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Université Paris 5 René Descartes

Ecole doctorale GC2ID
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THESE
Pour obtenir le grade de
DOCTEUR DE L'UNIVERSITE PARIS 5 RENE DESCARTES

L'immunité innée dans le diabète sucré

Présentée et soutenue publiquement le 26 novembre 2013 par

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Pr. Christian Boitard	Président du jury
Dr. Stéphanie Hugues	Rapporteur
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Dr. Sylvaine You	Examinateur
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A Paloma Fernández Quiros qui m'a beaucoup appris…

A ma soeur jumelle, mon cats et ma famille…

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RESUME

Le diabète de type 1 (T1D) est une maladie auto-immune caractérisée par la destruction des cellules β du pancréas par les lymphocytes T auto-réactifs. Durant ma thèse, nous nous sommes intéressés au rôle des cellules de l'immunité innée dans le T1D à l'aide d'un modèle murin de la maladie : la souris NOD. Au contraire des cellules du système adaptatif (lymphocytes T et B), les cellules de l'immunité innée constituent la première ligne de défense de l'organisme lors d'une infection. Cette population est constituée entre autre de neutrophiles, cellules dendritiques plasmacytoïdes (pDC), macrophages, mais aussi de lymphocytes T et B non conventionnels tel que les cellules iNKT et B-1a.

Précédemment, notre laboratoire a mis en lumière le rôle des lymphocytes iNKT dans le développement du T1D. Durant la première partie de ma thèse, nous avons démontré que les lymphocytes iNKT17, une sous-population des lymphocytes iNKT, ont un rôle délétère dans le T1D chez la souris NOD. Ces cellules infiltrent le pancréas et y produisent de l'IL-17, une cytokine pro-inflammatoire. Grâce à des expériences de transferts, nous avons mis en évidence que les lymphocytes iNKT17 exacerbent la maladie via la production d'IL-17.
Dans la deuxième partie de ma thèse, nous nous sommes intéressés aux mécanismes qui induisent l'activation des lymphocytes T auto-réactifs. Nous avons observé chez la souris NOD, que la mort physiologique des cellules β conduit à l'activation de cellules de l'immunité innée : les neutrophiles, les lymphocytes B-1a et les pDC. La coopération entre ces cellules conduit à l'activation des pDC qui produisent de l'IFNα. Cette cytokine active les lymphocytes T auto-réactifs qui vont détruire les cellules β du pancréas.

Nos résultats montrent que l'immunité innée est un acteur important dans la physiopathologie du diabète *sucré.*

PUBLICATIONS

Cette thèse est basée sur les articles suivants:

- Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes.
Diana J, **Simoni Y**, Furio L, Beaudoin L, Agerberth B, Barrat F, Lehuen A.
Nature Medicine. 2013

- Therapeutic manipulation of NKT cells in autoimmunity: Are we close to reality?
Simoni Y,Diana J, Ghazarian L, Beaudoin L and Lehuen A.
Clin. Exp. Immunol. 2013

- NOD mice contain an elevated frequency of iNKT17 cells that exacerbate diabetes.
Simoni Y, Gautron AS, Beaudoin L, Bui LC, Michel ML, Coumoul X, Eberl G, Leite-de-Moraes M, Lehuen A.
Eur. J. Immunol. 2011

- Innate immunity in type 1 diabetes.
Diana J, Gahzarian L, **Simoni Y**, Lehuen A.
Discovery medicine 2011

Articles non inclus dans la thèse:

- Plasmacytoid dendritic cells license regulatory T cells upon iNKT cell stimulation preventing autoimmune diabetes
Beaudoin L, Diana J, **Simoni Y**, Ghazarian L, Lehuen A
Submitted

- Rôle régulateur des lymphocytes NKT dans la prévention du diabète de type 1
Ghazarian L, **Simoni Y**, Beaudoin L, Pingris K Lehuen A
Medecine&sciences 2013

- Prevention or acceleration of type 1 diabetes by viruses.
Ghazarian L, Diana J, **Simoni Y**, Beaudoin L, Lehuen A
Cell. Mol. Life Sci. 2012

Dans un souci de clarté, cette thèse est divisée en deux parties distinctes. La première partie traite de l'immunité innée dans le diabète de type 1, la seconde partie de l'immunité innée dans le diabète de type 2.
La première partie est divisée en deux chapitres. Le chapitre 1 s'intéresse aux lymphocytes iNKT17, le chapitre 2 aux pDC, neutrophiles et lymphocytes B-1.
La seconde partie se compose du chapitre 3 et s'intéresse aux lymphocytes MAIT.
Chacun de ces chapitres possède sa propre discussion.

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ABREVATIONS

APC: antigen presenting cell
BB: rat biobreeding
BM: bone marrow
CCL: C-C chemokine ligand
CCR: C-C chemokine receptor
cDC: conventional dendritic cell
CXCL: chemokine (C-X-C motif) ligand
CXCR: chemokine (C-X-C motif) receptor
CMH: Complexe majeur d'histocompatibilité
DC : dendritic cell
EAE: experimental autoimmune encephalomyelitis
Flt3L: fms-like tyrosine kinase 3 ligand
GM-CSF: granulocyte macrophage-colony stimulation factor
IDO: indoleamine 2,3-dioxygenase
IFN: interferon
IL: interleukin
iLN: inguinal lymph node
KO: knock out
LPS: lipopolysaccharide
mDC: myeloid dendritic cell
mLN: mesenteric lymph nodes
MΦ: macrophage
NOD: nonobese diabetic
pDC: plasmacytoid dendritic cell
PD-L1: programmed death ligand 1
pLN: pancreas draining lymph node
SCID: severe combined immunodeficiency
SLE: systemic lupus erythematosus
T1D: Type 1 Diabetes
T2D : Type 2 Diabetes
TGF: transforming growth factor
TLR: toll-like receptor
TNF: tumor necrosis factor

Préambule Le Diabète sucré

A l'inverse des plantes, les hommes – et plus généralement les métazoaires – tirent leur énergie de leur alimentation. Les glucides sont des sucres qui représentent la principale source d'énergie de l'organisme. Les différents sucres (i.e. galactose, fructose, ribose) vont être décomposés en glucose afin d'être utilisé par les cellules. Pour permettre un apport constant et stable d'énergie aux cellules de l'organisme, les métazoaires ont développé la caractéristique de pouvoir stocker le glucose. Ainsi, le glucose pourra être stocké à court terme sous forme de glycogène (dans le foie et les muscles) et à long terme sous forme de lipide (dans le tissu adipeux).

Ce mécanisme de stockage/libération du glucose fait intervenir deux hormones clé : le glucagon et l'insuline[a]. Ces hormones sont produites dans le pancréas au niveau des îlots pancréatiques. Le glucagon est produit par les cellules α et l'insuline par les cellules β. Lorsque la glycémie chute, les cellules α des îlots pancréatiques produisent du glucagon. Celui-ci va induire la libération du glucose stocké dans l'organisme via la glycogénolyse hépatique (conversion du glycogène en glucose) et la lipolyse. A l'inverse, lorsque la glycémie augmente, les cellules β produisent de l'insuline. Celle-ci induit le stockage du glucose via la glycogénogenèse (conversion du glucose en glycogène) et la lipogenèse.

Lorsque l'organisme perd sa capacité à stocker le glucose, l'hyperglycémie chronique va conduire à l'apparition du diabète sucré.

Le terme diabète est apparu au IIIème siècle av. JC, il provient du grec *dia-baino* qui signifie : passer au travers. Les médecins grecques de l'école d'Hippocrate de Cos ont observé que : "des malades étaient frappés d'une soif continuelle, et qu'ils semblaient uriner aussitôt ce qu'ils venaient de boire, comme s'ils étaient traversés par l'eau sans pouvoir la retenir " [1]. Proxagoras de Cos, disciple d'Hippocrate, observa que parmi ces malades on pouvait en distinguer certains dont les urines était sucrées (diabète sucré ou diabète *mellitus*) et d'autres dont les urines n'avaient pas de goût (diabète insipide) [1].

Le diabète insipide est une maladie rare (1-9/1 000 000 en France en 2009) dont la cause est une anomalie dans la sécrétion ou la reconnaissance de l'hormone antidiurétique. A l'opposé, le diabète sucré (mot latin signifiant: bonbon de miel) comprend un large spectre de maladies

[a]D'autres hormones peuvent aussi induire une libération de glucose lors d'un stress comme l'adrénaline et le cortisol.

ayant pour conséquence une hyperglycémie chronique, résultant d'un défaut dans la sécrétion et/ou l'action de l'insuline.

L'hyperglycémie chronique affecte la fonction de différents organes et peut conduire à la cécité, l'insuffisance rénale ou l'amputation. De plus, l'hyperglycémie chronique augmente les risques d'accident cardiovasculaire et d'accident vasculaire cérébral.

En 2000, le diabète *sucré* touchait 2.8% de la population mondiale (171 millions de personnes). En 2030, il est estimé que 4.4% de la population mondiale sera atteinte par cette maladie (366 millions de personnes) (Figure 1) [2].

