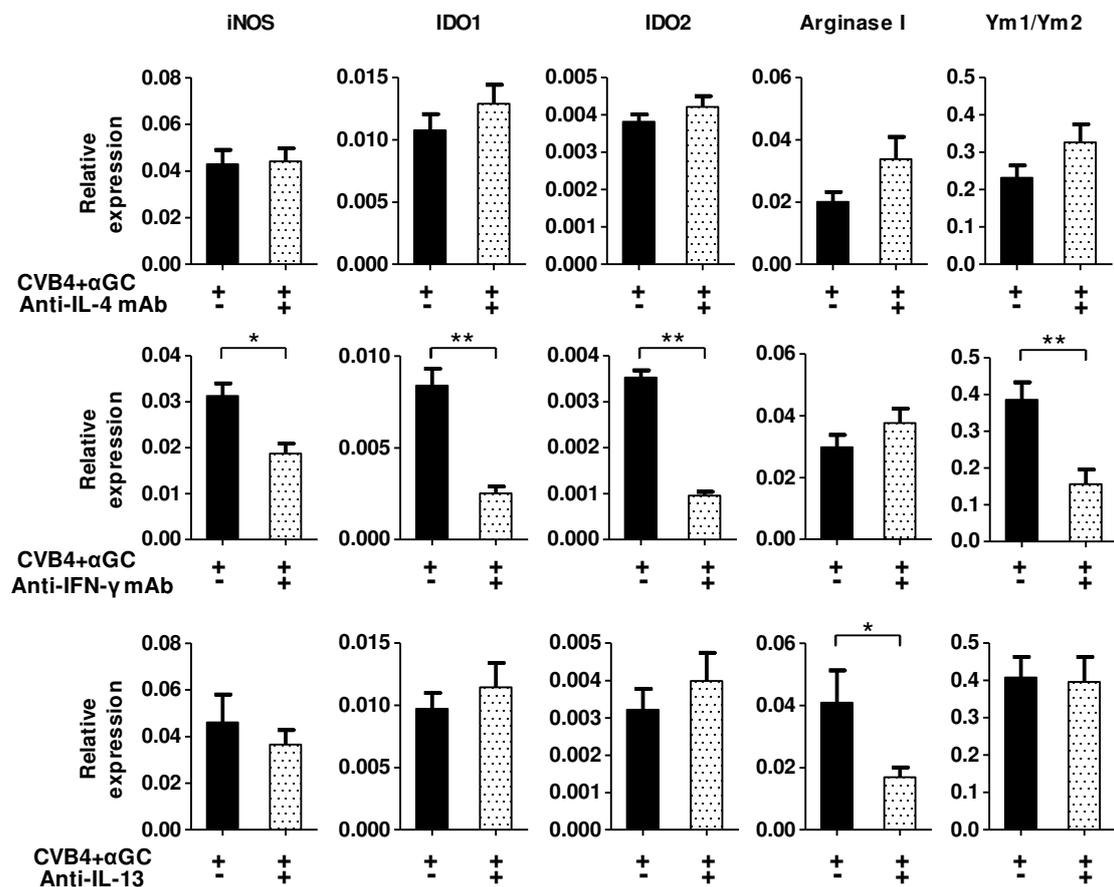


Figure 34. Critical role of IFN- γ and IL-13 in the induction of suppressive enzymes. CVB4 infected and α GalCer treated female Pro-ins2^{-/-} mice were injected with blocking anti-IL-4 mAb or anti-IFN- γ mAb or respective isotype control antibodies on days -1 and +1 of infection. To block IL-13, mice 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 qPCR was performed with total islet RNA. Data are presented as the mean of specific gene expression relative to GAPDH \pm SD. Data represent 2 independent experiments with 3 mice per group. * p <0.05, ** p <0.005, Mann Whitney.

CD11b⁺/CD11c⁻ myeloid cells express the suppressive enzymes and Ym1/Ym2

We next characterized the suppressive populations that infiltrated the islets in CVB4+ α GalCer group. iNOS, IDO and arginase I have been mostly described to be expressed by MDSC; however IDO expression is not limited to MDSC and has also been detected in DCs, pDCs, endothelial cells and even NK cells. Additionally, arginase I combined with Ym1/Ym2 can be expressed by AAM ϕ . To identify which population expressed the suppressive enzymes, we sorted out different cell populations from the islets and performed qPCR analysis. The sorted populations were : CD45⁻ non immune cells and among CD45⁺ cells CD11b⁺/CD11c⁺/120G8⁻ DCs, CD11b⁻/CD11c^{int}/120G8⁺ pDCs, CD11b⁺/CD11c⁻/120G8⁻ myeloid cells and CD11b⁻/CD11c⁻ non myeloid cells comprising all other cells such as T and B lymphocytes, NK and NKT cells (Fig. 35).

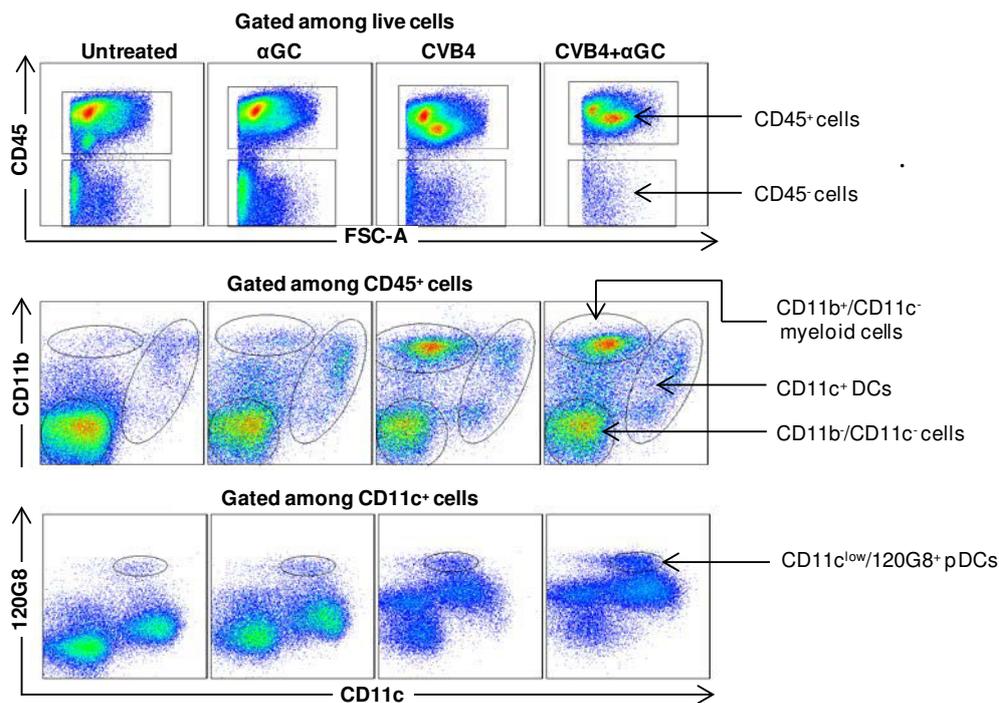


Figure 35. Representative staining of sorted cells from pancreatic islets. Dot plots correspond to a representative staining in pancreatic islets with gates used for sorting. Female Pro-ins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, dissociated into a single cell suspension, stained with antibodies and sorted out using BD ARIA II sorter: CD45⁻, and among CD45⁺ cells DCs, pDCs, myeloid cells and CD11b⁻/CD11c⁻ cells.

The results of qPCR analysis showed that the main cell population expressing these enzymes and Ym1/Ym2 was comprised of CD11b⁺/CD11c⁻ myeloid cells (Fig. 36). The expression of these molecules in sorted CD11b⁺/CD11c⁻ cells from CVB4+ α GalCer group was higher compared to sorted cells from CVB4 group, which is reminiscent to results obtained with total islets. The frequency of myeloid cells strongly increased on the second day of the infection and gradually decreased by day 8 of infection (Fig. 37A). No significant differences were observed in their frequency between mice from CVB4 and CVB4+ α GalCer groups (Fig. 37B).

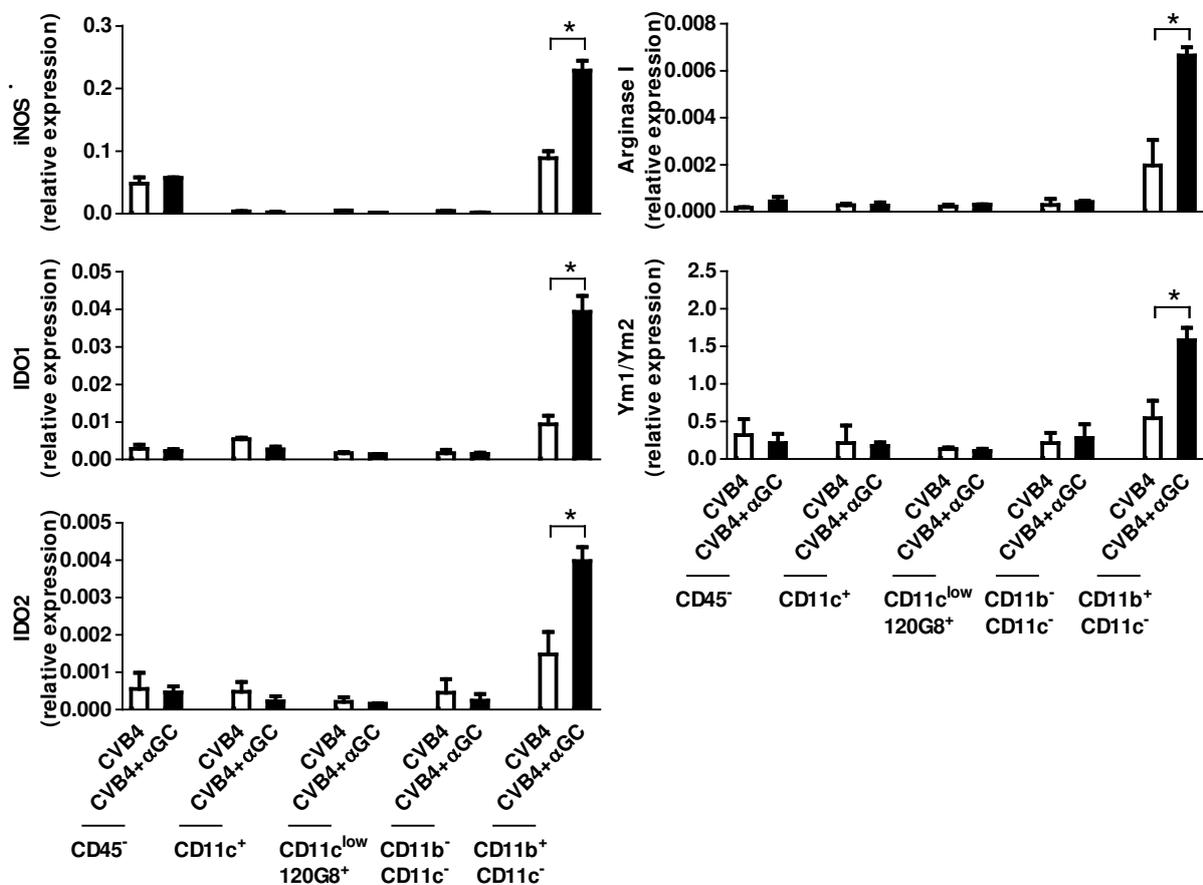


Figure 36. Suppressive enzymes are expressed by islet myeloid CD11b⁺/CD11c⁺ cells. Pro-*ins2*^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. RNA was isolated from different cell populations sorted among pancreatic islet CD45⁺ cells on day two post-infection (as described in Fig. 35) and mRNA levels were measured by qPCR. Data are presented as the mean of specific gene expression relative to GAPDH \pm SD. Data were obtained from 3 independent experiments with 3 mice in each group. * $p < 0.05$, Mann Whitney.

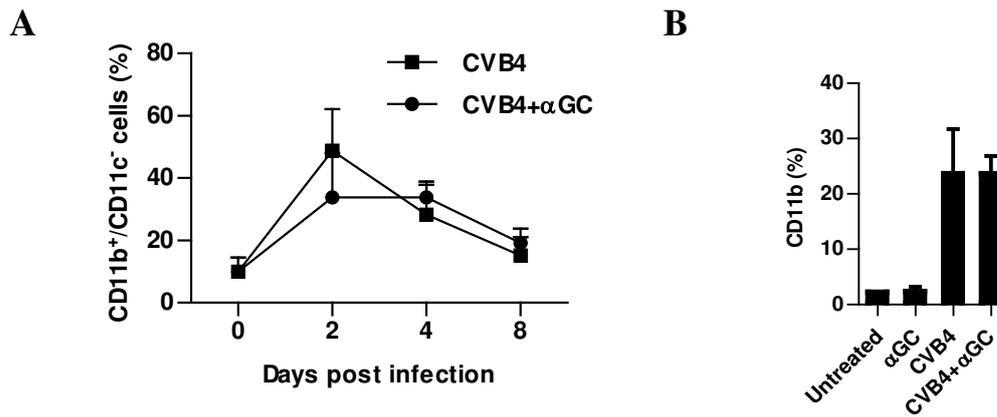


Figure 37. CD11b⁺/CD11c⁻ myeloid cells strongly infiltrate pancreatic islets after CVB4 infection. Pro-ins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with αGalCer or control vehicle. (A) On days 2, 4 and 8 post-infection, pancreatic islets were harvested, dissociated into a single cell suspension, stained with different surface antibodies and CD11b⁺/CD11c⁻ cells were analyzed by flow cytometry gated among CD45⁺ cells. Data was obtained from 3 independent experiments with 3 mice in each group ± SD. Untreated mice are represented as day 0. (B) Frequency of pancreatic CD11b⁺/CD11c⁻ cells in uninfected mice and on day 2 of infection gated among CD45⁺ cells.

MDSC were found in the draining lymphoid organs or the spleen during pathologies such as infection or cancer. Therefore, we also performed qPCR analysis with these same populations sorted from PLN and the spleen on days 0, 2, 4, 6, 8 and 10 post CVB4 infections with or without αGalCer treatment. However, the expression of these molecules was not upregulated in these organs (data not shown). Overall our data show that CD11b⁺/CD11c⁺ myeloid cells strongly infiltrate the pancreatic islets of CVB4 infected mice and highly upregulate several suppressive enzymes when iNKT cells are stimulated with αGalCer at the time of infection.

Pancreatic suppressive enzymes are expressed by macrophages

Since CD11b⁺/CD11c⁻ myeloid cells can be comprised of macrophages and of neutrophils, we stained them with antibodies specific for these two populations, F4/80 and Ly-6G respectively to determine the origin of these cells. The results of the staining showed that almost 90% of CD11b⁺/CD11c⁻ cells expressed F4/80 and only 10% expressed low levels of Ly-6G showing that CD11b⁺/CD11c⁻ cells were primarily macrophages (Fig. 38). We next

wanted to specifically isolate MDSC, so we stained islet immune cells by mAbs against Ly-6C and CD115 since they were also described to be upregulated by MDSC infiltrating some tumors or sites of infections. Ly-6C and CD115 were expressed by only 20% of pancreatic macrophages in untreated and α GalCer treated uninfected mice showing that the majority of macrophages in steady state are resident macrophages. On the contrary both Ly-6C and CD115 were strongly upregulated on macrophages infiltrating the islets after the infection. However, no differences were found in the level of expression of these markers between pancreatic islet macrophages of mice from CVB4 and CVB4+ α GalCer groups. Since Ly-6C and CD115 are also two markers of blood circulating monocytes, their high expression by islet infiltrating macrophages, therefore, indicates that these cells are monocytes that were recruited recently into pancreas as a consequence of CVB4 infection.

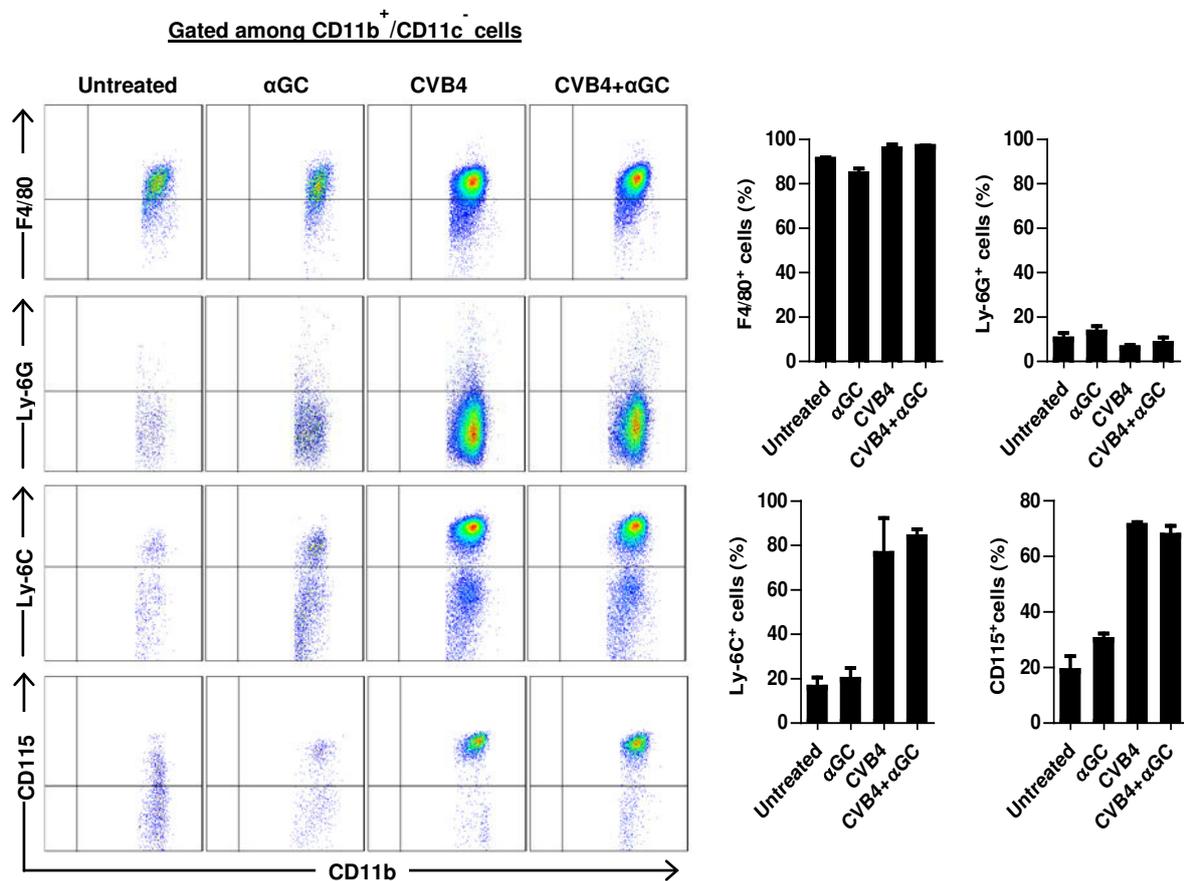


Figure 38. Pancreatic islet CD11b⁺/CD11c⁻ cells are inflammatory macrophages. Female Proins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, dissociated, stained with different antibodies and analyzed by flow cytometry. Dot plots correspond to a representative staining of CD11b⁺/CD11c⁻ macrophages in pancreatic islets gated among CD45⁺ cells. On the right, summary of the frequency of CD11b⁺/CD11c⁻ cell expressing different markers gated among CD45⁺ cells obtained from 3 independent experiments with 3 mice in each group \pm SD.

To further determine whether the suppressive enzymes were expressed by newly infiltrating macrophages or resident macrophages, we sorted out pancreatic islet F4/80⁺/Ly-6C⁺/CD115⁺ inflammatory and Ly-6C⁻/CD115⁻ resident macrophages from day 2 infected CVB4 and CVB4+ α GalCer treated mice and analyzed them by qPCR. Our results showed that the highest expression of iNOS, IDO, arginase I and Ym1/Ym2 was found in Ly-6C⁺/CD115⁺ inflammatory rather than the resident Ly-6C⁻/CD115⁻ macrophages (Fig. 39 and data not shown). Reminiscent to total CD11b⁺/CD11c⁻ population, Ly-6C⁺/CD115⁺ macrophages expressed lower levels of proinflammatory cytokines and higher levels of suppressive enzymes in mice from CVB4+ α GalCer group compared to CVB4 group. Of note, Ly-6C⁺/CD115⁺ macrophages did not express IFN- γ , IL-13, IL-4, TGF- β or IL-10 (data not shown).

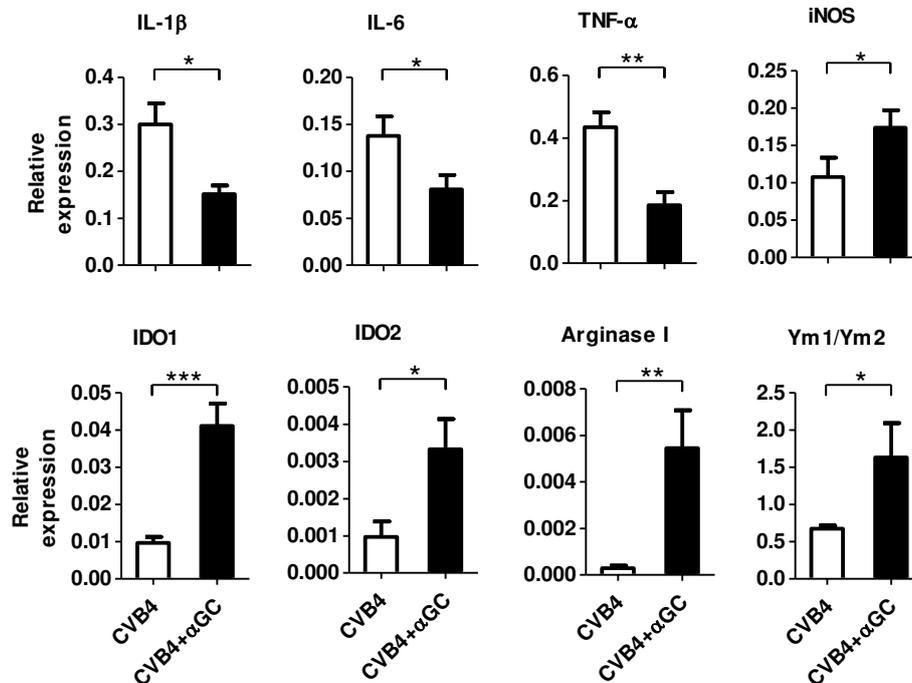


Figure 39. Inflammatory and suppressive molecules are expressed by CD11b⁺/F4/80⁺/Ly-6C⁺/CD115⁺ cells. At day 2 post-infection, pancreatic islet CD45⁺/CD11b⁺/F4/80⁺/Ly-6C⁺/CD115⁺ cells were sorted out from CVB4 infected mice treated or not with α GalCer. Total RNA was extracted from these cells and mRNA levels were measured by qPCR. Data are presented as specific gene expression relative to GAPDH \pm 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.

To confirm that the suppressive enzymes were only expressed in pancreatic islets, we sorted out F4/80⁺/Ly-6C⁺/CD115⁺ macrophages from pancreas, PLN and the spleen of mice treated or not with α GalCer on days 2, 4, 6, 8 and 10 and performed qPCR analysis. The results showed that compared to pancreatic islets, the expression of these enzymes was not upregulated in macrophages from the spleen and PLN (Fig. 40). Overall our results show that myeloid cells, recruited into pancreatic islets after CVB4 infection are Ly-6C⁺/CD115⁺ macrophages that express inflammatory cytokines IL-1 β , IL-6 and TNF- α and resemble CAM ϕ . On the contrary, in mice, whose iNKT cells were activated by α GalCer, macrophages express lower levels of inflammatory molecules and upregulate the expression of suppressive enzymes. These results, therefore, strongly suggest that α GalCer stimulated iNKT cells favor the differentiation of macrophages into MDSC after CVB4 infection.

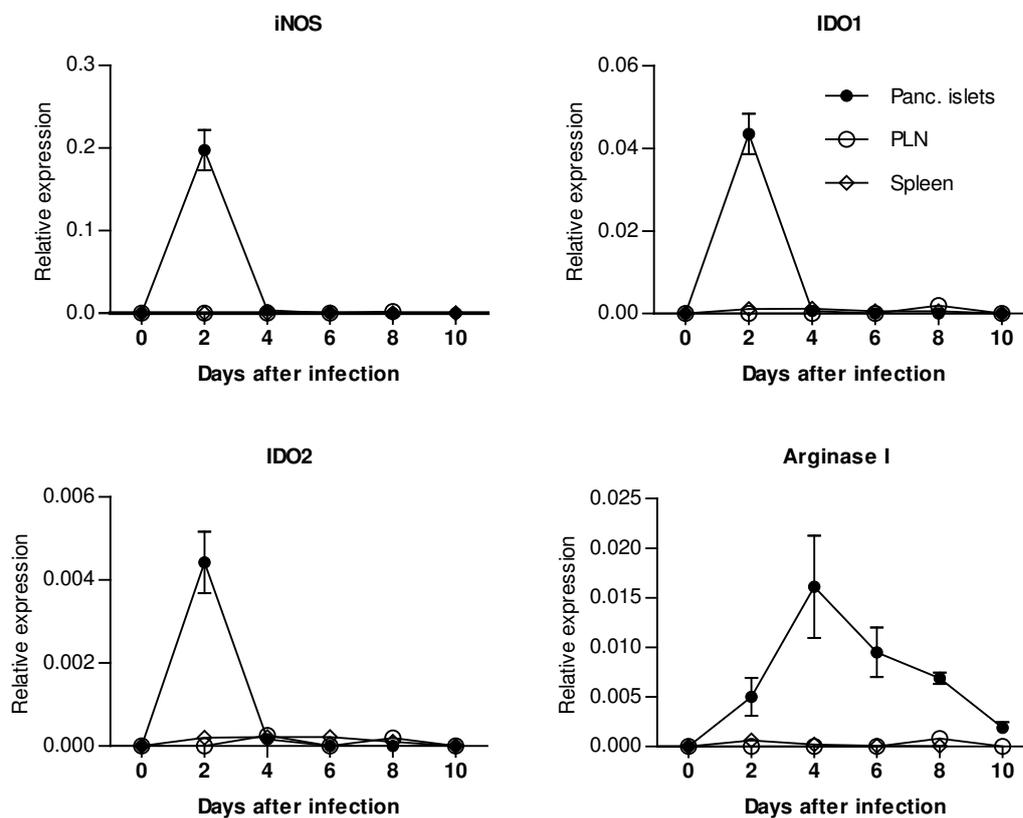


Figure 40. Kinetics of the expression of suppressive enzymes in pancreatic islets, PLN and spleen. Female Pro-ins2^{-/-} mice at 5-6 weeks of age were inoculated i.p. with CVB4 and treated with α GalCer. On days 0 (uninfected mice), 2,4,6,8 and 10 post-infection, pancreatic islets, PLN and spleen were harvested, dissociated into single cell suspensions and stained with antibodies against CD45, CD11b, CD11c, F4/80 and Ly-6C. CD45⁺/CD11b⁺/F4/80⁺/Ly-6C⁺ cells were then sorted with a FACS Aria II sorter. Total RNA was extracted from sorted cells and mRNA levels were measured by qPCR. Data are presented as specific gene expression relative to GAPDH. Data represents means of 4 mice from two independent experiments \pm SEM.

Inhibition of MDSC differentiation does not restore CAM ϕ population

Since both CAM ϕ and MDSC can be detected in CVB4 as well as CVB4+ α GalCer treated mice, we hypothesized that these two populations were generated in each group of mice, however, at different ratios. Thus, mostly CAM ϕ were generated and infiltrated pancreatic islets during CVB4 infection, while after CVB4+ α GalCer treatment, the infiltrating macrophages were mostly MDSC. We, therefore, investigated whether the inhibition of MDSC differentiation in CVB4+ α GalCer treated mice could result in the restoration of CAM ϕ pool. To inhibit MDSC differentiation, we used IFN- γ or IL-13 inhibitors to prevent the upregulation of IDO, iNOS and arginase I respectively. We then assessed the level of expression of inflammatory cytokines IL-1 β , IL-6 and TNF- α , which characterize CAM ϕ . The analysis of total islets by qPCR revealed that, while the inhibitors induced the downregulation of the suppressive enzymes (Fig. 34), the expression of IL-1 β , IL-6 and TNF- α was not upregulated (Fig. 41). These results show that inhibition of MDSC differentiation was not sufficient to induce CAM ϕ in pancreatic islets and that other factors must be present for their differentiation.

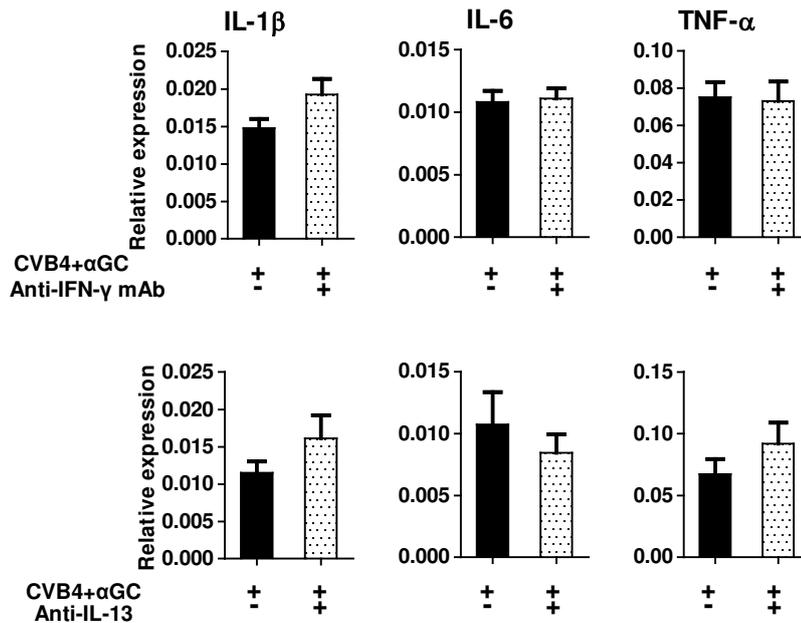


Figure 41. Inhibition of MDSC differentiation was not sufficient for the induction of CAM ϕ in pancreatic islets. CVB4 infected and α GalCer treated female Pro-ins2^{-/-} mice were injected with blocking anti-IFN- γ mAb or the isotype control on days -1 and +1 of infection. To block IL-13, mice were 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 qPCR was performed with total islet RNA. Data are presented as the mean of specific gene expression relative to GAPDH \pm SD. Data represent 2 independent experiments with 3 mice per group.

IDO suppresses diabetes development in CVB4+ α GalCer group

To determine the role of suppressive enzymes in the protection against diabetes, we studied the diabetes incidence in Pro-ins2^{-/-} mice from CVB4+ α GalCer group treated with specific inhibitors of iNOS, IDO and arginase I. Treatment with the iNOS inhibitor, 1400W, or the arginase I inhibitor, nor-NOHA, did not abrogate the protection against diabetes (Fig. 42A). However, the incidence of diabetes rose to 86% in the group of mice treated with the inhibitor of IDO, 1-methyl-D-tryphophan, compared to the 27% diabetes rate in CVB4+ α GalCer mice that were left untreated. These results showed the critical role of IDO in the protection of mice from CVB4+ α GalCer group from diabetes. Importantly, because the treatment with 1MT did not result in 100% of diabetes, we treated the mice with the combination of all three inhibitors to test whether iNOS and arginase I, while not preventing diabetes on their own, could enhance the inhibitory effect of IDO. While diabetes incidence

was slightly accelerated with this treatment, it did not reach statistical significance compared to CVB4+ α GalCer group that had only received 1MT suggesting that iNOS and arginase I did not play a significant role in T1D prevention in CVB4+ α GalCer group. Consistent with the high incidence of diabetes, the insulinitis severity was increased in mice from CVB4+ α GalCer group treated with 1MT compared to untreated mice (Fig. 42B).

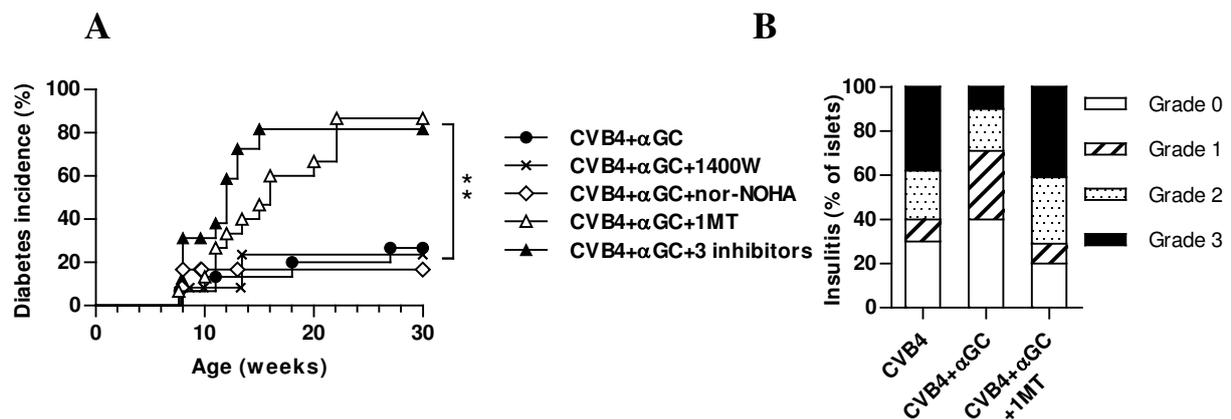


Figure 42. IDO is required for the inhibition of diabetes onset. Pro-*ins2*^{-/-} females, infected with CVB4 and injected with α GalCer, received treatments as indicated; 1400W, 1MT and nor-NOHA to inhibit iNOS, IDO and arginase I respectively. **(A)** Incidence of diabetes following different treatments. (CVB4+ α GC n=15, CVB4+ α GC+1400W n=12, CVB4+ α GC+nor-NOHA n=12, CVB4+ α GC+1MT n=15, CVB4+ α GC+1400W+nor-NOHA+1MT n=12). ***p*<0.005 using log-rank test analysis. Data represent 2 pooled independent experiments. **(B)** Histological scoring of insulinitis was performed on pancreatic sections of Pro-*ins2*^{-/-} females from days 7-10 post-infection stained with haematoxylin/eosin (n=6 mice).

Since IDO expression in islets was induced by IFN- γ , we tested the role of this cytokine in the protection against diabetes by treating mice with an anti-IFN- γ mAb. IDO expression in the islets is very transitory. It peaks on the second day of CVB4 infection and α GalCer treatment and is back to basal by the fourth day of infection (Appendix 3). We, therefore, chose a protocol with a short-term IFN- γ blockade i.e. last injection of anti-IFN- γ was done on the third day of infection. Importantly, with this short term treatment, IFN- γ is only blocked in the beginning of the anti-viral response without interfering with the later IFN- γ secretion by T lymphocytes. Our results showed that that the inhibition of IFN- γ in mice from CVB4+ α GalCer group increased diabetes incidence from 20 to almost 80% (Fig. 43). Of note, the inhibition of both IDO and, particularly, of IFN- γ increased the diabetes

development in CVB4 group, even though the increase did not reach statistical significance. Thus, these results show the protective role of IDO and of IFN- γ in CVB4 induced diabetes.

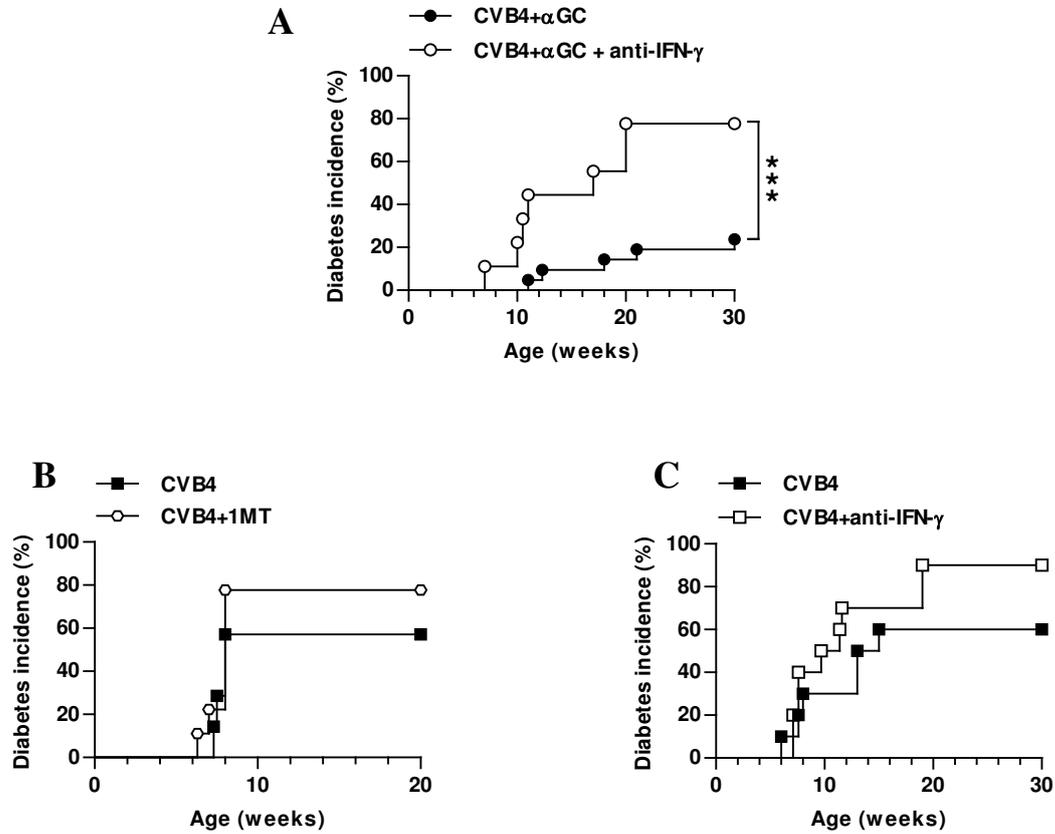


Figure 43. IFN- γ is critical for the prevention of diabetes in mice from CVB4+ α GalCer group. (A) Incidence of diabetes of Pro-ins $2^{-/-}$ females from CVB4+ α GalCer group treated with control isotype mAb (n=15) or an anti-IFN- γ mAb (n=9) or on days -1, +1 and +3 of infection. ***p<0.0005 using log-rank test analysis. (B and C) Incidence of diabetes of Pro-ins $2^{-/-}$ females infected with CVB4 at 5-6 weeks of age and treated with (B) 1MT or control vehicle 2 days before the infection and for up to 8 days after (CVB4=9, CVB4+1MT n=7) or (C) an anti-IFN- γ mAb or control isotype mAb injected on days -1, +1, +3 of virus infection (CVB4+isotype control n=10, CVB4+anti-IFN- γ n=10).

The role of macrophages in preventing diabetes

To specifically study the role of MDSC during CVB4 infection, we first assessed their capacity to suppress T cell proliferation *in vitro* by sorting them out from pancreas of CVB4+ α GalCer treated mice on the second day of infection and culturing them with diabetogenic BDC2.5 CD4⁺ T cells stimulated with CD3/CD28 beads. The results of this experiment showed that MDSC effectively suppressed T cell proliferation compared to macrophages sorted out from CVB4 group (Fig. 44). Moreover, the addition of IDO inhibitor to the cell culture with MDSC restored T cell proliferation confirming that the suppression by MDSC was dependent on IDO. Similar results were obtained with sorted cells from NOD mice (data not shown). Of note, as expected, splenic F4/80⁺/Ly-6C⁺ macrophages sorted out from CVB4+ α GalCer treated mice on the second day of infection did not suppress T cell proliferation confirming that splenic macrophages are not suppressive (data not shown).

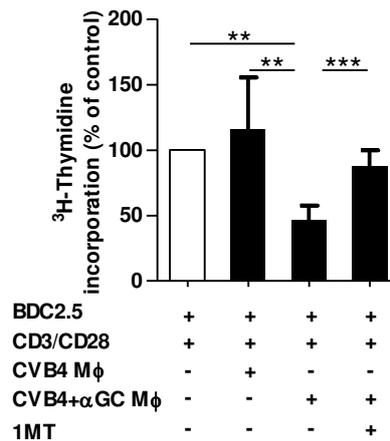


Figure 44. IDO expressing MDSC suppress T cell proliferation *in vitro*. CD11b⁺/F4/80⁺/Ly-6C⁺ macrophages were sorted out among pancreatic islet CD45⁺ cells of Pro-ins2^{-/-} females from CVB4 or CVB4+ α GalCer groups on the second day of infection and cultured (3×10^4 /well) with sorted CD62L⁺ BDC2.5 CD4 T cells (3×10^4 /well) in the presence of anti-CD3/CD28 beads \pm 1MT (200 μ M). 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 \pm SD for 3 independent experiments. **p<0.005, ***p<0.0005, Mann Whitney.