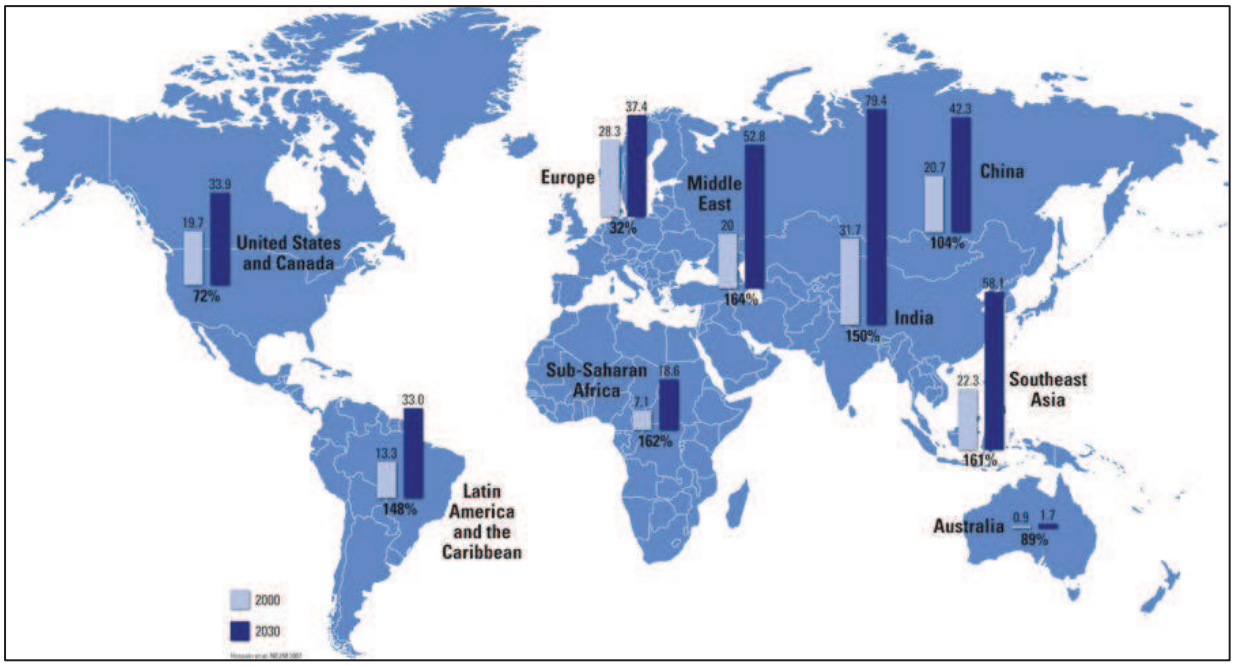

Figure 1. Prévalence du diabète *sucré* en 2000 et projection pour 2030 (millions de cas)
Données de Wild *et al*.[2]

Différentes formes de diabète *sucré* ont été répertoriées en fonction de leur étiologie. Les formes les plus répandues sont :

- Le diabète de type 1 (5-10% des cas de diabète sucré) [3]
- Le diabète de type 2 (90-95% des cas de diabète sucré) [2]
- Le diabète LADA (2-10% des cas de diabète sucré) [4]
- Le diabète gestationnel (7% des femmes enceintes) se caractérise par une intolérance au glucose due à la production d'hormones placentaires. Après la gestation, le diabète disparait [5].

D'autres formes de diabète *sucré* ont des prévalences beaucoup plus rares.

- Les diabètes MODY (Maturity Onset type Diabetes of the Young). Maladies mono génétiques caractérisées par la mutation de certains gènes (i.e. IPF-1) (1/400 000) [6].
- Les diabètes secondaires à des maladies du pancréas (i.e. cancer du pancréas) ou du foie (i.e. cirrhose, infection par l'Hépatite C).
- Le diabète fulminant [7]

Durant ma thèse, nous nous sommes intéressés uniquement au diabète de type 1 et de type 2.

Diabète de type 1, diabète de type 2: différences et similarités.

- Le diabète de type 1 (T1D) est une maladie auto-immune se développant principalement chez l'enfant et l'adolescent. Les cellules immunitaires détruisent les cellules β du pancréas qui produisent l'insuline. En absence d'insuline, les cellules ne peuvent plus absorber le glucose afin de l'utiliser en énergie. Par conséquent, l'organisme met en place la cétogenèse. Ce processus énergétique va utiliser les graisses comme source d'énergie. Ce procédé va entrainer une perte de poids importante chez le patient. En absence de traitement, le déficit aigü en insuline conduit au coma puis à la mort. A partir de la détection des premiers signes de la maladie (présence d'auto-anticorps), le T1D se déclare en quelques mois ou plusieurs années suivant les individus (>20 ans) [8]. Actuellement, deux traitements existent : l'injection d'insuline et la greffe d'îlots pancréatiques. Les progrès dans le traitement et le suivi de la maladie ont permis d'augmenter l'espérance de vie des patients diabétiques. Avant la découverte de l'insuline, l'espérance de vie après apparition de la maladie était de quelques mois. En 2012, une étude a estimé à 4 ans la différence d'espérance de vie entre patients diabétiques (68.8 ans) et le reste de la population (73 ans) [9].

- Le diabète de type 2 (T2D) correspond à un déséquilibre métabolique se développant chez l'adulte. Le T2D se caractérise par une hyperglycémie chronique résultant d'une résistance à l'insuline et d'un défaut de sécrétion d'insuline par les cellules β du pancréas. La résistance à l'insuline correspond à un défaut de signalisation des récepteurs à l'insuline dans les cellules. Celle-ci va induire l'apparition d'une hyperglycémie chronique chez les individus pré-diabétiques. Afin de diminuer l'hyperglycémie, les cellules β du pancréas, vont augmenter leur capacité de production d'insuline. Lorsque l'expansion fonctionnelle des cellules β ne parvient plus à compenser l'hyperglycémie chronique, le T2D apparait. Au niveau des

vaisseaux sanguins, l'hyperglycémie chronique conduit à un stress oxydatif puis à un dysfonctionnement de l'endothélium, qui se caractérise par une vasoconstriction, thrombose, inflammation, formation de plaques et lésions [10]. Sur l'organisme, ces défauts vont conduire à des complications macro-vasculaires (athérosclérose, amputation) et micro-vasculaires (rétinopathie, néphropathie, neuropathie). Suivant l'âge du diagnostic du T2D, l'espérance de vie des patients est réduite en moyenne de 5 à 8 ans [11, 12].

- Le diabète LADA (Latent Auto-immune Diabetes in Adult) présente des caractéristiques propres au T1D et T2D. Tout comme le T1D, le diabète LADA est une maladie auto-immune. Cependant, il apparait chez l'adulte comme le T2D. De plus les patients LADA peuvent présenter une résistance à l'insuline [13]. Des études génétiques ont montré que les patients atteints du diabète LADA présentent des similarités dans les facteurs de risques génétiques présents chez les patients atteints du T1D et T2D [14]. Cependant, dû à son origine auto-immune le diabète LADA est classé dans le T1D.

	Diabète de type 1	**Diabète de type 2**
Apparition	Enfant, adolescent Adulte pour le diabète LADA	Adulte
Prévalence parmi les patients diabétiques	5-10%	90-95%
Mécanismes	Auto-immun : Destruction des cellules β	Métabolique : Résistance à l'insuline Défaut fonctionnel des cellules β
Concordance entre jumeaux monozygote/dizygote	50% / 5-6%	80% / 20-30%
Facteurs de risques	Génétiques*, environnementaux	Génétiques*, surpoids, régime alimentaire
Traitements	-Injection d'insuline -Greffe d'îlots pancréatiques	-Régime et activité physique -Metformine

Table 2. Comparaison des caractéristiques associées au T1D et T2D.
*Les facteurs génétiques associés à ces maladies sont différents entre T1D et T2D.
Table modifiée d'après Donath *et al* [15].

Partie I

Immunité innée et diabète de type 1

Introduction	Le diabète de type 1
Chapitre 1	Rôle des lymphocytes iNKT17
Chapitre 2	Rôle des pDC, lymphocytes B-1a et neutrophiles
Conclusion	

Introduction Le diabète de type 1

I-0.a Caractéristiques du T1D.

La présence de cellules immunitaires dans les îlots pancréatiques fut décrite dès 1925 [16]. Cependant, jusqu'en 1960, le T1D et T2D étaient reconnus comme une même maladie pouvant se déclarer chez l'enfant ou l'adulte. Willy Gepts fut le premier à décrire que l'insulite (infiltration de cellules immunitaires autour des l'îlots pancréatiques) était une caractéristique spécifique aux enfants diabétiques [17]. Cette découverte majeure a permis de poser les premières hypothèses concernant le rôle du système immunitaire dans le T1D.

A partir des années 1980, la mise au point et l'optimisation de nouveaux procédés (immunohistochimie, cytométrie en flux, nouveaux anticorps, souris NOD) ont permis de caractériser cet infiltrat de cellules immunitaires. L'ensemble des études ont montré que les lymphocytes T CD4, T CD8, Treg, lymphocytes B, macrophages et cellules dendritiques infiltrent les îlots pancréatiques (Figure I-0.a) [18].