To demonstrate the role of MDSC in diabetes prevention *in vivo*, we depleted macrophages in CVB4 and CVB4+ α GalCer treated mice and followed the diabetes incidence

afterwards. Similar to the anti-IFN- γ treatment, macrophage depletion was short-term to cover the period of IDO expression in islets. Macrophages were depleted by dichloromethylene bisphosphonate (or clodronate Cl2/MBP) containing liposomes, which are phagocytized by macrophages and monocytes. Inside the cytoplasm, the clodronate is transformed into an ADP analog, which inhibits ATP production and causes apoptosis of cells. Of note, while clodronate treatment can induce apoptosis of DCs as well, we did not see a decrease in the numbers of islet DCs in clodronate treated mice compared to PBS treated mice. Clodronate treatment significantly reduced the infiltration of macrophages into pancreatic islets in both CVB4 and CVB4+ α GalCer treated mice (Fig. 45A). While we expected that MDSC depletion by clodronate treatment would result in increased diabetes incidence in CVB4+ α GalCer treated mice, we found that, on the contrary, clodronate treatment significantly decreased diabetes incidence in both CVB4 and CVB4+ α GalCer treated mice compared to untreated infected mice (Fig. 45B). In line with this observation, we found that the expression of different inflammatory molecules expressed by macrophages was strongly decreased in the islets of clodronate treated mice (Fig. 46). These results showed that macrophages are critical for the diabetes development after CVB4 infection.

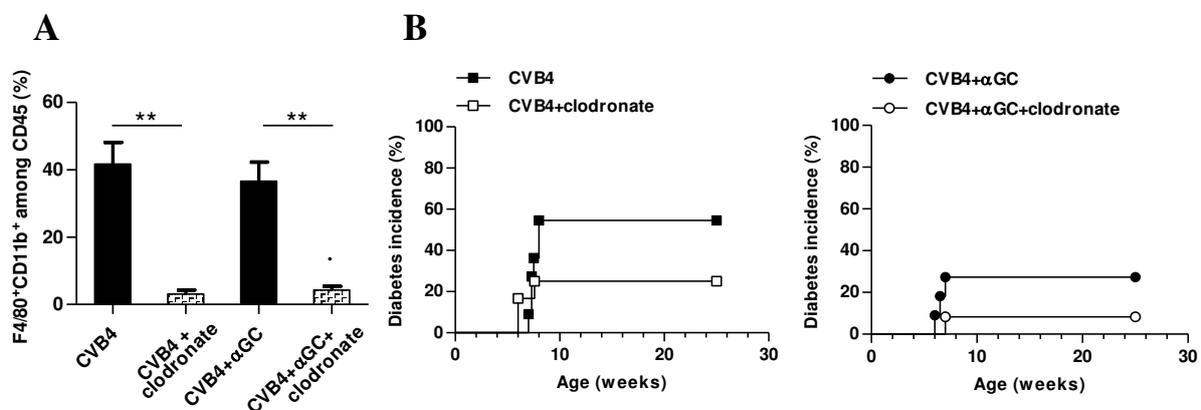


Figure 45. Macrophages are critical for diabetes development after CVB4 infection. Pro-ins2^{-/-} females at 5-6 weeks of age were inoculated i.p. either with CVB4 or PBS and treated with α GalCer or control vehicle. To deplete macrophages, mice were injected i.v. with 200 μ l of clodronate or control PBS loaded liposomes on days -1, +1 of virus infection. **(A)** On day 2 post-infection, pancreatic islets were harvested, dissociated and stained with antibodies. Data represent the frequency of F4/80⁺/CD11b⁺/CD11c⁻ cells gated among CD45⁺ cells from 2 pooled independent experiments. (CVB4 n=9, CVB4+clodronate n=8, CVB4+ α GC n=9, CVB4+ α GC+clodronate n=8). **p< 0.005, Mann Whitney. **(B)** Diabetes incidence of 5-6 weeks old Pro-ins2^{-/-} females inoculated i.p. with CVB4 or PBS and treated with α GalCer 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+ α GalCer n=11, CVB4+ α GalCer+clodronate n=12). Data represent 2 pooled independent experiments.

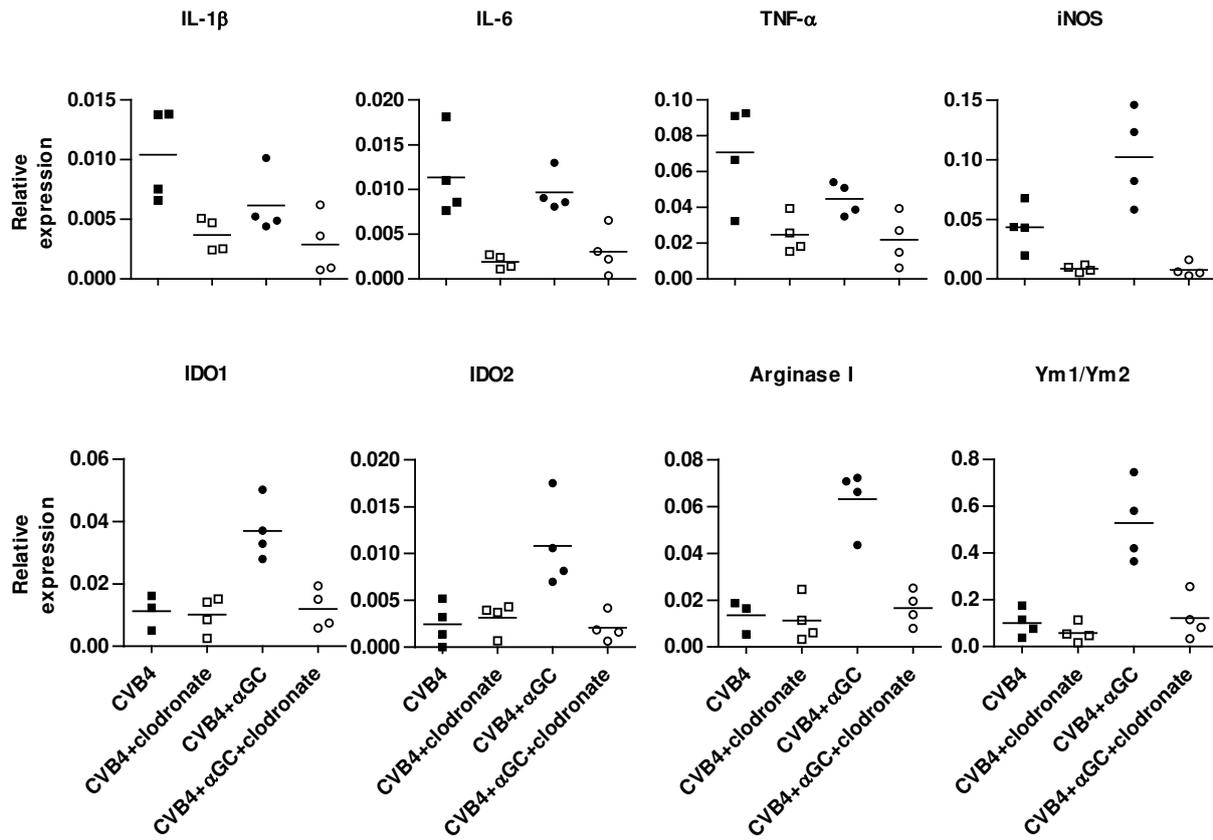


Figure 46. The expression of inflammatory and suppressive molecules in islets is strongly decreased after macrophage depletion. Pro-*ins2^{-/-}* females at 5-6 weeks of age were inoculated i.p. either with CVB4 or PBS and treated with α GalCer or control vehicle. To deplete macrophages, mice were injected i.v. with 200 μ l of clodronate or control PBS loaded liposomes on days -1, +1 of virus infection. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by qPCR. Data are presented as specific gene expression relative to GAPDH. Each symbol represents pooled islets of an individual mouse.

Since macrophage depletion *in vivo* did not allow to demonstrate the role of MDSC in diabetes prevention *in vivo*, we chose to perform transfer experiments. Pancreatic islet macrophages from mice of CVB4 or CVB4+ α GalCer groups were sorted out on the second day of infection when the expression of both pro-inflammatory and suppressive molecules is the highest and were transferred into CVB4 infected recipient mice (Fig. 47A). We chose to infect the recipient mice one day before macrophage transfer to allow enough time for CVB4 to infect pancreas, induce inflammation as well as chemokine secretion and favor macrophage recruitment into this organ. Importantly, macrophages of CVB4 infected mice do not yet infiltrate pancreas on day one post-infection. This protocol, therefore, favors the recruitment of both self and donor macrophages into the islets. The results of transfer experiment showed

that macrophages isolated from CVB4 donor mice significantly increased the incidence of diabetes of CVB4 infected recipients (Fig. 47B). On the contrary, macrophages from CVB4+ α GalCer donor mice significantly decreased diabetes development of CVB4 infected recipients. Thus, these experiments, on one hand, demonstrate that islet infiltrating CAM ϕ favor diabetes development and, on the other hand, they show that MDSC effectively prevent the development of CVB4 induced diabetes.

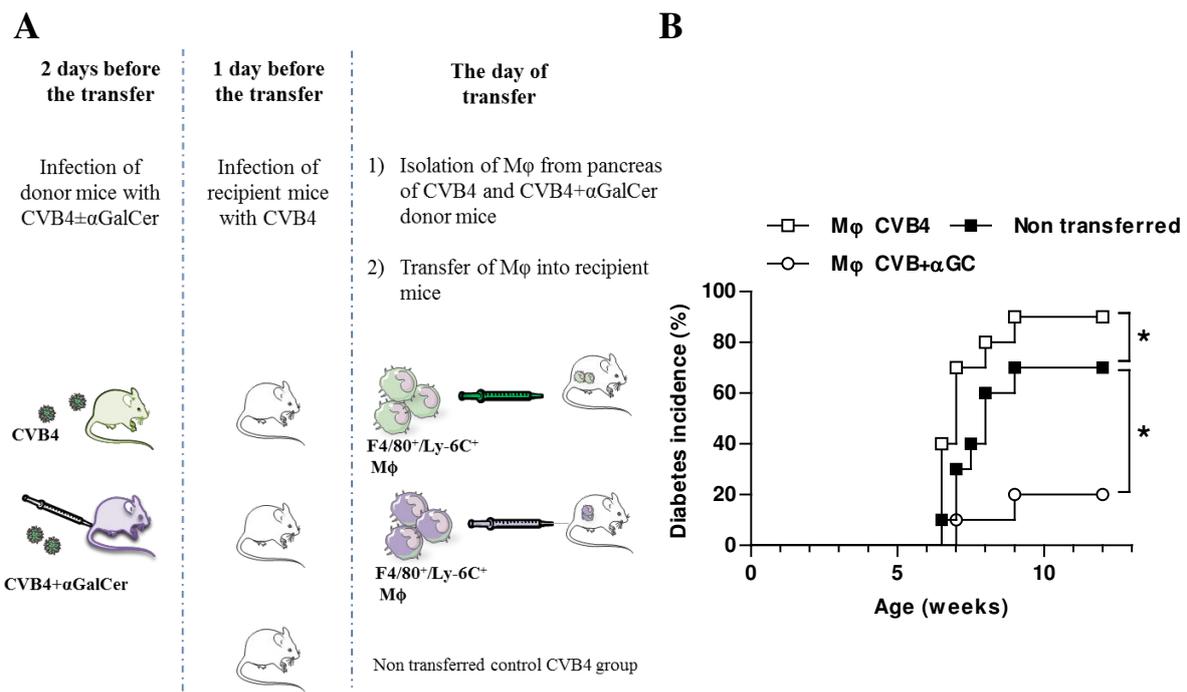


Figure 47. CAM ϕ increase, while MDSC decrease diabetes incidence of CVB4 infected mice. (A) Experimental design. Pro-ins2^{-/-} NOD females at 5-6 weeks of age were inoculated i.p. with CVB4 and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islet CD45⁺/CD11b⁺/F4/80⁺/Ly-6C⁺ macrophages were sorted out and injected i.v. (2x10⁵ macrophages (M ϕ) per mouse) into Pro-ins2^{-/-} females infected with CVB4 one day earlier. **(B)** 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.

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

To further decipher the immune mechanisms involved in the development of diabetes upon CVB4 infection and its inhibition by iNKT cell activation, we evaluated specific anti-

islet T cell responses. We first evaluated the infiltration of T lymphocytes into pancreatic islets in the period from 1 week post-infection (when diabetes starts) for up to three weeks post-infection (time point when most diabetes has already developed). Untreated and α GalCer treated mice were tested in parallel. Both CD4 and CD8 T cells highly infiltrated the islets after the infection as compared to untreated and α GalCer treated mice. However, no differences were found in their total numbers or the frequency between different infected groups of mice (Fig. 48A and B).

To detect autoreactive T cells, we chose to analyze CD8 T cells that recognize IGRP. IGRP was chosen as the large fraction of islet CD8 T cells in NOD mice recognize IGRP₂₀₆₋₂₁₄ peptide, and these cells are diabetogenic (32; 33; 437). IGRP specific IFN- γ producing CD8 T cells were not detected in untreated or α GalCer treated mice (Fig. 48C and D). However, they formed between 3 to 15% of all islet CD8 T cells in Pro-ins2^{-/-} and NOD mice that became diabetic after CVB4 infection regardless of the treatment received. By contrast, no such cells were detected in the islets in CVB4 infected mice that did not develop diabetes despite high infiltration of CD8 T cells. Similar results were observed with NOD mice (Appendix 5). The absence of these cells in the islets could have been due to their retention in lymphoid organs. However, we did not detect any IFN- γ producing IGRP specific CD8 T cell in the spleen or PLN of infected non diabetic or even diabetic mice. Importantly, the treatment with IDO inhibitor, that abolished diabetes protection, increased the frequency of anti-IGRP effector CD8 T cell. Altogether, our results strongly support the notion that diabetes development after CVB4 infection is caused by anti-islet T cell responses.

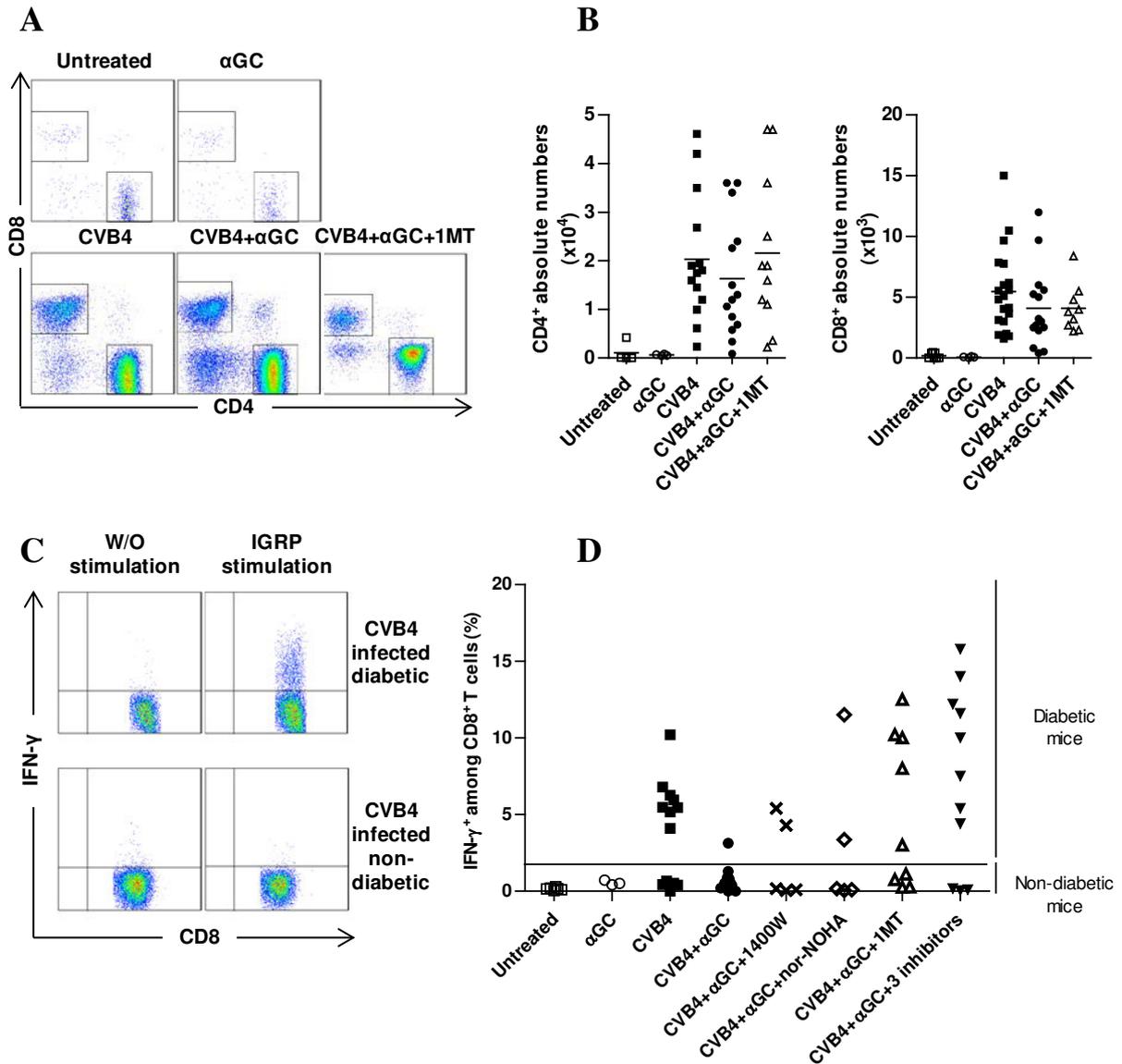


Figure 48. Anti-islet T cell response in the development of diabetes. (A and B) Representative CD8 versus CD4 dot plots of T cells from islets of female Pro-ins2^{-/-} mice two weeks after the treatments as indicated on the figure (left panel) and summary of CD8 and CD4 T cell counts gated among CD45⁺/TCRβ⁺ cells 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 CD45⁺ 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 IGRP₂₀₆₋₂₁₄ peptide. (D) Graph showing the percentage of islet CD8 T cells secreting IFN-γ after IGRP₂₀₆₋₂₁₄ peptide stimulation gated among islet CD45⁺/TCRβ⁺ CD8 T cells 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.

The phenotype of DCs

The frequency of DCs:

The absence of IGRP specific IFN- γ producing CD8 T cells in non-diabetic infected mice could be due to defects in T cell priming in lymphoid organs. MDSC were shown to decrease the migratory capacity of DCs (438), which could result in their retention in islets. Thus, fewer DCs would migrate into lymphoid organs and, therefore, less autoantigens would be presented to T lymphocytes. However, CD11b⁺/CD11c⁺ DCs left the pancreatic islets by day 4 of infection in both CVB4 and CVB4+ α GalCer treated mice showing that MDSC did not induce the retention of DCs in pancreas (Fig. 49).

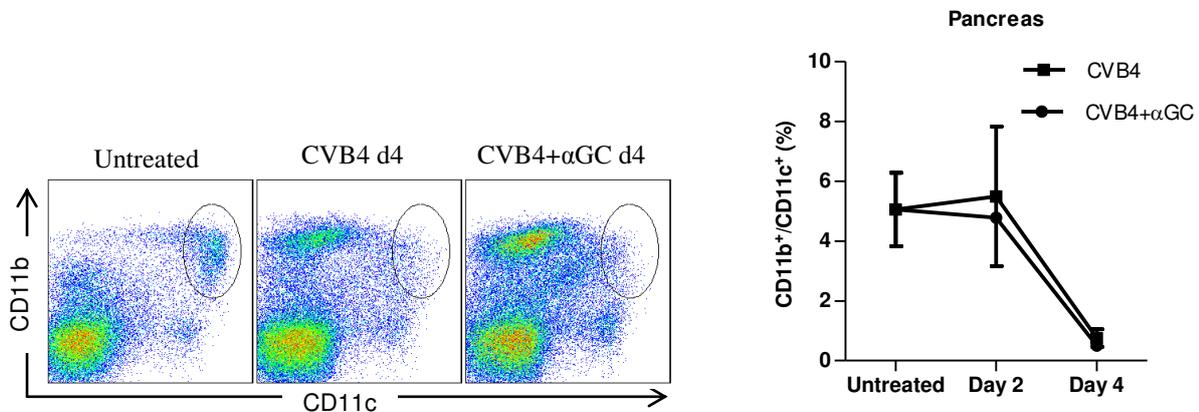


Figure 49. DCs leave pancreatic islets by day 4 of infection in both CVB4 and CVB4+ α GalCer treated mice. Female Pro-ins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On days 0, 2 and 4 post-infection, pancreatic islets were harvested, dissociated into a single cell suspension and stained with different antibodies. Dot plots correspond to a representative staining of CD11b⁺/CD11c⁺ DCs gated among CD45⁺ cells in pancreatic islets. On the right, summary of the frequency of CD11b⁺/CD11c⁺ DCs in pancreatic islets from 3 independent experiments with 2 mice in each group \pm SD.

The function of DCs:

MDSC can also induce tolerogenic DCs that are inefficient in T cell priming (438). Such DCs have an intermediate level of expression of stimulatory MHC-II and CD80/CD86 molecules but express high levels of suppressive PD-L1 and PD-L2. While we did not see upregulation of MHC II in DCs in the spleen and PLN of infected mice, the expression of both activatory CD86 and inhibitory PD-L1 were increased after the infection and gradually

decreased by day 8 of infection (Fig. 50). However, no differences were observed in the level of expression of CD86 or PD-L1 between mice from CVB4 and CVB4+ α GalCer groups.

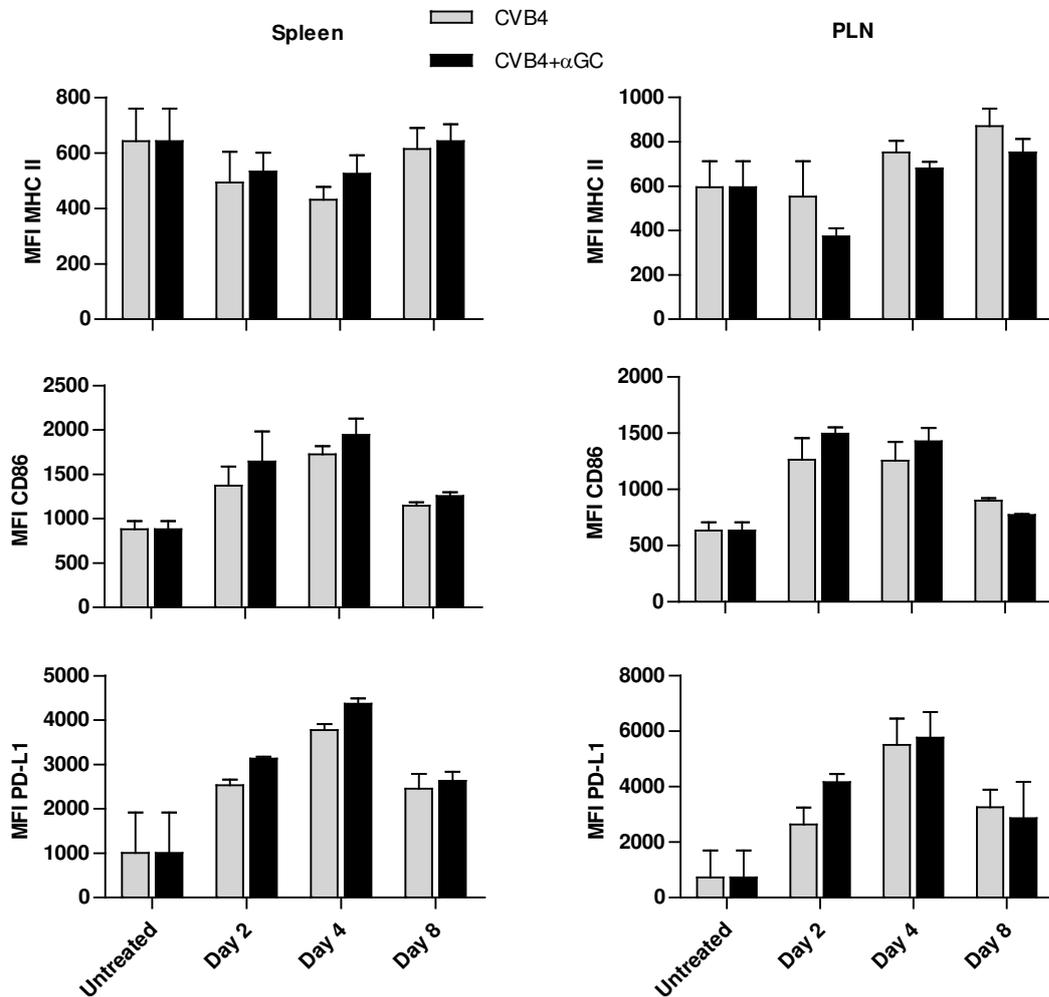


Figure 50. DC phenotype does not differ in the spleen and PLN of CVB4 and CVB4+ α GalCer treated mice. Female Pro-ins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On days 2, 4 and 8 post-infection, spleen and PLN were harvested, dissociated into a single cell suspension and stained with different antibodies. CD11b⁺/CD11c⁺ DCs were gated among islet CD45⁺ cells. Data represent 3 independent experiments with 2 mice per group \pm SD.

The secretion of a pro-Th1 cytokine IL-12 has been associated with diabetes development (439). CVB4 infection increased IL-12 production among CD45⁺ cells of the spleen and PLN on day 6 post-infection. The level of IL-12 production, however, was similar between CVB4 and CVB4+ α GalCer treated mice (Fig. 51). Of note, IL-12 was not secreted

by CD11c⁺ cells or CD11b⁺ cells. Thus, we did not detect major differences in DCs between mice of CVB4 and CVB4+ α GalCer groups.

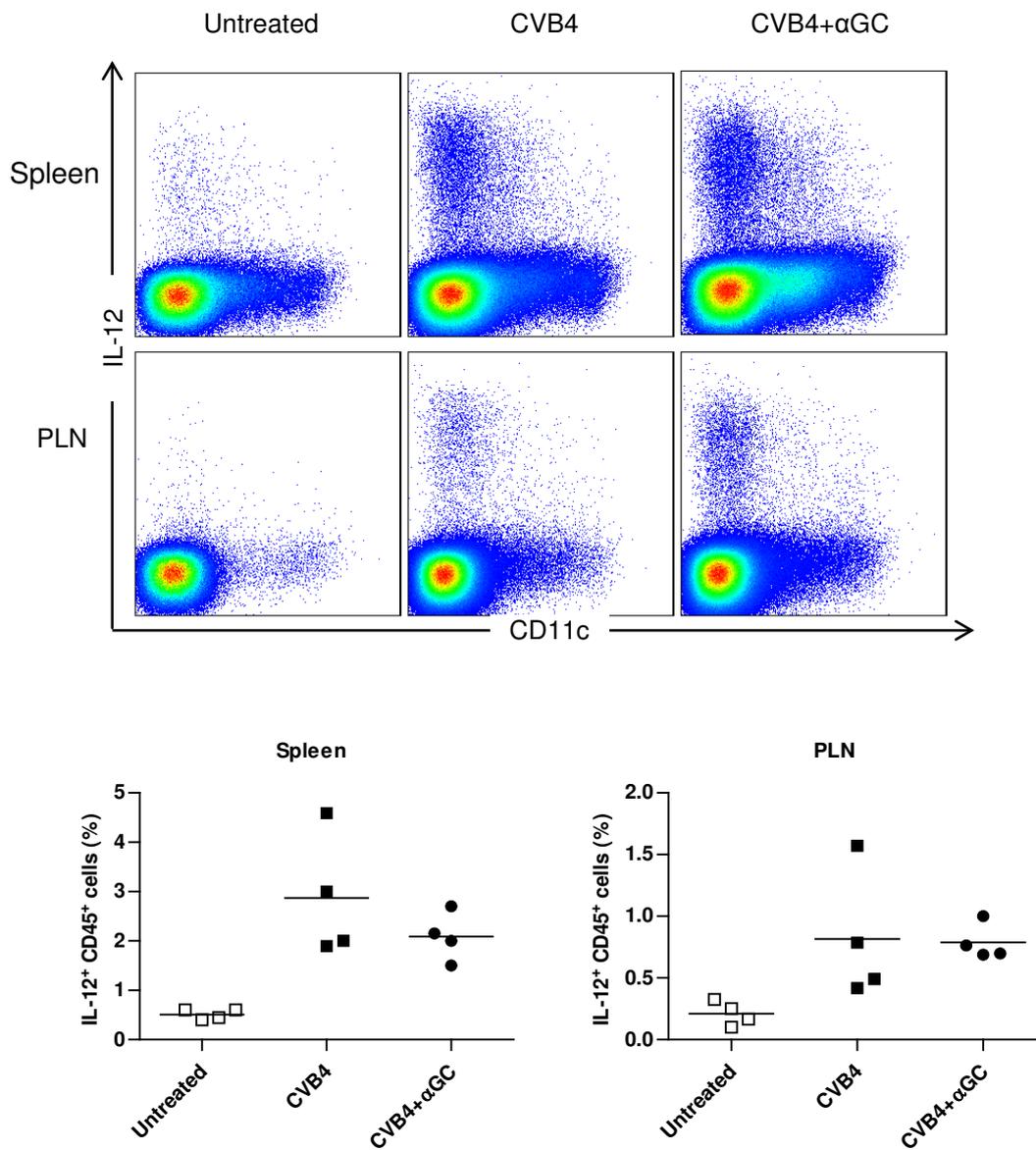


Figure 51. IL-12 level did not differ in the spleen and PLN of CVB4 and CVB4+ α GalCer treated mice. Female Pro-ins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On day 6 post-infection, spleen and PLN were harvested, dissociated into a single cell suspension and stained with different antibodies. Dot plots correspond to a representative staining of total CD45⁺ cells stained with anti-IL-12 mAb after stimulation with PMA and ionomycin for 4 h in the presence of Brefeldin A. On the bottom, summary of IL-12⁺ cells gated among CD45⁺ cells obtained from 2 independent experiments with 2 mice per group. Each symbol represents an individual mouse.

The role of Foxp3 cells in diabetes prevention after CVB4 infection

Foxp3⁺ Treg cells are major regulators of autoimmunity. Since IDO and IDO generated tryptophan metabolites were shown to induce regulatory Foxp3⁺ cells, we compared the frequency and numbers of Foxp3⁺ cells in pancreatic islets, PLN and spleen of CVB4 infected mice treated or not with α GalCer between one and three weeks post-infection. While the percentage of Foxp3⁺ cells remained similar in all three organs before and after the infection, their absolute numbers increased in both CVB4 and CVB4+ α GalCer treated mice compared to uninfected or α GalCer treated mice. The increase in Foxp3⁺ cell numbers was consistent with the increase of total CD4 numbers in infected mice but was not different between CVB4 and CVB4+ α GalCer treated mice (Fig. 52 and data not shown). We did not observe any upregulation of TGF- β and IL-10 in infected mice in the pancreas, spleen or PLN compared to uninfected mice. Then, we stained Foxp3 wells with antibodies against CTLA-4, OX-40, CD103 and GITR all of which could reflect their function. Similar to results with TGF- β and IL-10, none of these molecules were upregulated after the infection. Importantly, no differences were found between diabetic and non-diabetic CVB4 infected mice. These data do not support the role of regulatory Foxp3⁺ cells in the decreased diabetes incidence in CVB4+ α GalCer group.

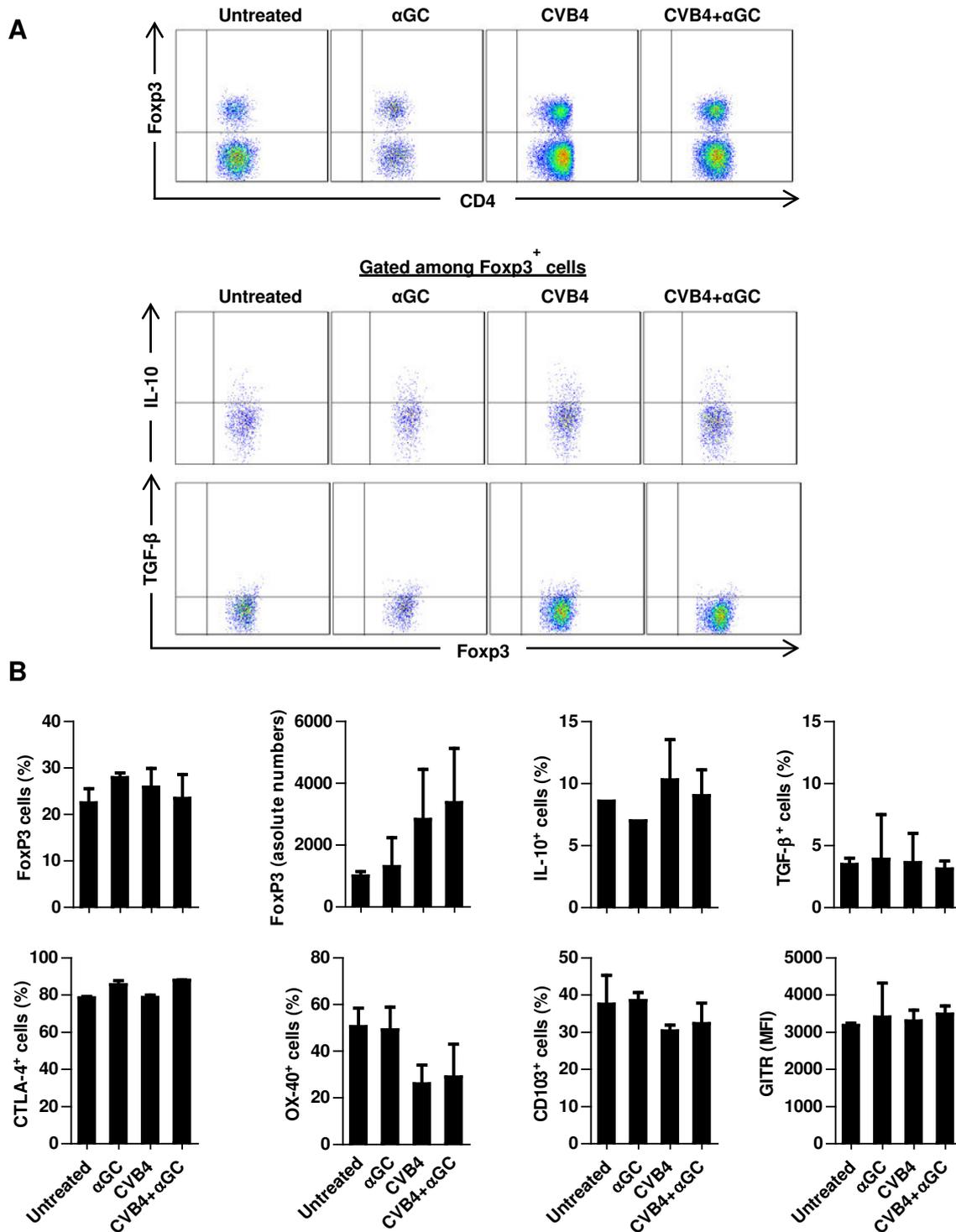


Figure 52. Foxp3 cells have similar frequency and phenotype in CVB4 and CVB4+αGalCer groups. Female Pro-ins2^{-/-} mice at 5-6 weeks of age were inoculated i.p. with CVB4 or PBS and treated with αGalCer or control vehicle. One to three weeks after the infection, pancreatic islets were isolated, dissociated into a single cell suspension and stained with different antibodies. **(A)** Dot plots correspond to a representative staining of islet CD4⁺/Foxp3⁺ cells gated among CD45⁺/TCRβ⁺ 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 the frequency of islet CD4⁺/Foxp3⁺ T cells expressing different markers gated among CD45⁺ cells from 3 independent experiments with 3 mice in each group ± SD.

Anti-islet T cells are suppressed locally in the pancreas

The absence of IFN- γ production by CD8 T cells stimulated by IGRP peptide in non-diabetic infected mice, prompted us to investigate whether IGRP specific CD8 T cells were present in the islets of these mice. We, therefore, stained pancreatic islet cells of Pro-ins2^{-/-} and NOD mice with IGRP loaded tetramers. Islets were isolated from untreated, α GalCer treated and CVB4 infected mice with or without α GalCer treatment in the period from 1 to three weeks post-infection. The results of the staining showed a heterogeneous infiltration of anti-IGRP CD8 T cells in the islets (Fig. 53). Importantly, while IFN- γ production was only detected in CVB4 infected diabetic mice, anti-IGRP CD8 T cells could be detected in both uninfected and infected mice. Moreover, the frequency of IGRP tetramer positive T cells did not reflect their diabetes status. Altogether, these results showed that IGRP specific autoreactive T cells are present in the pancreatic islets of Pro-ins2^{-/-} and NOD mice, however, they produce IFN- γ only in mice that are diabetic.

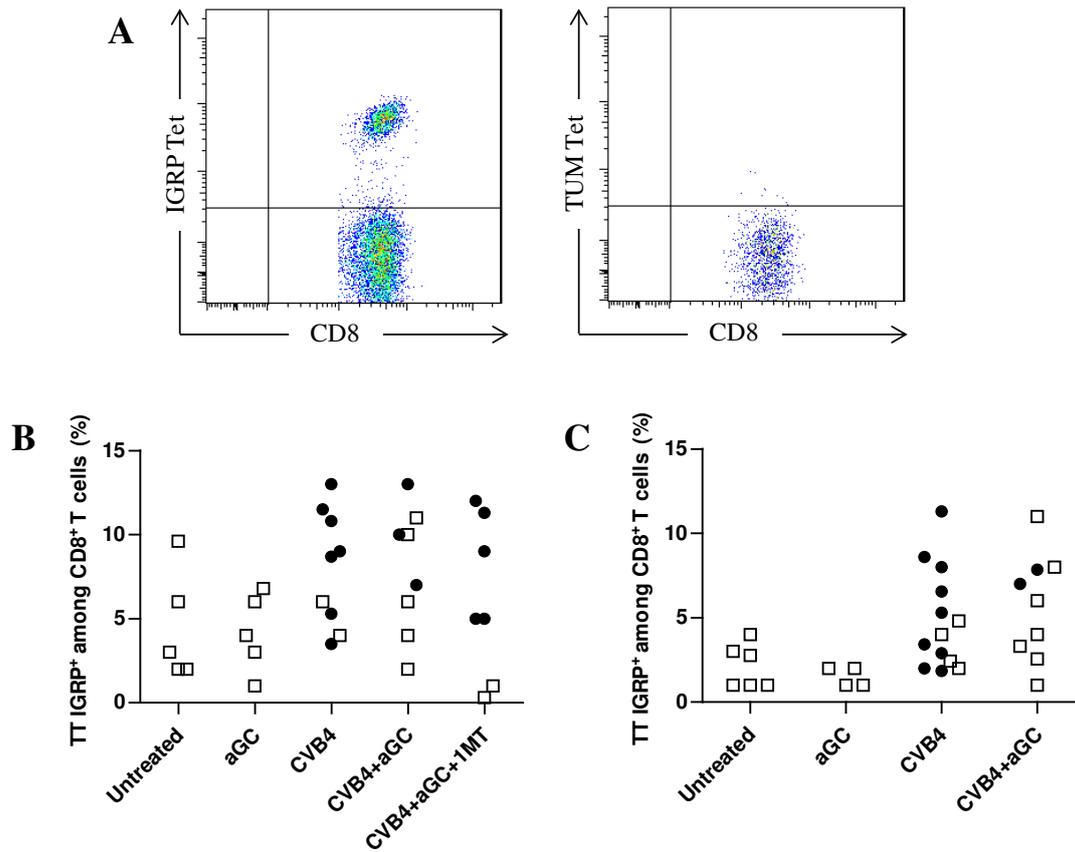


Figure 53. IGRP specific T cells similarly infiltrate pancreas of mice from CVB4 and CVB4+ α GalCer groups. Pancreatic islets were recovered, dissociated into a single cell suspension and stained with NRP-V7 (IGRP₂₀₆₋₂₁₄ mimotope; KYNKANVFL) loaded Kd tetramer and unrelated TUM peptide (KYQAVTTTL) loaded Kd tetramer. **(A)** Representative IGRP and TUM tetramer stainings of pancreatic islet CD8 T cells of a diabetic Pro-ins2^{-/-} mouse from CVB4 group 2 weeks after the infection gated among islet CD45⁺/TCR β ⁺ cells. **(B and C)** Summary of IGRP tetramer positive islet CD8 T cells of Pro-ins2^{-/-} **(B)** and NOD **(C)** females gated among islet CD45⁺/TCR β ⁺/CD8⁺ cells, 7 days to 3 weeks post CVB4 infection and treatments as indicated on the figure. Untreated, α GalCer treated or CVB4 infected non-diabetic mice were tested in parallel with diabetic mice. Non diabetic mice are represented by white squares and diabetic mice are represented by black circles. Data represent three independent experiments.

Discussion and Perspectives

Our study allowed to show 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 (Fig. 54). 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.

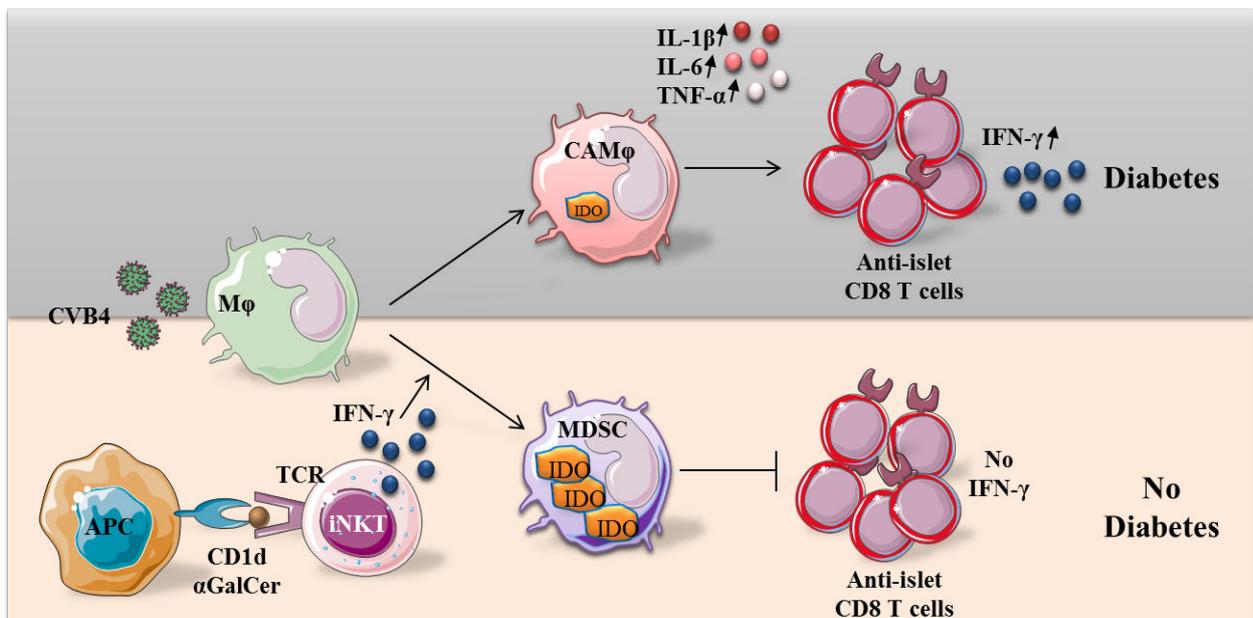


Figure 54. Schematic view of the immune cell interplay in the islets after infection. After CVB4 infection macrophages strongly infiltrate pancreatic islets and resemble classically activated macrophages (CAM ϕ) with low IDO expression and high secretion of proinflammatory cytokines IL-1 β , IL-6 and TNF- α . In this setting, anti-islet T cells strongly infiltrate the islets and produce IFN- γ , which can kill islet β -cells. However, when iNKT cells are activated by α GalCer 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.

CVB4 infection induced a very strong inflammation in pancreatic islets with the upregulation of numerous inflammatory cytokines and a massive infiltration of macrophages. These macrophages expressed Ly-6C and CD115 suggesting that these were blood monocytes that had recently infiltrated the site of CVB4 infection. They had a CAM ϕ phenotype expressing pro-inflammatory cytokines IL-1 β , IL-6 and TNF- α . The pathogenic role of macrophages in T1D development has been suggested in several publications (395; 400; 403; 415) with a particular emphasis of CAM ϕ derived cytokines (404; 406; 440). Thus during the infection by EMC-D virus, the depletion of macrophages or the inhibition of macrophage associated toxic mediators IL-1 β , TNF- α and iNOS allowed to strongly decrease diabetes development (152). Macrophage depletion strongly decreased diabetes incidence after CVB4 infection. However, CVB4 infected NOD Scid mice did not develop diabetes. This was not due to the absence of macrophage infiltration into the pancreas of NOD Scid mice, as we found increased numbers of macrophages producing IL-1 β , IL-6, TNF- α or iNOS in pancreatic islets (data not shown). These results show that macrophages alone do not provoke diabetes after CVB4 infection. Still, they can favor diabetes development by inducing the death of some islet β -cells through secretion of inflammatory cytokines (Fig. 55). Apoptotic and necrotic bodies can then be captured by DCs and be presented to autoreactive T cells, thereby initiating the anti-islet autoimmune response. In addition to DCs, macrophages were shown to engulf islet cell debris in CVB4 infected mice (208); consequently, they can present autoantigens as well. It is, therefore, not surprising that as a consequence of macrophage depletion by clodronate containing liposomes and a strong decrease in inflammatory cytokine expression in pancreas, fewer mice developed diabetes after CVB4 infection. While clodronate liposomes can also kill DCs and prevent antigen presentation and activation of autoreactive T cells, we did not see any decrease in absolute numbers of DCs in clodronate treated islets suggesting that diabetes was prevented because of macrophage depletion. Interestingly, while CAM ϕ , sorted from CVB4 infected mice, did not significantly enhance the proliferation of T cells in vitro, they significantly increased diabetes incidence when transferred into CVB4 infected mice. These results suggest that CAM ϕ do not directly enhance T cell proliferation but rather promote diabetes development by other mechanisms that are mentioned above. All these data support the pathogenic role of CAM ϕ in CVB4 induced diabetes.

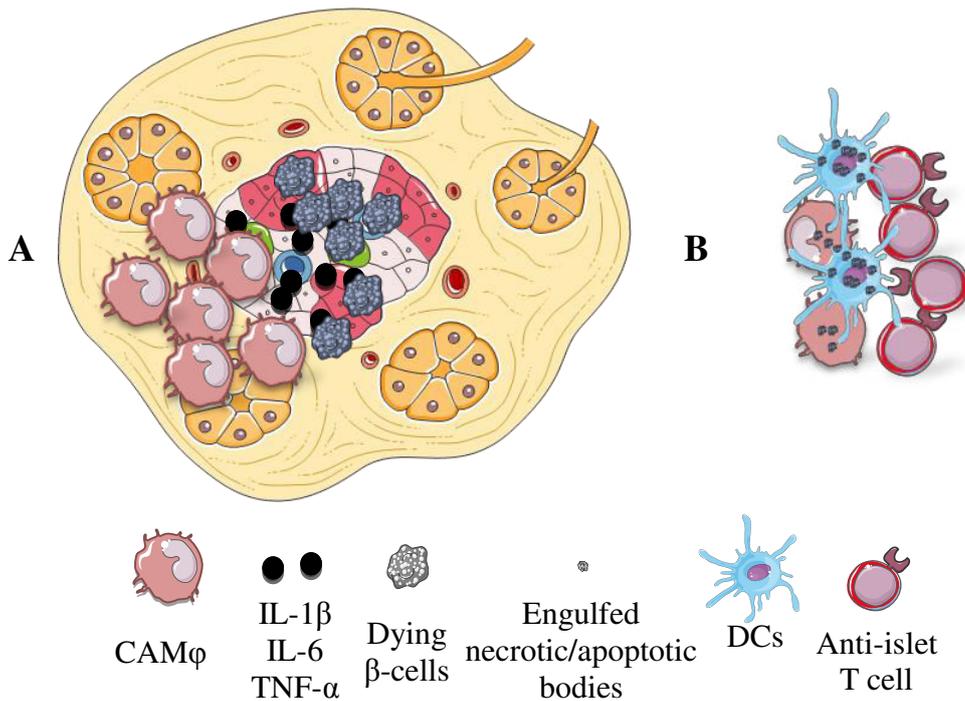


Figure 55. Possible role of CAMφ in T1D development in CVB4 infected mice. (A) CAMφ, infiltrating the pancreas after CVB4 infection, could induce the death of islet β-cells through secretion of pro-inflammatory cytokines. **(B)** Macrophages and DCs could then engulf necrotic and apoptotic bodies and present islet antigens to anti-islet T cells, thus, favoring T1D development.

Besides the strong upregulation of inflammatory cytokines, macrophages of CVB4 infected mice had also upregulated the enzymes iNOS and IDO as part of an anti-viral immune response. The expression of iNOS and IDO in CVB4 group was independent of iNKT cells as they were also detected in the islets of NOD Scid mice, which lack NKT cells. The enzyme iNOS has previously been shown to be important for CVB4 elimination as iNOS deficient mice die shortly after CVB4 infection due to uncontrolled viral replication that damages the heart (204). Thus, even though iNOS might act as a suppressive enzyme, its primary role during CVB4 infection might be the control of viral replication. The expression of the suppressive enzyme IDO during CVB4 infection might be somewhat surprising, since IDO induced suppression of the immune system can potentially lead to viral escape. However, CVB4 infected mice did not die after the infection and virus was undetectable in pancreas by PFU technique by the 8th day of infection despite high IDO expression. Therefore, IDO did not seem to interfere with the natural course of anti-viral immune response and was perhaps upregulated as part of a negative feedback control. This can be particularly important for

tissues in which the excessive activation of the immune system can lead to major organ damage.

In contrast to the inflammatory environment in CVB4 infected pancreas, the environment in pancreas of CVB4+ α GalCer treated mice was suppressive with the moderate downregulation of numerous pro-inflammatory cytokines. This suppressive environment was consistent with low diabetes incidence in these mice. IFN- α was among the downregulated cytokines, which was unexpected in regard to the previous study conducted in the laboratory by Diana et al. with LCMV infection (230). Thus, the molecular mechanisms leading to diabetes prevention by iNKT cells can be quite different depending on the virus. LCMV infection prevents diabetes development in NOD mice. This previous study had revealed that during the infection with LCMV, iNKT promoted high IFN- α production by pancreatic pDCs. This resulted in the control of viral replication locally in pancreas that could contribute to diabetes prevention. While contrasting LCMV, the downregulation of IFN- α production by iNKT cells in CVB4+ α GalCer group and the low diabetes incidence in these mice actually support the proposed pathogenic role of IFN- α in T1D. Indeed, low type I interferon secretion is suggested to be protective from T1D in humans (219-221). In line with this, IFN- α was detected more frequently in newly diagnosed T1D patients, half of which were infected by coxsackieviruses, compared to healthy controls (222).

As mentioned previously, the high IFN- α production after LCMV infection allowed to effectively control the viral replication in pancreas. This can be critical for diabetes prevention, since the infection can result in β -cell death and presentation of autoantigens by APCs. In line with this, Kanno et al. have demonstrated that T1D onset after the infection with the diabetogenic CVB3 was favored by the increased viral replication rate and high infectious dose (201). By contrast to LCMV and CVB3, CVB4 titers did not differ between mice having high or low IFN- α level. This could either suggest that even at low dose IFN- α can effectively control the viral replication, or that, perhaps, other mechanisms compensate for low IFN- α production. Either way, CVB4 by itself does not seem to explain differences in T1D incidence between CVB4 and CVB4+ α GalCer treated mice.

Besides the downregulation of proinflammatory cytokines, iNOS, IDO and arginase I enzymes were upregulated significantly higher in the islets of CVB4+ α GalCer group compared to pancreas of CVB4 group. By contrast to iNOS and IDO, Arginase I was not upregulated in CVB4 infected mice, but was highly upregulated in α GalCer treated mice with

or without CVB4 infection indicating that α GalCer is the inducer of arginase I. The expression of these enzymes, thus, required different stimuli. Of note, the high expression of iNOS in CVB4+ α GalCer group might be one of the mechanisms by which mice of this group cope with low IFN- α expression.

Any of these enzymes, alone or combined, might have been suppressive enough to mediate T1D prevention in CVB4+ α GalCer treated mice. We, therefore, used specific inhibitors to determine their role in the T1D prevention. While inhibition of iNOS or arginase I did not result in increased diabetes incidence, the inhibition of IDO, on the contrary, significantly increased diabetes incidence in CVB4+ α GalCer treated mice. Therefore, IDO was the main enzyme responsible for low diabetes development in this group. The main cell population expressing IDO in the islets was comprised of infiltrating Ly-6C⁺/CD115⁺ macrophages, which strongly resemble MDSC. Thus, the stimulation of iNKT cells by α GalCer allows to inhibit the differentiation of islet infiltrating macrophages into CAM ϕ and rather promotes their differentiation into suppressive MDSC that prevent diabetes.

Both inflammatory IL-1 β , IL-6 and TNF- α and the suppressive IDO were expressed in CVB4 and CVB4+ α GalCer treated mice. However, in each group one feature dominated over the other. Thus, while inflammatory cytokines were strongly expressed in pancreatic islets of CVB4 infected mice, some IDO expression could also be detected. Similarly, lower levels of inflammatory cytokines were found in CVB4+ α GalCer treated mice along with the strong upregulation of IDO. This could mean that inflammatory and suppressive macrophages formed two distinct populations that infiltrated the pancreas at different ratios. However, we did not detect any phenotypical differences between macrophages from CVB4 and CVB4+ α GalCer treated mice in terms of their size or granulocytosis, the upregulation of activation markers CD69 or CD86 or the suppressive PD-L1 molecule. Their differences are therefore only functional.

The high upregulation of IDO in CVB4+ α GalCer treated mice required the stimulation of iNKT cells by α GalCer and could not be compensated by increased iNKT cell numbers. Interestingly, iNKT cells were activated in CVB4 infected mice. They did not produce IL-4 or IL-13 that typically characterize iNKT cells, but produced low levels of IFN- γ . By contrast, iNKT cells produced all these cytokines in α GalCer treated mice. Among these cytokines, IFN- γ was critical for the high upregulation of IDO and diabetes prevention since the inhibition of IFN- γ downregulated IDO expression and increased diabetes incidence. Paradoxically, the strong upregulation of IFN- γ in CVB4+ α GalCer treated mice could have favored diabetes development since IFN- γ can be toxic to islet β -cells. However, in this

context, IFN- γ , on the contrary played a protective role. The induction of a potent suppression by high levels of an inflammatory cytokine such as IFN- γ is reminiscent to the study by Baban et al. showing that the higher the dose of CpG treatment, the higher the level of IDO in spleen and PLN (367). Thus, the strong inflammation can promote a strong induction of suppressive mechanisms. Of note, the inhibition of IDO and, particularly, of IFN- γ also increased the diabetes incidence of CVB4 group. The low upregulation of IDO in CVB4 infected mice was independent of iNKT cells since IDO could also be detected in NOD Scid and Pro-ins2^{-/-} Scid mice. Therefore, the source of IFN- γ in these mice could have been other immune cells such as NK cells.

While α GalCer treatment of uninfected mice induced high levels of IFN- γ , it neither induced IDO expression in islets nor diabetes prevention. Thus, the viral infection is an absolute requirement for IDO induction. However, despite the presence of these three factors i.e. IFN- γ , macrophages and CVB4 infection, in the spleen and PLN, we did not detect any upregulation of IDO in macrophages or other immune cells. Therefore, the fourth parameter, the pancreatic tissue, must be present for the IDO induction (Fig. 56). α GalCer activated iNKT cells were shown to induce the expansion and recruitment of suppressive MDSC into central nervous system during EAE, but were also shown to induce the conversion of MDSC into immunogenic APCs in tumor model (427; 433). We did not detect any particular role for unstimulated iNKT cells during CVB4 infection. By comparison, iNKT cells effectively inhibited MDSC expansion in the lungs of mice infected with influenza virus (253). Therefore, whether iNKT cells will inhibit MDSC, or, on the contrary, promote their differentiation seems to highly depend on the type of pathology and the tissue. When comparing the contrasting roles of iNKT cells during infection with influenza virus and CVB4, it is perhaps possible to hypothesize that when the most damage to the organism can come from the virus, then iNKT cells will inhibit MDSC differentiation, which could otherwise interfere with viral clearance. However, when the viral infection by itself is not damaging, but can favor an autoimmune reaction, then MDSC differentiation is favored.

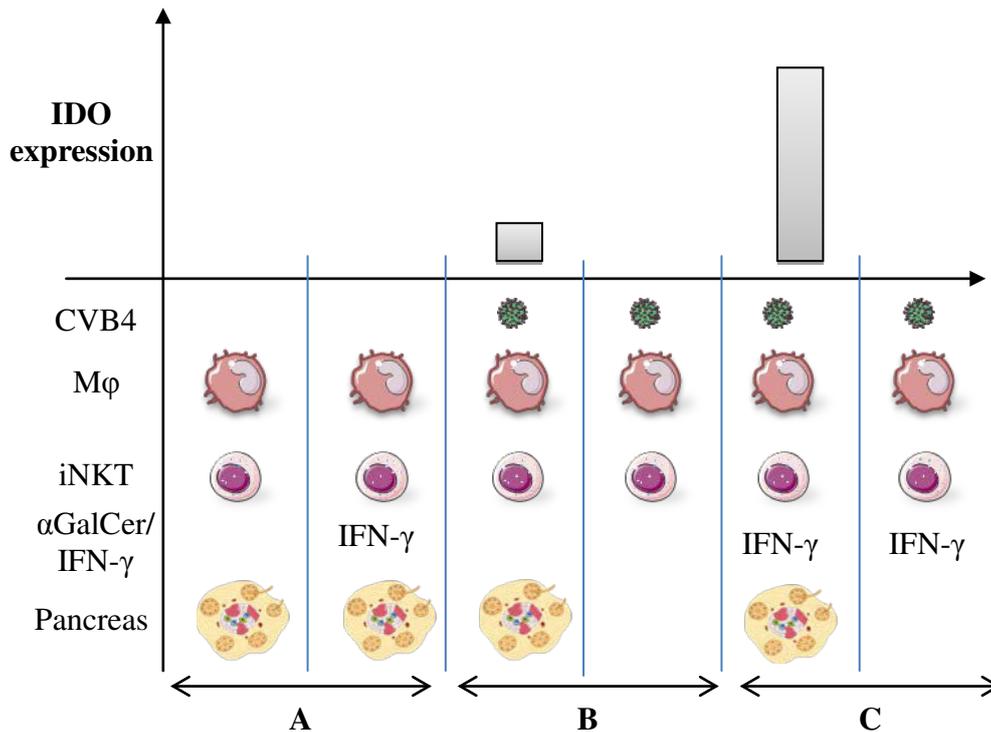


Figure 56. Stimuli needed for IDO induction. (A) Without CVB4 infection, IDO is not induced in pancreatic macrophages even after stimulation of iNKT cells with α GalCer that results in high levels of IFN- γ production. (B) After CVB4 infection, macrophages upregulate IDO, however, this upregulation is restricted to pancreas. (C) α GalCer induced IFN- γ strongly increases IDO expression by macrophages; yet again high IDO expression is limited to pancreas.

Diabetes development and long term prevention

When comparing two CVB4 strains, one that protects from diabetes and the CVB4 E2 strain that is diabetogenic, Yap et al. have found that the protective strain induced less damage to the exocrine tissue compared to the diabetogenic strain. After the primary immune response, new pancreatic islets regenerated in the less destructed exocrine tissue thereby preventing diabetes. On the contrary, the exocrine pancreatic tissue of mice infected with CVB4 E2 strain was so damaged that new islets did not regenerate and mice developed diabetes. While the suppressive environment of CVB4+ α GalCer treated mice could potentially have favored the preservation of the exocrine tissue and further islet regeneration, in reality, no differences were observed in the level of destruction of pancreatic exocrine tissue between CVB4 and CVB4+ α GalCer treated mice. Drescher et al. have observed that even a non-diabetogenic CVB strain can induce diabetes, if it infects pancreatic islets (209). While we detected CVB4 in the exocrine tissue, we found that pancreatic islets were free of

virus. Therefore, the difference in CVB4 incidence between these two groups of mice is not based on the level of destruction of the exocrine tissue or the virus.

NOD Scid, Pro-ins2^{-/-} Scid or Cα^{-/-} NOD mice, that only lack T lymphocytes, did not become diabetic after CVB4 infection (data not shown), suggesting that pancreatic islet destruction after CVB4 infection is mediated by T lymphocytes. Numerous publications have shown that IDO induces tolerance by inhibiting T cell proliferation through tryptophan depletion and induction of apoptosis through by-products of tryptophan degradation, such as kynurenins (387). Consistent with these data, MDSC effectively suppressed T cell proliferation in our in vitro experiment by a mechanisms involving IDO as the use of IDO inhibitor restored T cell proliferation. However, in our vivo data showed that diabetes prevention in CVB4+αGalCer treated mice did not depend on the suppression of T cell proliferation. CD4 and CD8 T cells strongly infiltrated pancreas by the 7th day of infection. Their absolute numbers and frequency were similar between CVB4 infected mice that had low IDO expression, CVB4+αGalCer treated mice with high IDO expression and CVB4+αGalCer mice treated with IDO inhibitor. Similarly, we found anti-IGRP CD8 T cells in pancreas of all infected mice regardless of treatment. Thus, diabetes prevention in IDO expressing mice could not be linked to the inhibition of T cell proliferation in vivo.

Interestingly, despite infiltrating pancreas of all CVB4 infected mice, anti-IGRP CD8 T cells produced IFN-γ only in mice that were diabetic, suggesting that anti-IGRP CD8 T cells were inhibited locally in the pancreas of CVB4 infected non-diabetic mice. Our data showing that IDO can induce inhibition of the function of CD8 T cells is reminiscent of a previous study in allograft setting (441; 442). Moreover local regulation by IDO has been previously observed during infections and in cancer (443). The inhibition of IDO by 1MT in CVB4+αGalCer treated mice resulted in an increase in frequency of IFN-γ producing anti-IGRP CD8 T cells and a high incidence of diabetes confirming the link between IDO and T cells in diabetes prevention.

It is curious as to why diabetes develops only in a subset of CVB4 infected mice while the remaining mice acquire a long term protection from developing diabetes. The previous studies by Serreze et al. and Horwitz et al. suggest that diabetes development or prevention depends on the level of insulinitis and the frequency of autoreactive T cells infiltrating the pancreas at the time of CVB4 infection (199; 200). Similarly, we observed that Pro-ins2^{-/-} mice infected at a younger age, did not develop diabetes. Based on previous publications and our new data we propose the following scenario to explain the acceleration and the long term protection from diabetes after CVB4 infection.

Diabetes initiation

CVB4 group: At the time of CVB4 infection around half of mice have already developed high insulinitis with the infiltration of autoreactive T cells into pancreas (Fig. 57). The inflammation provoked by CVB4 infection activates the cells of the innate immune system as well as anti-islet CD4 and CD8 T cells present in the pancreatic tissue that kill some islet β -cells. APC then engulf the apoptotic bodies, migrate to secondary lymphoid organs and present islet β -cell antigens to T cells. In the last phase, activated pathogenic T cells infiltrate the pancreas, kill islet β -cells and induce T1D. Importantly, IDO is strongly downregulated in the islets after the second day of infection; therefore the IFN- γ , produced by newly infiltrating autoreactive T cells, will not be able to induce IDO upregulation and to initiate suppression. On the contrary, IFN- γ , produced by anti-islet T cells, would be pathogenic to islet β -cells. In the second half of infected mice, the level of insulinitis is moderate at the time of CVB4 infection. As a result, after CVB4 infection very few islet β -cells are killed, which limits the presentation of islet antigens in lymphoid organs, thereby preventing diabetes development.

CVB4+ α GalCer group: In CVB4+ α GalCer group, the mice with insufficient insulinitis are still protected from diabetes. α GalCer treatment induces a strong burst of IFN- γ early after viral infection and upregulates IDO expression in order to dampen the inflammation after viral infection. As a consequence, the subset of mice with high insulinitis, that otherwise would have developed diabetes, is now protected by high IDO expression that immediately inhibits T cell function and prevents the killing of islet β -cells. Interestingly, the inhibition of IDO, by 1MT, not only abolishes the diabetes prevention of the “high insulinitis” group but also of the “low insulinitis” group. Therefore, having low insulinitis does not necessarily protect mice from developing diabetes after CVB4 infection. Rather, these results suggest that the low IDO upregulation in CVB4 group is also important for diabetes prevention.

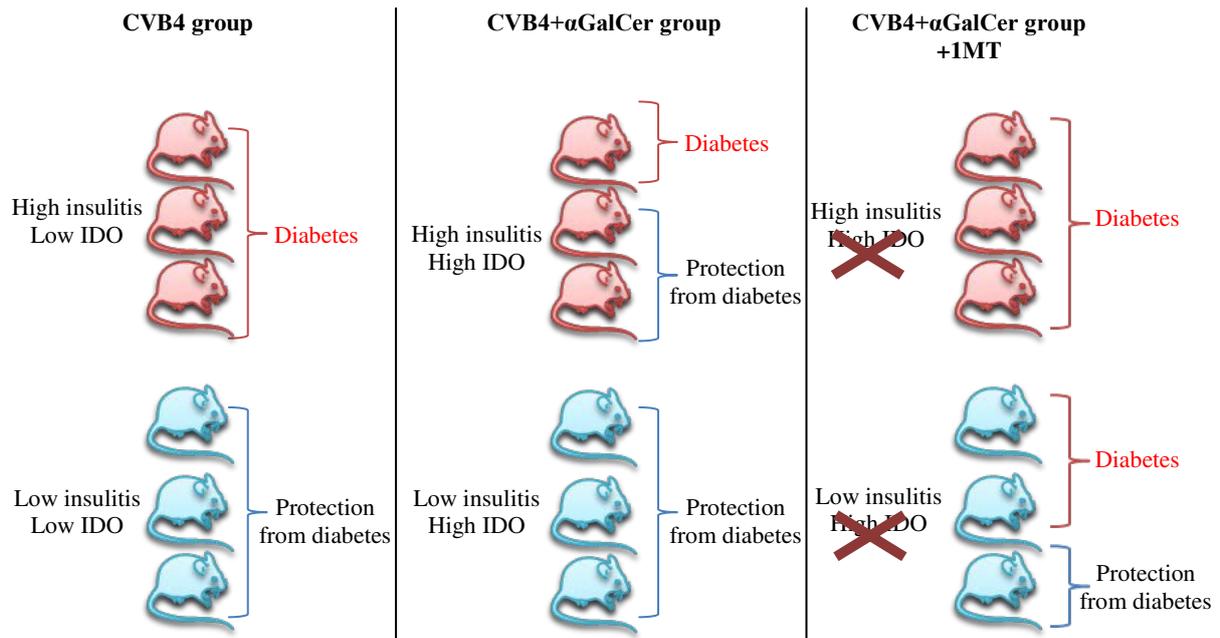


Figure 57. Diabetes development after CVB4 infection depends on the level of insulinitis and IDO. Low insulinitis and IDO allow prevention of diabetes development after CVB4 infection. Diabetes development in mice that have high insulinitis at the time of infection, needs much stronger upregulation of IDO.

Long term diabetes prevention

After the acute phase of infection, mice that did not become diabetic, remain diabetes free for a very long period of time. This long term protection from diabetes was also observed in $J\alpha 18^{-/-}$ $Pro-ins2^{-/-}$ mice indicating that this phenomenon was independent of iNKT cells. CD4 and CD8 T cells still highly infiltrated the pancreas of $Pro-ins2^{-/-}$ mice fifteen weeks after CVB4 infection. Anti-IGRP CD8 T cells were also present in the pancreas of these mice; however, they did not produce any $IFN-\gamma$ when stimulated with IGRP mimotope suggesting that long term suppression of diabetes development was not due to the absence of autoreactive T cells in pancreas but was due to continuous local suppression (data not shown). Whether long-term diabetes prevention is a direct consequence of IDO expression in islets or whether it is independent of IDO remains an open question. It is possible that, the transitory IDO expression, low and, particularly, high expression, could induce a permanent upregulation of some suppressive molecules in pancreas, which would further inhibit the effector functions of all pancreatic infiltrating T cells. For example, the suppressive molecule HLA-G can be upregulated by IDO (200). On the other hand, IDO could only condition the first phase of diabetes development: that is, whether a mouse would become diabetic or not. After this step, diabetes development in all mice could be suppressed by a mechanism that is completely

independent of IDO. When we treated mice from both CVB4 and CVB4+ α GalCer groups with 1MT, we still had some mice that remained diabetes-free for a very long time. While, this could indicate that the long term diabetes prevention is independent of IDO, we cannot exclude that the administration of 1MT in the drinking water could create these differences. Indeed, we do not know if mice drank the same volume of water and received the same amount of 1MT or not. To better determine the role of IDO in the establishment of long-term diabetes prevention, we would need to use a different method of 1MT administration such as pellet implantation that will continuously release 1MT as long as it is implanted (during the period of IDO expression). Thus, if all mice become diabetes, then we could conclude that IDO is the inducer of long term suppression of T cells.

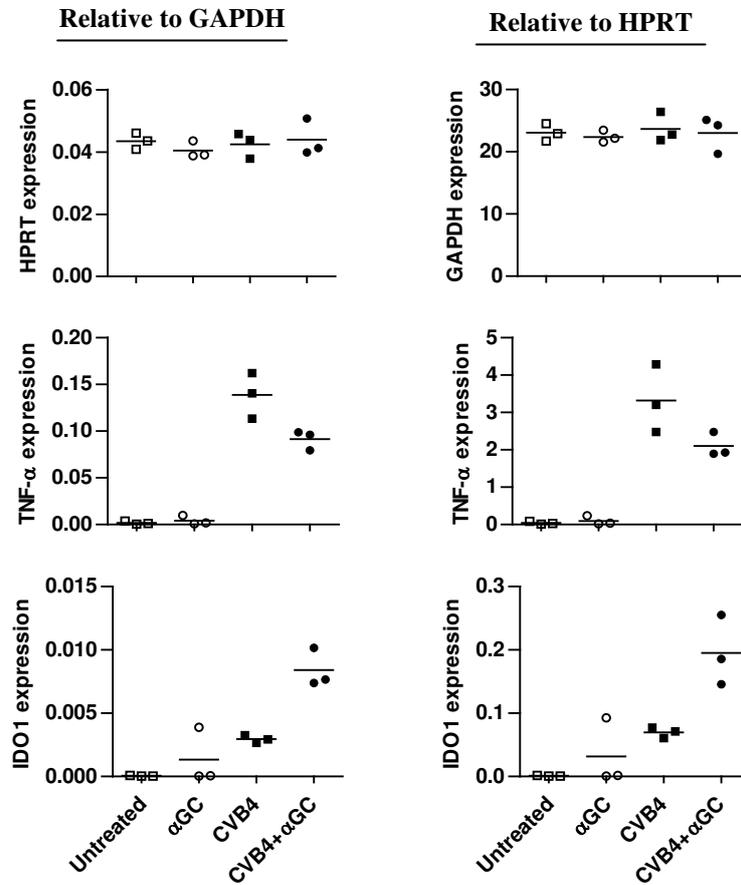
The long term suppression that is established after CVB4 infection could involve several mechanisms including regulatory Foxp3⁺ T cell induction in pancreatic islets particularly since IDO was shown to induce Foxp3 cells (200; 210). However in our study, while the absolute numbers of Foxp3⁺ CD4 T cells slightly increased after the infection, their expression of CTLA-4, CD103, GITR and OX40 remained unchanged and, most importantly, did not differ between CVB4 and CVB4+ α GalCer treated mice tested from a week to three weeks after the infection among newly diabetic and non-diabetic mice. Richer et al. have reported that transgenic expression of TGF- β in islet β -cells or administration of TGF- β after CVB4 infection induces a strong infiltration of Foxp3⁺ cells into pancreatic islets that inhibit diabetes development (444). We have also previously published that infection with LCMV induced the expansion of Treg cells producing TGF- β . However, we did not detect an increase in the production of suppressive cytokines IL-10 and TGF- β by Foxp3⁺ cells in the islets, PLN and spleen of infected Pro-ins2^{-/-} and NOD mice.

Tryptophan metabolites can render DCs tolerogenic which are characterized by intermediate expression of stimulatory MHC-II, CD86 and upregulation of the suppressive PD-L1 and PD-L2 (365). 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 α GalCer.

With constant increase in T1D incidence worldwide, research on T1D prevention is vital. iNKT cells are potent regulators of T1D and can be used in therapies since they can also be activated in humans through α GalCer administration. The previous study performed in our group showed the capacity of iNKT cells to maintain their regulatory role in T1D during LCMV infection. However, in humans T1D in susceptible individuals is suggested to be promoted by enteroviruses. We, therefore, performed this study to determine if the

manipulation of iNKT cells with α GalCer during coxsackievirus infection would still preserve the capacity of iNKT cells to prevent T1D. Our study allowed to determine that the manipulation of iNKT cells can lead to the induction of suppressive macrophages in the pancreas after CVB4 infection, which suppress T1D development. Thus, the regulatory role of iNKT cells could be boosted by α GalCer treatment. Second, it highlights the role of macrophages in the development or prevention of diabetes after CVB4 infection. Importantly, it shows that iNKT cells do not interfere with the natural course of coxsackievirus clearance. This is an extremely important parameter since, if iNKT cell induced MDSC expressed IDO during a longer period, this could have favored viral escape by suppressing anti-viral T cells. If one day a vaccination protocol with enteroviruses and iNKT cells is designed, it is likely to be administered to very young subjects; therefore, high enterovirus titers would, of course, be unacceptable. It is interesting to imagine a vaccination protocol with enteroviruses and α GalCer stimulation of iNKT cells that could result in the infiltration of suppressive macrophages in human pancreas and induce long term diabetes prevention. However, first we would need to determine, whether this mechanism could be duplicated in humans. The numbers of iNKT cells in humans are very heterogeneous, and, therefore, α GalCer treatment might not benefit individuals with low iNKT cell frequency. Next, the pattern of CVB4 infection in mouse models is quite different from humans. Thus, in experimental settings, where CVB4 is administered by intraperitoneal injection, the murine exocrine tissue is severely damaged. By contrast, infection with enteroviruses in humans pass by the oral route, and enteroviruses are rarely detected in the exocrine tissues of human pancreatic biopsies. By contrast, they can be detected in the islets. The choice of the virus or the combination of different viruses seems to be the most difficult choice. Mouse models showed that viruses with efficient replication reaching high dose can promote T1D. However, even the lowest viral dose could harm individuals with inefficient anti-viral immune response. Therefore, research must continue in view of designing novel therapeutic approaches consisting of manipulation of iNKT cells and suppressive macrophages.

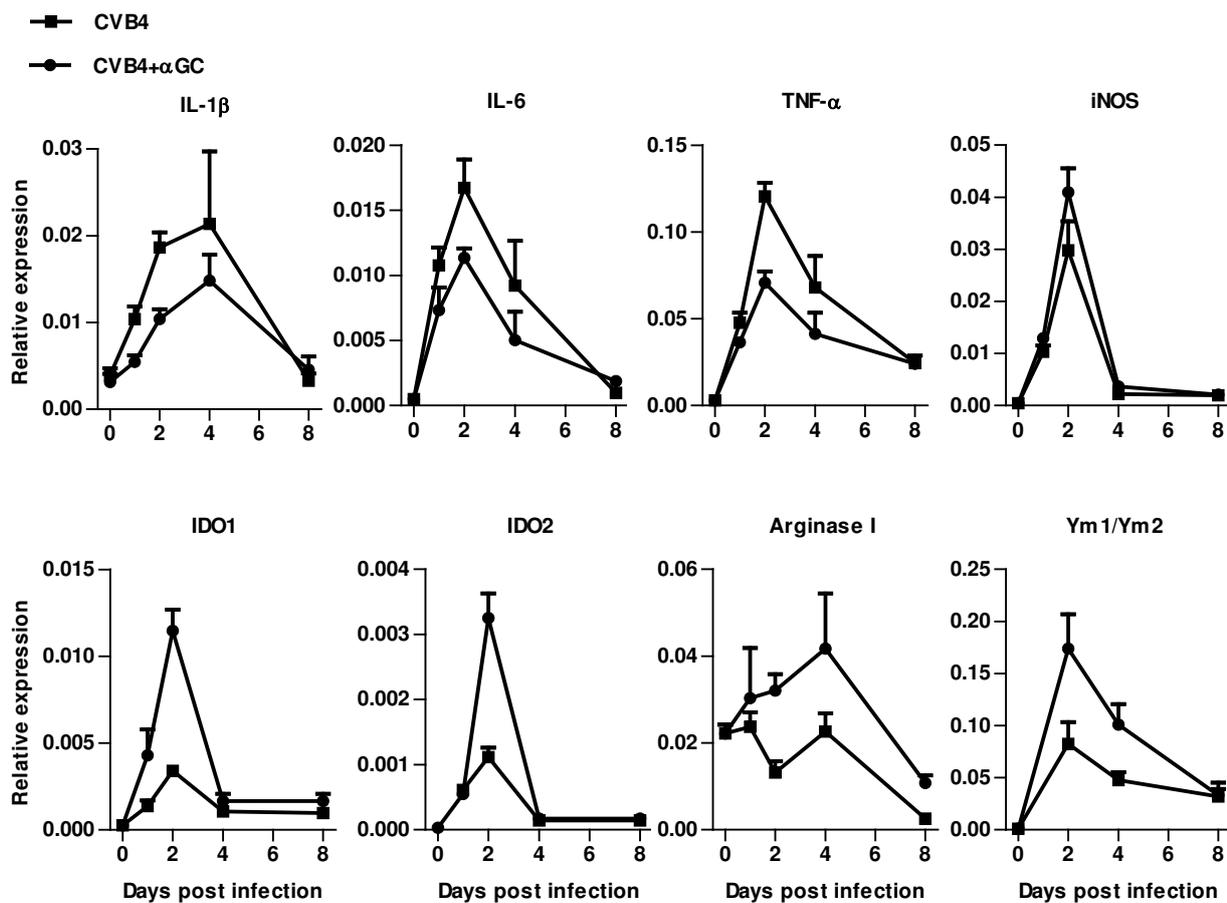
Appendix



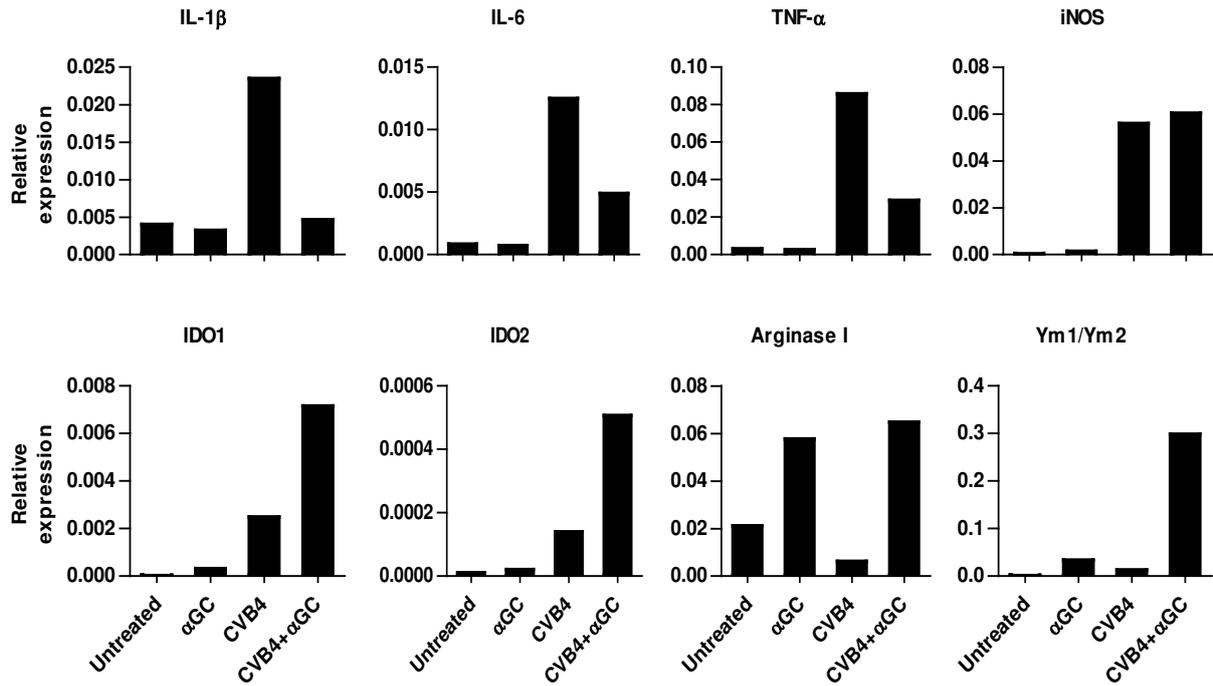
Appendix 1. Comparison between GAPDH and HPRT consistency in different groups of mice. Female Pro-ins2^{-/-} mice at 5-6 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by qPCR. Data are presented as specific gene expression relative to GAPDH and HPRT. Each symbol represents pooled islets of an individual mouse.