Chez l'homme, les lymphocytes T CD8 sont les cellules majoritaires autour de l'îlot pancréatique. Le pic de recrutement des lymphocytes T CD8 est étroitement associé avec le degré de destruction des cellules β. Ces lymphocytes T CD8 disparaissent une fois l'ensemble des cellules β détruites. L'effet cytotoxique des lymphocytes T sur les cellules β semble faire intervenir différents mécanismes : granzyme/perforine [19], récepteurs de mort [20] et des cytokines pro-inflammatoires (i.e. IFNγ, IL-1β). La présence de lymphocytes T CD8 est étroitement associée à une forte expression de la molécule du CMH de classe I (CMH I) par les cellules β du pancréas [21]. Les lymphocytes T CD4, lymphocytes B, macrophages et cellules dendritiques sont présents dans l'insulite. Cependant, leur nombre reste faible par rapport aux lymphocytes T CD8 [22, 23]. Les lymphocytes T régulateurs (Treg) et cellules NK ne sont que très rarement observés dans l'insulite des patients T1D [18, 24].

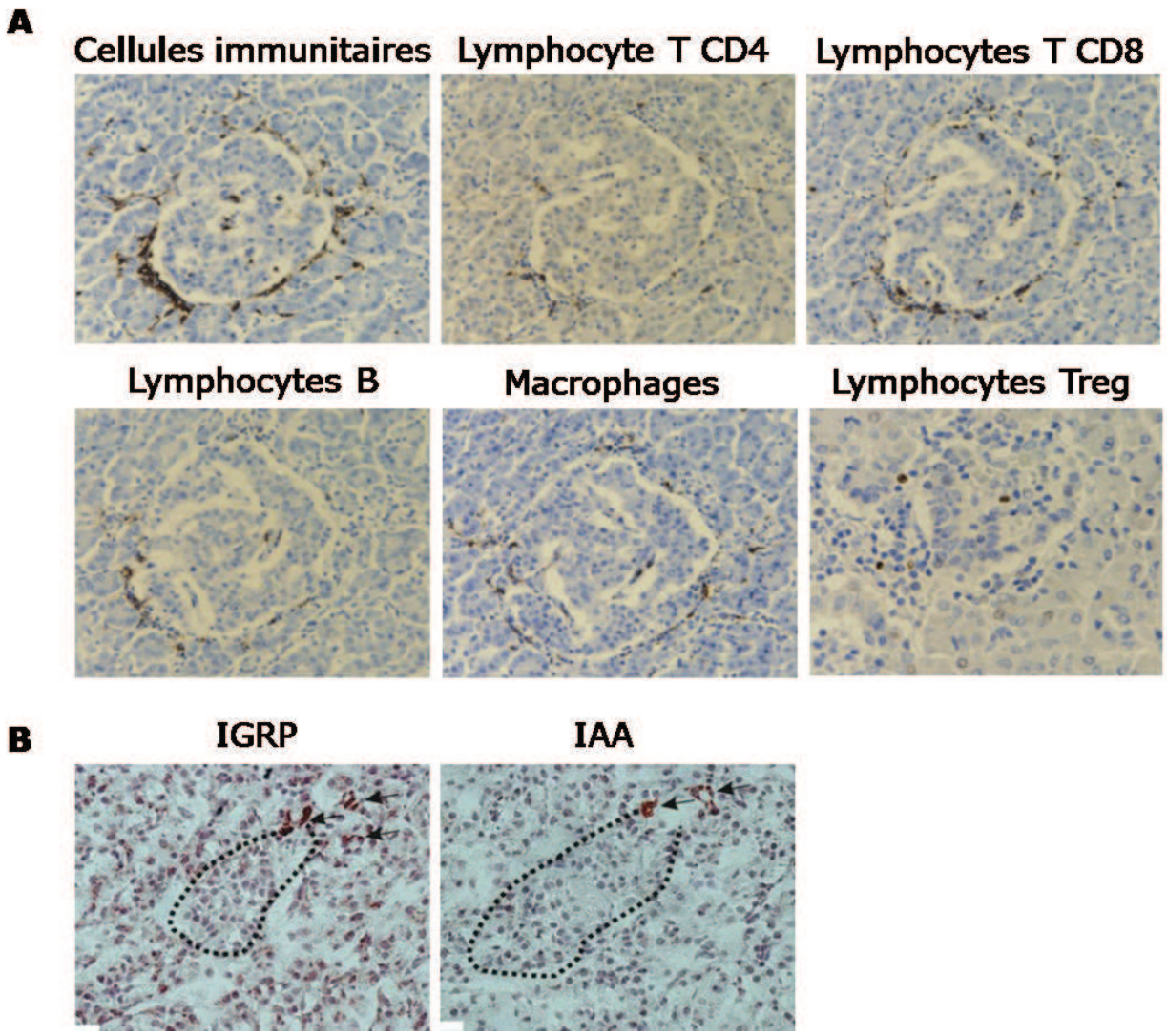

Figure I-0.a. (A) Immunomarquage d'un pancréas de patient T1D. Cellules immunitaires (CD45+) infiltrant l'îlots pancréatiques d'un patient T1D expriment des marqueurs membranaires spécifiques aux lymphocytes T CD4 (CD4+), CD8 (CD8+), régulateur (FOXP3+), lymphocytes B (CD20+) et macrophages (CD68+). Photographie publiée par Willcox *et al* [18]. **(B) Lymphocytes T CD8 auto-réactifs dans l'îlot pancréatique d'un patient T1D.** Marquage tétramère des lymphocytes T auto-réactifs contre les auto-antigènes IAA et IGRP. Photographie publiée par Coppieters *et al* [25].

L'analyse des peptides présentés par les molécules du CMH des patients T1D démontre que les lymphocytes T CD4 et CD8 observés dans l'insulite sont spécifiques pour des auto-antigènes de la cellule β : Insulinoma-associated Antigen-2 (I-A2), Glutamic Acid Decarboxylase 65 (GAD65) et Islet-specific Glucose-6-phosphatase catalytic subunit Related Protein (IGRP) (Figure I-0a) [26]. De même, les auto-anticorps présents chez les patients T1D sont spécifiques pour ces protéines.

Dû à l'impossibilité de pratiquer des biopsies de pancréas chez l'homme, l'étude des modèles animaux a permis de mieux comprendre la maladie. De la même façon que chez l'homme, la souris NOD présente des prédispositions génétiques au développement du T1D. Il a été identifié différents locus de susceptibilité à la maladie. Parmi ces locus, on trouve des gènes codant pour des protéines intervenant directement dans la réponse auto-immune (i.e. CMH, CTLA-4, IL-2) [27]. Tout comme chez l'homme, les lymphocytes T auto-réactifs détruisent les cellules β du pancréas. L'infiltration des cellules immunitaires commence à partir de 4 semaines d'âge. L'insulite est constituée de macrophages, cellules dendritiques, lymphocytes B, lymphocytes T et NKT. L'apparition du T1D est observée à partir de 10 semaines d'âge. En raison des fortes homologies entre la maladie humaine et celle de cette souris, la souris NOD représente un très bon outil pour étudier les premières étapes du T1D.

Les premiers auto-antigènes vont être libérés suite à la mort physiologique des cellules β du pancréas [28][b]. Cette mort cellulaire est un processus physiologique qui a été observé chez les rongeurs, le cochon et l'homme [29, 30]. Chez la souris NOD, cette vague de mort cellulaire se produit entre 14 et 17 jours après la naissance. A cette même période sont également détectés les premiers signes d'activation des lymphocytes T auto-réactifs.

Les lymphocytes T auto-réactifs, qui ont échappé à la sélection thymique ont besoin d'être activés par les auto-antigènes provenant de cellules β. Cette activation se produit dans les ganglions drainants du pancréas (pLN). Il a été montré que la mort physiologique des cellules β participe au recrutement de cellules dendritiques et de macrophages dans le pancréas [30]. Ces cellules vont capturer les auto-antigènes et migrer vers les pLN. Dans les pLN, ces cellules présentent les antigènes dérivés des cellules β aux lymphocytes T naïfs. Après activation, les lymphocytes T auto-réactifs migrent dans le pancréas et tuent les cellules β [30]. L'ablation des pLN chez la souris NOD a cette période conduit à une protection contre le développement du T1D [31].

L'ensemble des observations faites chez l'homme et la souris ont conduit à proposer le mécanisme suivant pour expliquer le développement du T1D chez l'homme:

Les lymphocytes T CD8 auto-réactifs sont les acteurs principaux dans la pathologie. Très précocement, ces cellules sont activées dans les pLN par les cellules dendritiques. Puis, les lymphocytes T CD8 vont infiltrer les îlots du pancréas. Les cellules β, par la présentation de peptides du soi par le CMH I, activent ces lymphocytes T CD8 qui tuent les cellules β par

[b] Le rôle de mort des cellules β dans l'initiation du T1D est développé en détail dans la partie discussion I-2.3

l'interaction Fas/Fas-L, la production de granzyme/perforine et la production de cytokines pro-inflammatoires. Une fois l'ensemble des cellules β détruites, les lymphocytes T CD8 partent du pancréas. Les macrophages présents dans l'insulite ont une fonction d'APC et produisent des cytokines pro-inflammatoires (i.e. TNF-α et IL-1β) qui favorisent l'expression de récepteurs induisant l'apoptose (i.e. Fas) sur les cellules β [22]. Les lymphocytes T CD4 permettent l'activation des lymphocytes B et T CD8 auto-réactifs, ils orchestrent la réponse auto-immune. Il est à noter que la fonction des lymphocytes B dans la pathologie du T1D reste floue. Il semblerait que ces cellules soient importantes pour la présentation antigénique et non pour leur production d'auto-anticorps.

Le rôle de l'immunité innée dans le T1D reste peu étudié. Nous avons décrit la fonction de ces cellules dans notre revue : Innate Immunity in Type 1 Diabetes (cf. partie revue) [32]. Durant ma thèse, nous nous sommes intéressés spécifiquement aux rôles des lymphocytes iNKT, neutrophiles, lymphocytes B-1 et pDC.

I-0.b Facteurs génétiques.

A partir de 1970, les gènes du HLA (Human Leukocyte Antigen) furent identifiés comme pouvant conférer une susceptibilité ou une résistance au T1D [33]. A partir des années 2000, l'apparition de nouveaux outils a permis la réalisation d'études génétiques sur un très grand nombre de patients. L'étude GWAS de 2009 (Genome-Wide Association Study) a comparé le génome[c] de 7500 patients T1D à 9000 sujets sains [34]. Cette étude a permis de confirmer et de révéler de nouveaux gènes de susceptibilité dans le T1D (Figure I-0b).

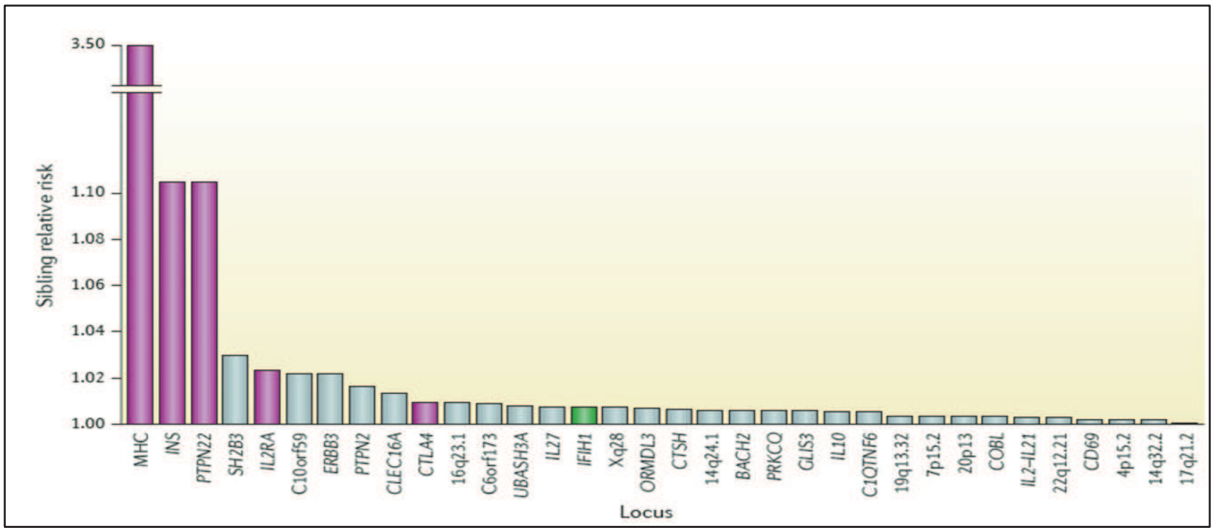

Figure I-0.b. Gènes de susceptibilité au T1D. Le Sibling relative risk (risque relatif entre frère et sœur) se définit comme le ratio de manifestation d'une maladie, étant donné que son frère/sœur est affecté, comparé avec la fréquence de maladie dans la population générale. Si le ratio est égal à 1 pour un gène donné, celui-ci ne confère aucune susceptibilité à la maladie. A contrario, un ratio supérieur à 1 pour un gène confère une susceptibilité à la maladie. Graphique publié par Polychronakos *et al* [35]

La majorité des gènes de prédisposition au T1D codent pour des protéines intervenant directement dans la réponse immunitaire (i.e. HLA, IL2RA, CTLA4). Parmi les gènes présentant un risque relatif élevé, on retrouve :

- Les gènes du HLA[d] sont un ensemble de gènes (≃200). Parmi ces gènes, certains codent pour le CMH I (HLA-A, HLA-B, HLA-C) ou le CMH II (HLA-DP, HLA-DQ, HLA-DR). Dans la population, différents allèles existent pour chacun de ces gènes. Certains allèles du CMH tels que *HLA-DR3, HLA-DR4 et HLA-A02* confèrent une susceptibilité au T1D. A l'opposé, l'allèle *HLA-DR2* confère une protection contre le développement de la maladie. Les études génétiques ont démontré que les gènes du HLA sont les acteurs principaux de la prédisposition au T1D (Figure I-0.b1).

[c] L'analyse porte sur les variants SNP (single-nucleotide polymorphism)
[d] Chez la souris, ces gènes sont nommés I-A

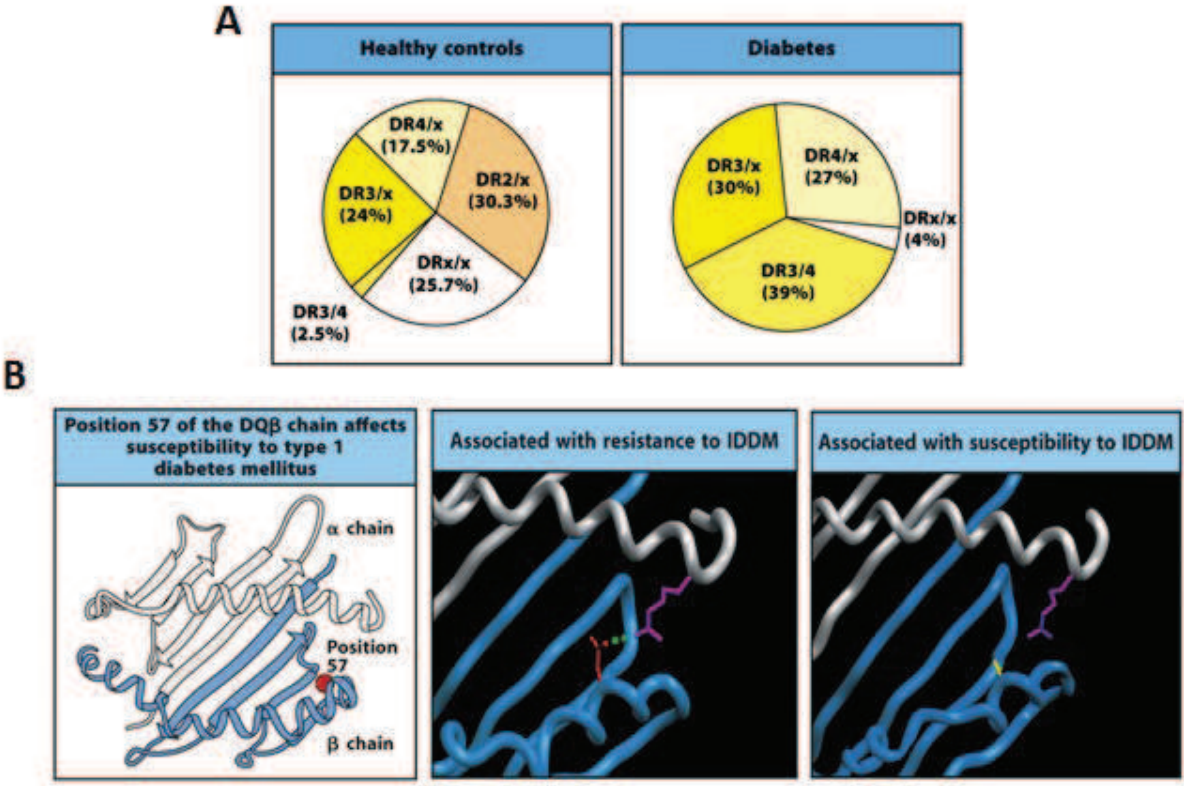

Figure I-0.b1. (A) Association entre T1D et HLA chez l'homme. Répartition des différents allèles du HLA-DR (CMH II) chez des patients sains ou T1D.
(B) Le polymorphisme d'un acide aminé du HLA-DQ est associé à la résistance ou la susceptibilité au T1D. Rouge : Asparagine en position 57 de la chaine β, Violet : Arginine en position 76 de la chaine α, Vert : liaison électrostatique, Jaune : Alanine.
Image issue de Janeway's Immunobiology (7ème édition, figure 14.34 et figure 14.36)

Plusieurs hypothèses peuvent expliquer le rôle du HLA dans le T1D. La première explication est que les allèles du HLA prédisposant à la maladie présentent un certain répertoire de peptides du soi, qui favorise l'émergence d'un pool de lymphocytes T auto-réactifs. Une autre explication de l'implication du HLA dans l'auto-immunité a été déterminée par l'analyse de la structure de ces molécules par cristallographie [36, 37]. Ces analyses ont montré que les allèles du HLA-DQ8 conférant une susceptibilité à la maladie, sont associés avec le polymorphisme d'un acide aminé en position 57 de la chaine β du CMH II. Dans la population caucasienne, cet acide aminé en position 57 est une asparagine. Celle-ci interagit avec une arginine de la chaine α et permet ainsi de stabiliser la molécule du CMH II. Chez les patients diabétiques, l'acide aminé en position 57 peut être une valine, sérine ou alanine. Ces acides aminés n'interagissent pas avec l'arginine de la chaine α (Figure I-0.b). Par conséquent, la stabilité de la molécule du CMH II est altérée. Cette instabilité du HLA pourrait entrainer une moins bonne délétion des lymphocytes T auto-réactifs lors de la sélection thymique [38].