IL-1 β	Forward	CAGCAGGTTATCATCATCATCC
	Reverse	ATCTCACAGCAGCACATCAAC
TNF- α	Forward	AGCCCCCAGTCTGTATCCTT
	Reverse	CTCCCTTTGCAGAACTCAGG
IL-6	Forward	AGTTGCCTTCTTGGGACTGA
	Reverse	TCCACGATTTCCCAGAGAAC
iNOS	Forward	CACCTTGGAGTTCACCCAGT ζ
	Reverse	ACCACTCGTACTTGGGATGC
IDO1	Forward	AAGGGCTTCTTCCTCGTCTC
	Reverse	AAAAACGTGTCTGGGTCCAC
IDO2	Forward	GTGCTGACAAACTGGACCAA
	Reverse	TCCTGACTGTGTTGCCGAAT
Arginase	Forward	TCCAAGCCAAAGTCCTTAGAG
	Reverse	ACAGACCGTGGGTCTTCAC
Ym1/Ym2	Forward	AGGCCAACAGAAGGGAGTTT
	Reverse	GTCCAAACTTCCATCCTCCA
IL-15	Forward	AAGGAATGTGAGGAGCTGGA
	Reverse	AGCTTAGTTTGCCAGCAGA
IFN- α	Forward	TACTACTGGTCAGCCTGTTCTC
	Reverse	GGGAGTCTTCTCATTCTTCC
IL-10	Forward	GGTTGCCAAGCCTTATCGGA
	Reverse	ACCTGCTCCACTGCCTTGCT
TGF- β	Forward	TTCCTGGCGTTACCTTG
	Reverse	CTGATCCCGTTGATTTC
GAPDH	Forward	AACGACCCCTTCATTGAC
	Reverse	TCCACGACATACTCAGCAC
HPRT	Forward	AAGCTTGCTGGTGAAAAGGA
	Reverse	TTGCGCTCATCTTAGGCTTT

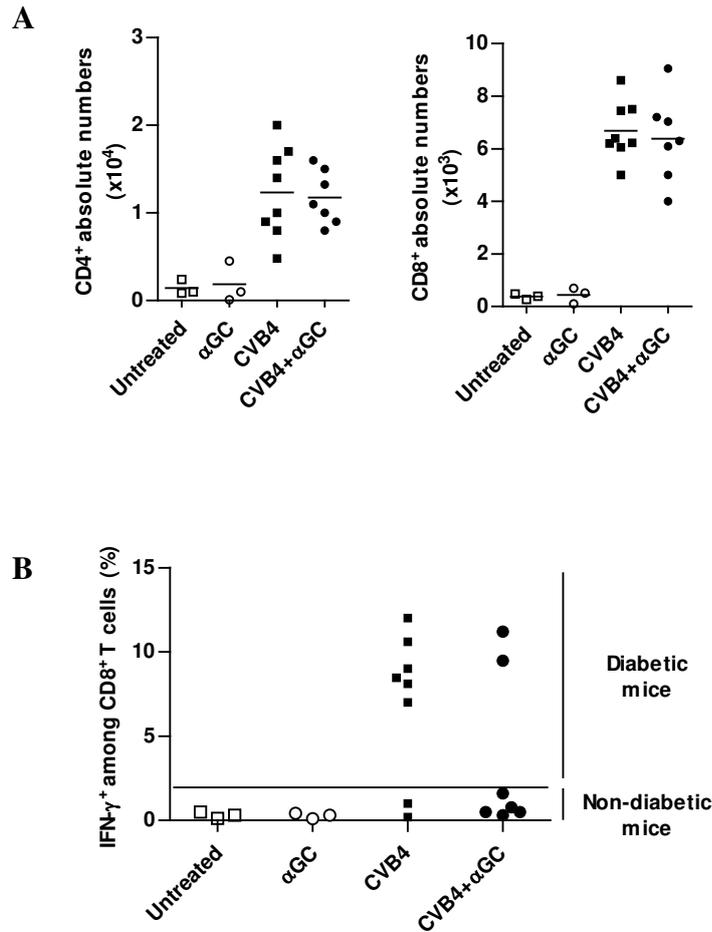
Appendix 2. Forward and reverse sequences of primers used for specific mRNA detection by qPCR.



Appendix 3. Kinetics of expression of different molecules post CVB4 infection. Pro-*ins2*^{-/-} females at 5-6 weeks of age were inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On days 0, 1, 2, 4 and 8 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by qPCR. Untreated mice are represented as day 0. Data are presented as specific gene expression relative to GAPDH. Data represent means of 6 pooled mice from two independent experiments \pm SEM.



Appendix 4. Expression of inflammatory and suppressive molecules in pancreatic islets. Female NOD mice at 10 weeks of age were either inoculated i.p. with CVB4 or PBS and treated with α GalCer or control vehicle. On day 2 post-infection, pancreatic islets were harvested, total RNA was extracted and mRNA levels were measured by qPCR. Data are presented as specific gene expression relative to GAPDH. Bars correspond to data obtained from 3 pooled mice.



Appendix 5. IFN- γ producing IGRP CD8 T cells infiltrate islets of infected diabetic mice. (A) Summary of CD8 and CD4 T cell counts of female NOD mice (infected at 10 weeks of age) from day 7 to 3 weeks post CVB4 infection of two independent experiments after the treatments as indicated on the figure. CD4 and CD8 T cells are gated among CD45⁺/TCR β ⁺ cells. **(B)** Graph showing the percentage of islet CD8 T cells secreting IFN- γ after IGRP₂₀₆₋₂₁₄ peptide stimulation of diabetic and non-diabetic NOD females receiving different treatments as indicated on the figure. IFN- γ producing CD8 T cells are gated among islet CD45⁺/TCR β ⁺/CD8 T cells. The horizontal line shows the limit between diabetic and non-diabetic mice. Each symbol represents a single mouse.

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Publications

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

Review: Prevention or acceleration of type 1 diabetes by viruses

Review: Regulatory role of NKT cells in the prevention of type 1 diabetes

Review: Innate Immunity in Type 1 Diabetes

Review: Therapeutic manipulation of natural killer (NK) T cells in autoimmunity: are we close to reality?

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

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Condensed title: Macrophages in CVB4 induced diabetes.

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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-ins^{2-/-}) NOD mice, the activation of iNKT cells by a specific agonist, α -Galactosylceramide (α GalCer), 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-1 β , 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; α GalCer or α GC α -Galactosylceramide; 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 insulinitis (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 V α 14-J α 18 (V α 14) in mice and V α 24-J α 18 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 α GalCer (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, V α 14 transgenic NOD mice expressing the V α 14-J α 18 TCR α chain and BDC2.5 C α ^{-/-} mice were previously described (15; 21; 25; 26). NOD V α 14 were crossed to Pro-ins2^{-/-} NOD mice to generate V α 14 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 1x10⁵ PFU/mouse. When indicated, mice were treated with a single i.p. injection of α GalCer (2 μ g/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 μ g 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 2x10⁵ macrophages were isolated from the pancreas of Pro-ins2^{-/-} mice treated with CVB4 or CVB4+ α GalCer 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. Insulinitis 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 (Me1-14), CD4 (GK1.5), CD8 (53-6.7) anti-human Ki-67 (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 α GalCer-loaded CD1d tetramer was prepared in our laboratory. For cytokine stainings, cells were stimulated with PMA (10 ng/ml) and ionomycine (1 μ g/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 $2^{-\Delta\Delta C_t}$ 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 IGRP₂₀₆₋₂₁₄ peptide (VYLKTNVFL; 10 μ M) 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⁺ CD4 T cells (3×10^4) were cultured with anti-CD3/CD28 beads (Invitrogen) and 3×10^4 macrophages, isolated either from the pancreas of mice infected with CVB4 alone or infected and treated with α GalCer. After 48 h culture, wells were pulsed with 1 μ Ci tritiated thymidine ($[^3\text{H}]\text{-TdR}$) overnight. $[^3\text{H}]\text{-TdR}$ 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 μ M.

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 insulinitis. Interestingly, one single injection of the iNKT cell agonist, α GalCer, at the time of infection strongly decreased diabetes incidence in both NOD and Pro-ins2^{-/-} mice (13% and 25% respectively). Insulinitis was also reduced in CVB4+ α GalCer 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 α GalCer (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-1 β , IL-6 and TNF- α was significantly decreased in Pro-ins2^{-/-} and NOD mice from CVB4+ α GalCer 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, α GalCer 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 α GalCer alone, the strongest upregulation of iNOS and IDO required both α GalCer and CVB4 infection. Interestingly, Ym1/Ym2 mRNA was also upregulated in the islets from mice of CVB4+ α GalCer 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+ α GalCer group compared to CD45⁺ DCs (CD11c⁺), plasmacytoid DCs (CD11c^{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+ α GalCer 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 α GalCer 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 (CAM ϕ) 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). V α 14 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, α GalCer 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+ α GalCer 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+ α GalCer 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+ α GalCer group since 86% of mice became diabetic compared to only 27% of mice from CVB4+ α GalCer 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 insulinitis in mice from CVB4+ α GalCer 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+ α GalCer 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^{-/-} mice of CVB4 and CVB4+ α GalCer 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+ α GalCer 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+ α GalCer treated Pro-ins2^{-/-} 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+ α GalCer

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, α GalCer 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 α GalCer (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 α GalCer. Pancreas of mice that developed diabetes after CVB4 infection harbored macrophages with classically activated phenotype expressing high levels of IL-1 β , 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+ α GalCer 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 α GalCer activated iNKT cells. IFN- γ alone was not sufficient for diabetes protection since α GalCer

treated mice had similar diabetes incidence as untreated mice despite high levels of IFN- γ . Importantly, while CVB4 infection did induce some IDO, α GalCer 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 I μ ^{-/-} (11) and NOD Ca^{-/-} (data not shown) mice do not develop diabetes after CVB4 infection. Similarly, CVB4 infected Pro-ins2^{-/-} Scid mice did not become diabetic (data not shown). Diabetes development only in a subset of CVB4 infected Pro-ins2^{-/-} mice could result from differences in insulinitis 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-IGRP 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+ α GalCer treated mice resulted in a strong anti-IGRP 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

allograft setting (45). 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 α GalCer. However, we cannot rule out that IFN- γ , produced by α GalCer 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+ α GalCer 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 α GalCer 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 α GalCer. 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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No potential conflicts of interest relevant to this article were reported.

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 (1×10^5 PFU/mouse) or PBS and treated with α GalCer (α GC) or control vehicle (untreated n=14, α GalCer n=15, CVB4 n=14, CVB4+ α GalCer 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 Pro-ins2^{-/-} mice inoculated i.p. with CVB4 (1×10^5 PFU/mouse) or PBS and treated with α GalCer or control vehicle at 5-6 weeks of age (untreated n=44, α GalCer n=10, CVB4 n=36, CVB4+ α GalCer 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 \pm 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 (1x10⁵ 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 (1x10⁵ 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 \pm 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 α GalCer. 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 \pm 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 V α 14 transgenic Pro-ins2^{-/-} female mice inoculated with CVB4 or PBS and treated with α GalCer 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 \pm 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 α GalCer 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 \pm 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 α GalCer, 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+ α GC n=15, CVB4+ α GC+1400W n=12, CVB4+ α GC+nor-NOHA n=12, CVB4+ α GC+1MT n=15, CVB4+ α GC+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 insulinitis 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 α GalCer 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+ α GalCer 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 \pm 1MT (200 μ M). 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 \pm SD for 3 independent experiments. ** $p < 0.005$, *** $p < 0.005$, Mann Whitney. **(F)** Sorted macrophages

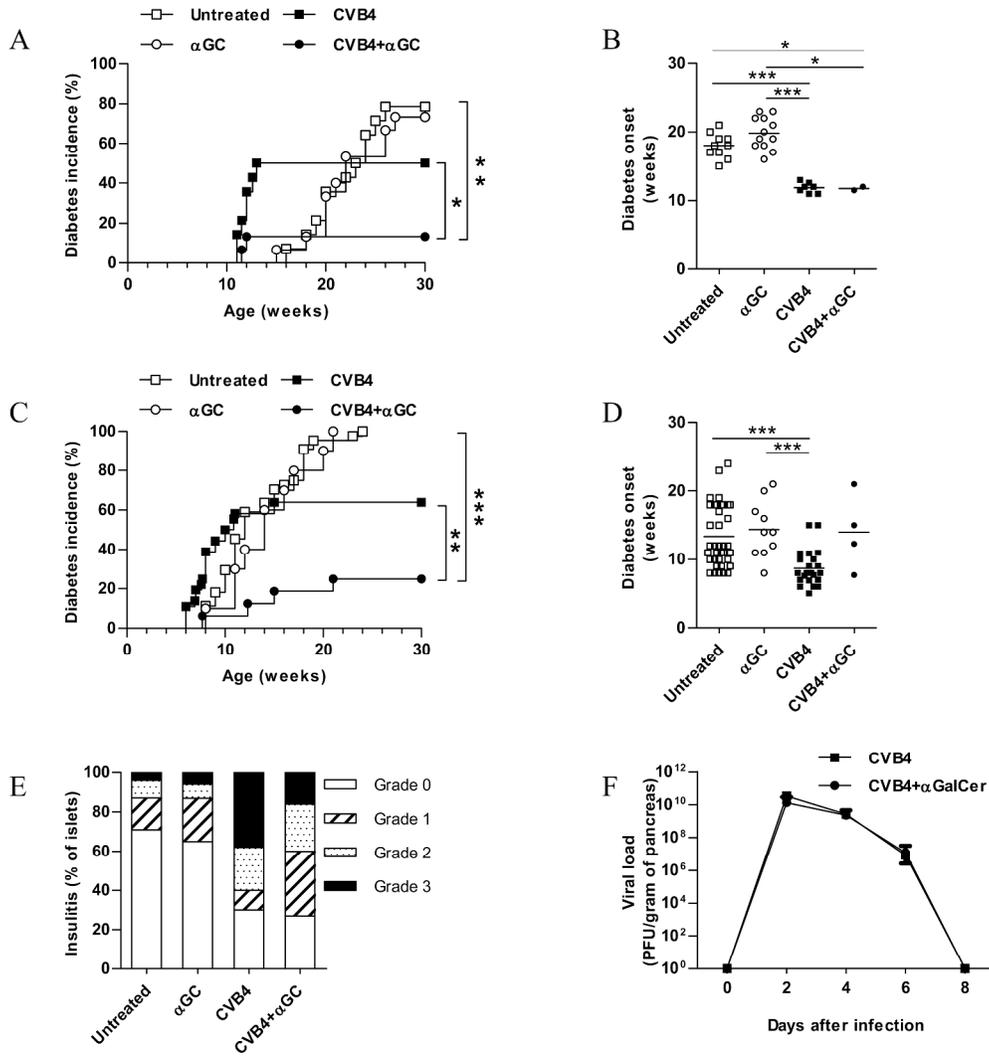
were injected i.v. (2×10^5 per mouse) into Pro-ins2^{-/-} females infected with a single dose of CVB4 (1×10^5 PFU/mouse) one day earlier. Data represent the incidence of diabetes of the three groups of mice (n=10 for each group) from two independent experiments. *p<0.05, using log-rank test analysis.

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 IGRP₂₀₆₋₂₁₄ peptide. **(D)** Graph showing the percentage of islet CD8 T cells secreting IFN- γ after IGRP₂₀₆₋₂₁₄ 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 (CAM ϕ) with low IDO expression and high secretion of proinflammatory cytokines IL-1 β , 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 α GalCer at the time of infection, they produce large amount of IFN- γ inducing a strong upregulation of IDO expression in islet infiltrating suppressive

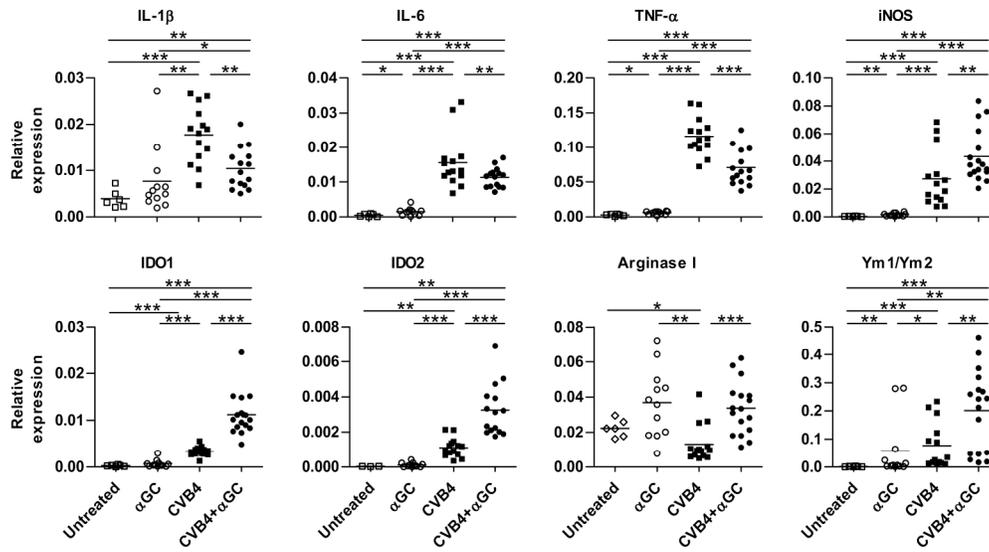
macrophages (MDSC). IDO expressing MDSC suppress IFN- γ production by anti-islet T cells in the pancreas, thereby preventing type 1 diabetes onset.

Figure 1



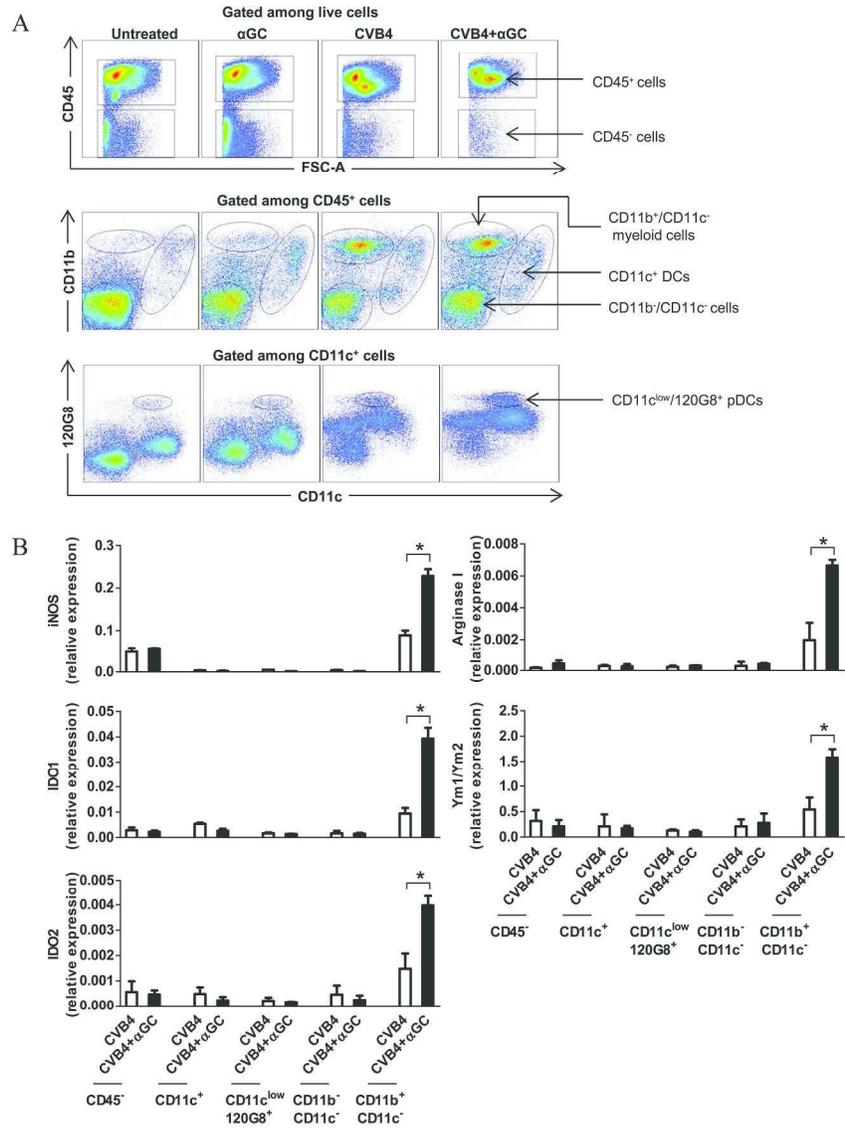
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Figure 2



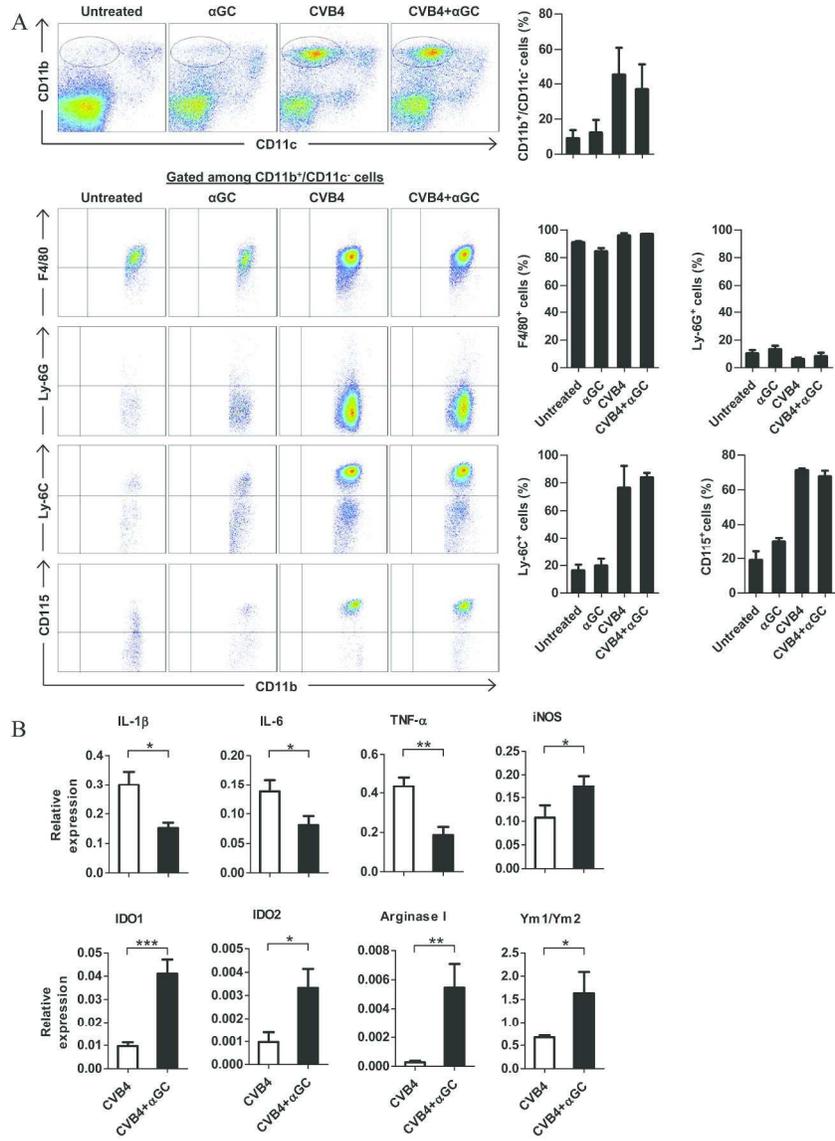
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Figure 3



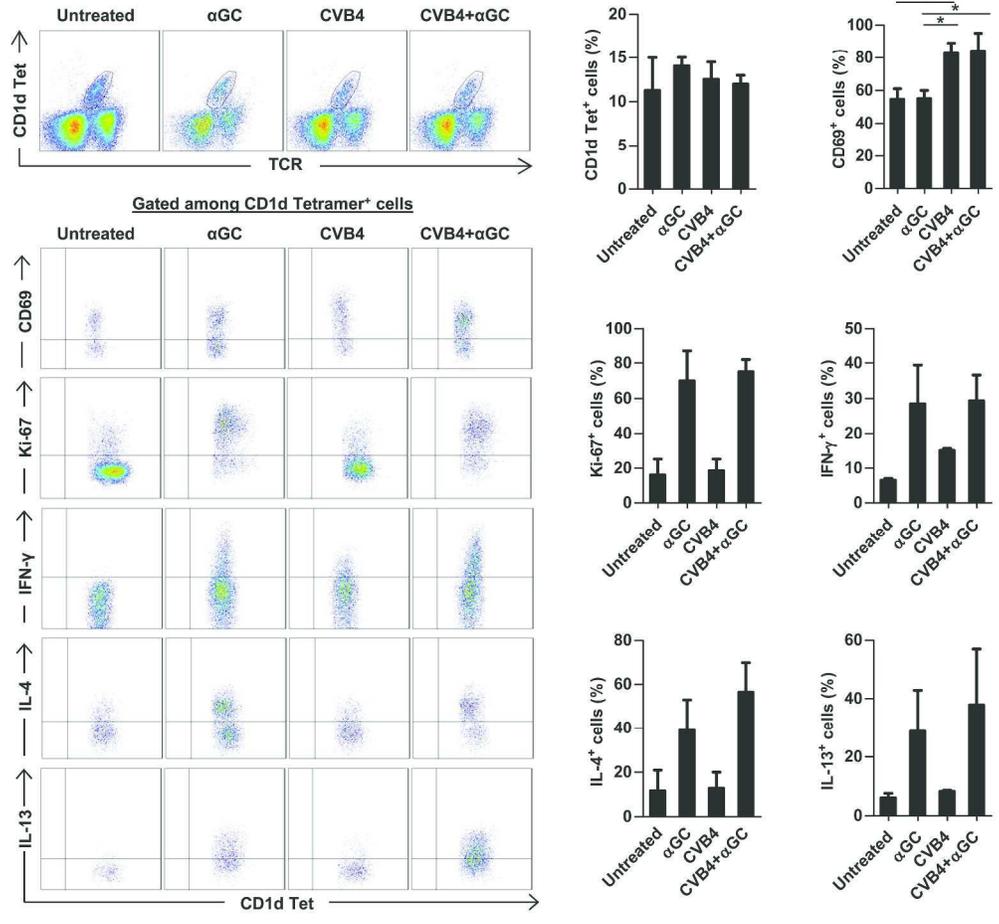
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Figure 4



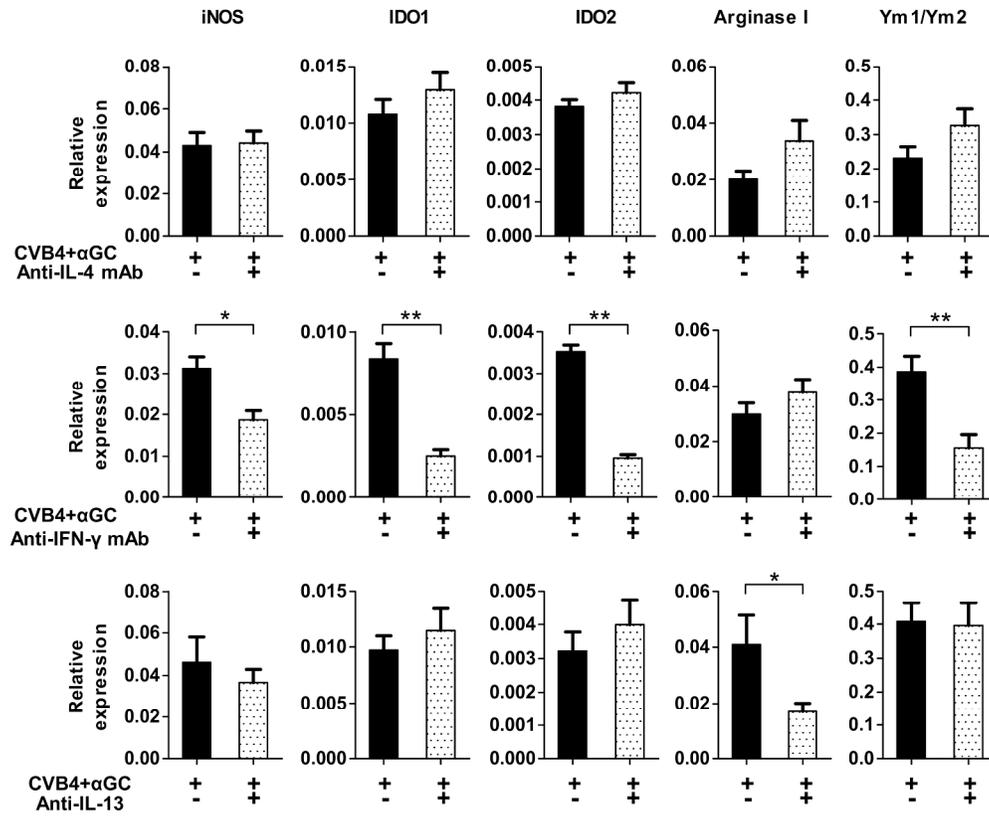
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Figure 5



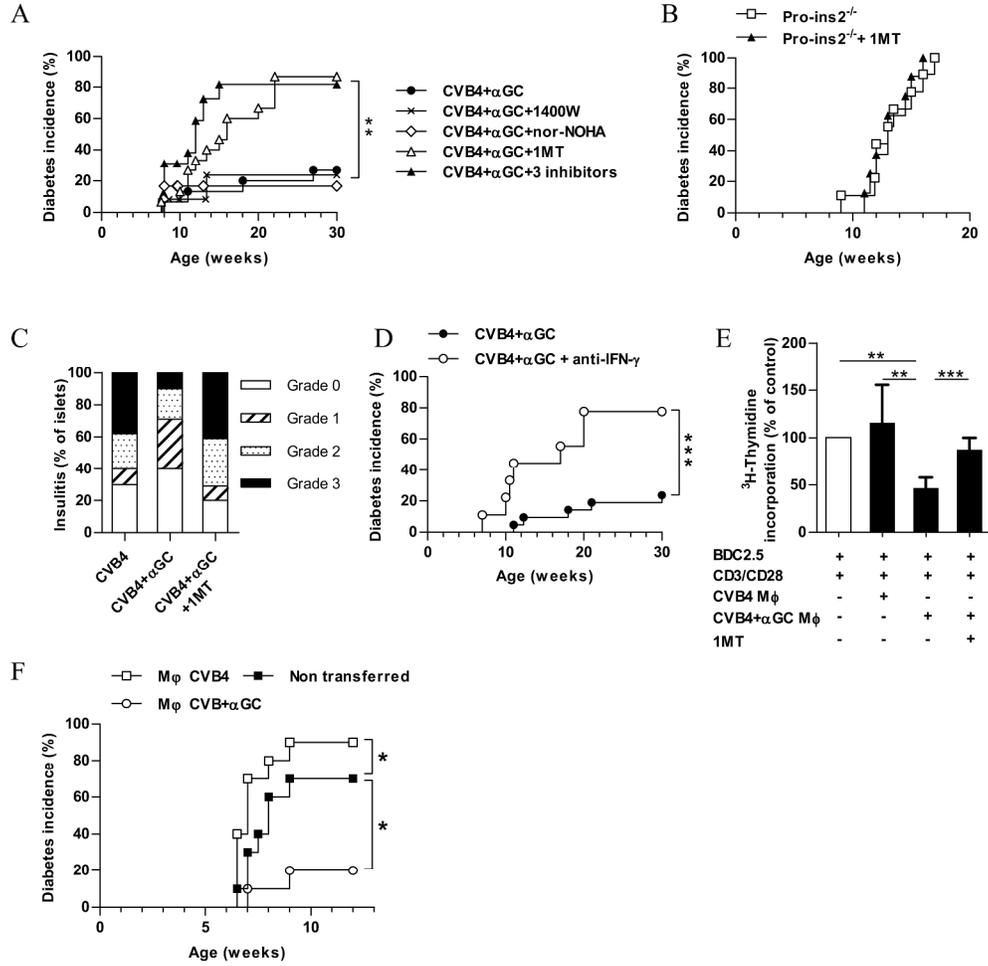
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Figure 6



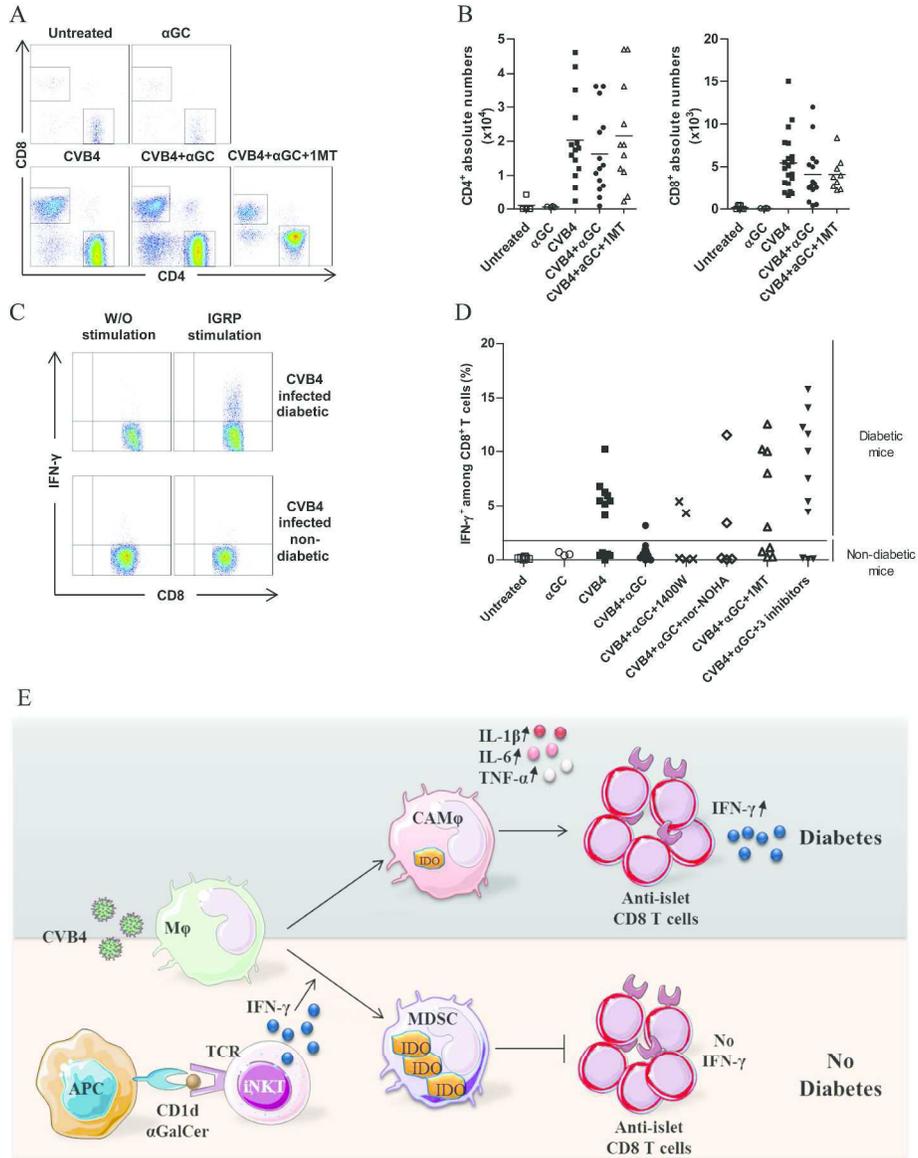
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Figure 7



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Figure 8



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Supplementary figure 1.

Female 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 α 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 (1×10^5 PFU/mouse) or PBS and treated with α 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 \pm 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 (1×10^5 PFU/mouse) or PBS and treated with α 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^{-/-} females at 5-6 weeks of age were inoculated i.p. with a single dose of CVB4 (1×10^5 PFU/mouse) or PBS and treated with α 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 \pm 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 α GalCer 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 \pm 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 α GalCer. 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 \pm 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 (1x10⁵ 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 (1x10⁵ 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 IGRP₂₀₆₋₂₁₄ 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.

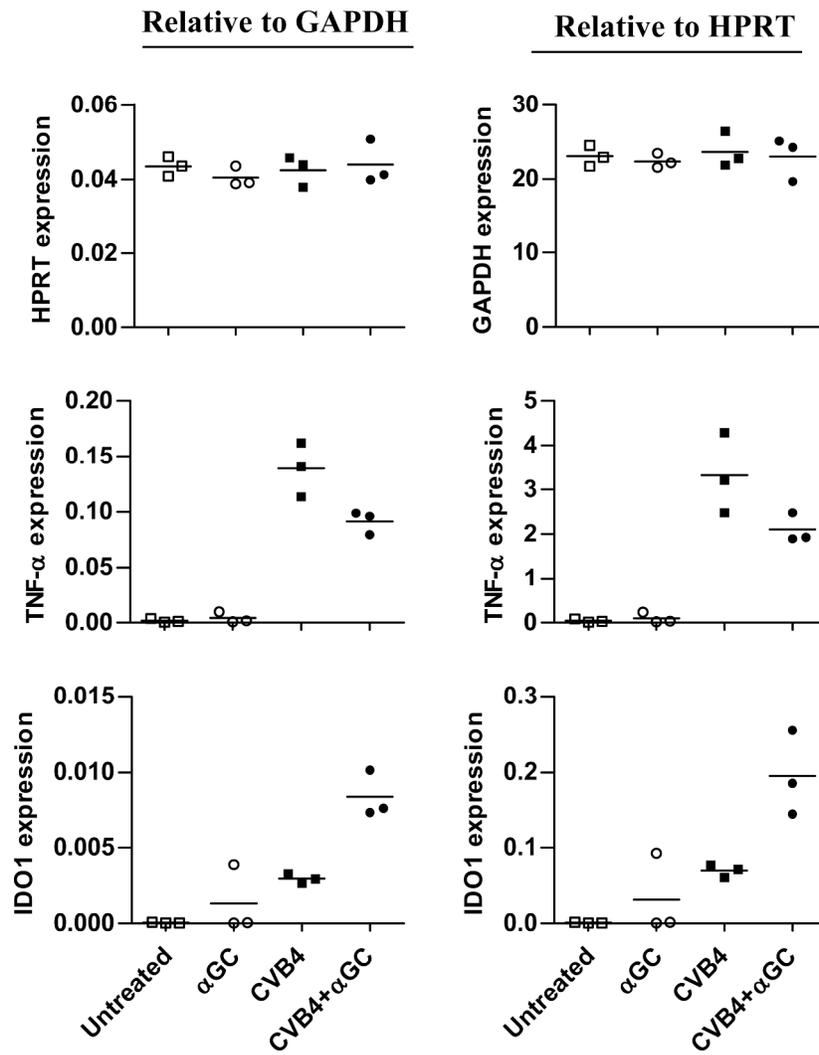
(A, B and C) Pro-ins2^{-/-} females at 5-6 weeks of age were inoculated i.p. with a single dose of CVB4 (1x10⁵ PFU/mouse) and treated with α GalCer or control vehicle. To deplete macrophages, mice were injected i.v. with 200 μ l of clodronate or control PBS loaded liposomes on days -1, +1 of virus infection (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 \pm SD of 2 pooled independent experiments. (CVB4 n=9, CVB4+clodronate n=8,

CVB4+ α GalCer n=9, CVB4+ α GalCer+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 (1x10⁵ PFU/mouse) and treated with α GalCer 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+ α GalCer+PBS n=11, CVB4+ α GalCer+clodronate n=12). Data represent 2 pooled independent experiments.

Supplementary figure 8.