- L'insuline est l'antigène majeur dans le T1D. L'analyse de ce gène a montré que les variants génétiques conférant une prédisposition touchent la région promotrice de ce gène (VTNR Type I). L'expression de l'insuline dans le thymus est réduite chez les patients ayant le VTNR Type I [39]. D'après les études menées chez la souris, il est démontré que la faible expression de l'insuline dans le thymus conduit à un défaut d'élimination des lymphocytes T auto-réactifs pour l'insuline lors de la sélection thymique [40, 41].
- *PTPN22* code pour une protéine de signalisation régulant négativement l'activation des lymphocytes via leur TCR ou BCR. Le rôle des différents variants de *PTPN22* dans l'activation des lymphocytes T et B dans T1D reste contradictoire [42, 43].
- *IL2RA* code pour la chaine α du récepteur à l'IL-2 (CD25). IL-2Rα est exprimé sur les lymphocytes T après activation et sur les lymphocytes Treg. Les variants de IL2-Rα ne semblent pas jouer sur la fréquence des lymphocytes Treg mais sur leur fonction suppressive à l'encontre des lymphocytes T auto-réactifs [44].
- *CTLA-4* code pour une protéine membranaire présente sur la surface des lymphocytes T. La reconnaissance des molécules B7-1 et B7-2 par CTLA-4 induit l'anergie des lymphocytes T via l'inhibition de la production d'IL-2 et l'arrêt du cycle cellulaire [45]. Par conséquent, un défaut dans l'expression de cette protéine peut favoriser l'augmentation du nombre de lymphocytes T auto-réactifs.

En conclusion, la génération de lymphocytes auto-réactifs est un phénomène naturel. Les gènes de susceptibilité au T1D conduisent à un défaut dans les mécanismes de tolérance immunitaire. Par conséquent, le pool de lymphocytes auto-réactifs s'expand et conduit à l'auto-immunité.

I-0.c Facteurs environnementaux.

Il est estimé que les gênes du HLA peuvent contribuer pour approximativement 50% de la susceptibilité génétique au T1D. Les autres polymorphismes génétiques représentant les 50% restant (Figure I-0.1) [46]. Cependant, parmi la population, seulement une faible proportion des individus présentant une susceptibilité génétique au T1D va progresser vers la maladie. Les études de jumeaux monozygotes ont montré une concordance de 30% à 60% (suivant les études) dans le développement de la maladie [47, 48]. De même, les études de la population caucasienne en Europe montrent des différences importantes dans l'apparition du T1D entre pays. Ainsi, la Finlande présente une incidence de la maladie 20 fois plus élevée que la Macédoine (63/100 000 contre 3.2/100 000) [49]. De plus, alors que l'incidence du T1D augmente rapidement depuis les 15 dernières années, la fréquence des patients présentant des allèles de HLA à risques diminuent alors que la fréquence des patients présentant des allèles protecteurs du HLA augmentent (figure I-0.c). L'ensemble de ces observations montrent qu'à côté des facteurs génétiques, l'environnement a un impact important sur le développement de la maladie.

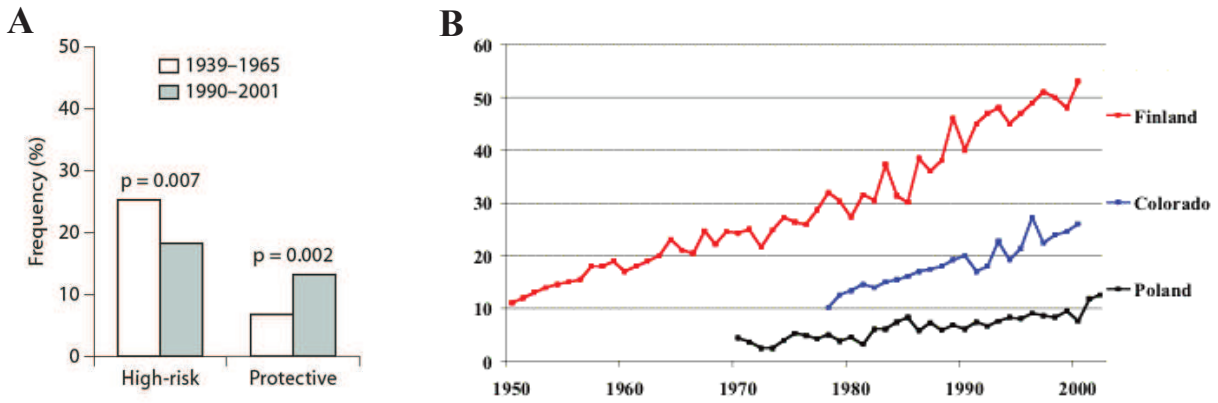

Figure I-0.c. (A) Distribution des allèles à risque ou protecteurs du HLA-DQ parmi les patients T1D entre 1939-1965 et 1990-2001. Figure modifiée d'après Knip *et al* 2011 [49].
(B) Prévalence du T1D dans différentes régions du monde. Nombre de cas pour 100 000 personnes en fonction de l'année. Figure : www.jdrf.org

Les infections virales[e].

Dès 1926, l'hypothèse d'un lien entre diabète et infections virales a été proposée. Les modèles animaux ont montré que les virus pouvaient déclencher le diabète par deux mécanismes : un effet direct sur la destruction des cellules β et/ou l'activation du système immunitaire qui ensuite détruit les cellules β. Parmi les différents virus suspectés de jouer un rôle dans l'initiation de la maladie (i.e. rotavirus, myxovirusparoditis, rubivirus), les entérovirus ont été les plus étudiés [51].

Les entérovirus sont des virus à ARN appartenant à la famille des *Picornaviridae.* Ces virus infectent l'individu via le système gastro-intestinal. L'ensemble de la population est susceptible à l'infection par les entérovirus. La plupart du temps, ces infections sont bénignes ou asymptomatiques. Parmi les entérovirus, la classe virus Coxsackie a été impliquée dans l'apparition du diabète.

En 1969, pour la première fois un titre élevé d'anticorps contre l'entérovirus CVB4[f] fut observé chez les patients diabétiques, suggérant ainsi un lien entre l'infection virale et l'apparition de la maladie [52]. En 1971, une étude a suivi la fréquence de diabétiques parmi la population de l'île de Pribilof en Alaska. Les habitants de cette île avaient été victime d'une épidémie par l'entérovirus CVB4. Cinq année après l'infection, il fut observé aucun changement dans l'incidence du diabète [53]. En 2007, l'entérovirus CVB4 a été détecté dans les cellules β de certains patients diabétiques. De façon intéressante, les patients présentant cette infection virale présentaient un infiltrat pancréatique majoritairement composé de cellules NK. Les auteurs n'ont pas réussi à mettre en évidence la présence de lymphocytes T auto-réactifs [54]. Le développement des technique (détection d'auto-anticorps) et du suivit (large cohorte de patients) a permis de mettre en place des études sur le lien entre entérovirus et T1D chez l'homme. L'étude DIPP portant sur le suivit de plus de 500 enfants à risque a montré un lien entre infection virale par les entérovirus et apparition des premiers auto-anticorps [55]. Cependant les études BABYDIAB [56] et DAISY [57] portant respectivement sur 120 et 70 enfants à risque, n'ont montré aucun lien entre infection virale et apparition des premiers auto-anticorps. Toutefois, l'étude DAISY a mis en évidence que parmi les patients présentant des auto-anticorps, l'infection virale par CVB4 est un facteur favorisant l'apparition du T1D [58].

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[e]Notre équipe a publié une revue très détaillée sur le rôle des infections virale dans le T1D.

50. Ghazarian L, Diana J, Simoni Y, Beaudoin L, Lehuen A. Prevention or acceleration of type 1 diabetes by viruses. Cell Mol Life Sci 2013; **70**:239-55.

[f] CVB4: Coxsackie Virus B 4

Alimentation.

Différents facteurs alimentaires semblent influencer le développement du T1D. Ainsi, l'allaitement, le lait de vache, les céréales, l'huile de foie de morue ou l'insuline bovine pourraient influencer le développement de la maladie. Cependant les études de cohortes montrent des résultats contradictoires sur l'effet de ces aliments dans l'apparition du T1D [46].

Vitamine D.

La vitamine D est une hormone synthétisée par l'organisme sous l'influence des UVB[g]. Cette hormone influence l'expression d'environ 200 gènes. Différentes études ont indiqué qu'un défaut en vitamine D chez l'enfant augmente les risques de développer le T1D. De ce fait, la faible exposition solaire en Finlande pourrait expliquer la forte prévalence de la maladie (figure I-0.c). Deux études finlandaises ont montré que la supplémentation en vitamine D par l'alimentation permet de diminuer les risques de développement de la maladie [59, 60]. Cependant, certains arguments contredisent le rôle de la déficience en vitamine D dans le T1D. La région de Karelia en Russie est frontalière et a la même latitude que la Finlande. Ces deux régions ont le même taux d'ensoleillement. Cependant l'incidence du T1D est 6 fois supérieure en Finlande (42/ 100 000 contre 7.8 /100 000 en Karelia) [61]. De plus l'étude portant sur la cohorte DAISY (2644 patients dont 198 devenus diabétiques) n'a montré aucune différence sur la concentration sanguine en vitamine D entre les enfants ayant développé le T1D et les sujets contrôles [62].

La flore bactérienne.

L'être humain est l'hôte d'environ 100 000 milliards de microorganismes [63]. Il est estimé que le tube digestif d'un adulte est colonisé par 1000 milliards de bactéries (soit dix fois plus que le nombre total de cellules dans notre corps). Cette flore bactérienne est composée d'environ 1000 espèces. Les phylums *Bacteroides*, *Firmicutes* et *Archaea* représentent 98% du nombre total des bactéries. Le tube digestif du fœtus est stérile. Après accouchement, la colonisation bactérienne commence. La composition de la flore intestinale est influencée par différents facteurs. Ainsi, le tube digestif d'un enfant né par césarienne sera colonisé par les bactéries présentes à la surface de la peau. Un enfant né par la voie naturelle sera colonisé par la flore vaginale et digestive de la mère. De ce fait, la flore intestinale se transmet de

[g] UVB: rayonnement Ultraviolet B (315-280nm)

génération en génération. De plus, l'alimentation et le contact physique avec d'autres individus modifient cette flore intestinale.

La mise au point des techniques de séquençage de l'ADN a permis de mettre en évidence que la composition de la flore intestinale est différente entre les individus "sains" et les individus présentant des maladies telles que l'obésité [64], des syndromes métaboliques [65] ou une colite ulcéreuse [66].

Des études sur les modèles animaux montrent que, chez le rat BB[h] et la souris NOD, l'ingestion d'antibiotiques permet de prévenir l'apparition du T1D via la modulation de la flore intestinale [67, 68]. Cette protection semble être dépendante des bactéries SFB (Segmented Filamentous Bacteria) [69]. Le groupe de D. Mathis a montré que dans une même colonie de souris NOD, l'incidence du T1D chez les souris SFB négatives est significativement supérieures (90% à 30 semaines) aux souris SFB positives (20% à 30 semaines) [70]. Les souris SFB- et SFB+ ne montrent aucune différence dans l'insulite pancréatique. Le mécanisme d'action des SFB sur le T1D semble faire intervenir l'immunité mucosale et les réponses Th17 [70]. Myd88 est une molécule impliquée dans les voies de signalisation des TLR (récepteurs impliqués dans la reconnaissance des pathogènes). La souris NOD Myd88-/- possèdent une composition de la flore intestinale modifiée et ne développe pas de T1D. Cette observation indique que l'interaction entre flore bactérienne et immunité innée est importante dans l'apparition du T1D [71].

Chez l'homme, une augmentation de la perméabilité intestinale a été observée chez les patients T1D [72]. Cette perméabilité, due à un défaut dans les jonctions serrées, pourrait favoriser l'activation de l'immunité mucosale. Le lien entre l'immunité mucosale et l'auto-immunité reste flou [73]. L'étude du microbiome a montré que la flore bactérienne des enfants atteints du T1D diffère de celle des patients contrôles [74]. Il est toutefois à noter que l'ensemble de ces études utilisent des techniques différentes et portent sur un faible nombre de patients (sur les trois études, 20 patients T1D et 20 patients contrôles). De plus, certains paramètres ne sont pas pris en compte (accouchement par la voie naturelle/césarienne, alimentation). Dans le futur, l'analyse génétique du microbiome sur de grandes cohortes de patients (i.e. DAYSY, DIPP) permettra de confirmer ou d'infirmer le rôle de la flore intestinale dans l'apparition du T1D.

[h]BB : Bio-Breed, modèle animaux du T1D

En conclusion, en dépit du fait que les facteurs environnementaux jouent un rôle prépondérant dans l'apparition du T1D, l'ensemble des études n'ont pas permis de mettre en évidence un facteur environnemental impliqué dans le développement de la maladie.

I-0.d La souris NOD : Un excellent modèle du T1D ?

Avant les années 1980, le T1D était induit chez les souris par l'injection de molécules toxiques (i.e. streptozotocine, alloxan). En 1980, fut décrit le premier modèle spontané de T1D chez la souris: la souris NOD (Non-Obese Diabetic mice). C'est en cherchant à produire des souris développant la cataracte, qu'un groupe de chercheurs japonais sélectionna cette souris [75]. L'utilisation de la souris NOD a permis de décrire les mécanismes cellulaires et moléculaires conduisant à la destruction des cellules β, d'étudier les causes potentielles de la maladie et de développer de nombreux traitements. Cependant, après 25 ans de recherche, aucune thérapie permettant de prévenir ou soigner le T1D n'a pu être transposée chez l'homme (i.e. anti-CD3, anti-IL-1β). Différents facteurs peuvent expliquer cet échec :

- Une première explication peut être les différences génétiques entre l'homme et la souris. Ces différences touchent le système immunitaire inné (i.e. fréquence en neutrophiles dans le sang, TLR) et adaptatif (i.e. molécules de co-stimulation, présentation antigénique) [76]. De plus, la différence homme/souris se retrouve au niveau de l'organisation tissulaire. Contrairement à l'homme, dans l'îlot pancréatique de la souris, les cellules α sont en périphérie et les cellules β au centre (Figure I-0.d).

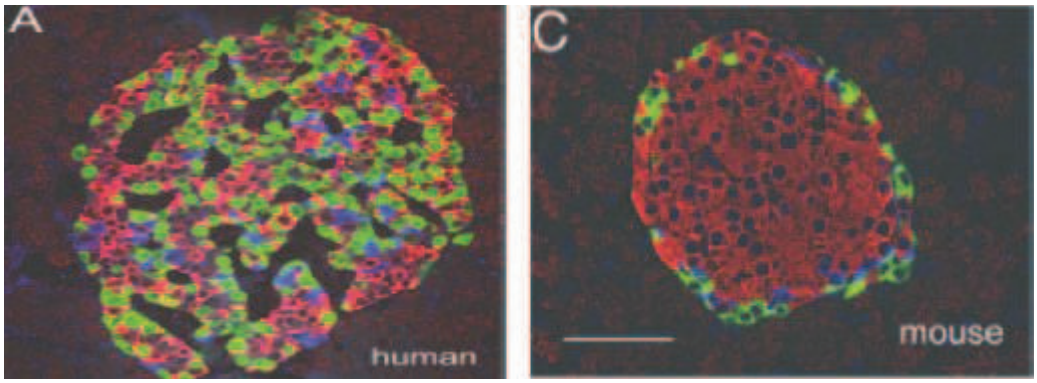


Figure I-0.d. Différence d'organisation d'un îlot pancréatique chez la souris et l'homme. Rouge : Cellules β, Vert : Cellules α, Bleu : Cellules δ.
Figure modifiée d'après Cabrera *et al* [77]

- Une seconde explication réside dans les facteurs de risques génétiques au T1D. L'analyse des gènes de prédisposition à la maladie a montré d'importantes similitudes entre la souris NOD et les patients T1D (i.e. l'homologie de structure entre les molécules du CMH II prédisposant au T1D : HLA-DQ8 et I-A^{g7}) [38]. Cependant, certains gènes de prédisposition sont spécifiques à l'homme (i.e *ifih1*), d'autres à la souris (i.e *vav3*) [27].

- Enfin, contrairement à la souris NOD, les patients T1D présentent une hétérogénéité au niveau génétique (i.e. HLA), du développement de la maladie (i.e. patients LADA), du répertoire T auto-réactif.