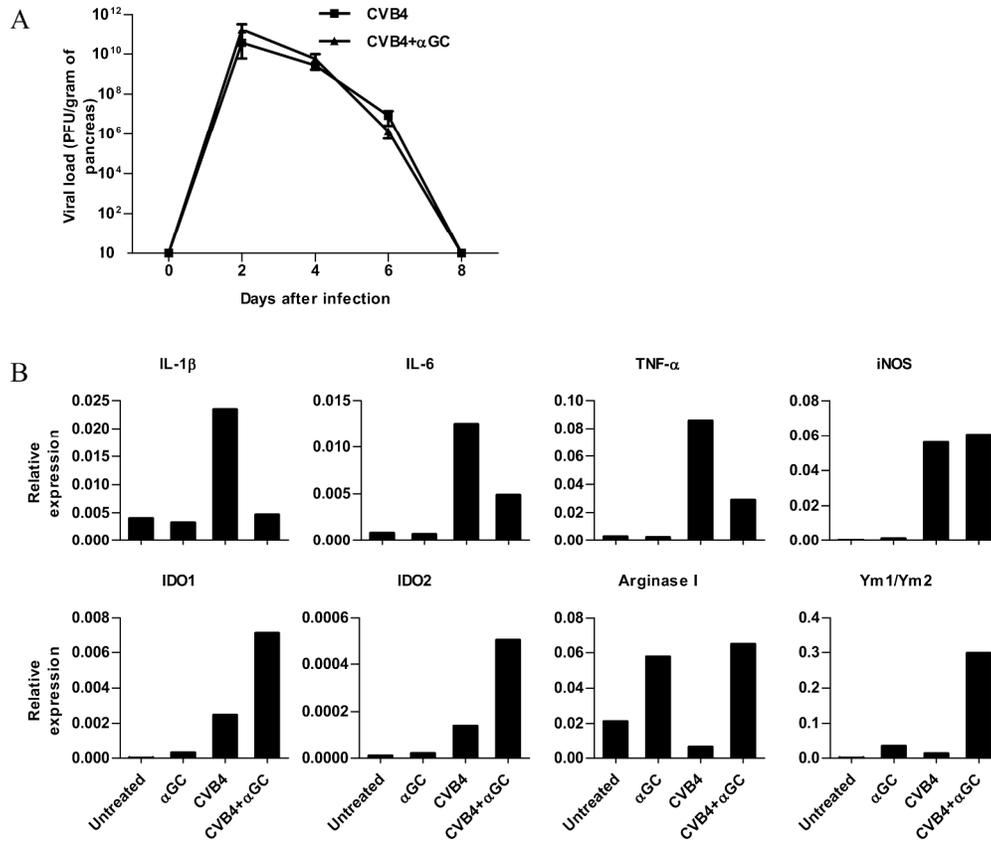
Female Pro-ins2^{-/-} mice at 5-6 weeks of age were inoculated i.p. with a single dose of CVB4 (1x10⁵ PFU/mouse) or PBS and treated with α GalCer or control vehicle. 7 days to three weeks after the infection, pancreatic islets were isolated, dissociated into single cell suspension and stained with different 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 \pm SD.

Supplementary online figure 1



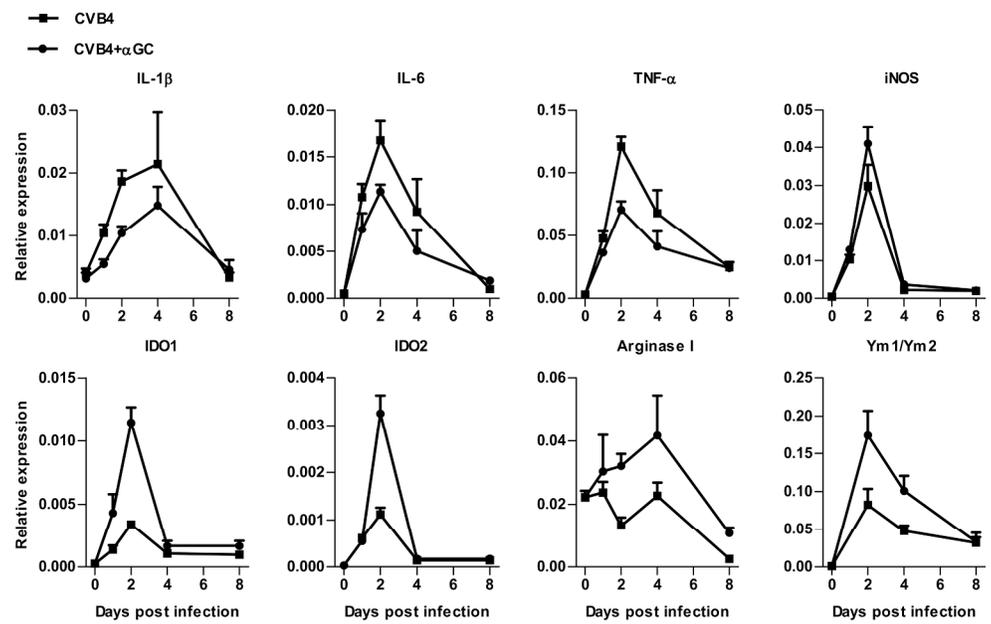
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Supplementary figure 2



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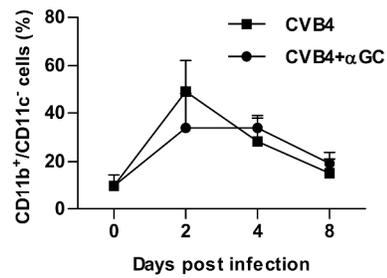
Supplementary figure 3



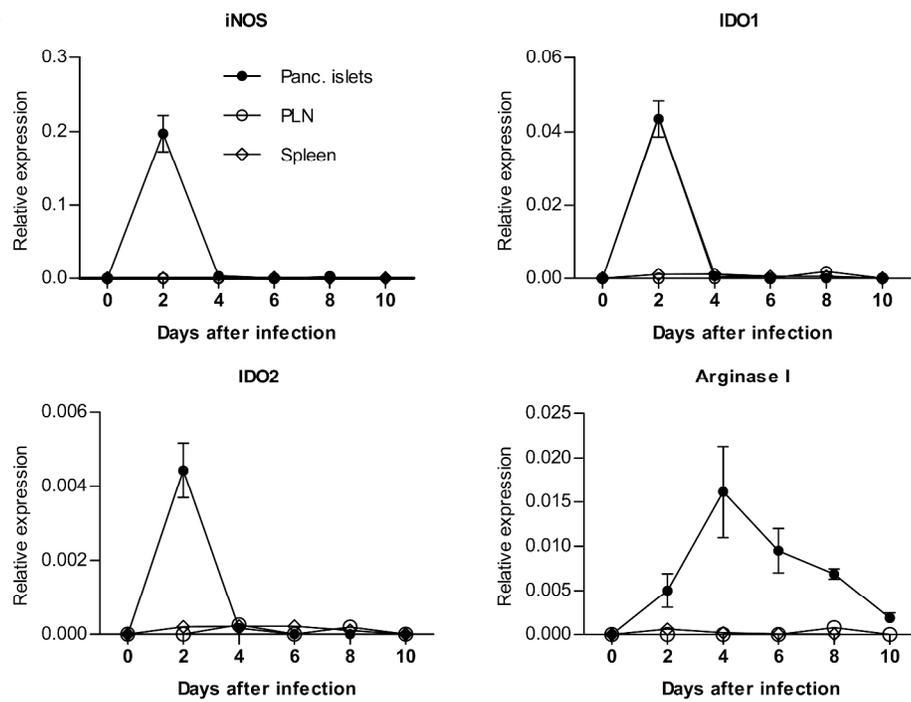
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Supplementary online figure 4

A

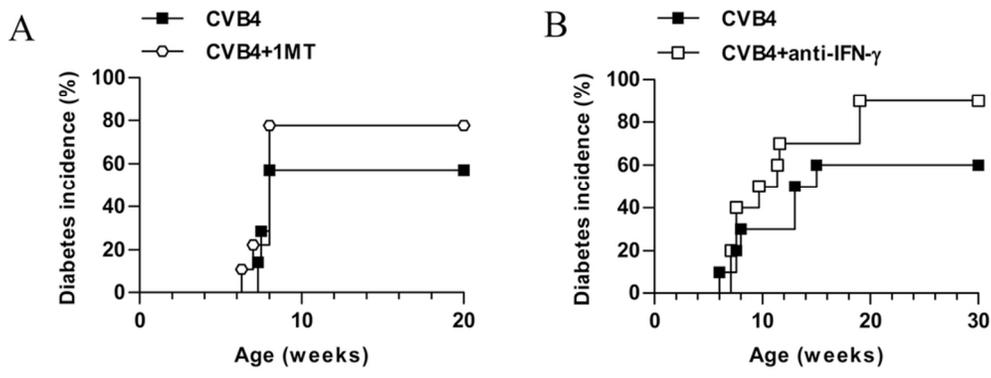


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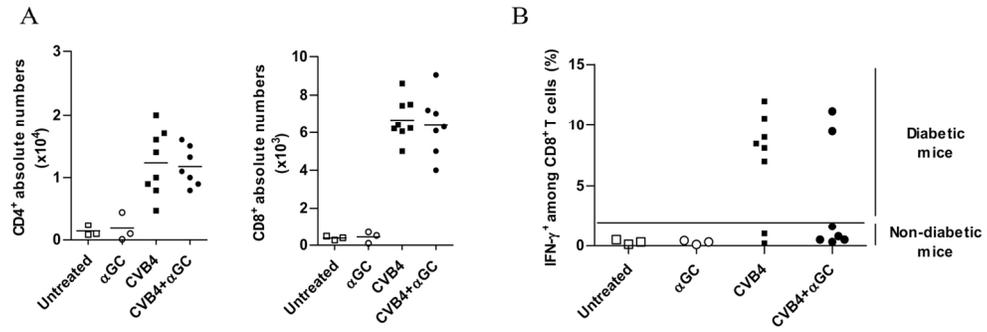
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Supplementary online figure 5



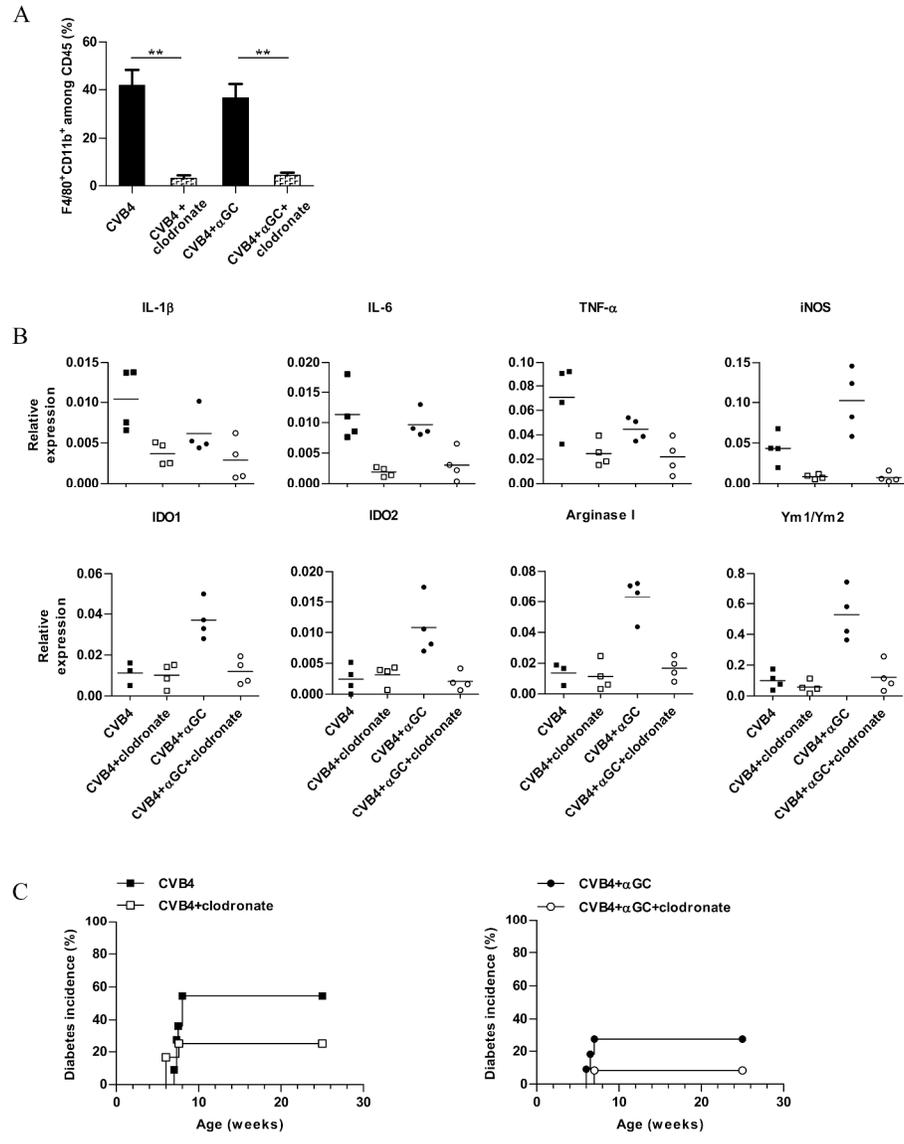
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Supplementary online figure 6



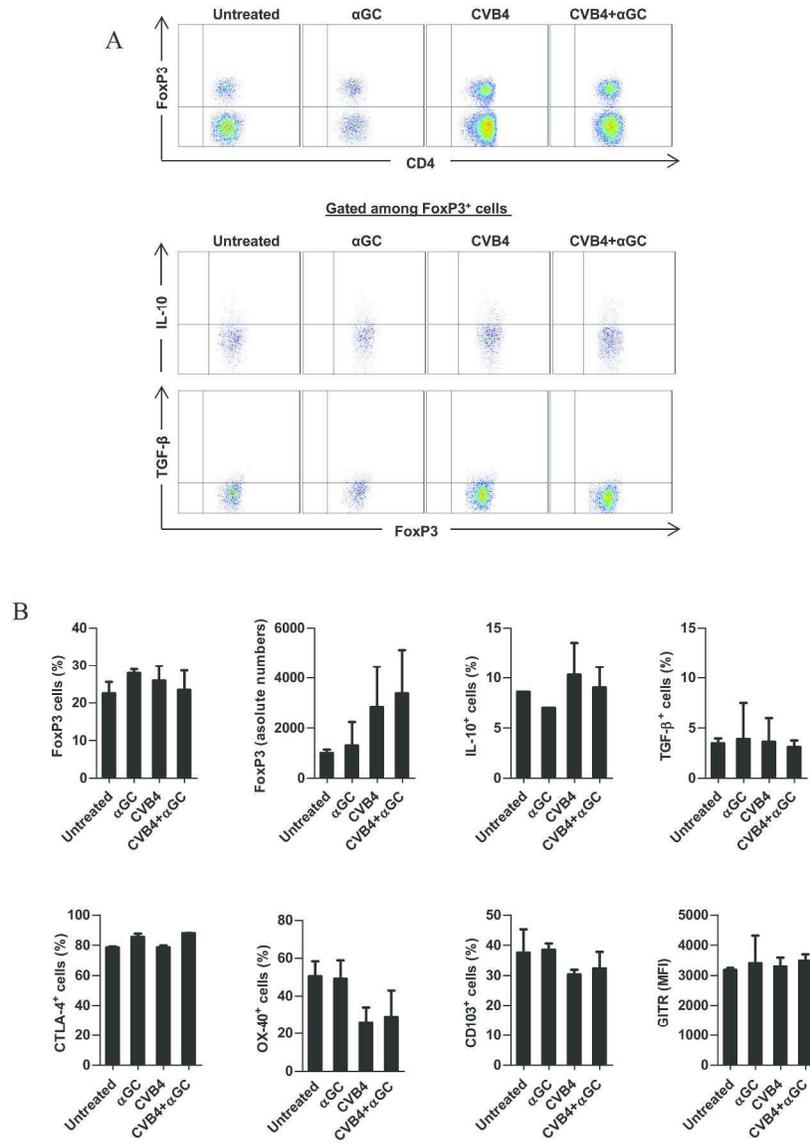
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Supplementary online figure 7



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Supplementary online figure 8



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Prevention or acceleration of type 1 diabetes by viruses

Liana Ghazarian · Julien Diana · Yannick Simoni ·
Lucie Beaudoin · Agnès Lehuen

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Abstract Type 1 diabetes is an autoimmune disease characterized by the destruction of insulin-producing pancreatic β -cells. Even though extensive scientific research has yielded important insights into the immune mechanisms involved in pancreatic β -cell destruction, little is known about the events that trigger the autoimmune process. Recent epidemiological and experimental data suggest that environmental factors are involved in this process. In this review, we discuss the role of viruses as an environmental factor on the development of type 1 diabetes, and the immune mechanisms by which they can trigger or protect against this pathology.

Keywords Diabetes · Environment · Virus · Coxsackievirus

Introduction

Type 1 diabetes (T1D) is an organ-specific autoimmune disease characterized by the destruction of β -cells within the islets of Langerhans in the pancreas. Once 80–90 % of the β -cells have been destroyed, insulin production becomes insufficient, resulting in hyperglycemia. T1D is considered a childhood disease because most patients develop T1D by 20 years of age, and accounts for 1–5 % of all diabetes cases. Studying diabetes is often difficult

because its development is very heterogeneous among patients. In some diabetic patients, it can develop rapidly without clear signs of autoimmunity, such as the presence of autoantibodies [1]. Other patients can have a subclinical phase of various durations, characterized by the presence of autoantibodies and autoreactive T cells recognizing islet antigens before the onset of overt diabetes [2]. Furthermore, some patients can harbor islet autoantibodies for many years without ever progressing to T1D [3]. The circulating autoantibodies and autoreactive T cells mostly target β -cell antigens such as proinsulin, glutamic acid decarboxylase (GAD), tyrosine phosphatase IA-2, islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) and chromogranin A [4]. It is not yet clear what initiates the breakdown of tolerance towards β -cells, but genetic and environmental factors have been implicated, both alone and in synergy.

Relationship between genetics, environment, and autoimmune diabetes

The frequency of autoimmune diseases has increased in recent decades. This rise includes both allergic diseases such as asthma, whose incidence has more than doubled since 1980 in the United States of America, rhinitis and atopic dermatitis [5, 6], and autoimmune diseases such as T1D [7, 8], multiple sclerosis [9] and Crohn's disease [10]. The annual increase in the frequency of T1D is estimated to be 3 % [11], although its global distribution is not homogenous. In fact, the distribution of many autoimmune diseases forms a gradient with the highest frequency in the north, which decreases towards the south of the northern hemisphere and from the south to the north in the southern hemisphere [12]. Such gradients have been observed for

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multiple sclerosis in the United States of America and Australia [13, 14] and for T1D, with the Canadian province of Newfoundland and Labrador and European countries having the highest rates [15]. In Europe, the highest incidence was observed in Nordic countries [16], with Finland being on top of the list (40.9/100,000/year) followed by Sweden (30/100,000/year) and Norway (20.8/1,000,000/year) while in most Asian countries the incidence was lower than 1/100,000/year. [17]. Genetic and environmental factors have been proposed to explain these differences and are discussed in this review.

Genetics

The development of T1D is under genetic control. Type 1 diabetes is particularly common in families with one or more diabetic siblings or first-degree relatives [18] and there is a high (40–60 %) concordance of diabetes in identical twins [19, 20]. More than 40 disease-susceptibility genetic loci have been identified in T1D, which include the genes coding insulin, cytotoxic T lymphocyte antigen (CTLA)-4, interleukin (IL)-2 receptor α , the tyrosine phosphatase PTPN22 and the intracellular viral RNA sensor IFIH1 [21]. However, the strongest risk for T1D is associated with HLA loci, also known as IDDM1 (insulin-dependent diabetes mellitus locus), particularly the HLA class II DR and DQ alleles, which is found in around 40 % of cases, although these loci can also confer protection against T1D. Interestingly, European countries with the highest incidence of T1D, such as Finland or Sardinia, also have the highest frequency of T1D-predisposing major histocompatibility complex (MHC) class II alleles, HLA DR3/4-DQ8 [22]. However, not all individuals carrying a susceptibility allele develop T1D, and the susceptibility alleles can have different effects in different nations. For example, while DR3/4-DQ8 alleles confer increased risk in Bahraini Arabs, they actually play a neutral role in the Lebanese population [23]. Meanwhile, Japanese individuals carry both susceptibility and protective alleles, and it is the balance between these alleles that contributes to the low incidence of T1D in Asia [24]. Thus, the distribution of different HLA alleles at least partly accounts for the differences in T1D incidence worldwide; however, other factors are also involved. For example, the frequency of susceptibility and protective HLA-DQ alleles is similar among children from Finland and Karelia, a neighboring region in Russia; however, the incidence of T1D in Finland is six times higher than that in Karelia [25]. Similarly, while the frequency of various alleles does not differ between Baltic States and Nordic countries, the incidence of T1D is higher in Nordic countries [26]. There is also a difference in the incidence of T1D between eastern and western Germany, even though both populations share the

same genetic background. Another striking observation concerns immigrant families. First-generation Pakistani children born in the United Kingdom have a similar rate of T1D as the local population (11.7/100,000/year), but this is ten times higher than in the incidence in Pakistan (1/100,000/year) [27]. Another recent study showed that children with non-Swedish parents but living in Sweden have an increased risk of T1D compared to children in their native countries [28]. All of these observations support the role of non-genetic factors in the etiology of T1D. Nevertheless, the genetic background is important because the incidence of T1D in Sardinian families migrating to a country with a lower incidence of T1D remains high, similar to that in their native Sardinia [29, 30]. Clearly, genetic susceptibility is a very strong factor underlying the development of T1D, but external factors might play a decisive role in either inducing or protecting against T1D.

Environment

It is clear that genetics is a risk factor that accounts for at least some of the pattern of T1D distribution; however, it cannot explain the rapid worldwide increase estimated to be 3 % per year [31]. If this rise was solely dependent on genes, one would expect an increase in the frequency of predisposing HLA alleles among newly diabetic patients. However, this is not the case. In fact, the frequency of susceptibility alleles has actually decreased while that of protective HLA alleles has increased among newly diagnosed children [32, 33]. Therefore, the role of changing environmental factors has been proposed and examined in epidemiological and animal studies. As a result, epidemiological studies have suggested an association between the incidence of T1D and socio-economic status, which reflects the exposure to microbial agents and dietary habits. Sun exposure and vitamin D intake have also been proposed to influence T1D onset.

Socio-economic status and the role of infectious diseases

The European north–south gradient not only correlated with T1D distribution but also with the degree of development and national growth income of the countries in Europe. The richest and most developed countries have the highest incidence of T1D [34]. There are numerous differences between poor and rich countries, and one particularly interesting finding relevant for T1D is the increase in hygiene and decrease in infection because both phenomena are very recent. Developed countries have better hygiene because they invest in general cleanliness of cities, in education, and in medical care such as vaccination. These approaches have eliminated the favorable niches where pathogens used to proliferate, such as sewage, thus limiting the numbers of pathogens. In addition, if an

infection occurs, its spread is often better controlled through greater accessibility to drugs and campaigns aimed at instilling people to follow basic rules of hygiene, such as frequent hand washing. Consequently, people are no longer exposed to the wide variety of pathogens that they used to be, and the age at which children encounter such pathogens has increased. Consistent with these observations, the increasing frequency of immune-mediated diseases such as T1D, allergy, and asthma in Europe has been correlated with the decreasing rate of infections with enteroviruses, tuberculosis, and hepatitis B or C viruses.

In 1989, David Strachan, a scientist studying the relationship between autoimmune hay fever, hygiene, and household factors, proposed that frequent encounters with parasites, bacteria, and viruses in early childhood favor the development of a balanced immune system [35]. Otherwise, the untrained immune system may develop inappropriate immune reactions to the self, thus provoking autoimmune diseases. His proposal was later coined “Hygiene Hypothesis”, and has since been applied to numerous autoimmune and inflammatory diseases including T1D, multiple sclerosis, Crohn’s disease, inflammatory bowel disease, allergy, and asthma. This hypothesis is supported by the observation that, in large families, the incidence of T1D is lower among the youngest children than in their oldest siblings, possibly because the youngest children are more exposed to pathogens brought home by their siblings. Most importantly this happens from a very young age. In a similar way, children who attend daycare less frequently develop autoimmune diseases compared to children who are kept at home and who do not socialize with other children as much.

Protective role of parasitic and bacterial infections

Parasites and bacteria can inhibit the development of T1D in animal models [36]. For example, infection with *Schistosoma mansoni* or injection of *S. mansoni* egg soluble antigen (SEA) prevents T1D in non-obese diabetic (NOD) mice [37]. Treatment with SEA induces a shift in the cytokine profile of dendritic cells (DCs) that produce less pro-inflammatory interleukin IL-12 and more suppressive tumor growth factor (TGF)- β . *Schistosoma mansoni* soluble egg antigen also promotes the differentiation of type 2 macrophages and the skewing of T lymphocytes towards the production of IL-4 and IL-10 [38]. Gastrointestinal parasites, such as *Trichinella spiralis* or *Heligmosomoides polygyrus*, can also inhibit the development of diabetes in NOD mice by diminishing insulinitis, inducing the secretion of cytokines such as IL-4, IL-10, and IL-13, and skewing T lymphocyte responses towards a T helper (Th)2 profile [39, 40]. Infection with the nematode filarial activates Th2 T cells and FoxP3⁺ Tregs and protects NOD mice from T1D [41]. These mice are

also protected from T1D by *Salmonella* infection, which upregulates the inhibitory programmed cell death one ligand one (PD-L1) receptor [42]. Recent studies have also highlighted the role of the gut microbiota in regulating the development of T1D in NOD mice [43]. Infection with a laboratory strain of *Mycobacterium avium* induced the expression of the death receptor Fas on autoreactive T lymphocytes, which enhanced their killing by other immune cells [44, 45]. Interestingly, the *M. avium* subspecies paratuberculosis is currently a focus of research as a possible trigger of T1D in humans [46–48]. Since many pathogens can prevent T1D, elucidation of the underlying mechanisms will provide new knowledge that could be used to develop therapeutic strategies to protect against T1D.

Cow’s milk

Early introduction of cow’s milk into the diet of children has been proposed to trigger T1D. Enhanced expression of antibodies to cow’s milk was observed in children who later develop T1D [49]. Elliott et al. [50] compared the consumption of milk proteins in 14 different countries and found that the incidence of diabetes increased with increasing consumption of milk protein β -casein A1 and the B variants, with Nordic countries having the highest intake. Iceland, where the consumption of these two proteins is lower than in Nordic countries, has a lower incidence of T1D despite similar environmental conditions [51]. Other milk proteins such as lactoferrin, bovine serum albumin, and immunoglobulin against bovine insulin are considered harmless [52]. However, other studies found no such associations and the causative role of cow’s milk remains unconfirmed. It is possible that the increased consumption of cow’s milk in young infants simply reflects the decreased tendency towards breastfeeding in wealthy Western countries.

Wheat and gluten

Type 1 diabetes and celiac disease, an immune disorder characterized by intolerance to gluten present in some cereals, share several common susceptibility HLA alleles and non-HLA alleles, such as CTLA-4 and C–C chemokine receptor type 5 [53]. Celiac disease is more frequent among T1D patients than in control subjects [54]. Therefore, dietary gluten was proposed as a link between the gut, immune activation in gut-associated lymphoid tissues, and the development of T1D. The German Babydiab Study of more than 1,600 children with T1D parents showed that the introduction of gluten before 3 months of age increased islet autoantibody risk [55]. The Babydiab investigators are currently investigating whether eliminating gluten in genetically susceptible newborns during the first year of life can delay or decrease T1D incidence.

Sun and vitamin D

The distribution of T1D along the north–south gradient is correlated with exposure to sunlight and, consequently, the amount of vitamin D produced in the presence of solar ultraviolet B rays. Vitamin D was suggested to play a protective role because of its immunosuppressive capacity, and countries with greater solar exposure and enhanced vitamin D synthesis generally show a reduced incidence of T1D [56–59]. Mohr et al. [60] reported that each time the daily recommended dose of vitamin D was lowered in Finland (from 4,500 to 2,000 IU in 1960s, to 1,000 IU in 1975, and to 400 IU in 1992), the incidence of T1D increased sharply. However, other researchers have presented contradictory results.

Dietary supplementation of vitamin D in pregnancy or in the first year of life in children with little sun exposure was reported to reduce the risk of T1D in some studies, but not all [61–65]. Importantly, sunlight exposure cannot explain the differences in T1D in adjacent Finland and Karelia or in eastern and western Germany, where sunlight exposure is the same. Moreover, the incidence of T1D in Sardinia remains high, despite high sunlight exposure. Therefore, the contribution of this factor to the development of T1D remains under investigation.

Role of viruses in T1D

Viruses have been documented as possible causative agents of T1D in humans, and can act via numerous mechanisms. One of these mechanisms is molecular mimicry in which a pathogen-derived peptide shows sequence homology with a self-peptide and the host's T cells mistakenly attack

self-tissue. Another mechanism is bystander activation of T cells. Viral infection can provoke significant inflammation and destruction of its target tissue with subsequent release of autoantigens that can activate autoreactive T cells. Inflammation can also induce stress in the endoplasmic reticulum, causing misfolding of proteins and the creation of new autoantigens. Even if the initial amount of autoantigen released is minimal, the small pool of autoreactive T cells, by killing target cells, could provoke the release of normally sequestered autoantigens from β -cells, a process known as antigen spreading [66].

Viruses can also protect against T1D by several mechanisms. First, when an organism is subjected to repetitive infections, its resources can be used in priority for the expansion and action of antipathogenic immune cells, which limits resources available for autoreactive cells and may therefore control their numbers and activation [67, 68, 12]. Second, natural selection has made some pathogens able to modify or dampen the host's immune responses to promote their own survival. For example, some viruses can alter the functions of macrophages and antigen-presenting cells [69, 70] while other viruses induce the proliferation and differentiation of regulatory T cells (Tregs) [71, 72]. These mechanisms favor the maintenance of peripheral tolerance and prevent T1D onset. Moreover, not only pathogens can directly alter the immune system, but the human body can also promote immune regulatory mechanisms to avoid exacerbated responses that could damage host tissues.

Viruses and acceleration of T1D

Several murine and human viruses such as rubella, mumps, rotavirus, and cytomegalovirus (CMV) can trigger the

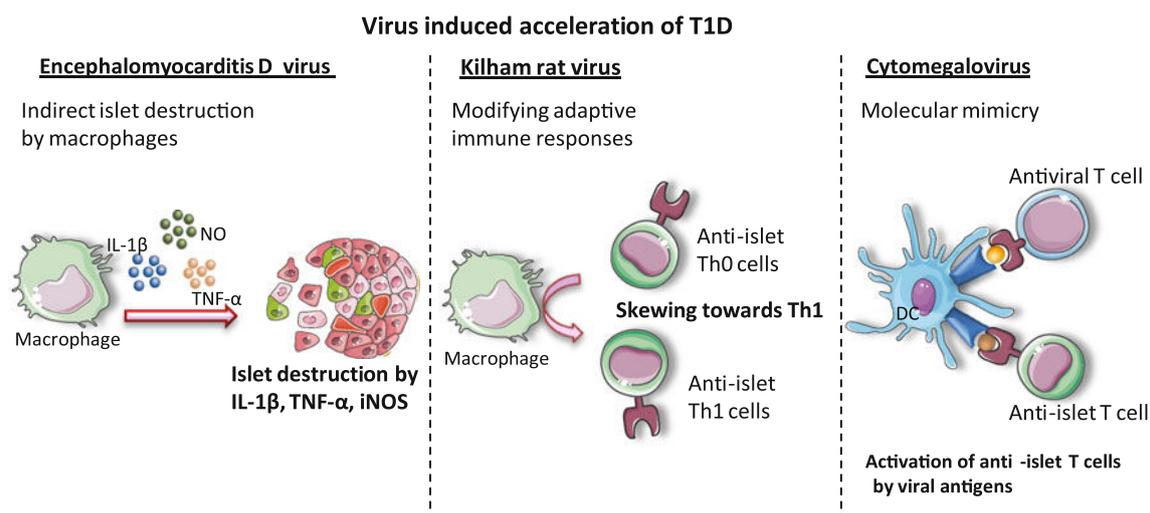


Fig. 1 Virus-induced acceleration of T1D. Viruses can accelerate T1D by inducing immune cells such as macrophages to produce proinflammatory cytokines that can kill pancreatic β -cells or by

skewing immune responses towards generation of pathogenic anti-islet Th1 cells. Molecular mimicry can be another mechanism leading to the activation of autoreactive anti-islet T cells

development of diabetes (Fig. 1). It is important to note that the induction of diabetes by these viruses involves various mechanisms, and the detection of anti-islet autoimmune responses remains elusive for rubella and mumps. The association with a particular HLA allele and/or the identification of antigenic epitopes shared by viruses and islet antigens suggests that specific anti-islet responses could occur.

Infection with rubella in the first trimester of pregnancy was associated with a 20 % higher incidence of diabetes in children born with congenital rubella infection [73]. A relationship between rubella and diabetes has been shown in rabbits and hamsters [73, 74]. In New York, the incidence of diabetes increased after a rubella epidemic affecting mostly genetically predisposed children. In a cohort of over 200 infected patients, there was a decrease in the frequency of the HLA-DR2 allele and an increase in the HLA-DR3 allele among diabetic patients [75]. Molecular mimicry between human GAD65 and rubella virus protein was suggested as a triggering factor [76]. Despite the possible influence of HLA alleles and the described epitope mimicry, rubella-induced diabetes might differ from classical T1D since rubella infection affects many other organs as well [77].

Mumps virus infection was suggested to be associated with the presence of pancreatic autoantibodies [78]. Interestingly, after virtually eliminating mumps infection through a vaccination campaign in Finland, a plateau in the T1D incidence was observed [79]. However, little is known about the autoimmune process induced by mumps virus.

Several studies by Honeyman and colleagues have pointed towards a role of rotaviruses in T1D induction. For example, the appearance of antiviral IgG was linked to the appearance of autoantibodies against GAD65, insulin, and IA-2 in the Australian BabyDiab Study involving at-risk children [80]. The same authors also showed that rotavirus could infect pancreatic cells from NOD mice and macaque monkeys [81]. Interestingly, the rotaviral VP7 protein shows high sequence homology with islet autoantigens, tyrosine phosphatase IA-2, and GAD65. T cells cross-reacting with VP7 peptides and epitopes of IA2 and GAD65 were recently characterized in T1D patients. These peptides bind strongly to HLA-DRB1*04, which confers susceptibility to T1D [82]. All of these findings support the hypothesis that molecular mimicry between VP7 and islet autoantigens could facilitate the development or exacerbation of anti-islet autoimmunity and T1D onset. However, a Finnish study failed to find a link between rotavirus and T1D [83]. Concerning mouse models, high replicative rate of rhesus monkey rotavirus strain RRV infection was shown to protect NOD mice from [84].

In humans, initial reports found a correlation between CMV infection and the presence of anti-islet autoantibodies [85]. Molecular mimicry between CMV peptide and GAD65

has been proposed [86]. However, other studies failed to find a link between CMV infection and T1D in genetically predisposed children [87–89]. Interestingly, CMV can trigger T1D in susceptible LEW.1WR1 and BioBreeding (BB) rats [90, 91], and a recent study showed that CMV can infect human β -cells and induce the expression of inflammatory cytokines and chemokines [92].

Besides these viruses that can infect both humans and rodents, two other viruses whose natural host is a rodent are used in laboratory studies to decipher the mechanisms of viral pathogenesis. Even though encephalomyocarditis D (EMC-D) virus can directly infect β -cells, the mechanism by which it induced T1D is mostly indirect. After low-dose EMC-D virus infection, the infected macrophages were shown to be involved in the development of diabetes in DBA/2 mice through the production of mediators such as IL-1 β , tumor necrosis factor (TNF)- α and inducible nitric oxide synthase (iNOS). The depletion of macrophages or the inhibition of these three mediators decreased the incidence of T1D [93]. Interleukin-1 β and tumor necrosis factor- α can induce the expression of the death receptor Fas on β -cells, and subsequent binding of Fas to its ligand can lead to β -cell apoptosis. Notably, nitric oxide produced by iNOS can directly induce β -cell apoptosis.

Several laboratories have analyzed the pathogenic role of the Kilham rat virus (KRV) in T1D induction in diabetes-resistant BB rats [94, 95, 90]. Even though the KRV does not infect β -cells, it skews the immune responses towards a deleterious Th1 profile [94]. Subsequent studies showed that KRV induces the production of the proinflammatory cytokines, IL-12 and IL-6. Interestingly, IL-12 production was dependent on Toll-like receptor (TLR) 9, and blocking of TLR9 by chloroquine prevented the development of T1D in BB rats [95].

Protective effects of viruses against T1D

Because the protective role of viruses in T1D in humans can only be observed through the correlation between infection and reduced T1D incidence, the mechanisms by which viruses confer protection against T1D were mainly studied in rodent models [36]. Non-obese diabetic mice have been widely used because they spontaneously develop diabetes with many characteristics of human T1D. Interestingly, these mice less frequently develop T1D in animal facilities that are not pathogen-free or after treatment with pathogen-derived molecules. For example, a single Bacillus Calmette-Guérin injection is sufficient to inhibit T1D when administered to young NOD mice [96]. Similarly, treating NOD mice with complete Freund's adjuvant reduced the number of diabetogenic T cells, while incomplete Freund's adjuvant skewed the T cell responses towards a Th2 profile protecting against T1D [97, 98].

The protection of T1D by viral infection has been extensively analyzed using lymphocytic choriomeningitis virus (LCMV) (Fig. 2). Twenty years ago, Oldstone and colleagues [99, 100] reported that LCMV infection in diabetes-prone rats and NOD mice decreased or prevented the development of autoimmune diabetes. Subsequent studies have shown that the protection against diabetes is associated with an interferon (IFN)- γ -induced protein-10 chemokine gradient, with the highest concentration in pancreatic lymph nodes resulting in the attraction of activated T lymphocytes in this tissue, followed by their apoptosis [101]. More recently, we reported that the protective role of LCMV is dependent on a small lymphocyte population called invariant natural killer T (iNKT) cells because of their expression of an invariant V α 14J α 18 T cell receptor and typical natural killer (NK) cell receptors. These innate-like T cells exhibit various regulatory and effector functions, and inhibit the development of spontaneous T1D by dampening pathogenic autoimmune responses [102–104]. In the context of LCMV infection, iNKT cells mediate protection against spontaneous diabetes by promoting the recruitment and the activation of plasmacytoid DCs (pDCs) into the pancreas. Plasmacytoid dendritic cells produce large amounts of IFN- α and rapidly

inhibit viral replication in the pancreas. This control of viral replication is a key factor in the inhibition of pancreatic tissue destruction and T1D in transgenic mice expressing the nucleoprotein of LCMV in β -cells [105]. In a second step, pDCs migrate from the pancreas to the pancreatic lymph node where they produce TGF- β and induce the conversion of naive T cells into Tregs. These induced Tregs migrate to the pancreas and locally produce TGF- β , which suppresses anti-LCMV and anti-islet T cell responses, thereby inhibiting the destruction of pancreatic islets and the development of LCMV-induced and spontaneous T1D [71].

Protection against T1D was also observed after infection with coxsackievirus B3 (CVB3). Two mechanisms act in synergy to delay and reduce the onset of T1D. First, CVB3 infection upregulates the expression of the inhibitory receptor PD-L1 on lymphoid cells, which hinders the expansion of PD-1-expressing autoreactive CD8⁺ T cells and delays T1D onset. Second, CVB3 infection enhances the proliferation of Tregs, which produce TGF- β to confer long-term protection against diabetes [72]. Studies of the CVB4 strain, which is able to both induce and protect against diabetes, depending on the context, will be discussed later in this review.

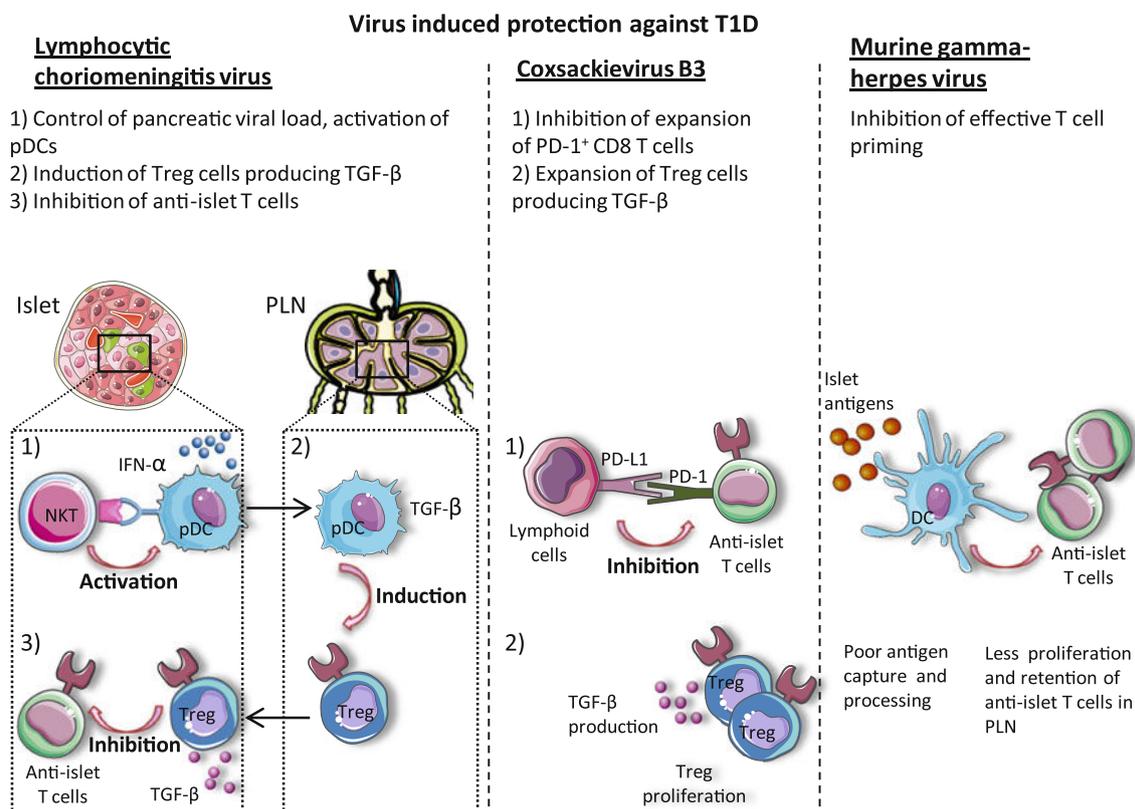


Fig. 2 Virus-induced protection against T1D. Viruses can induce protection from T1D by eliciting immune mechanisms such as induction of Treg cells, inhibition of anti-islet T cells through

suppressive receptors like PD-1 and suppressive cytokines like TGF- β or by blocking the efficient antigen capture and processing by antigen-presenting cells

Infection of NOD mice with the murine gammaherpesvirus-68 delays the onset of T1D by reducing the capacity of CD11c⁺ DCs to capture and process autoantigens. This results in the retention of autoreactive T cells in the spleen and pancreatic lymph node, limiting their homing to the pancreas and the killing of islet β -cells. The numbers of Tregs do not change during the course of infection [69].

Even though the reovirus strain type 3 Abney (T3A) can infect β -cells and induce their apoptosis, the infection of newborn NOD mice with this virus reduces or delays the onset of T1D without preventing insulinitis [106]. While the exact mechanism by which the T3A strain inhibits T1D has not been determined, the authors proposed two non-exclusive scenarios. The first one is based on the observation that the T3A strain infects various organs, including the thymus. Infection of the thymus of young animals could somehow eliminate autoreactive T cells in this tissue. In the second scenario, the infection and destruction of pancreatic islets could lead to the release of β -cell antigens and the establishment of an active tolerance through Treg cells.

Chronic mouse hepatitis virus (MHV) infection can prevent the development of T1D in NOD mice [107]. Studies outside of the context of T1D have shown that MHV can, in some cases, suppress the activation of splenic T lymphocytes and reduce the functions of antigen-presenting cells, such as macrophages, DCs, and B lymphocytes [108, 109].

Finally, lactate dehydrogenase virus infection also suppresses T1D onset in NOD mice by reducing the numbers of inflammatory macrophages in the peritoneum [70].

Dual role of enteroviruses in T1D

The role of enteroviruses in T1D is of great interest. They seem to represent a perfect illustration of the hygiene hypothesis. In times when enterovirus infections were frequent, the incidence of diabetes was low [110, 111]. Currently, however, the rarity of enterovirus infection is thought to make them more aggressive insusceptible individuals and can favor the onset of T1D.

The enterovirus genus belongs to the Picornaviridae family. Their genome is composed of a single positive RNA molecule encapsulated in capsid without an envelope. Human enteroviruses include polioviruses, echoviruses, rhinoviruses, enterovirus 71, and coxsackieviruses, which is the most prevalent group after the introduction of poliovirus vaccination. Coxsackieviruses are divided into two groups, A and B, with 24 and six serotypes, respectively. Coxsackievirus group B (CVB), particularly CVB4, is widely implicated in T1D. Infections with enteroviruses are mostly asymptomatic and only rarely cause complications such as hand, foot, and mouth disease, acute hemorrhagic conjunctivitis, aseptic meningitis, myocarditis, and severe neonatal

sepsis-like disease. They are predominantly transmitted via the oral–fecal route (i.e., consumption of food or water containing contaminated feces).

Epidemiological perspectives of enteroviruses

Studies examining the relationship between enteroviruses and T1D probably started with two articles published by Gamble and Taylor in 1969. In their first article, they reported that patients with a recent onset of T1D (<3 months) had higher antibody titers against CVB4 compared to control individuals and patients whose T1D was not recent [112]. In their second article, they reported a seasonal onset of T1D with peaks in late fall/early winter, which followed the seasonal outbreak of CVB infection in late summer/early fall [113]. Since then, many studies have analyzed the role of CVB in T1D. The major difficulty in providing direct proof for the pathogenic role of enteroviruses is the interval between enteroviral infection and onset of T1D, meaning the enteroviral infection may be undetectable. Enteroviral infection is mostly identified by the detection of viral RNA in the serum. However, in healthy individuals, enteroviral RNA is detectable for only a few days after infection and most studies analyzed serum samples at intervals of several months. Nevertheless, with the exception of a few studies [114, 115], more than 24 retrospective and prospective studies have detected a link between enteroviruses and T1D [116].

Prospective studies

In 1995, the Finnish (DiMe) study group showed that enterovirus infections, identified by the presence of antibodies against enteroviral antigens, were almost two times more frequent in siblings who developed clinical T1D compared to siblings who remained diabetes-free [117]. Similarly, in the Finnish Diabetes Prediction and Prevention (DIPP) Study, anti-enteroviral antibodies were detected in 26 % of children who developed diabetes compared to 18 % of children in the control group. They reported a temporal relationship between enteroviral infection and the onset of T1D since 57 % of cases had autoantibodies present within 6 months after infection [118]. Similar results were observed in the Finnish trial to reduce IDDM in genetically at-risk (TRIGR) project, which revealed that enteroviral RNA was more frequently detected in children developing autoantibodies than in children who did not have autoantibodies [119]. The diabetes and autoimmunity study in the young (DAISY) conducted in Denver, CO, USA, followed 2,365 at-risk children, of which 140 subsequently developed autoantibodies. Among them, 61 % of children with enteroviral RNA developed T1D as compared to 28 % of children who

did not have enteroviral RNA [120]. Even in Cuba where the incidence of T1D is low, the presence of enteroviral RNA was reported to be associated with T1D [121]. Thus, enterovirus infection is often considered a pivotal event that shifts the balance from chronic subclinical autoimmunity towards destructive autoimmunity.

Retrospective studies

In the United Kingdom, the study by Nairn et al. [122] found that 27 % of newly diagnosed patients harbored enteroviral RNA in serum samples taken within 1 week after confirmation of diagnosis, as compared to only 4.9 % of healthy controls. A recent study by Schulte et al. [123] also detected enteroviral RNA at the onset of T1D whereas no enteroviral RNA was detected in healthy controls. This study also underlined the importance of assessing peripheral blood mononuclear cells because 4/10 patients with T1D were positive for enteroviral RNA in peripheral blood mononuclear cells as compared to 2/10 in serum [123]. In a Swedish study, anti-CVB4 neutralizing antibodies were more frequently detected in patients with newly diagnosed T1D as compared to healthy controls [124].

Because detecting enteroviral presence in the pancreas requires biopsies or pancreatic samples from postmortem donors, such analyses have been limited. However in 1979, Yoon et al. [125] isolated a virus from the pancreas of a deceased patient diagnosed with diabetic ketoacidosis. This virus was later identified as being related to the diabetogenic CVB4 strain. When injected into mice, this virus had infected pancreatic islets and caused β -cell loss. Similarly, Dotta et al. [126], using immunohistochemistry, detected enteroviral VP1 capsid protein in islets from patients with T1D, whereas islets from healthy donors were virus-free. They sequenced the viral genome present in the pancreatic islets of one out of three VP1-positive diabetic donors and identified this virus as CVB4. The isolated CVB4 could infect human islets from healthy donors and reduce insulin secretion [126]. Richardson et al. [127] detected enteroviral capsid VP1 protein in the pancreatic islets of 44/72 (66 %) patients with recently diagnosed T1D, while this protein was virtually non-existent in healthy controls. In this study, VP1 was also detected in 42 % of T2D patients. Interestingly, the authors propose that enteroviral infection could also play a role in T2D, since enteroviral infection of β -cells in vitro decreases glucose-induced insulin secretion. Thus, in individuals with increased insulin resistance and higher insulin requirements, enteroviral infection could contribute to the development of T2D. Enteroviral RNA was also detected by in situ hybridization of pancreatic islets from two deceased donors with fulminant CVB3 infection and in some CVB3-infected patients with T1D [128]. Indirectly, the presence of a virus has also been

suggested by the detection of the antiviral cytokine IFN- α in the pancreas and β -cells of patients with T1D [129, 130].

Finally, echoviruses 4, 6, 9, 14, and 30, other members of the enterovirus family, have been implicated in the etiology of T1D and were shown to impair or kill human β -cells in vitro [131–137]. However, studies focusing on echoviruses remain very scarce.

Understanding the relationship between enteroviral infections and T1D: studies in NOD mice

Because the sequence of P2-C protein of CVB4 is very similar to that of the highly immunogenic GAD65 expressed in β -cells, molecular mimicry was proposed to explain the pathogenic effects of CVB4 in T1D in humans [138]. This hypothesis implies that anti-CVB4 T lymphocytes cross-react with GAD65, destroy β -cells, and induce T1D. However, molecular mimicry was ruled out in later studies [139].

Coxsackievirus group B 4 infection in NOD mice seems to yield contradictory results. The discrepancy might reflect the complexity of the interactions among CVB4, the immune system and pancreatic β -cells, which could lead to the induction or prevention of T1D. Indeed, several parameters, including timing of infection, type of mice, viral dose, and viral strain with its particular virulence, play important roles in the outcome of CVB infection in animal models (Fig. 3). All of these factors, in combination, might explain why CVB4 could accelerate T1D in some individuals while remaining asymptomatic in others.

Age and associated numbers of autoreactive T cells

Using BDC2.5 transgenic NOD mice, Horwitz et al. [140] showed that CVB4 infection of prediabetic mice, harboring a greater number of anti-islet BDC2.5 CD4⁺ T cells compared to younger mice, induces the acceleration of T1D through bystander activation of T cells. This study suggested that the number of anti-islet T cells at the time of infection is a critical factor. Two subsequent studies strengthened this hypothesis by infecting non-transgenic NOD mice at different ages. Coxsackievirus group B 4 infection of young NOD mice, aged 4–8 weeks, did not accelerate or induce T1D [141, 142]. However, CVB4 infection of older mice accelerated T1D onset in 61 % of mice [142]. Interestingly, it seems that both the number and location of autoreactive T cells are important factors. F1 mice, obtained by crossing BDC2.5 NOD mice with BALB/c or C57BL/6 mice, had the same numbers of peripheral autoreactive T cells. However, these T cells only infiltrated the pancreas in BDC2.5NOD \times BALB/c mice. Upon CVB4 infection, BDC2.5NOD \times BALB/c mice, but not BDC2.5NOD \times C57BL/6 mice, developed diabetes, indicating that infiltration of pancreas by diabetogenic

Factors involved in enteroviral induced T1D (mouse models)

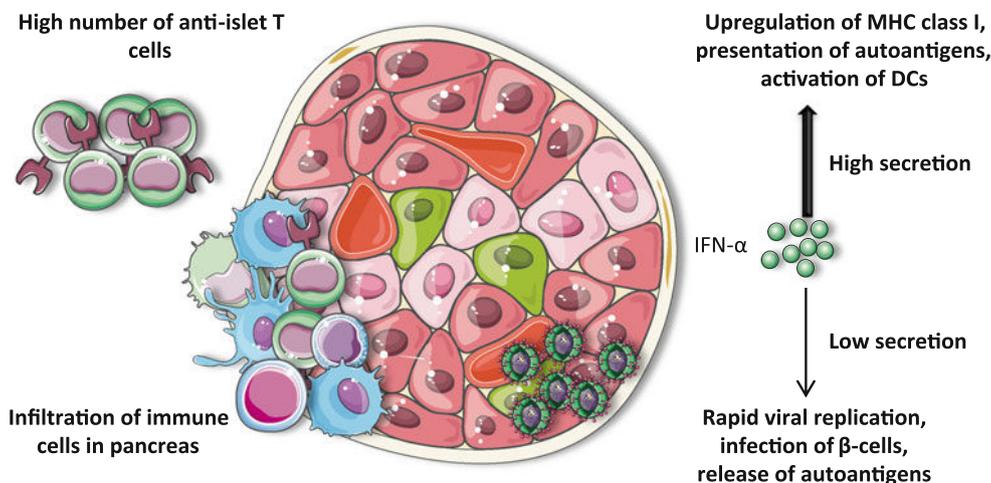


Fig. 3 Factors involved in enteroviral-induced T1D. Studies in mouse models have revealed that in order for enteroviruses to accelerate T1D, a high number of autoreactive anti-islet T cells are necessary. In addition, pancreas must be infiltrated by cells of the immune system. As for the IFN- α , its high secretion can result in a

strong activation of the immune system favoring the presentation of islet autoantigens. On the other hand, if IFN- α secretion is too low, viruses will replicate freely, infect islet β -cells, and provoke the release of autoantigens leading to the activation of anti-islet T cells and provoking diabetes

T cells before infection is an important factor that promotes the onset of diabetes [143].

Based on these experimental results, the pre-existence of a pool of anti-islet T cells and the infiltration of the pancreas by immune cells in children could determine whether T1D will develop following enteroviral infection.

Viral titer

A comparison of non-diabetogenic CVB3/GA and diabetogenic CVB3/28 strains prompted Tracy and coworkers [144] to propose that viral dose and replication rate play an important role in the initiation of T1D. Infection of 12-week-old NOD mice with 5×10^5 TCID₅₀ (50 % tissue culture infective dose) per mouse of the non-diabetogenic CVB3/GA strain did not induce diabetes, whereas a 100-fold higher dose induced diabetes in 30 % of mice. On the other hand, the diabetogenic CVB3/28 strain induced T1D in up to 70 % of NOD mice, although this rate decreased when a lower dose of CVB3/28 was administered. These results indicate that the pathogenicity of the strain could be dependent on its dose. Thus, children infected with a high viral dose or whose immune system would allow viruses to quickly reach high titers, could be at increased risk of developing T1D.

CVB4, β -cell infection and the antiviral response

Some CVB strains have been reported to infect, proliferate, affect the metabolism, and destroy human pancreatic islet

cells in vitro. In human pancreas, CVB4 was detected in islets but not in the exocrine tissue, whereas it was mostly detected in pancreatic exocrine tissue in mice [145–147]. The effects of infection of mouse β -cells with CVB4 are contradictory. It has been described that CVB4 primarily uses the coxsackie and adenovirus receptor (CAR) to enter cells [128, 148]. Although CAR and CVB4 were not detected in some studies [149, 150], others have detected CVB4 in murine islets. Importantly, the permissiveness of islet β -cells was suggested to determine the outcomes of infections with different CVB strains. Using immunohistochemistry, Horwitz et al. [143, 151] demonstrated the presence of CVB4 in pancreatic islets at 7 days post-infection in BDC2.5 mice, and the pathogenic effect of the CVB4 strain was attributed to its capacity to infect β -cells whereas CVB3 could not. Meanwhile in old prediabetic NOD mice, even the non-diabetogenic CVB3 could infect islets and accelerate diabetes [152]. If infection of islet β -cells is an important factor, the efficacy of an individual's response to the virus could determine the progression to T1D.

Interferon- α , interferon- β and interferon- γ are the major cytokines that are rapidly secreted after viral infection. The expression of IFN- γ in pancreatic islet β -cells of IFN- γ —deficient mice allows them to control viral replication and survive after CVB4 infection [153]. Similarly in transgenic NOD mice in which IFN signaling is inhibited in pancreatic β -cells, CVB4 induces diabetes in 95 % of mice while non-transgenic mice remain diabetes-free. This inability to

respond to IFNs rendered β -cells permissive to CVB4 infection resulting in their killing by activated NK cells [154]. It has been shown that during coxsackievirus infection, IFN- α increases the expression of intracellular double-stranded RNA sensor 2-5AS, which activates the enzyme RNase L that cleaves viral RNA and therefore protects β -cells. Interferon- γ activates the enzyme PKR (dsRNA-dependent protein kinase), which can disturb protein synthesis and block CVB replication [155]. Thus, the rapid islet response to viral infection through IFN secretion has a strong influence on diabetes outcome because it determines whether enteroviruses can infect islet β -cells. As described above, uncontrolled viral replication, which can yield high viral titers very quickly, can make an otherwise harmless viral strain highly pathogenic.

Islet neogenesis

Islet neogenesis has been proposed to be a factor determining the outcome of CVB infection in mice. Yap et al. [150] used two CVB4 strains, the diabetogenic CVB4/E2 strain and the non-diabetogenic CVB4/JVB strain, and found that the severity of pancreatic acinar tissue damage caused by viral infection influences T1D development. While the E2 strain caused massive destruction of acinar tissue without islet destruction, the JVB strain caused only minor damage. This allowed regeneration of the acinar tissue and new pancreatic islets, which were not observed in the severely destroyed pancreas of CVB4/E2-infected NOD mice [150]. Although it is unknown whether islet neogenesis occurs in the human exocrine tissue, a marker for cell proliferation, Ki-67, was expressed in human pancreatic islets positive for enteroviral VP1 protein [156]. Therefore, the capacity of islet cells to proliferate after the infection might protect against T1D.

Ambivalent roles of enteroviruses in T1D

Hygiene hypothesis

According to the hygiene hypothesis, the rarity of enteroviral infections could lead to decreased immunity against these viruses, thus increasing their invasiveness and pathogenicity. To estimate the frequency of enteroviral infections, Viskari et al. [157] studied the prevalence of enteroviral meningitis in Finnish children and concluded that the frequency of enteroviral infection in children aged ≥ 6 months had decreased. However, they found that the infection rate was actually increasing in younger children aged 0–6 months. One explanation for this rise in young infants could be the lack of protective anti-enteroviral antibodies of maternal origin that would pass to the child through the placenta and breastfeeding. Indeed, Sadeharju

et al. [158] found that children who were breastfed for >2 weeks after the birth had a lower incidence of enteroviral infections than children breastfed for <2 weeks. Moreover, the percentage of women lacking antibodies against the CVB4 strain increased from 6 to 17 % between 1983 and 1995 while the percentage of women lacking antibodies against enteroviral peptides increased from 13 to 42 % during the same period [110, 111].

With fewer infections, pregnant women nowadays might lack anti-enteroviral antibodies or may have a repertoire that covers fewer serotypes compared to women at the beginning of the 20th century. Thus, young children would receive fewer or no protective antibodies from their mothers. In children with a weaker immune defense against enteroviruses, a single strong infection could then allow increased virus replication, promoting the development of T1D. Interestingly, this scenario proposing the increased pathogenic role of CVB in T1D is supported by observations of polioviral infections, another member of the enteroviral family. At the end of the 19th century, poliovirus infections became rarer, and the number of children developing a severe complication of polioviral infection, paralytic poliomyelitis, increased drastically. It is suggested that with frequent polioviral infections children encountered the virus at an early age when they still had protective antibodies transmitted by their mothers. These antibodies protected against paralysis by forming an immediate barrier and blocking the invasion of the central nervous system by poliovirus. Thus the infected individual had time to make his own protective antibodies to further eliminate the virus. However, because of improved sanitary conditions, the virus became rare and children were infected at an older age when the level of maternal antibodies had strongly decreased. Without any immediate protection, poliovirus attacked the central nervous system easier, thus increasing the chances to develop paralytic poliomyelitis [159].

The reduced frequency of enteroviral infection could favor delayed enteroviral encounters in genetically predisposed older children. The older the child is at the time of infection, the more likely the child is to have accumulated anti-islet T cells. It is important to remember that studies of NOD mice have shown that the number of anti-islet T cells and the degree of islet inflammation are critical factors and could explain why some children develop T1D while others do not.

RNA sensors and T1D

To further support the pathogenic role of enteroviruses, the genomic region coding the viral RNA sensor IFIH1 (IFN induced with helicase C domain 1), otherwise known as MDA5 (melanoma differentiation-associated protein 5),

has been identified as a T1D susceptibility locus. This is particularly important because MDA5 is critical for the recognition of picornaviruses to which the enterovirus genus belongs [160]. This intracellular receptor recognizes double-stranded viral RNA that forms during viral replication, induces the expression of type I IFNs (IFN- α and β), and activates the immune system. Four rare mutations that reduce the expression of IFIH1 and type I IFNs were reported to have a protective role in T1D [161–163]. These findings suggest that wild-type IFIH1 and effective recognition of viral infection actually predispose individuals to T1D. This role of MDA5 is supported by several previous studies. For example, IFN- α was detected in plasma samples of 70 % of newly diagnosed T1D patients, of whom 50 % carried enteroviral RNA in their blood samples; none of the IFN- α -negative patients had enteroviral RNA [164]. The scenario proposes that after viral RNA binding to IFIH1, large amounts of type I IFNs are secreted, leading to the upregulation of MHC class I molecule expression on β -cells, increased presentation of autoantigens, and activation of DCs [165]. Type I IFNs could also regulate cells of the innate and adaptive immune system, which could facilitate the killing of β -cells and destruction of pancreatic islets [166, 167].

Even though much less documented than MDA5, it has been proposed that another RNA sensor, TLR7, could be implicated in the etiology of T1D [168]. Toll-like receptor 7, mainly expressed by pDCs, can recognize coxsackieviruses and initiate an antiviral immune response by inducing the production of type I IFNs [169]. Treatment with a TLR7 agonist, particularly in combination with other immune stimulatory molecules such as CD40, induced T1D in transgenic NOD mice bearing an increased number of autoreactive T cells [170]. Interestingly, NOD mice deficient for Myd88, an adaptor molecule required for the signaling of most TLRs, including TLR7, do not develop diabetes in a specific pathogen-free mouse facility [43].

While an efficient MDA5-mediated response is associated with T1D susceptibility in humans, several studies have highlighted the critical role of IFN- α production in diabetes prevention in infected mice [105, 154, 171, 172]. The amount and the environment in which IFN- α is secreted could perhaps account for this phenomenon. IFN- α is required to block viral replication, but excessive secretion in the context of an ongoing autoimmune response could render IFN- α pathogenic.

Could pollutants promote virus-induced diabetes?

Since the frequency of autoimmune diseases, including T1D, is higher in more industrialized countries, it is tempting to speculate that pollutants present in the environment might be

involved. Heavy metals such as mercury or cadmium worsen the course of autoimmune diseases in mouse models [173–175]. Concerning T1D, arsenic present in drinking water in western Bangladesh and Taipei city, Taiwan, was associated with an increased incidence of T1D in these populations [176, 177]. Pollutants have also been found at higher levels in diabetic mothers [178]. In a mouse model, exposure to mercury caused dysfunction and apoptosis of pancreatic β -cells [179, 180]. However, other studies in Bangladesh and the United States found no link between arsenic and T1D [181, 182], and mercury was found to delay T1D onset in NOD mice [183]. The discrepancy between these results may be due to the fact that the pollutants by themselves do not have a noticeable effect on autoimmune diseases in humans. Instead, they may create an environment that is aggravated by an infection. For example, exposing mice to mercury and bacterial lipopolysaccharide exacerbated the onset of autoimmune disease in genetically susceptible mice or rendered resistant mice susceptible to autoimmunity [184]. Funseth et al. [185] showed that CVB3 infection can cause an accumulation of an environmental pollutant in the viral target organ, such as the pancreas, and aggravate the inflammation by its toxicity. However, additional epidemiological and experimental studies are required to determine the precise role of pollutants in virus-induced T1D.

Conclusions

Many recent studies in humans and animal models have implicated viruses in the development of T1D. The interactions between viruses, the immune system, and β -cells are probably key in determining their pathogenic or beneficial roles. Most importantly, the conditions in which the disease develops might have changed in recent years because the role of HLA is becoming less prominent. Other genes are clearly involved and can interfere with environmental factors such as infection, diet, and pollutants. Interdisciplinary studies in the fields of genetics, immunology, microbiology, nutrition, and epidemiology are essential to elucidate the etiology of T1D and develop efficient preventive strategies.

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► Le diabète de type 1 est une maladie auto-immune résultant de la destruction des cellules β pancréatiques par le système immunitaire. Les lymphocytes NKT (*natural killer T cell*) sont des cellules régulatrices qui inhibent le développement du diabète de type 1 dans les modèles murins, tout en favorisant les réponses contre les agents infectieux. Les mécanismes régulateurs impliquent la production de cytokines et des modifications fonctionnelles des cellules dendritiques. La sélection de molécules ciblant spécifiquement les lymphocytes NKT représente une étape importante pour l'utilisation thérapeutique de ces lymphocytes afin d'inhiber le développement du diabète de type 1 chez les patients. ◀

Le diabète de type 1 est une maladie auto-immune résultant de la destruction des cellules β pancréatiques productrices d'insuline par les cellules du système immunitaire. Cette maladie est caractérisée par la présence d'auto-anticorps et de lymphocytes T autoréactifs dirigés contre des antigènes produits par les cellules β pancréatiques. Bien que de nombreuses études soient consacrées à la caractérisation de ces réponses auto-immunes, il devient de plus en plus évident que d'autres cellules du système immunitaire jouent un rôle déterminant dans le développement du diabète de type 1 [1, 2]. L'étude des mécanismes conduisant à l'apparition de cette pathologie a révélé le rôle crucial des mécanismes de régulation immunitaire. Parmi les cellules régulatrices, une population particulière, les lymphocytes NKT (*natural killer T cell*), pourrait devenir une cible thérapeutique de choix dans la prévention du diabète de type 1.

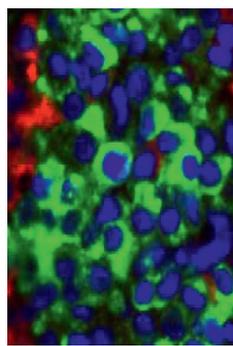
Les cellules NKT

Les cellules NKT sont des lymphocytes T non conventionnels, car ils ne reconnaissent pas les molécules du CMH (complexe majeur d'histocompatibilité), mais la molé-

Diabète : approches thérapeutiques émérgentes

Rôle régulateur des lymphocytes NKT dans la prévention du diabète de type 1

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cule non polymorphe CD1d. Le terme NKT reflète leurs caractéristiques phénotypiques et fonctionnelles partagées avec des cellules *natural killer* (NK), comme l'expression du marqueur NK1.1 ou CD161 chez l'homme. Les lymphocytes NKT sont considérés comme des lymphocytes T innés ayant un phénotype de cellules activées/effectrices, et ils sont très conservés chez la souris et chez l'homme [3]. Alors que les molécules classiques du CMH (comme les molécules HLA [*human leukocyte antigen*]-DR) présentent des antigènes peptidiques aux lymphocytes T, les molécules non classiques du CMH (comme le CD1d) présentent des antigènes non peptidiques [3]. De nombreux travaux ont mis en évidence le rôle régulateur des lymphocytes NKT, et particulièrement leur capacité à inhiber le développement du diabète de type 1 dans les modèles murins [4]. Les cellules NKT modulent les réponses immunitaires en produisant rapidement de grandes quantités de cytokines et en induisant l'expression de différentes molécules de surface. Elles influencent le développement des réponses immunitaires innées et adaptatives. Classiquement, les lymphocytes NKT sont divisés en trois groupes : NKT invariants (iNKT), NKT de type II et NKT-like, selon l'expression de leur récepteur T (TCR) et leur spécificité antigénique (Figure 1) [4].

Les lymphocytes iNKT expriment un TCR α invariant, V α 14-J α 18 chez la souris et V α 24-J α 18 chez l'homme, et reconnaissent des glycolipides

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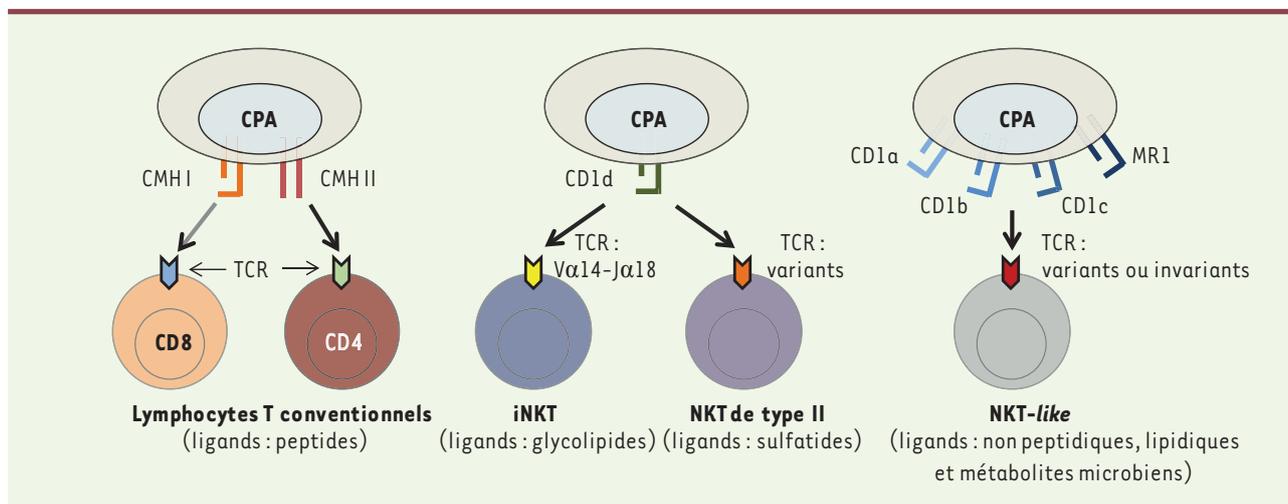


Figure 1. Lymphocytes T et lymphocytes NKT. Alors que lymphocytes T conventionnels reconnaissent des peptides présentés par le CMH I ou CMH II (gauche), les lymphocytes NKT expriment un TCR restreint par les molécules non classiques du CMH. Les lymphocytes iNKT et NKT de type II sont restreints par la molécule CD1d qui présente des glycolipides ou des sulfatides (centre). Les lymphocytes NKT-like sont restreints par d'autres molécules non polymorphes comme CD1a, b, c ou MR1 (*MHC related protein 1*) (droite). CPA : cellules présentatrices d'antigène ; CMH : complexe majeur d'histocompatibilité ; TCR : *T cell receptor*.

présentés par CD1d, en particulier l' α -galactosylcéramide (α GalCer). Les lymphocytes NKT de type II sont également restreints par la molécule CD1d, mais ils ne reconnaissent pas l' α GalCer et ils expriment un TCR α variable [3]. Parmi cette population, certains lymphocytes reconnaissent des sulfatides ou le lysophosphatidylcholine. Plus récemment, une autre population de lymphocytes T $\alpha\beta$ non conventionnels, reconnaissant la molécule non polymorphe MR1 (*MHC-related protein 1*), a été caractérisée. Les souris transgéniques V α 14-J α 18 et les outils permettant la détection spécifique des lymphocytes iNKT (tétramères CD1d- α GalCer et anticorps 6B11) ont permis une étude approfondie de ces cellules et, en particulier, de déterminer leur rôle régulateur dans le diabète de type 1.

Les lymphocytes iNKT dans le diabète de type 1

L'implication des lymphocytes iNKT dans la régulation de maladies auto-immunes a été proposée suite à l'observation d'anomalies de cette population cellulaire chez les patients affectés de cette pathologie, ainsi que chez les modèles animaux correspondants. Le rôle régulateur des cellules iNKT dans le diabète de type 1 a été démontré puis analysé grâce aux souris génétiquement modifiées qui sont, soit dépourvues en lymphocytes NKT, soit au contraire expriment une fréquence accrue de cette population lymphocytaire.

La fréquence des lymphocytes iNKT

Le modèle animal le plus utilisé pour l'étude du diabète de type 1 est la souris NOD (*non obese diabetic*), car elle présente des signes cliniques similaires à ceux observés chez l'homme [1]. Les souris femelles NOD développent spontanément un diabète à partir de 12-15 semaines d'âge. La caractérisation des lymphocytes iNKT chez ces

souris a montré une fréquence et un nombre plus faibles de cellules iNKT dans le thymus et la rate par rapport aux souches de souris contrôles (BALB/c, C57BL/6, AKR et NOR) [5, 6]. De plus, notre équipe a montré que l'augmentation du nombre des lymphocytes iNKT, provoquée par l'expression de la chaîne V α 14-J α 18 par transgénèse, diminue l'incidence du diabète des souris NOD [7]. L'incidence du diabète diminue également quand les cellules iNKT s'accumulent dans les ganglions drainants le pancréas suite à la surexpression de la molécule CD1d par les cellules β pancréatiques [8]. Inversement, l'incidence du diabète est plus élevée et accélérée chez les souris déficientes en cellules NKT (souris NOD CD1d^{-/-}) comparées aux souris NOD sauvages [9, 10].

Le rôle fonctionnel des cellules iNKT

Le développement du diabète est associé à la présence de lymphocytes T anti-cellules β pancréatiques de type Th1, alors que la protection contre cette pathologie est fréquemment associée à des réponses de type Th2 [1]. Bien que les cellules iNKT puissent produire diverses cytokines dont l'IFN γ (interféron γ) et l'IL-4 (interleukine-4), de nombreuses études ont montré que leur production d'IL-4 inhibe les réponses auto-immunes pathogènes de type Th1 (Figure 2). Dans ce contexte, il est intéressant de noter que les cellules iNKT des souris NOD produisent moins d'IL-4 que celles de souris non diabétiques, comme les souris BALB/c [5]. En revanche, la correction d'un déficit en

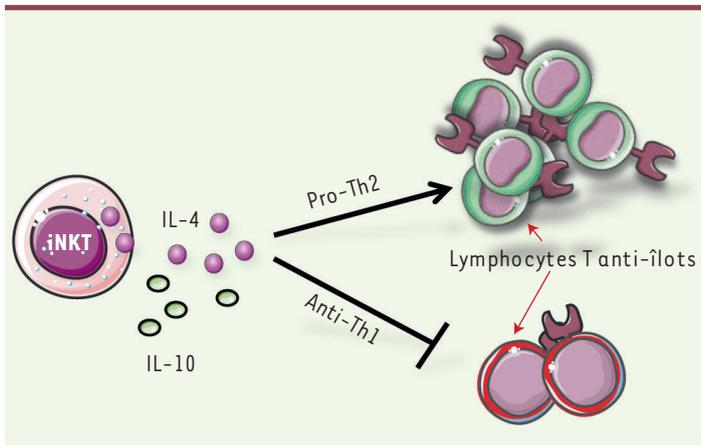


Figure 2. Rôle protecteur des cytokines produites par les lymphocytes iNKT. Les lymphocytes iNKT peuvent inhiber le développement du diabète de type 1 par la production de cytokines IL-4 et/ou IL-10. Ces deux cytokines inhibent les réponses Th1 diabétogènes, et l'IL-4 favorise les réponses Th2, non pathogènes dans le diabète.

IL-4 par l'administration d'IL-4 exogène [11] ou l'expression d'IL-4 sous le contrôle du promoteur de l'insuline prévient l'apparition du diabète chez les souris NOD. De façon similaire, nous avons montré que la protection du diabète chez les souris $V\alpha 14-J\alpha 18$ transgéniques est associée à une augmentation de la production d'IL-4 et une diminution de celle d'IFN γ localement dans le pancréas [12]. Une autre étude confirme le rôle des cytokines dans la protection du diabète par des thymocytes α/β TCR $^+$ CD4 $^+$ CD8 $^-$, fortement enrichis en cellules iNKT. Dans ce système, l'effet protecteur des cellules iNKT est dépendant de l'IL-4 et de l'IL-10, car l'administration d'anticorps neutralisant l'IL-4 et l'IL-10 abolit la protection contre le diabète [13].

Il est important de noter que les cellules iNKT peuvent également inhiber le développement du diabète par d'autres mécanismes d'immunorégulation indépendants de l'IL-4 et de l'IL-10. Des expériences de transfert de populations monoclonales de lymphocytes T diabétogènes, soit CD4 $^+$, soit CD8 $^+$, ont révélé la capacité des cellules iNKT à inhiber la différenciation de ces lymphocytes T anti-cellules β pancréatiques en cellules effectrices (Figure 3). Dans ces études, les lymphocytes T autoréactifs ne dévient pas vers une réponse Th2, mais ils deviennent anergiques ou non fonctionnels [14, 15]. L'activation abortive des lymphocytes T anti-cellules β pancréatiques, en présence de lymphocytes iNKT, est associée à des anomalies des cellules dendritiques dans les ganglions pancréatiques [14, 16]. Nous analysons actuellement les capacités régulatrices des lymphocytes T anti-cellules β pancréatiques anergiques. L'inhibition de l'activation des lymphocytes CD4 autoréactifs nécessite un contact cellulaire entre ces lymphocytes, les cellules iNKT et les cellules présentatrices d'antigènes [17, 18]. Bien que ces contacts cellulaires ne soient pas dépendants de la molécule CD1d, la surexpression par transgénèse de cette molécule à la surface des cellules β pancréatiques induit l'accumulation dans les ganglions drainants des cellules iNKT, qui inhibent l'activation des lymphocytes T autoréactifs [8].

Malgré le rôle protecteur des lymphocytes iNKT dans leur globalité, une sous-population de cellules iNKT produisant de l'IL-17 joue un rôle délétère dans le diabète. En effet, une nouvelle sous-population de lymphocytes iNKT (CD4 $^-$ NK1.1 $^-$) producteurs d'IL-17 a été récemment décrite [19]. Nous avons observé que les souris NOD possèdent un nombre plus élevé de ces cellules iNKT17 par rapport aux souches contrôles (C57BL/6 et BALB/c) [20]. De plus, des expériences de transfert ont montré que ces cellules accentuent le développement du diabète induit par les lymphocytes T CD4 diabétogènes, alors que la population iNKT CD4 $^+$ NK1.1 $^+$, dépourvue de cellules NKT17, prévient très efficacement le diabète (Figure 4).

Les lymphocytes iNKT chez les patients diabétiques

Différentes études chez des patients présentant diverses pathologies auto-immunes ont montré le lien entre des anomalies des lymphocytes iNKT et une dérégulation du système immunitaire [4]. Une première étude, publiée dans *Nature* en 1998, a décrit des défauts numériques et fonctionnels des lymphocytes iNKT chez des patients diabétiques comparés à des sujets contrôles [21]. De plus, il a été observé un défaut de la sous-population CD4 $^+$ des lymphocytes iNKT dans le sang des patients diabétiques [22]. Contrairement à la souris, la présence des lymphocytes iNKT17 n'a pas encore été décrite parmi les lymphocytes circulants chez l'homme, bien que ceux-ci puissent être obtenus dans certaines conditions de culture *in vitro*. L'analyse des lymphocytes iNKT dans les ganglions pancréatiques a révélé un défaut de production d'IL-4 par ces cellules chez les patients diabétiques par rapport aux sujets contrôles [23]. Il faut toutefois noter que des résultats divergents ont été publiés en ce qui concerne la fréquence des sous-populations et la production de cytokines par les lymphocytes iNKT dans le sang des sujets ayant un diabète de type 1. Ces différences pourraient en partie s'expliquer par la très grande hétérogénéité de la fréquence des lymphocytes iNKT chez l'homme et par les différentes techniques utilisées pour l'analyse de cette population [4]. Par conséquent, il serait intéressant de réitérer ces études avec un grand nombre de patients et une méthode standardisée.

Par ailleurs, du fait de l'impossibilité de pratiquer des biopsies de pancréas chez l'homme, la présence de lymphocytes iNKT dans ce tissu n'a pas été analysée. Cependant, la création du programme nPOD (*network for pancreatic organ donors with diabetes*) pourrait permettre l'étude des lymphocytes iNKT à partir d'une banque de pancréas de patients qui avaient un diabète de type 1.