L'ensemble de ces facteurs aboutit au fait que le T1D chez la souris NOD présente beaucoup de similarités avec la maladie humaine mais aussi des différences.
Parmi ces différences, on trouve un ratio Homme:Femme différent. Celui-ci est de 1:1 pour l'humain et de 1:3 pour la souris NOD. Cette différence chez la souris semble faire intervenir la flore bactérienne et la testostérone [78]. L'implication des lymphocytes B dans la pathologie du T1D est différente chez la souris NOD et chez l'homme. Ces cellules sont indispensables au développement du T1D chez la souris. Chez l'homme, il a été rapporté l'apparition du T1D chez un patient déficient en lymphocyte B [79]. Cette étude prouve que les lymphocytes B ne sont pas indispensables pour l'apparition du T1D chez l'homme. Cependant, elle ne permet pas d'écarter un rôle délétère de ces cellules dans la pathologie.
Les auto-antigènes impliqués dans le T1D présentent de nombreuses similarités entre l'homme et la souris (i.e. Insuline, GAD65, IGRP). Dû au polymorphisme du HLA chez les patients T1D, le répertoire antigénique des lymphocytes T auto-réactifs est différent d'un individu à un autre. Par conséquent, il est difficile de comparer le répertoire T entre homme et souris NOD. Cependant, il semble admis que les lymphocytes T CD8 auto-réactifs de la souris NOD reconnaissent majoritairement IGRP alors que chez l'homme, ces cellules reconnaissent l'insuline [26]. Comparer au répertoire T de la souris NOD, le spectre d'auto-antigènes reconnus par les lymphocytes T humains est plus important (i.e. amylin, Znt8, ICA69) [80]. De plus, les épitopes dominants reconnus par un lymphocyte T auto-réactif sont différents entre l'homme et la souris NOD pour une même protéine. Pour l'insuline, le peptide immunodominant est B:9-23 chez la souris NOD et PPI14-33 pour l'homme [80].
Les lymphocytes Treg sont des cellules immunosuppressives qui constituent un acteur majeur dans la tolérance périphérique. Ces cellules se caractérisent par l'expression du facteur de transcription FoxP3. Les patients IPEX (Immunodeficiency Polyendocrinopathy and Enteropathy X-Linked Syndrome), qui n'expriment pas la protéine FoxP3, n'ont pas de lymphocytes Treg. Ces patients développent de nombreuses maladies auto-immunes. Environ 60% des patient IPEX développent un T1D [81]. Cette observation met en lumière le rôle prépondérant des lymphocytes Treg dans le T1D. Il montre aussi que ces cellules ne sont pas l'unique acteur de la tolérance périphérique dans le T1D car tous les patients IPEX ne développent pas de diabète.

Les études portant sur les lymphocytes Treg chez les patients T1D montrent des résultats contradictoires. Deux études observent un défaut fonctionnel des lymphocytes Treg dans le sang [44, 82], et dans les pLN [83] des patients T1D. Cependant, une autre étude n'a pas réussi à mettre en évidence un défaut numérique et fonctionnel des lymphocytes Treg chez les patients T1D [84]. Tout comme chez l'homme, certaines études montrent que la souris NOD présente un défaut en lymphocytes Treg [85, 86], d'autres études ne confirment pas ce résultat [87, 88]. En dépit du fait qu'il n'y ait pas de consensus sur un défaut numérique et/ou fonctionnel des lymphocytes Treg dans la pathologie du T1D, l'utilisation de la fonction immunosuppressive de ces cellules peut être utilisée pour le développement de traitement. Ainsi, le traitement de souris NOD avec l'anti-CD3 ou la rapamicyne stabilise et augmente la population de lymphocytes Treg. Ces traitements protègent la souris NOD du T1D [89, 90]. Les premiers essais cliniques avec l'anti-CD3 (teplizumab ou otelixizumab) ont montré une préservation du peptide C chez les patients T1D par rapport au groupe contrôle (le dosage du peptide C dans le sang permet de mesurer la fonction des cellules β)[91, 92]. Cependant, l'essai clinique de phase III avec teplizumab n'a pas permis de confirmer ce résultat [93].
Une autre différence importante est l'efficacité des traitements. Contrairement à l'homme ou aucune thérapie existe, plus de 195 thérapies protègent ou réduisent l'incidence du T1D chez la souris NOD [94].

L'ensemble de ces observations montre que l'utilisation de la souris NOD a des limites dans la compréhension du T1D. Cependant, son utilisation a été indispensable pour comprendre la pathologie du T1D. De plus, la souris NOD demeure un excellent outil pour étudier certaine phase de la maladie, tel que l'initiation, le rôle de la flore bactérienne ou des infections virales.

Table 1 | **Comparison of autoimmune diabetes in NOD mice and humans**

Similarities	**Humans**	**Mice**
Genetic predisposition and polygenetic trait	Yes	Yes
MHC-loci contribution	Multiple	Multiple
Environmental influence	Yes	Yes
Defective peripheral immune regulation	Yes	Yes
Impaired dendritic-cell maturation and function	Possibly	Yes
Disease transmission with bone-marrow transplantation	Yes	Yes
Autoantigens	GAD65, IA2, insulin and 38 kD	GAD65, IA2, insulin and 38 kD
Initiating autoantigen	Unknown	Unknown
Delayed onset with immunosuppression	Yes	Yes
Islet autoimmunity linked to early gluten exposure	Yes	Yes
Differences		
Endogenous retrovirus in β-cells	Unclear	Yes*
T-cell-driven insulitis	Mild	Severe
Humoral reactivity to β-cells	GAD65, IA2 and insulin	Insulin
Insulin gene	One	Two
GAD65 expression by β-cells	Yes	No
Incidence	0.25–0.40%	> 80%
Incidence in genetically susceptible subjects	5–30%	> 80%
Gender bias	No	Females
Peri-insulitis	No	Yes
Lymphocytic infiltrates in other tissues	Minority of individuals	All mice
Susceptibility of β-cells to STZ or NO *in vitro*‡	Only at high concentrations	Very susceptible
Maternal autoantibodies	Potentially reduced risk of T1D	Diabetogenic
B cells required	No	Yes
Successful intervention therapies	Pending	> 195

Figure X. Comparaison des caractéristiques du T1D chez l'homme et la souris NOD
Figure d'après Roep *et al* [95].

Chapitre 1 Rôle des lymphocytes iNKT17

I-1.1a Les lymphocytes NKT.

En 1986, le groupe de M. Taniguchi clone et séquence une chaîne α invariante Vα14Jα18, exprimée par un hybridome T suppresseur [96]. En parallèle, B.J Fowlkes et R. Budd décrivent des thymocytes doubles négatifs (CD4- CD8-), dont le TCR se compose préférentiellement d'une chaîne Vβ8. Ces cellules sont des lymphocytes T CD3+ $CD5^{high}$ exprimant la molécule NK1.1, caractéristique des cellules NK, et la molécule CD44, retrouvée sur les cellules activées ou mémoires [97]. Tout d'abord identifiées dans le thymus, ces cellules sont ensuite détectées dans d'autres organes comme le foie et la moelle osseuse. L'intérêt des chercheurs pour cette nouvelle population grandit quand il fut découvert qu'elle pouvait produire simultanément de l'IL-4 et de l'IFNγ après stimulation [98, 99].

C'est en 1994 que O. Lantz et A. Bendelac révèlent que les hybridomes Vα14Jα18 et les thymocytes Vβ8 $CD44^{high}$ NK1.1+ sont une même population cellulaire, appelée NKT (Natural Killer T). Ils démontrent que ces lymphocytes sont sélectionnés positivement par une molécule non classique du CMH I: CD1d [100]. Une population homologue est découverte chez l'homme. Les lymphocytes NKT humains expriment une chaîne invariante Vα24-Jα18 [101].

Dans les années 1990, le terme NKT désigne une population de cellules T non conventionnelles, CD4+ ou DN, exprimant à leur surface des marqueurs de cellules activées ($CD5^{high}$ HSA^{low} $CD44^{high}$ $CD69^{int}$ $CD62L^{low}$), des molécules caractéristiques de cellules NK (NK1.1, Ly49, NKG2A, NKG2D), une chaîne α du TCR invariante (Vα14Jα18 chez la souris et Vα24-Jα18 chez l'homme) et une chaîne β peu diversifiée, ce TCR étant restreint par la molécule CD1d [102].

L'étude des cellules NKT qui expriment la chaîne α invariante Vα14Jα18, aussi dénommées iNKT (invariant NKT), a été facilitée par l'apparition d'outils spécifiques pour ces cellules : souris mutantes et transgéniques, découverte d'un ligand et construction d'un tétramère.

En 2000, les équipes de M. Kronenberg et A. Bendelac ont tiré profit du fait que ces lymphocytes lient par le biais de leur TCR invariant, les glycolipides présentés par la molécule du CD1d. Ils ont construit un tétramère composé de quatre molécules CD1d associées au glycolipide α-GalCer et couplées à un fluorochrome [103].

Ce tétramère a permis de mettre en évidence qu'une fraction des cellules iNKT n'exprime pas le marqueur NK1.1 et à l'inverse, certaines cellules TCR+ NK1.1+ ne fixent pas le tétramère CD1d/α-GalCer et n'expriment pas la chaîne Vα14Jα18.

Aujourd'hui, une nouvelle nomenclature est utilisée pour classer les cellules NKT : iNKT, NKT de type II et NKT-*like*[i] (Figure I-1.1a).

Dans la suite de l'introduction, nous nous intéressons uniquement aux lymphocytes iNKT.