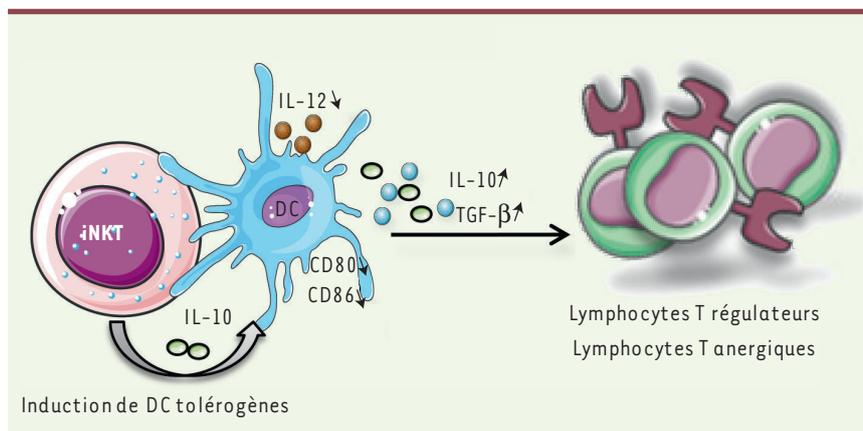


Figure 3. Régulation du diabète de type 1 par le biais de cellules dendritiques. Les lymphocytes iNKT peuvent induire des cellules dendritiques (DC) tolérigènes qui expriment faiblement CD80 et CD86 et sécrètent de l'IL-10 et/ou du TGF β (*transforming growth factor β*), favorisant la conversion des lymphocytes T naïfs en lymphocytes T régulateurs ou anergiques. Cette induction est indépendante de la sécrétion d'IL-4 par les lymphocytes iNKT, mais dépendante des contacts cellulaires entre les lymphocytes iNKT et les DC et/ou de la production d'IL-10 par les lymphocytes iNKT.

Rôle protecteur des lymphocytes iNKT dans le diabète lors d'une infection virale

La dualité fonctionnelle des lymphocytes iNKT nous a incités à étudier leur rôle régulateur dans le diabète de type 1 au cours des infections virales. En effet, parallèlement aux études qui ont montré le rôle tolérigène des lymphocytes iNKT dans le diabète de type 1 et dans d'autres pathologies auto-immunes, plusieurs laboratoires ont observé un rôle activateur des lymphocytes iNKT au cours des infections [3]. Lors d'infections virales, bactériennes ou parasitaires, les lymphocytes iNKT sont activés très rapidement et stimulent à leur tour les réponses immunitaires anti-infectieuses. Par conséquent, une question fondamentale se pose : les lymphocytes iNKT peuvent-ils effectuer ces deux fonctions, maintien de la tolérance immunitaire et réponse anti-infectieuse efficace, en même temps et sans préjudice pour l'intégrité de l'organisme ? Le modèle privilégié pour répondre à cette question a été le diabète induit par une infection par le virus de la chorioméningite lymphocytaire (LCMV). Dans ce contexte, les lymphocytes iNKT favorisent la réponse immunitaire antivirale, tout en protégeant les souris contre le développement du diabète [24, 25]. Dans la rate, les cellules iNKT activent les cellules dendritiques conventionnelles qui produisent de l'IL-12 et induisent une forte réponse CD8 anti-LCMV, indispensable à l'élimination du virus par la lyse des cellules infectées. Dans le pancréas, les lymphocytes iNKT n'interagissent pas avec les cellules dendritiques conventionnelles, mais avec les cellules dendritiques plasmacytoïdes (pDC). Au cours de l'infection par le LCMV, les lymphocytes iNKT pancréatiques sont activés et ils induisent le recrutement des pDC dans ce tissu. De plus, les lymphocytes iNKT activent la production par les pDC de grandes quantités d'IFN α , qui inhibe la réplication virale dans le pancréas et limite ainsi la destruction du tissu pancréatique [26]. Dans un second temps, les pDC migrent dans les ganglions pancréatiques où elles produisent du TGF β et induisent la conversion des lymphocytes T naïfs en cellules T régulatrices (FoxP3⁺) (Figure 3). Ensuite, ces cellules T régulatrices migrent dans le pancréas où elles sécrètent du TGF β qui inhibe les lymphocytes T anti-cellules β pancréatiques. Ces deux mécanismes complémentaires mettent en jeu les lymphocytes iNKT et les pDC qui contrôlent la réplication virale, tout en prévenant l'apparition du diabète [24]. Il est intéressant de noter que ces mécanismes régulateurs sont également impliqués dans l'inhi-

bition du diabète spontané après infection par le LCMV. Il était connu depuis de nombreuses années que certaines infections inhibaient le développement du diabète de type 1 dans les modèles animaux [25], et nos travaux ont révélé les mécanismes cellulaires et moléculaires impliqués dans la protection induite par le LCMV. Il est donc très intéressant de constater que l'activation du système immunitaire peut être protectrice dans les maladies auto-immunes. Par ailleurs, nos résultats préliminaires sur le rôle des lymphocytes iNKT lors d'une infection par le virus Coxsackie B4 montrent également que les lymphocytes protègent contre le développement du diabète induit par cette infection virale. Toutefois, les mécanismes de protection sont différents de ceux mis en jeu lors de l'infection par le LCMV. L'ensemble de ces résultats est encourageant pour le développement de stratégies thérapeutiques contre le diabète de type 1, au moyen de l'activation des lymphocytes iNKT.

Manipulation thérapeutique des lymphocytes iNKT dans le diabète

Activation des lymphocytes iNKT par des ligands spécifiques

Traitement par l' α GalCer

Le glycolipide α GalCer, initialement purifié à partir de l'éponge marine *Agelas mauritanus*, stimule spécifiquement les lymphocytes iNKT chez l'homme et la souris [3]. La reconnaissance du complexe CD1d- α GalCer par le TCR des lymphocytes iNKT aboutit à la production rapide de cytokines. Des injections multiples d' α GalCer à des souris NOD prédiabétiques préviennent le développement du diabète [27]. Ce traitement induit l'accumulation des lymphocytes iNKT produisant de l'IL-4 dans les îlots pancréatiques ainsi que dans les ganglions pancréatiques. Cette cytokine pourrait être impliquée dans la protection contre le diabète. Cette

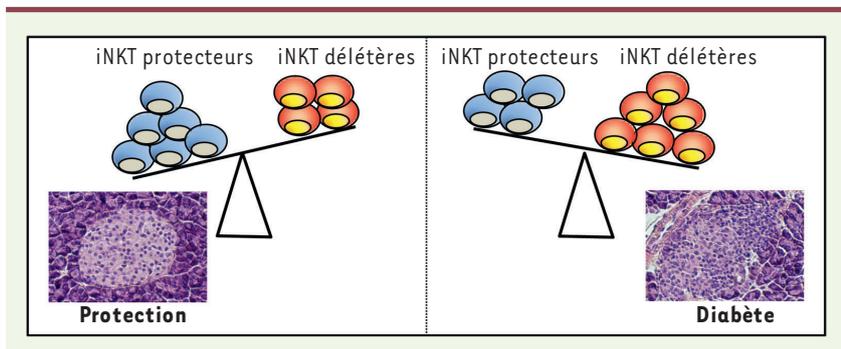


Figure 4. Rôle ambivalent des lymphocytes iNKT dans le diabète de type 1. Chez la souris NOD, une sous-population de lymphocytes iNKT (iNKT CD4⁺) exerce un rôle protecteur, alors qu'une autre sous-population (CD4⁻, iNKT17) stimule la maladie. Par conséquent, la protection (gauche) ou la stimulation (droite) de la réponse auto-immune anti-îlot(s) pourrait être due à un déséquilibre quantitatif entre ces différentes sous-populations. Photographies : coupe de pancréas en coloration H&E (hématoxyline-éosine) montrant un îlot pancréatique sain (gauche : protection) ou infiltré (droite : diabète).

prévention n'est plus observée dans les souris NOD déficientes en IL-4, ce qui démontre le rôle régulateur de cette cytokine. Toutefois, comme décrit précédemment, la production d'IL-4 n'est probablement pas le seul paramètre impliqué dans la prévention du diabète par l'activation des lymphocytes iNKT. Plusieurs équipes ont observé qu'un traitement itératif par l' α GalCer induit une modification de la fonction des cellules présentatrices d'antigène. Ainsi, des cellules dendritiques tolérogènes, de phénotype CD11c⁺/CD8 α ⁻, s'accumulent dans les ganglions pancréatiques. Ces cellules produisent des quantités réduites d'IL-12, une cytokine pro-Th1 favorisant les réponses T diabétogènes. Par ailleurs, ces cellules dendritiques expriment aussi plus faiblement les récepteurs de costimulation CD80 et CD86 que les cellules de souris ayant reçu une seule injection d' α GalCer [28]. Toutes ces modifications des cellules dendritiques peuvent induire une absence de réponse immune ou des réponses régulatrices. De façon intéressante, l'effet suppresseur de ce type de traitement semble spécifique des lymphocytes T autoréactifs anti-cellules β pancréatiques, car la stimulation des splénocytes par de la concanavaleine A, un activateur polyclonal des lymphocytes T, induit leur production d'IL-4 et d'IFN γ . Malgré ses effets bénéfiques contre le développement du diabète, il a été montré que l' α GalCer pourrait exacerber d'autres pathologies auto-immunes, comme le lupus [4]. De plus, les traitements répétés par de l' α GalCer entraînent la sécrétion d'IL-4 par les lymphocytes iNKT, et cette cytokine pourrait favoriser certaines réactions allergiques (comme l'asthme) chez des sujets prédisposés. Ces données suggèrent qu'un traitement par l' α GalCer ne serait pas le plus approprié, et de nouveaux agonistes des lymphocytes iNKT sont actuellement produits et testés dans différents modèles animaux.

Les analogues de l' α GalCer

Des modifications structurales de l' α GalCer influencent le profil des cytokines produites par les lymphocytes iNKT. Un analogue de l' α GalCer, dénommé C16:0, possède la particularité de n'induire qu'une

très faible production de cytokines, dont l'IL-4, par les lymphocytes iNKT. Il est particulièrement intéressant d'observer que cette molécule C16:0 prévient plus efficacement le diabète chez les souris NOD que l' α GalCer [29]. À nouveau, ces données illustrent le rôle régulateur des lymphocytes iNKT indépendamment de l'IL-4. Les mécanismes de prévention mis en jeu par un traitement avec du C16:0 sont en cours d'analyse dans notre laboratoire. Par ailleurs, il serait important de tester l'efficacité de cette molécule dans la prévention du diabète induit par des infections virales, le LCMV et également le virus Coxsackie B4 qui a été proposé comme agent étiologique dans le diabète de type 1 [30].

Augmentation du nombre de lymphocytes iNKT

Chez la souris, nous avons démontré que l'augmentation du nombre de lymphocytes iNKT permet de réduire significativement la progression du diabète de type 1 [7]. Augmenter le nombre de lymphocytes iNKT semble donc une stratégie intéressante à transposer à l'homme.

Expansion *in vitro* des lymphocytes iNKT

Plusieurs laboratoires ont développé des méthodes *in vitro* permettant d'accroître le faible nombre de lymphocytes iNKT humains présents dans le sang périphérique. Un essai clinique de phase 1 a montré que l'injection de lymphocytes iNKT obtenus par expansion *in vitro* était bien tolérée et sans danger [31]. Cette stratégie présente l'avantage de pouvoir activer puis sélectionner spécifiquement certaines sous-populations de lymphocytes iNKT. Par analogie avec les données chez la souris, on pourrait proposer de sélectionner, grâce à leurs marqueurs de surface, les lymphocytes iNKT CD4⁺ au fort pouvoir régulateur, et d'exclure les lymphocytes iNKT17 qui ont un rôle délétère dans le diabète [4].

Augmenter la présentation antigénique

Dans le diabète de type 1, le rôle des antigènes du soi (glycolipides) dans l'activation des lymphocytes iNKT reste encore inconnu. Récemment, il a été montré que certaines infections bactériennes induisent l'expression d'enzymes, comme la glucosylcéramide synthase, qui permettent la synthèse de glycolipides du soi. Ces glycolipides sont ensuite présentés aux lymphocytes iNKT et induisent leur prolifération [32]. Il serait intéressant d'induire l'expression de ces enzymes chez les patients diabétiques afin d'augmenter le nombre de

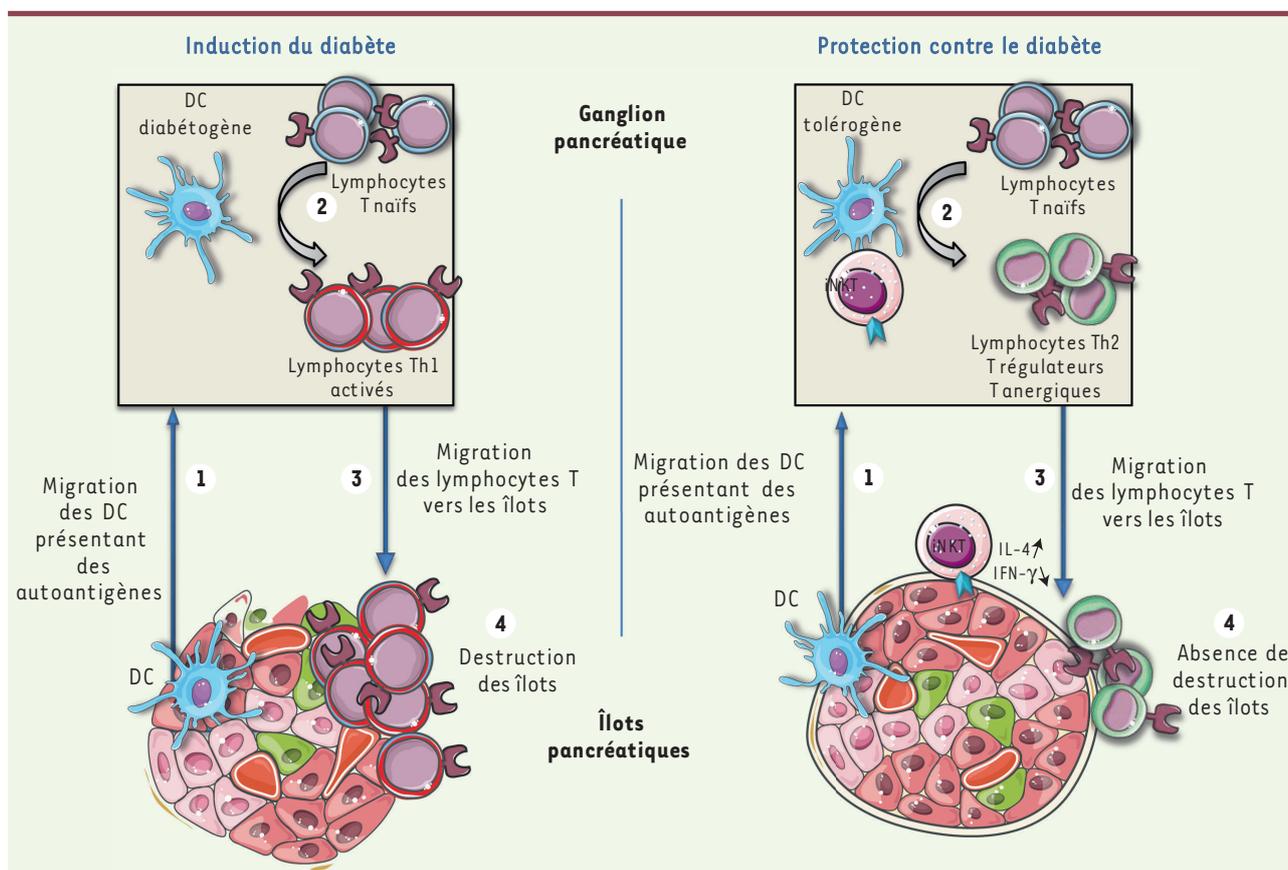


Figure 5. Mécanismes de régulation du diabète de type 1 par les lymphocytes iNKT. Au cours de l'induction du diabète, les cellules dendritiques (DC) diabétogènes migrent des îlots pancréatiques vers les ganglions drainant le pancréas (gauche). Dans les ganglions, les DC activent des lymphocytes T naïfs anti-îlots qui deviennent des lymphocytes Th1. Ces derniers migrent vers le pancréas où ils détruisent les cellules β , induisant ainsi le diabète. Toutefois, les lymphocytes iNKT peuvent induire des DC tolérogènes qui favorisent la différenciation des lymphocytes T naïfs en lymphocytes Th2, régulateurs ou anergiques (droite). Ces lymphocytes migrent vers le pancréas, mais ne détruisent pas les îlots.

lymphocytes iNKT. Cependant, il faudrait au préalable vérifier que cette technique augmente sélectivement les lymphocytes iNKT aux propriétés régulatrices et non pas ceux qui auraient un rôle délétère, comme les iNKT17.

Conclusion

Un ensemble de données démontrent donc le rôle régulateur des lymphocytes iNKT dans les modèles animaux du diabète de type 1 (Figure 5). Des études *in vitro* avec des lymphocytes iNKT humains confortent le rôle régulateur de cette population lymphocytaire chez l'homme [33]. De plus, les premiers essais cliniques basés sur la manipulation des lymphocytes iNKT ont démontré l'absence de toxicité de ces cellules. Il est raisonnable de penser que la production et la sélection de nouveaux agonistes des lymphocytes iNKT devraient permettre de proposer des composés susceptibles d'être testés dans des essais thérapeutiques de prévention du diabète de type 1. Les recherches futures doivent avoir pour objectif une meilleure connaissance de cette population de cellules particulières, pour utiliser leur fonction régulatrice dans un but thérapeutique. \diamond

SUMMARY

Regulatory role of NKT cells in the prevention of type 1 diabetes

Type 1 diabetes is an autoimmune disease resulting from the destruction of pancreatic β cells by the immune system. NKT cells are innate-like T cells that can exert potent immuno-regulatory functions. The regulatory role of NKT cells was initially proposed after the observed decreased frequency of this subset in mouse models of type 1 diabetes, as well as in patients developing various autoimmune pathologies. Increasing NKT cell frequency and function prevent the development of type 1 diabetes in mouse models. Several mechanisms including IL-4 and IL-10 production by NKT cells and the accumulation of tolerogenic dendritic cells are critical for the dampening of pathogenic anti-islet T cell responses by NKT cells. Importantly, these cells can at the same time prevent diabetes and promote efficient immune responses against infectious agents. These results strengthen

the potential role of NKT cells as a key target for the development of therapeutic strategies against type 1 diabetes. ♦

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LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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Innate Immunity in Type 1 Diabetes

JULIEN DIANA, LIANA GAHZARIAN, YANNICK SIMONI, AND AGNÈS LEHUEN

Abstract: Type 1 diabetes (T1D) is a complex autoimmune disease that is untimely caused by the destruction of insulin-producing pancreatic β -cells by autoreactive T cells. The development of the pathology involved several cell types of both the innate and adaptive immune systems. This disease is under the control of several genetic loci of susceptibility but it is also influenced by environmental factors such as infectious agents. Studies in animal models, such as the non-obese diabetic (NOD) mouse, reveal that during the development of T1D multiple interactions occur between macrophages, dendritic cells (DC), natural killer (NK) cells, NKT cells, and lymphocytes. As a consequence, the various components of the immune system can be of peculiar interest as therapeutic targets for disease prevention or cure. This review focuses on the involvement of innate immune cells in the development and the prevention of T1D. [*Discovery Medicine* 11(61):513-520, June 2011]

Introduction

T1D is a chronic autoimmune disease caused by the specific destruction of pancreatic β -cells, which produce insulin. The lack of insulin leads to hyperglycemia and despite daily insulin injections this pathology can induce several complications, mainly at the vascular level in organs such as the kidney, the eye, and the foot

(Maahs and Rewers, 2006). The incidence of T1D, which is particularly high in developed countries in Europe and North America, is dramatically increasing and reaching up to 65.2/100,000 in Finland (Harjutsalo *et al.*, 2008). The development of diabetes is under polygenic control with a 40-60% concordance rate between identical twins (Redondo *et al.*, 1999). This variation in genetic identical individuals indicates the role of the environment in the disease onset. Epidemiologic studies and experimental data obtained in animal models suggest the pathological role of certain viruses, such as enteroviruses, as precipitating agents (Hober and Sauter, 2010). On the contrary, it has been clearly shown in animal models that several infections with viruses, bacteria, and parasites can prevent the development of diabetes (Lehuen *et al.*, 2010). In parallel, the increasing incidence of T1D in populations with decreased exposure to pathogens fits with the protective role of infections against the development of this autoimmune disease. It is interesting to associate this observation with the hygiene hypothesis proposed for the increased incidence of asthma and allergy in the developed countries (Strachan, 1989).

T1D is characterized by the presence of autoantibodies recognizing islet antigens. Despite the critical role of antibodies for the diagnostic of the disease in patients, many data suggest that T cells are the key players in the autoimmune attack of β -cells (Bluestone *et al.*, 2010). Anti-islet T cells, both CD4 and CD8 T cells, have been identified in type 1 diabetic patients as well as in the animal models. Two spontaneous murine models have been extensively analyzed — the biobreeding (BB) rat and the NOD mouse (Lehuen *et al.*, 2010). Importantly, transfer of anti-islet specific CD4 or CD8 T cells induces diabetes to immuno-incompetent recipient NOD mice. In contrast, antibodies do not transfer the disease. CD8 T cells can directly kill β -cells that express MCH class I, through perforin/granzyme secretion. CD4 T cells that recognize peptides presented by

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MHC class II molecules usually participate in carrying out β -cell destruction directly by the production of IFN- γ and indirectly by the activation of local innate cells such as macrophages and dendritic cells (Mathis *et al.*, 2001). Conversely to effector T cells, another T cell population dampens autoimmune pathological responses. Regulatory CD4 T cells, expressing the molecule forkhead box P3 (Foxp3), inhibit the development of diabetes (Tang and Bluestone, 2008). The protective role of this population has been clearly demonstrated in the NOD mouse. Patients harboring mutations in the Foxp3 gene can develop several autoimmune diseases including T1D (Wildin and Freitas, 2005). These observations confirm the role of this regulatory T cell population in humans. Even though B cells are not required for the effector phase of T1D, several studies have revealed the role of these cells in the development of the disease. B cell deficiency by gene targeting and B cell depletion by specific antibodies prevent the development of the disease in NOD mice (Hu *et al.*, 2007). Similar treatment improves the β -cell function in newly diagnosed patients (Pescovitz *et al.*, 2009).

Innate Immune Cells in Type 1 Diabetes

Macrophages

As mentioned above, diabetogenic T cells are key players in the induction of T1D in humans and NOD mice. However, they represent only one piece of the puzzle of the innate and adaptive immune systems implicated in this pathology. The role of macrophages in the pathogenesis of T1D has been suggested in early studies (Hutchings and Cooke, 1990). First, these cells are detected in the islet infiltrates of young NOD mice and inhibition of this macrophage influx into the pancreas, by inhibiting an adhesion-promoting-receptor on this cell, or their depletion, prevents the development of T1D (Hutchings *et al.*, 1990; Jun *et al.*, 1999). *In vivo* and *in vitro* studies in rodents demonstrated that macrophages could play a pathogenic role on β -cells through their production of pro-inflammatory cytokines TNF- α and IL-1 β (Arnush *et al.*, 1998; Dahlen *et al.*, 1998). Indeed compared to other control strains such as non-obese resistant (NOR) mice, macrophages from NOD mice produce higher levels of the inflammatory IL-12, IL-1 β , and TNF- α cytokines after stimulation with CD40L or LPS, or after engulfment of apoptotic cells (Alleva *et al.*, 2000; Uno *et al.*, 2007). Moreover, NOD macrophages are less efficient at engulfing apoptotic cells leading to a defective clearance of apoptotic cells and this accumulation of dying cells can promote inflammatory responses (O'Brien *et al.*, 2002). This

accumulation of dying cells and their products can be related to the physiological apoptosis of pancreatic β -cells in neonatal NOD mice. Consequently, these products released by dying cells have been suggested to initiate T1D development in this strain (Trudeau *et al.*, 2000) possibly by activating other innate cells such as dendritic cells (DCs). Together, these studies support a pathogenic role for macrophages in both the initiation and destruction phases of T1D.

Conventional dendritic cells

The first suggestion of the implication of DC in T1D was the description of myeloid cells in transplanted pancreatic islets in mice (Lacy *et al.*, 1979). Moreover, the depletion of these cells facilitated graft survival in mice suggesting that antigen-presenting cells (APC) could take up and present β -cell-derived antigens to T cells, thereby inducing the diabetogenic response (Faustman *et al.*, 1984). Additional studies confirmed that self-antigens released after β -cell death are taken up by cDCs in the pancreatic islets, processed, and presented to islet-specific T cells in the pancreatic lymph nodes to initiate the diabetogenic response (Marleau *et al.*, 2008; Turley *et al.*, 2003). Importantly, a wave of β -cell death could occur physiologically in the NOD mice, at two weeks of age for tissue remodeling, at weaning due to a metabolic change, or through injury mediated by viral infections (Turley *et al.*, 2003; von Herrath *et al.*, 2003). Toll-like receptor 2 (TLR2) could participate in this process (Kim *et al.*, 2007); however, a recent study revealed that wild-type and TLR2 deficient NOD mice harbor the same incidence of T1D (Wen *et al.*, 2008). Of note, several reports have suggested that cDCs from NOD mice have increased their ability to activate T cells through higher IL-12 production and co-stimulatory molecule expression compared to C57Bl/6 mice (Poligone *et al.*, 2002; Steptoe *et al.*, 2002). Together, these studies support a diabetogenic role for cDCs in the initiation steps of this disease.

A protective role of cDC in T1D is strongly supported by the fact that mice with cDC deficiency develop autoimmune diseases (Ohnmacht *et al.*, 2009). Actually, cDCs control the peripheral tolerance in physiological and pathological conditions as these cells can induce T cell deletion, T cell anergy, or the expansion of antigen-specific Treg cells (Ueno *et al.*, 2007). In the context of T1D, cDCs can induce the expansion of self-antigen specific Treg cells that are key players in the prevention of T1D (Tang and Bluestone, 2008) and are promising therapeutic targets in this disease. Treatment of NOD mice with granulocyte colony-stimulating fac-

tor (G-CSF) increases the numbers of cDCs and pDCs in the spleen and subsequently expands Treg cells that suppress diabetogenic T cells through the production of TGF- β (Kared *et al.*, 2005). In the same line, FMS-like tyrosine kinase (Flt3)-ligand treatment administered in young NOD mice prevents the development of T1D. This molecule promotes a tolerogenic subtype of cDC that enhances Treg cell frequency in the pancreatic lymph nodes (Chilton *et al.*, 2004; O’Keeffe *et al.*, 2005). Of note, Flt3-ligand treatment is protective only when administered in the early stage of diabetes development in NOD mice when islet-specific T cells are still at low frequency (van Belle *et al.*, 2010). Importantly, G-CSF and Flt3-ligand modulate the development of both cDC and pDC populations. Consequently both types of DCs potentially play a role in the prevention of T1D.

Plasmacytoid DCs

Plasmacytoid DCs (pDCs) are professional antiviral cells that are able to detect viral RNA or DNA through TLR7 and TLR9 and in turn produce large amounts of antiviral cytokines, such as type 1 IFNs (Lande and Gilliet, 2010). A pathogenic role of pDCs in T1D is supported by observations in both humans and rodent models that type 1 IFN is produced in pancreatic islets and it could induce or promote the development of the disease (Huang *et al.*, 1995; Stewart *et al.*, 1993). Indeed the blockade of type 1 IFN pathway by antibody treatment prevents the development of T1D in NOD mice. Two other reports further strengthen a diabetogenic role of pDCs in NOD mice. One study revealed an increased frequency of type 1 IFN-producing pDCs in the pancreatic lymph nodes during the initiation of T1D (Li *et al.*, 2008). The other study showed that Flt3-ligand treatment, which resulted in expanding both cDC and pDC populations, enhanced T1D development in old NOD mice (van Belle *et al.*, 2010). In humans, conflicting results have been obtained regarding the frequency of pDCs in the blood of diabetic patients compared to healthy controls (Chen *et al.*, 2008; Vuckovic *et al.*, 2007). However, pDC from early-diagnosed patients are able to present antigen to T cells and activate them (Allen *et al.*, 2009).

Contrary to their potentially diabetogenic role, pDCs could also be protective through the expression of various molecules implicated in tolerance induction such as programmed cell death 1 ligand 1 (PD-L1), inducible T-cell costimulator (ICOS), and indoleamine 2,3-dioxygenase (IDO). One study described a protective role for pDCs in T1D using transfer of naïve diabetogenic

CD4⁺ T cells in NOD.Scid mice (Saxena *et al.*, 2007). It was shown that pDCs prevented T1D onset likely by inducing IDO production in the pancreas that inhibited the diabetogenic T cell response. IDO regulates effector T cell expansion by catalyzing oxidative catabolism of tryptophan, as free tryptophan is an essential nutrient for T cells. Interestingly, one study described that young NOD mice appear to be defective in IDO expression (Grohmann *et al.*, 2003) and over-expression of IDO extends islet graft survival (Alexander *et al.*, 2002). As detailed in the last part of this review, our group described two complementary pathways of T1D prevention by pDCs in a context of viral infection (Diana *et al.*, 2011; 2009). These studies support a protective role of pDCs in T1D and strengthen their potential use in new therapeutic strategies.

NK cells

NK cells are involved in antiviral and anti-tumor responses mainly through direct killing of target cells or indirectly by producing IFN- γ . NK cells have been described to infiltrate the pancreas of NOD mice and they have also been detected in the pancreas of diabetic patients (Brauner *et al.*, 2010; Dotta *et al.*, 2007; Poirot *et al.*, 2004). In diabetic patients, their presence in the pancreas has been associated to coxsackievirus B infection. Interestingly this cell type has been involved in diabetes induced in mouse models by coxsackievirus infection or transgenic expression of type 1 IFN (Alba *et al.*, 2008; Flodstrom *et al.*, 2002). Recent studies suggest that NK cell ligands, recognized by NKG2D and NKp46, are expressed by the pancreatic β -cells of NOD mice upon the development of diabetes as well as in β -cells from patients (Gur *et al.*, 2010; Ogasawara *et al.*, 2004). These molecules could play a key role in the destruction of pancreatic β -cells by NK cells. Of note, the depletion of Treg cells in the NOD mouse precipitates disease onset through an exacerbation of NK cell activation in the pancreas (Feuerer *et al.*, 2009). On the other hand, several studies have reported a protective role of NK cells in mouse models of T1D. Prevention of diabetes in NOD mice induced by complete Freund adjuvant injection is dependent on the presence of NK cells that produce IFN- γ (Lee *et al.*, 2004). Interestingly, impaired NK cell function has been observed in the blood of diabetic patients and in lymphoid tissues of NOD mice (Carnaud *et al.*, 2001; Ogasawara *et al.*, 2003; Rodacki *et al.*, 2007). The deleterious or beneficial role of NK cells in the development of diabetes might depend on the infectious context and the insulinitis stage.

iNKT cells

iNKT cells are non-conventional $\alpha\beta$ T cells that are restricted by the non-polymorphic CD1d molecule presenting glycolipids. These cells express an invariant TCR α chain (V α 14-J α 18 in mice and V α 24-J α 18 in humans) associated to a limited set of β chains and they harbor an activated phenotype. Upon TCR activation, these innate-like T cells promptly produce large amounts of various cytokines, thereby influencing the downstream network of other immune cells including DCs, NK cells, and lymphocytes. Many studies have demonstrated the protective role of iNKT cells against autoimmune diseases and particularly T1D (Novak *et al.*, 2007; Novak and Lehuen, 2011). The incidence of diabetes is decreased in NOD mice containing an elevated frequency of iNKT cells, either by introduction of a V α 14-J α 18 transgene or adoptive cell transfer (Hammond *et al.*, 1998; Lehuen *et al.*, 1998). The activation of iNKT cells, with specific agonist such as α -galactosylceramide or its analogues, also inhibits the development of T1D in NOD mice (Forestier *et al.*, 2007; Hong *et al.*, 2001; Mizuno *et al.*, 2004; Sharif *et al.*, 2001). It was initially proposed that iNKT cell-mediated protection was associated with the induction of Th2 cell responses to islet autoantigens (Hong *et al.*, 2001; Laloux *et al.*, 2001; Sharif *et al.*, 2001). However, following studies analyzing the protection against diabetes induced by the transfer of anti-islet CD4⁺ and CD8⁺ T cells revealed that iNKT cells impaired the differentiation of these pathogenic T cells. Instead these autoreactive T cells become anergic and did not destroy pancreatic islets (Beaudoin *et al.*, 2002; Chen *et al.*, 2005). The abortive priming of anti-islet T cells in pancreatic lymph nodes could be explained by the ability of iNKT cells to promote the recruitment of tolerogenic DCs (Chen *et al.*, 2005). A second type of NKT cells expressing variable TCRs can also inhibit the development of diabetes in NOD mice; however, the protective mechanism is still under investigation (Duarte *et al.*, 2004). iNKT cells from NOD mice are defective in number and function and this defect could contribute to T1D susceptibility (Carnaud *et al.*, 2001; Jordan *et al.*, 2007). Several reports on iNKT cell analysis in type 1 diabetic patients have been published and there is no consensus since some authors described a decreased frequency and function of iNKT cells in these patients but it has not been confirmed by other investigators (Kukreja *et al.*, 2002; Oikawa *et al.*, 2002). Despite this complexity of iNKT cell analysis in humans, it has been extensively shown that manipulations of iNKT cells prevent and even cure T1D in vari-

ous mouse models. These observations are encouraging to further develop new therapeutic strategies based on iNKT cell targeting.

Prevention of T1D by iNKT cell-pDC interactions

The use of Ins-NP model has enabled the identification of a new immune cell crosstalk regulating the development of T1D upon viral infection (Diana *et al.*, 2011; 2009). In this transgenic mouse model the pancreatic β -cells constitutively express the lymphocytic choriomeningitis virus (LCMV) nucleoprotein (NP) and consequently the viral infection triggers a rapid development of diabetes after the destruction of β -cells by antiviral T effector cells (Oldstone *et al.*, 1991). Using this model we unveiled two complementary mechanisms of regulation of T1D by innate immune cells. First, upon infection with LCMV, iNKT cells promote the recruitment of pDCs specifically in the pancreas and their production of type 1 IFN, the main antiviral cytokines (Diana *et al.*, 2009). This local iNKT-pDC crosstalk is dependent on the OX40-OX40L pathway. iNKT cells specifically express OX40 in the pancreatic tissue but not in lymphoid tissues, and experiments with blocking antibodies, as well as the transfer of wild-type iNKT cells into OX40-deficient mice, have shown the critical role of OX40-OX40L molecules in iNKT cell-pDC interaction in this tissue. As a result, in the pancreas and not in the spleen, the viral replication is rapidly controlled preventing inflammatory-mediated tissue damage and T1D development. These results reveal that a prompt and efficient antiviral response by innate cells is required to prevent diabetes when caused directly by a viral infection of the pancreatic tissue. We further observed that following viral infection, iNKT cell-pDC crosstalk dampens diabetogenic CD8⁺ T cell responses in the pancreas. In this infectious context, iNKT cells producing IL-10 in the pancreatic lymph nodes promote the production of TGF- β by pDCs. Subsequently, these tolerogenic pDCs induce the conversion of naïve CD4⁺ T cells in Foxp3⁺ Treg cells, which migrate to the pancreatic islets and are critical for preventing T1D (Diana *et al.*, 2011). Collectively, these studies support a protective role of pDCs in T1D and strengthen their potential use in new therapeutic strategies.

Concluding Remarks and Future of T1D Therapy

The role of innate cells in the development of T1D appears to be complex and varies depending on the genetic background, the environmental factors (such as viral infections) and the phase of the development of

the pathology (Figure 1). Indeed, based on studies of mouse models, these innate cells often seem to be protective in early phases of the disease, whereas at later stages, when diabetogenic T response is already initiated, innate cells may precipitate the disease. Studies of

the pathogenesis of T1D have largely focused on the analysis of diabetogenic T cells and their control by Treg cells and several clinical trials in humans are targeting this cell type. However, there is increasing evidence that innate cells play critical roles in T1D onset.

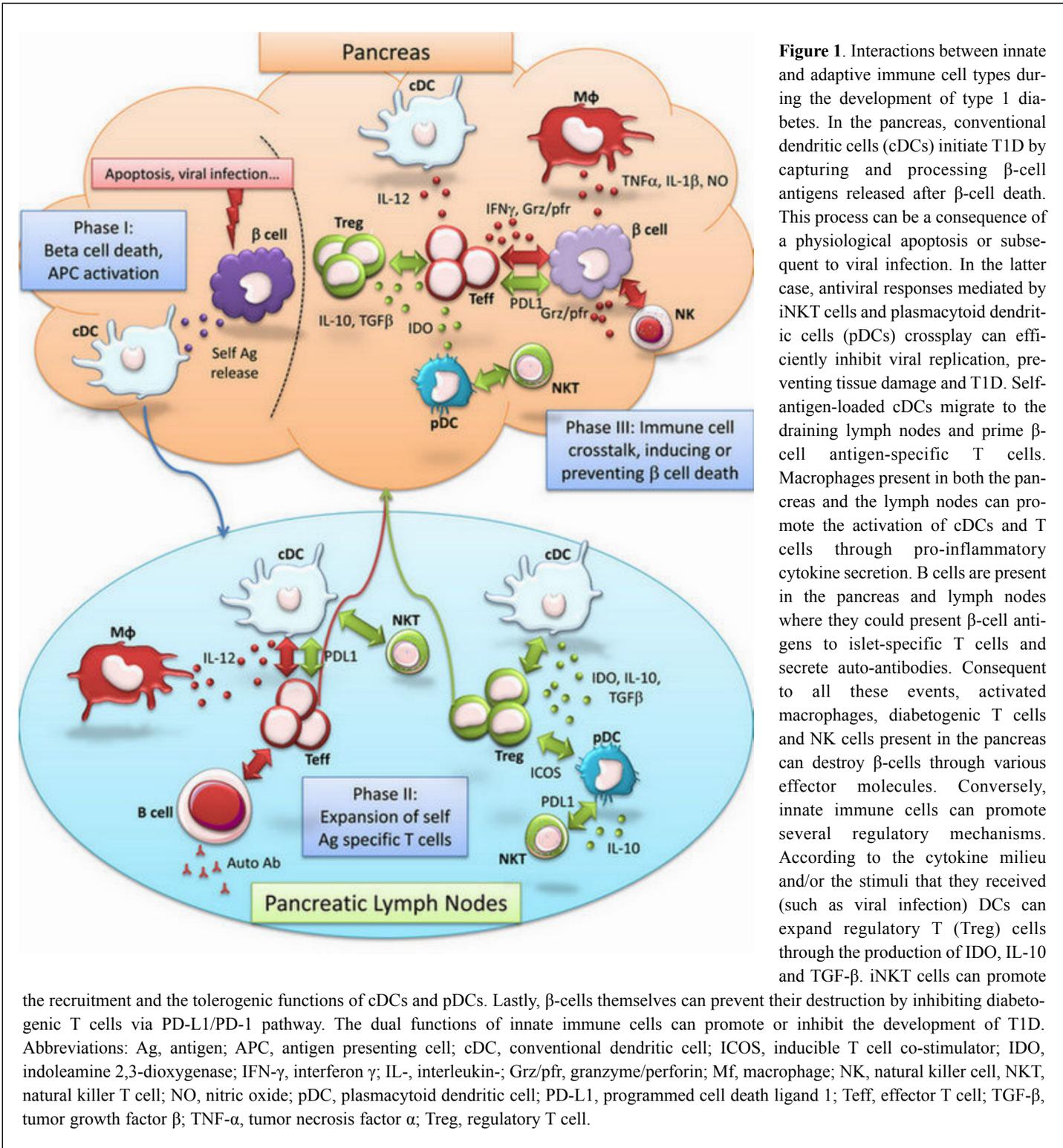


Figure 1. Interactions between innate and adaptive immune cell types during the development of type 1 diabetes. In the pancreas, conventional dendritic cells (cDCs) initiate T1D by capturing and processing β -cell antigens released after β -cell death. This process can be a consequence of a physiological apoptosis or subsequent to viral infection. In the latter case, antiviral responses mediated by iNKT cells and plasmacytoid dendritic cells (pDCs) crossplay can efficiently inhibit viral replication, preventing tissue damage and T1D. Self-antigen-loaded cDCs migrate to the draining lymph nodes and prime β -cell antigen-specific T cells. Macrophages present in both the pancreas and the lymph nodes can promote the activation of cDCs and T cells through pro-inflammatory cytokine secretion. B cells are present in the pancreas and lymph nodes where they could present β -cell antigens to islet-specific T cells and secrete auto-antibodies. Consequent to all these events, activated macrophages, diabetogenic T cells and NK cells present in the pancreas can destroy β -cells through various effector molecules. Conversely, innate immune cells can promote several regulatory mechanisms. According to the cytokine milieu and/or the stimuli that they received (such as viral infection) DCs can expand regulatory T (Treg) cells through the production of IDO, $IL-10$ and $TGF-\beta$. iNKT cells can promote

Many observations support a protective role of these cells following their triggering, by specific agonist or upon microbial infection in the early phase of the disease. Further investigations are needed to decipher why these cells are implicated in the development of T1D in absence of exogenous stimulus. T1D might be associated with some immune deficiency of innate cells rendering them unable to induce tolerance against islet antigens. Moreover, chronic low activation of these innate immune cells in the pancreas through continued β -cell death and/or persistent virus infection promote their pathogenic functions. Increasing the knowledge of regulating mechanisms of T1D by innate cells would open promising therapeutic approaches. New strategies could specifically target innate cell types such as pDCs and NKT cells, to preferentially induce protection against T1D. However, treatments targeting DC might be preferentially performed in at risk subjects at early stage of disease development, which do not yet exhibit strong anti-islet responses to avoid exacerbation on already ongoing pathogenic responses. One could also expect that new studies analyzing the functions of pDCs would identify particular pathways promoting their tolerogenic function. In the same line, many investigations are under way to generate new iNKT cell agonists that favor their regulatory functions in order to prevent the development of autoimmune diseases and particularly T1D.

Disclosure

The authors declare no competing financial interests.

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Therapeutic manipulation of natural killer (NK) T cells in autoimmunity: are we close to reality?

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Natural killer T cells

Natural killer T (NK T) cells were first described in the 1990s. These cells were characterized as a subset of T cells that share some characteristics with innate NK cells. NK T cells are present in mice, humans and other mammalian species [1]. Classically, NK T cells are divided into three subsets: type I, or invariant NK (iNK) T, type II NK T and NK T-like cells (Fig. 1). NK T cells represent a heterogeneous class of cells restricted by major histocompatibility complex (MHC) class I-like molecules such as CD1a,b,c,d, and MR1. These non-polymorphic molecules present non-protein antigens such as glycolipids and induce NK T cell activation [2]. NK T cells modulate immune responses by producing large amounts of cytokines and by the expression of various surface molecules. NK T cells influence the development of innate and adaptive immune responses. It is essential to understand more clearly the role of each NK T cell subset in the protection or exacerbation of various pathologies, and to determine if they can be manipulated therapeutically in autoimmune diseases.

Type I NK T or iNK T cells

Type I NK T cells, or iNK T cells, express an invariant T cell receptor α chain (TCR- α), V α 14-J α 18 in mice and V α 24-J α 18 in humans, and are associated with a limited set of

Summary

T cells reactive to lipids and restricted by major histocompatibility complex (MHC) class I-like molecules represent more than 15% of all lymphocytes in human blood. This heterogeneous population of innate cells includes the invariant natural killer T cells (iNK T), type II NK T cells, CD1a,b,c-restricted T cells and mucosal-associated invariant T (MAIT) cells. These populations are implicated in cancer, infection and autoimmunity. In this review, we focus on the role of these cells in autoimmunity. We summarize data obtained in humans and preclinical models of autoimmune diseases such as primary biliary cirrhosis, type 1 diabetes, multiple sclerosis, systemic lupus erythematosus, rheumatoid arthritis, psoriasis and atherosclerosis. We also discuss the promise of NK T cell manipulations: restoration of function, specific activation, depletion and the relevance of these treatments to human autoimmune diseases.

Keywords: autoimmunity, CD1, MAIT, NK T cells, therapy

TCR- β chains (V β 2, 7 or 8-3 in mice and V β 11 in humans). This T cell subset recognizes glycolipids presented by the MHC class I-like molecule, CD1d. iNK T cells specifically recognize the glycolipid α -galactosylceramide (α -GalCer) presented by CD1d [3,4]. As shown in Fig. 1, iNK T cells can be divided into distinct CD4⁺, CD4⁻CD8⁻ double-negative (DN) or CD8⁺ (in humans only) subsets [5]. Not all NK T cells express the NK1-1 (CD161 in humans) marker [1]. In humans, CD4⁺ iNK T cells produce T helper type 1 (Th1) and Th2 cytokines and CD4⁻ iNK T cells produce primarily Th1 cytokines. This dichotomy is not observed in mice [6,7]; however, functional subsets have been identified: iNK T NK1-1⁻ [8], iNK T interleukin (IL)-17⁺ (iNK T17) [9,10] and iNK T IL-17RB⁺ [11]. CD4, usually considered as a co-receptor for binding to MHC class II, is thought to interact with CD1d, thereby potentiating iNK T cell activation [12]. A new subset of iNK T cells has been described recently. These cells are reactive to α -GalCer, express the TCR- α chain V α 10-J α 50, the NK1-1 marker and secrete interleukin (IL)-4, IL-10, IL-13, IL-17 and interferon (IFN)- γ after TCR activation [13].

Type II NK T cells

Type II NK T cells express a more diverse TCR- α chain repertoire (such as V α 3-2-J α 7/9 or V α 1-J α 7/9 in mice), a limited TCR- β chain (such as V β 8 in mice) [14], and are

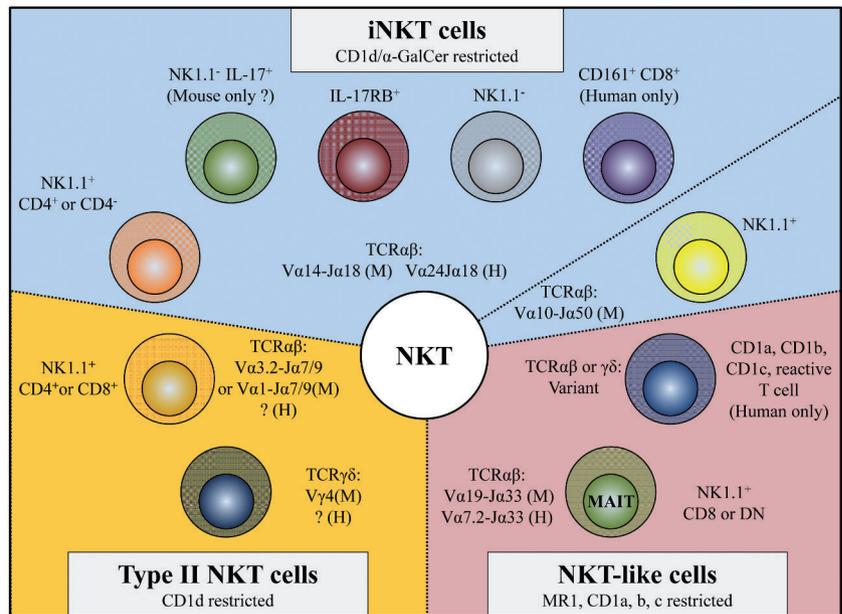


Fig. 1. Natural killer (NK T) cell populations. NK T cells can be divided into three groups: invariant NK (iNK) T (blue background), type II NK T (yellow background) and NK T-like cells (pink background). Each group is composed of distinct subsets.

present in humans [15]. Like type I NK T cells, these cells are CD1d-restricted. However, they do not recognize α -GalCer, but instead recognize other antigens such as sulphatide [16], lysophosphatidylcholine [17] or non-lipid small molecules [18]. A subset of $\gamma\delta$ T cells, expressing TCR V γ 4 in mice, is restricted to CD1d, but their antigen specificity has not been identified [19].

NK T-like cells

Mucosal-associated invariant T (MAIT) cells express an invariant TCR- α chain (V α 19-J α 33 in mice and V α 7.2-J α 33 in humans) and are restricted to the non-polymorphic MHC class I-like MR1 molecule [20]. In humans, a monoclonal antibody allows the specific detection of MAIT cells, which are primarily CD8⁺ (but may be DN), express CD161 and secrete tumour necrosis factor (TNF)- α , IFN- γ and IL-17 [21]. The nature of the antigen(s) presented by MR1 remains to be determined. One study suggested that synthetic α -mannosyl ceramide derivatives activate MAIT cells [22]. However, a subsequent study did not confirm this original observation [23].

CD1a, CD1b and CD1c MHC class I-like molecules present lipid antigens [24]. These molecules, well defined in humans, are absent in mice. In human blood, 10% of T cells are restricted to these molecules (2% are reactive to CD1a, 1% to CD1b and 7% to CD1c) [25–27]. These T cells express $\alpha\beta$ TCR [27] or $\gamma\delta$ TCR [28], but their role in autoimmunity remains unknown, as no cell-type specific markers were available until recently [27,29].

NK T cells in autoimmunity

There are two primary phases in the development of autoimmune disease: the initiation phase and the chronic phase

(Fig. 2). Because of the difficulty in determining the cause of tolerance breakdown in these pathologies, studies on the role of NK T cells in autoimmune disease initiation are limited. One interesting study suggests that iNK T cells are key players in the initiation of primary biliary cirrhosis (PBC). PBC is a chronic lethal autoimmune disease characterized by the destruction of small intrahepatic bile ducts by autoreactive T cells. In a PBC mouse model, iNK T activation upon infection by *Novosphingobium aromaticivorans* initiates liver injury [30]. Unfortunately, it will be difficult to interfere in established PBC by modulating iNK T cell function because,

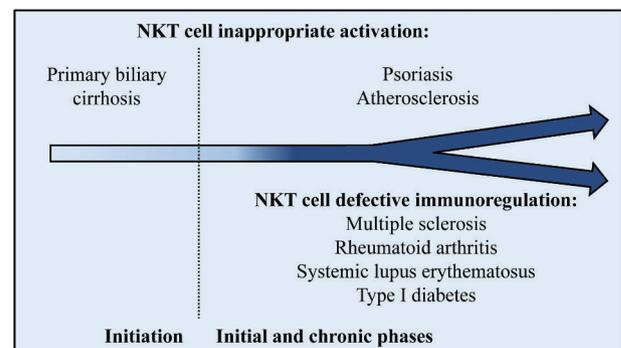


Fig. 2. Implication of natural killer (NK) T cells in human autoimmune diseases. The development of autoimmune diseases (blue arrow) can be divided into an initial and chronic phase. In primary biliary cirrhosis, invariant NK (iNK) T cells play a key role in the initial phase, whereas in other autoimmune diseases NK T cells can be involved at different phases of pathogenesis (e.g. psoriasis or multiple sclerosis). While some autoimmune diseases are associated with a defective pool of NK T cells (e.g. multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus or type 1 diabetes), others are associated with inappropriate activation (e.g. psoriasis, atherosclerosis).

at the time of diagnosis, iNK T cells are no longer required. For other autoimmune diseases, the contribution of NK T cells may be due to defective immunoregulation by NK T cells or inappropriate NK T cell activation (Fig. 2; Table 1).

Diseases linked to a defective pool of NK T cells

A functionally defective pool of NK T cells has been described in several autoimmune diseases, such as multiple sclerosis (MS), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), type 1 diabetes (T1D), Crohn's disease, Graves' disease and Sjögren syndrome [31,32].

MS. MS is characterized by neurological symptoms, including muscle spasms, muscle weakness and difficulty of movement. In MS, autoreactive T cells induce damage in the myelin sheath around the axons of the brain and spinal cord. In experimental autoimmune encephalomyelitis (EAE), a mouse model of MS, iNK T cells infiltrate the central nervous system (CNS). Mice devoid of iNK T cells ($J\alpha 18$ -deficient mice) develop a more severe EAE than control mice [33]. We have shown that increasing the number of iNK T cells protects mice from EAE by inhibiting Th1 and Th17 autoimmune responses [34,35]. This protection is independent of CD1d [35]. Recently, another group showed that iNK T cells, producing IL-4 or IL-10, inhibit Th1 responses and reduce EAE severity [33]. In the blood of MS patients, total iNK T cell frequency is decreased [31,36]. Under remission, CD4⁺ iNK T cells secrete large amounts of IL-4 that could favour a Th2 bias, suggesting a beneficial role of this subset [36]. In contrast to mouse models, iNK T cells have not been detected in human CNS lesions [37].

An increased number of type II NK T cells are observed in the CNS during EAE, and treatment of mice with sulphatide prevents development of the disease [16]. Increasing the number of MAIT cells ($V\alpha 19$ TCR transgenic mice) protects mice against the induction and progression of EAE. Mice devoid of MAIT cells (MR1-deficient mice) present an exacerbated form of EAE. In $V\alpha 19$ transgenic mice, as well as in wild-type mice subjected to adoptive transfer with MAIT cells, these cells modulate EAE severity by reducing the production of inflammatory cytokines and enhancing B cell IL-10 secretion in an inducible T cell co-stimulatory (ICOS)-B7RP-1 manner [38]. Polymerase chain reaction (PCR) analysis suggests that MAIT cells accumulate in human CNS [39]. More recently, flow cytometry analysis shows that MS patients harbour a lower frequency of MAIT cells in blood compared to healthy controls. The authors observed a positive correlation between clinical recovery and increase in MAIT cell frequency and that MAIT cells suppress IFN- γ production by T cells *in vitro* in a contact-dependent manner [40].

CD1b-reactive T cells are more frequent in the blood of MS patients than in healthy individuals. These cells respond to several glycolipids from the CNS and release IFN- γ and

TNF- α [41]. Their role, as well as the role of CNS self-lipids (e.g. ganglioside, sulphatide) in NK T cell activation, remains to be investigated [42].

SLE. SLE is characterized by a range of symptoms, including arthritis, facial rash, pleuritis, pericarditis and photosensitivity. Inappropriate activation of autoreactive T cells and autoantibody production cause acute and chronic inflammation of various tissues such as skin, kidney, joints and the nervous system. Two SLE mouse models (MRL-*lpr* and SLE pristane-induced) exhibit a reduced number of iNK T cells at disease onset in secondary lymphoid organs [43,44]. However, New Zealand black/white (NZB/W) F₁ mice do not have a defect in NK T cell frequency and iNK T cells are hyperactive, as indicated by cytokine production (IFN- γ and IL-4) [45]. Treatment of 3-month-old (NZB/W) F₁ mice with anti-CD1d blocking antibodies decreases disease severity, wherein iNK T cells interact with B cells to promote production of autoantibodies [45,46]. Paradoxically, CD1d-deficient (NZB/W) F₁ mice develop an exacerbated disease [47], similar to CD1d-deficient MRL-*lpr* [48]. The regulatory role of iNK T cells on B cell activation has also been described in another SLE mouse model. Injection of apoptotic cells induces autoreactive B cell activation and production of anti-DNA immunoglobulin (Ig)G in C57BL/6 mice. Autoimmune responses are increased in CD1d- and $J\alpha 18$ -deficient mice, which present immune complex deposition in the kidneys. CD1d expression on B cells is required for their suppression by iNK T cells [49]. These observations suggest that in the early phase of SLE development iNK T cells are protective, but promote autoantibody production later.

iNK T cell numbers decrease in the blood of SLE patients compared to healthy controls [31,50]. The reduced numbers affect DN, CD4⁺ and CD8⁺ subsets [50]. In addition, iNK T cells from SLE patients are functionally defective [50,51]. iNK T cell defect is associated with a defect of lipid antigen presentation by immature B cells from SLE patients [50].

CD1c-restricted T cell lines derived from SLE patients are more activated than cells from healthy individuals. These cells provide help to B cells in secreting pathogenic IgG antibodies [52], suggesting a pathogenic role.

RA. RA is characterized by joint deformity and loss of movement. RA autoantibodies and autoreactive T cells induce chronic inflammation in the synovial membrane of the joint. There is no evidence of a decrease in iNK T cell numbers in a collagen-induced arthritis (CIA) RA mouse model [53]. On the contrary, mice devoid of iNK T cells ($J\alpha 18$ -deficient mice) present an attenuated form of RA [54,55]. Recently, it has been shown that iNK T cells are activated in early-stage CIA and anti-CD1d blocking antibody treatment improves the clinical signs of arthritis [56]. The pathogenic mechanism of iNK T cells is unclear. One report demonstrated that antibodies activate iNK T cells directly through Fc γ RIII in an antibody-induced arthritis

Table 1. Role of invariant natural killer (iNK) T, type II NK T and NK T-like cells in autoimmune diseases.

	iNK T cells			Type II NK T cells		NK T-like cells	
	Mouse		Human	Mouse	Human	Mouse	Human
	Mouse	Human	Human	Mouse	Human	Mouse	Human
Multiple sclerosis	Inhibit autoreactive T cells by IL-4 or IL-10 secretion Infiltrate lesions	Defect of DN subset in blood	Defect of DN subset in blood	Infiltrate lesions		MAIT cells decreased EAE severity in an IL-10-dependent manner	MAIT cells infiltrate lesions and inhibit autoreactive T cells Enhanced frequency of CD1b-restricted T cells
Systemic lupus erythematosus	Low frequency is associated with pathology except in (NZB × NZW) _{F1} mice Induce autoantibody production Hyperactive Disease amelioration in CD1d-deficient mice	Low frequency (CD4, DN) in blood of patients	Regulate autoantibody production	Protective role			CD1c-restricted T cells induce autoantibody production
Rheumatoid arthritis	Anti-CD1d treatment delays pathology Activation through FcγRIII Suppress TGF-β production	Infiltrate synovial junction	Low frequency in blood Functional defect	Protective role		MAIT cells promote inflammation and exacerbate disease	
Type I diabetes	Infiltrate pancreas Protective role of CD4 ⁺ subset Reduced number (CD4 ⁺ subset) Lower CD1d expression Inhibit autoreactive T cells Promote 'tolerogenic DC' Pathogenic role of iNK T17 cells	Controversial data on iNK T cell frequency and function		Protective role Present in pancreas		Protective role of MAIT cells (mechanism unknown)	
Psoriasis	Infiltrate lesions	CD4 ⁺ subset infiltrate lesions High CD1d expression by keratinocytes IFN-γ secretion					
Atherosclerosis	Infiltrate lesions Attenuate disease in CD1d-deficient mice	CD4 ⁺ subset infiltrate lesions High CD1d expression in lesions IL-8 secretion More active in lesions					CD1a,b,c expression in atherosclerotic lesion

■ Protective role
■ Deleterious role
■ Ambivalent role
 Absence of data or controversial data

DC: dendritic cells; IL: interleukin; IFN: interferon; TGF: transforming growth factor; EAE: experimental autoimmune encephalomyelitis; MAIT: mucosal-associated invariant T; DN: double negative; NZB/NZW: New Zealand white/New Zealand black.

mouse model [55]. In that model, iNK T cells inhibit TGF- β production and promote arthritis by producing IL-4 and IFN- γ [57]. In contrast to mouse models, low numbers of circulating iNK T cells (particularly the DN subset) have been described in RA patients [51,58–60]. iNK T cells were detected in the synovium of patients and are biased towards Th0-like cytokine profiles upon α -GalCer activation [61]. Interestingly, in RA patients treated with anti-CD20, iNK T cell numbers increased, suggesting a beneficial role for these cells [60].

Another recent study showed that, in mice, an immunodominant peptide of mouse collagen presented by CD1d activates type II NK T cells, which inhibits the development of CIA [62]. However, additional studies are still needed to characterize NK T cells more clearly in mice and humans.

Mice devoid of MAIT cells (MR1-deficient mice) develop a milder disease than control mice, suggesting that MAIT cells promote inflammation and exacerbate RA. However, the mechanism remains unknown [63].

T1D. T1D is characterized by hyperglycaemia, polyuria, polydipsia, polyphagia and weight loss, and is lethal in the absence of insulin treatment. T1D is a chronic autoimmune disease in which insulin-secreting pancreatic β cells are destroyed selectively. It is thought to be a Th1-mediated disease with involvement of CD8⁺ T cells and macrophages. Several mouse model studies provide a converging picture of a protective role for iNK T cells in T1D [3]. iNK T cell numbers are reduced in young non-obese diabetic (NOD) mice [64,65], and increasing their number by adoptive transfer [66,67] or via the introduction of a V α 14-J α 18 transgene inhibits development of T1D [66]. Moreover, CD1d deficiency exacerbates diabetes in NOD mice [68]. Early reports suggest that iNK T cell protection is associated with induction of a Th2 response to islet autoantigens [69–72]. However, experiments based on the transfer of monoclonal anti-islet T cells showed that iNK T cells inhibit differentiation of autoreactive T cells into effector cells during their priming in pancreatic lymph nodes [73,74]. Defective priming of autoreactive T cells could reflect the ability of iNK T cells to promote recruitment of tolerogenic dendritic cells [74,75]. We described recently the functional dichotomy between CD4⁺ and DN iNK T cell subsets in the regulation of T1D. While CD4⁺ iNK T cells strongly protect NOD mice against diabetes, DN iNK T cells (containing iNK T17 cells) increase diabetes incidence. Importantly, exacerbation of diabetes by DN iNK T cells is abrogated by treating with an anti-IL-17 blocking antibody [76]. Interestingly, NOD mice contain a higher frequency of iNK T17 cells and fewer CD4⁺ iNK T cells compared to non-autoimmune C57BL/6 mice [76,77].

Contrary to autoimmune diseases cited previously, there is no clear evidence of a role for iNK T cells in T1D aetiology. PCR analysis found a lower frequency of DN iNK T cells in diabetic blood compared to discordant diabetic twins

without the disease [78]. However, flow cytometry analysis showed similar V α 24⁺ CD1d-tetramer⁺ iNK T cell frequency in discordant monozygotic twins [79]. Other studies reporting either low or high iNK T cell numbers in the blood of diabetic patients have been published [80,81]. Of note, analysis of several mouse strains showed that iNK T cell frequency in blood is not correlated with their frequency in lymphoid tissues [82]. Functional studies show that iNK T clones from pancreatic lymph nodes of diabetic patients exhibit defective IL-4 production [83].

Type II NK T cells inhibit diabetes progression in NOD mice. Diabetes protection was observed in V α 3-2-V β 9 TCR transgenic NOD mice harbouring elevated numbers of type II NK T cells as well as by adoptive transfer [84]. These type II NK T cells dampen the diabetogenic T cell response through regulatory mechanisms involving programmed cell death ligand 1 (PD-L1) and ICOS molecules [85].

Similarly, increasing the number of MAIT cells via the introduction of a V α 19-J α 33 TCR transgene in NOD mice reduces T1D onset significantly [86]. However, the mechanism by which MAIT cells prevent diabetes, as well as the role of these cells in T1D patients, remains to be elucidated.

Diseases linked to an inappropriate activation of NK T cells

Psoriasis. Psoriasis is characterized by the presence of red dry plaques on the skin. In psoriasis, innate and autoreactive T cells induce inflammation through TNF- α production, leading to abnormal proliferation of skin cells. In mouse models, NK T cells infiltrate the psoriatic plaques [87,88]. Similarly, human studies revealed an increased iNK T cell number, particularly of the CD4⁺ subset, in psoriatic lesions compared to healthy skin [87,89]. Furthermore, CD1d expression is higher in keratinocytes from psoriasis patients, and NK T cells co-cultured with keratinocytes from psoriasis patients produce IFN- γ [87]. Together, these mouse and human data suggest the involvement of NK T cells in psoriatic skin lesions.

Atherosclerosis. Atherosclerosis is involved in the development of cardiovascular diseases and exhibits aspects of autoimmune disease, including the presence of autoantibodies and autoreactive T cells against heat shock protein 60 (HSP60) [90]. In atherosclerosis, accumulation of immune cells and lipid particles in blood vessels leads to narrowing of the arterial lumen and causes thrombosis. Mouse models have shown the pro-atherogenic effect of iNK T cells [91–93]. ApoE-CD1d double-deficient mice exhibit a 25% decrease in lesion size [93]. CD4⁺ iNK T cells appear to be responsible for the proatherogenic activity of iNK T cells due to production of more proinflammatory cytokines (IL-2, TNF- α , IFN- γ) than DN iNK T cells [94]. In humans, iNK T cells are present and CD1d expression is enhanced in atherosclerotic plaques [95–97]. As observed in the mouse model,

CD4⁺ iNK T cells infiltrated human atherosclerotic lesions. Infiltrating iNK T cells secrete large amounts of IL-8, a chemoattractant for immune cells [97]. Furthermore, enhanced CD1a,b,c expression in macrophages was observed in atherosclerotic plaques compared to healthy controls [98]. These observations suggest a role for NK T-like cells in atherosclerosis.

Human NK T cell deficiency and autoimmunity

Genetic defects affecting lymphocyte signalling pathways (e.g. *ITK*, *XIAP*, *SH2D1A*), lipid transfer and processing proteins (e.g. *MTP*, *NCP2*) are associated with dysfunction of iNK T cells (reduced/absence of function and number) in humans. Patients with these disorders seem more susceptible to selective viral infections (e.g. Epstein–Barr virus), but do not present with autoimmune disorders. It is possible that development of autoimmune disorders is hampered by the fact that patients affected by these mutations experience a shortened life expectancy, wherein treatments such as stem cell transplantation may be performed to ameliorate symptoms [99].

NK T cells as therapeutic agents in autoimmunity

Harnessing of iNK T cell using specific ligands

Several autoimmune diseases exhibit a defective pool or function of NK T cells. During the past 10 years, many molecules have been tested for their ability to activate iNK T cells.

α-GalCer treatment. The glycolipid α -GalCer stimulates iNK T cells in mice and humans. Recognition of the CD1d– α GalCer complex by the semi-invariant TCR of iNK T cells results in rapid production of cytokines. Single or repeated injections of α -GalCer in mice give different outcomes. A single injection of α -GalCer induces IL-12 production and CD40 up-regulation by dendritic cells (DC) [100] and CD40L up-regulation on iNK T cells. The interaction between these two cell types induces a strong secretion of IFN- γ and IL-4 by iNK T cells and DC maturation [101]. This cross-talk leads to activation of NK cells (through IFN- γ produced by iNK T cells) and conventional CD4 and CD8 T cells (through mature DCs) [102]. On the contrary, repeated α -GalCer injections biased DC maturation towards a tolerogenic phenotype in an IL-10 dependent manner [103]. Furthermore, iNK T cells become unable to produce IFN- γ and IL-17 but their IL-4 production, although weaker, persists [76]. Both mechanisms probably contribute to inhibition of pathogenic autoreactive T cell responses. Therefore, repeated α -GalCer treatments may represent an attractive strategy for preventing autoimmune diseases as treatment in mice is protective against EAE [104,105], SLE [106], RA [53,107] and T1D [70–72,108]. However, depending on the timing and frequency of injections, age and sex of the mice,

α -GalCer may exacerbate some autoimmune diseases [57,105,109,110]. α -GalCer could also be deleterious in the context of atherosclerosis [92,93], allergic reaction [111] and asthma [112]. IL-4 secretion by iNK T cells during repeated α -GalCer treatment could promote the development of asthma through IgE induction and eosinophil recruitment [111–113], although the precise role of iNK T cells in asthma remains controversial [114]. Together, these data suggest that α -GalCer treatment might not be the most appropriate to prevent autoimmune diseases. In this regard, other iNK T cell agonists have been generated and tested in mouse models.

α-GalCer analogues: a perspective. Structural modifications of α -GalCer influence the iNK T cytokine secretion profile towards Th1 or Th2 [115]. The analogue OCH skews T cell responses towards Th2 through the production of IL-4 by iNK T cells, and a single OCH injection inhibits EAE [116]. This protective effect has been confirmed in other autoimmune diseases such as CIA [117], T1D [108] and colitis [118] in mice. Another Th2-biased analogue, C20:2, protects NOD mice against diabetes. The C20:2 molecule seems to favour the generation of tolerogenic DCs and inhibits IL-12 production by DCs [70,119]. However, as OCH and C20:2 skew T cell responses towards a Th2 profile, these molecules could promote the development of asthma. A new analogue, C16:0, that induces only moderate IFN- γ and IL-4 production by iNK T cells, is more efficient than α -GalCer in preventing T1D in NOD mice [120]. Because C16:0 induces very little IL-4 production, it may be a good candidate for a T1D clinical trials. It would be interesting to evaluate further the efficacy of C16:0 in other T1D models, such as virus-induced diabetes and other autoimmune diseases. Moreover, it would be important to determine the ability of C16:0 to reverse an established disease.

NK T cell agonists in clinical trials. Phase I cancer clinical trials revealed that soluble α -GalCer treatment is safe, but exerts moderate immunostimulatory effects [91–93]. This difference between humans *versus* mice might reflect the lower frequency of iNK T cells in humans. iNK T cells represent 0.2–0.5% of blood lymphocytes in mice *versus* 0.01–1% in humans [94] and 30% of liver lymphocytes in mouse *versus* 1% in humans. This lower frequency in humans suggests that α -GalCer analogue therapy might be less efficient in humans than in mice. Because iNK T cell numbers are quite variable in humans, individuals with a higher iNK T cell number should be favoured for iNK T cell-specific therapy. However, it will be important to investigate the expansion ability of iNK T cells from individuals exhibiting different iNK T cell frequencies. Moreover, it will be important to analyse iNK T cell subsets in patients before and during iNK T cell therapy to determine the effect of iNK T cell analogues on different subsets. Further investigation is required on type II NK T cells and MAIT

cells before using them for therapeutic purposes. Furthermore, the interplay between type I, type II and NK T-like cells during glycolipid treatment remains poorly characterized. Interestingly, researchers have noted the activation of type II NK T cells by sulphatide-induced energy in type I NK T cells in a mouse model of inflammatory liver disease [121].

Restoration of iNK T cell numbers

In vitro iNK T cell expansion. Increasing the number of iNK T cells by adoptive transfer reduces significantly the progression of autoimmune diseases in mouse models [66,67]. In humans, a Phase I clinical trial showed that injection of *in vitro* expanded iNK T cells is safe and well tolerated [122]. This strategy could have the advantage of expanding and selecting defined subsets of iNK T cells (e.g. CD4⁺ iNK T cells in MS).

Enhanced self-ligand presentation. The role of self-antigen(s) and the mechanisms triggering NK T cell activation in autoimmune diseases remain unknown. Mouse NK T cell clones can be activated by endogenous tumour cell ligands [123]. Microbial infections enhance the expression of glucosylceramide synthase, leading to the synthesis of β -glucosylceramide (β -GlcCer). This self-glycolipid presented by CD1d activates iNK T cells and induces their proliferation [124]. iNK T cell function may be promoted by enhancing the expression of glucosylceramide synthase (or other enzymes) that increases presentation of self-glycolipids capable of activating iNK T cells.

Interestingly, IFN- β treatment ameliorates the disease in MS patients [125]. This treatment increases the frequency and enhances the function of iNK T cells (IL-4, IL-5 and IFN- γ production) in the blood of MS patients. This iNK T cell modulation is mediated by DCs that up-regulate CD1d and CD40 expression [126].

Therapeutic approach for autoimmune diseases associated with inappropriate NK T cell activation

Diseases with inappropriate NK T cell activation (e.g. psoriasis or atherosclerosis) are characterized by elevated CD1d

expression in lesions. Antibodies against CD1d have been developed, and could be used to block NK T cell activation. However, anti-CD1d antibodies added to human PBMC cultures induce IL-12 production by DC [127]. Therefore, such antibodies might not be effective for inhibiting the development of autoimmune diseases. Another approach could be the depletion of NK T cells by using specific antibodies. Recently, a monoclonal antibody recognizing human iNK T cells has been generated [128], and could be modified to induce depletion, rather than activation.

Conclusion and perspectives

Studies in patients and animal models of autoimmune diseases describe different roles for NK T and iNK T cell subsets. For example, CD4⁺ iNK T cells prevent T1D in NOD mice, whereas iNK T17 cells aggravate the disease. In MS patients under remission, CD4⁺ iNK T cells secrete large amounts of IL-4, suggesting a beneficial role of these cells. In contrast, CD4⁺ iNK T cells infiltrate lesions in psoriasis and atherosclerosis, and might be pathogenic. All these data suggest that protection or exacerbation of autoimmune diseases by iNK T cells may be due to disequilibrium between the different subsets (Fig. 3). As highlighted in previous reviews [129,130], most studies used methods that do not identify iNK T cells clearly (e.g. V α 24 PCR, TCR⁺ NK1.1⁺ or CD56⁺ CD3⁺ staining). CD8⁺ iNK T cells, representing 20% of human iNK T cells in blood, were rarely analysed and most of the studies focused on CD4⁺ or DN cells. Similarly, the only cytokines produced by NK T cells were IL-4 and IFN- γ , and only a few studies explored the secretion of IL-2, IL-5, IL-13, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), TGF- β or chemokines. It is important to note that iNK T cell number is higher in mice than in humans, whereas type II NK T [131] and MAIT cells [132] are more abundant in humans than in mice. CD1a,b,c-restricted T cells are present in humans, but not mice, due to deletion of CD1 genes in mice, which suggests that CD1d-restricted NK T cells might compensate for these cell populations. Humanized mice expressing these molecules have been generated [133], and it would be interesting to cross them into genetic backgrounds susceptible to

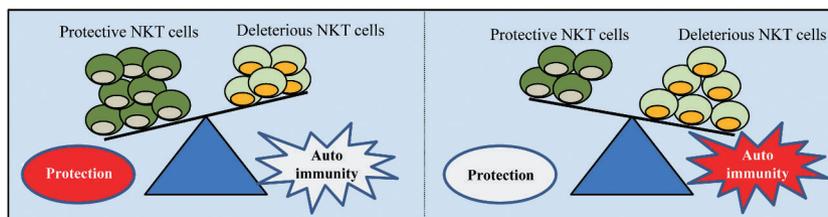


Fig. 3. Hypothesis regarding the effect of various natural killer (NK) T subsets in autoimmunity. NK T cell subsets exert different roles in autoimmune diseases. For example, in non-obese diabetic (NOD) mice developing type 1 diabetes CD4⁺ invariant NK (iNK) T cells are protective, whereas iNK T17 cells enhance disease incidence. Therefore, protection or exacerbation of autoimmune diseases by NK T cells could be due to disequilibrium between different cell subsets.

autoimmune disease. Future human studies should focus on less characterized innate T cells. Similarly, the role of self-ligands, cytokine environment and accessory molecules (e.g. NKG2D) required for NK T cell activation should be investigated further [130,134,135]. More extensive research must be performed on specific tissues. For example, the characterization of NK T cells in the pancreas of type 1 diabetic patients remains to be investigated. New mouse models of autoimmune diseases would be useful, in particular, to understand apparent discrepancies in the role of NK T cells between mouse models and human diseases [136]. For example, in mouse models of RA, iNK T cells have a deleterious role, whereas in human RA, iNK T cells seem to exhibit a protective role. It would be interesting to analyse the behaviour of human iNK T cells in humanized mice reconstituted with human stem cells.

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Disclosure

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Scientific summary

iNKT cells are non-conventional T lymphocytes that are restricted to glycolipid presenting CD1d molecule. iNKT cells express an invariant TCR α chain (V α 14-J α 18 in mice and V α 28-J α 18 in humans). Their particularity is to rapidly produce copious amounts of cytokines (IFN- γ and IL-4) after activation and to activate other cells of the immune system such as dendritic cells, NK cells and T lymphocytes. iNKT cells, therefore, form a bridge between innate and adaptive immune responses.

Type 1 diabetes is an autoimmune disease characterized by the destruction of pancreatic β cells whose role is to produce insulin. While diabetes development can clearly be associated with genetic polymorphisms, environmental factors were also implicated in the etiology of the disease. Numerous studies suggest that viral infections, particularly infections with Coxsackievirus B4 (CVB4), could be implicated in the development of type 1 diabetes.

Our study was performed with NOD mice that develop type 1 diabetes around 15 weeks of age and with proinsulin 2 knockout NOD mice (Pro-ins2^{-/-}) which become diabetic around 8 weeks of age. Our results show that CVB4 infection induces accelerated diabetes in around half of NOD and Pro-ins2^{-/-} mice compared to uninfected mice. However, the activation of iNKT cells with their agonist, α Galactosylceramide (α GalCer), at the time of infection greatly decreases diabetes incidence. CVB4 infection induces a strong recruitment of macrophages into the pancreas. Interestingly, iNKT cell activation modifies the function of these macrophages. Indeed, pancreatic macrophages of CVB4 infected mice strongly express IL-1, IL-6 and TNF- α , indicating their pro-inflammatory character. On the contrary, macrophages of mice infected with CVB4 and treated with α GalCer express low levels of these cytokines, but strong levels of suppressive enzymes iNOS (inducible NO synthase), IDO (Indoleamine 2,3-dioxygenase) and arginase I. The use of inhibitors of these enzymes showed that diabetes prevention is induced by IDO. We have also observed that autoreactive T cells strongly infiltrate the pancreatic islets after CVB4 infection. It is interesting to note that the high diabetes incidence of CVB4 infected mice is associated with an increased frequency of IFN- γ producing autoreactive T cells in pancreatic islets. On the contrary, the frequency of these cells is very low in infected mice treated with α GalCer. The inhibition of IFN- γ production is dependent on IDO enzyme, since the use of its inhibitor strongly increases IFN- γ production by anti-islet T cells and diabetes incidence.

To summarize, our results show that iNKT cell activation during the infection with CVB4 induces immunosuppressive macrophages in the pancreas. These cells inhibit the function of autoreactive T cells and prevent diabetes development.

Résumé scientifique

Les cellules NKT invariantes (iNKT) sont des lymphocytes T non conventionnels restreints par la molécule CD1d qui présente des glycolipides. Les cellules iNKT expriment un TCR avec une chaîne α invariante, V α 14-J α 18 chez la souris et V α 28-J α 18 chez l'homme. Elles ont la particularité de produire de grande quantité de cytokines (IFN- γ et IL-4) rapidement après leur activation et peuvent à leur tour stimuler d'autres cellules du système immunitaire comme les cellules dendritiques, les cellules NK et les lymphocytes T. Elles représentent ainsi un pont entre les réponses immunitaires innées et adaptatives.

Le diabète de type 1 est une maladie autoimmune caractérisée par la destruction des cellules β pancréatiques productrices d'insuline. Bien que l'apparition de diabète de type 1 soit associée à des polymorphismes génétiques, les facteurs environnementaux ont également été impliqués dans l'étiologie de cette maladie. De nombreuses études suggèrent que les infections virales, en particulier les infections par le virus de coxsackie B4 (CVB4), pourraient être impliquées dans le développement de cette maladie.

Notre étude a été réalisée avec des souris NOD qui développent un diabète de type 1 vers 15 semaines d'âge et des souris NOD déficientes pour la proinsuline 2 (Pro-ins2^{-/-}) développant un diabète vers 8 semaines d'âge. Nos résultats montrent qu'après infection par CVB4, la moitié des souris NOD et Pro-ins2^{-/-} développent un diabète accéléré par rapport à des souris non infectées. Toutefois, une injection de l'agoniste des cellules iNKT, la molécule α Galactosylceramide (α GalCer), au moment de l'infection des souris, diminue fortement l'incidence de diabète. L'infection par CVB4 induit un fort recrutement de macrophages dans le pancréas et l'activation des cellules iNKT modifie la fonction de ces macrophages. En effet, les macrophages pancréatiques des souris infectées par CVB4 expriment fortement les cytokines IL-1 β , IL-6 et TNF- α , révélant leur caractère pro-inflammatoire alors que les macrophages des souris infectées et traitées par α GalCer expriment faiblement ces cytokines inflammatoires et fortement des enzymes immunosuppressives iNOS (inducible NO synthase), IDO (Indoleamine 2,3-dioxygenase) et arginase I. L'utilisation d'inhibiteurs de ces enzymes montre que la protection contre le diabète est induite par IDO. Nous avons également observé une forte infiltration de lymphocytes T autoréactifs dans les îlots pancréatiques des souris infectées. De façon intéressante, l'incidence accrue de diabète du groupe CVB4 est associée à une fréquence élevée de cellules T autoréactives produisant de l'IFN- γ dans le pancréas, alors que la production d'IFN- γ par les cellules T autoréactives est très faible dans les souris du groupe CVB4+ α GalCer. Cette inhibition de la production d'IFN- γ est dépendante de l'enzyme IDO, car l'utilisation d'un inhibiteur d'IDO augmente fortement la production d'IFN- γ par les lymphocytes T anti-îlots et l'incidence de diabète.

Dans l'ensemble nos résultats montrent, que l'activation des cellules iNKT lors de l'infection par CVB4 induit des macrophages immunosuppresseurs dans le pancréas, ces cellules inhibant la fonction des lymphocytes T autoréactifs et ainsi le développement du diabète.