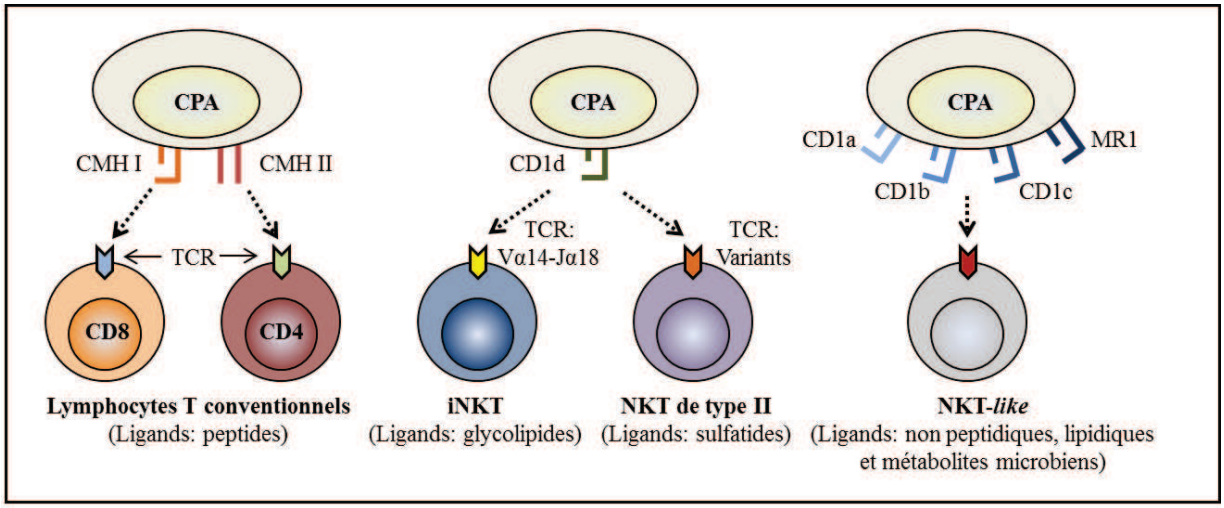


Figure I-1.1a. Lymphocytes T et lymphocytes NKT. A la différence des lymphocytes T conventionnels qui reconnaissent des peptides présentés par le CMH I ou CMH II (à gauche), les lymphocytes NKT expriment un TCR restreint par les molécules non classiques du CMH. Les lymphocytes iNKT et NKT de type II sont restreints par la molécule CD1d qui présente des glycolipides ou des sulfatides (au centre). Les lymphocytes NKT-*like* sont restreints par d'autres molécules non polymorphes comme CD1a, b, c ou MR1 (à droite). Figure d'après Ghazarian *et al* [105].

Chez l'homme, différentes sous-populations de cellules iNKT ont été identifiées, les lymphocytes iNKT CD4+ secrètent des cytokines de type Th1 et Th2 (IFNγ, TNF, IL-4, IL-10, IL-13) alors que les cellules iNKT CD8+ et DN secrètent des cytokines de type Th1 (IFNγ, TNF). Par contre, chez la souris, aucune observation similaire n'a été décrite [106].

En 2007, l'équipe de MC. Leite-de-Moraes a décrit une nouvelle population de cellules iNKT ayant la caractéristique fondamentale de secréter de l'IL-17 (iNKT-17). Cette population possède un phénotype CD4- NK1.1- CD103+ CCR6+ IL-23R+ et exprime le facteur de transcription RORγt. De plus, ces cellules ne possèdent pas le récepteur à l'IL-15, cette cytokine étant indispensable à la maturation des cellules iNKT classiques. Egalement, ces cellules peuvent secréter de l'IL-17 suite à l'engagement de leur TCR ou uniquement en présence d'IL-23. Ces deux stimuli induisent un effet synergique sur la sécrétion d'IL-17

[i]Une caractérisation détaillée des différentes sous-populations de cellules NKT est présente dans la revue 104. Simoni Y, Diana J, Ghazarian L, Beaudoin L, Lehuen A. Therapeutic manipulation of natural killer (NK) T cells in autoimmunity: are we close to reality? Clin Exp Immunol 2013; **171**:8-19.(cf : partie Revue)

[107-109]. Différentes études ont montré que les cellules iNKT-17 suivent une voie de différenciation thymique différente des cellules iNKT conventionnelles [106].

I-1.1b Les lymphocytes iNKT et le T1D.

Les lymphocytes NKT sont impliqués dans différentes pathologies auto-immunes. Nous avons fait une description détaillée de la fonction de ces cellules aussi que de leur rôle thérapeutique dans l'auto-immunité dans notre revue (cf. partie revue) [104].
Différentes études conduites sur la souris NOD ont montré que les cellules iNKT peuvent prévenir le développement du diabète de type 1. La souris NOD présente un déficit numérique en lymphocytes iNKT [110]. De plus, les souris NOD CD1d-/-, qui n'ont pas de lymphocytes NKT, ont un diabète accéléré [111]. Notre groupe a démontré que l'augmentation du nombre de iNKT par transfert adoptif [112, 113] ou via l'introduction du transgène Vα14Jα18 chez la souris NOD, protège les souris de l'apparition du diabète [113].
Les premières études ont suggéré que la protection par les cellules NKT était associée à une production d'IL-4 qui induisait un profil Th2 sur les lymphocytes T auto-réactifs [114-116]. Cependant, l'utilisation d'un modèle de diabète basé sur le transfert de cellules T diabétogènes, a montré que les cellules iNKT inhibent la prolifération et la différenciation des cellules T auto-réactives [117, 118]. Dans ce cas, les fonctions immuno-régulatrices des cellules iNKT ne font pas intervenir l'IL-4 mais plutôt des contacts cellulaires indépendants de la molécule CD1d [119, 120]. Le défaut d'activation des lymphocytes T, reflète la capacité des lymphocytes iNKT à recruter des cellules dendritiques tolérogènes [118, 121].
Chez l'homme, les études portant sur le rôle des lymphocytes iNKT montrent des résultats contradictoires. Une étude comparant des jumeaux monozygotes discordant pour l'apparition du T1D a observé une diminution de la fréquence en iNKT dans le sang des jumeaux diabétiques (analyse par PCR) [122]. Cependant, une étude utilisant la cytométrie en flux et le tétramère CD1d-αGalCer n'a montré aucune différence dans la fréquence en iNKT dans le sang des jumeaux discordants [123]. D'autres études portant sur des cohortes de patients, ont rapporté une fréquence plus faible ou plus forte des lymphocytes iNKT dans le sang des patients diabétiques [124, 125]. Il est à noter, que l'analyse de différentes souches de souris a montré que la fréquence sanguine en cellules iNKT n'avait pas de corrélation avec la fréquence de ces cellules dans les tissus [126]. Par conséquent, il semblerait plus indiqué d'étudier la présence de ces cellules dans les pLN et le pancréas de patients diabétiques (i.e. marquage en coupe sur pancréas).

Récemment, une étude fonctionnelle a montré que les clones de cellules iNKT provenant des ganglions pancréatiques de patients diabétiques, présentent un défaut de production en IL-4 [127]. Ce résultat semble confirmer les premières observations faite sur la souris NOD, ou la fonction protectrice des iNKT était associé à leur production d'IL-4 [114-116].

Jusqu'à la publication de notre article [128], aucune étude ne s'était intéressée au rôle de la sous-population cellulaire iNKT-17 dans le développement du T1D.

I-1.1c IL-17 et diabète de type 1.

L'IL-17 est une interleukine impliquée dans de nombreuses maladies inflammatoires et auto-immunes. Essentielle dans la défense de l'hôte contre les infections, cette cytokine influence le développement de l'auto-immunité et joue un rôle ambivalent dans la réponse allergique. L'IL-17 fut identifiée en 1993 par E. Rouvier [129]. L'IL-17 ou IL-17A, fait partie d'une famille composée de six cytokines : IL-17A à IL-17F. La fixation de l'IL-17A à son récepteur induit une voie de transduction aboutissant à l'activation de facteurs de transcription tels que NF-κB et AP-1, qui conduisent à l'expression de cytokines pro-inflammatoires telles que l'IL-8 et l'IL-6 [130]. Dans un premier temps, il fut mis en évidence que l'IL-17 jouait un rôle dans le recrutement des neutrophiles via l'IL-8 (ou CXCL1/2 chez la souris) [131]. De nouvelles études montrent que l'IL-17 peut stimuler l'activité phagocytaire des macrophages [132] ou induire le recrutement de lymphocytes T CD4+ [133]. Enfin l'IL-17 peut interagir fonctionnellement avec d'autres cytokines afin d'amplifier une réponse inflammatoire. Elle peut stimuler la sécrétion de TNFα et d'IL-1β par les monocytes et agir en synergie avec ces cytokines pour accroitre la libération de chimiokines (CXCL10, CXCL6) [134].

De multiples études chez la souris montrent le rôle pathogène des cellules sécrétrices d'IL-17 (Th17, lymphocytes Tγδ) dans des maladies auto-immunes telles que l'EAE ou l'arthrite. Les souris déficientes en IL-17 ou traitées avec un anticorps bloquant, présentent une inflammation réduite [135-137]. Le T1D étant l'une des maladies auto-immunes les plus étudiées, de nombreuses équipes ont recherché si cette cytokine pouvait avoir un rôle dans cette maladie. En 2008, H. Zaghouani montre dans un modèle thérapeutique de prévention du diabète, que les lymphocytes T Th17 favorisent la maladie à un stade avancé. La neutralisation de l'IL-17 avec un anticorps bloquant prévient totalement l'apparition du diabète [138]. En 2009, A. Cooke confirme en partie ces résultats en montrant que le transfert de lymphocytes T Th17 dans des souris NOD SCID induit un diabète. Cependant, la

neutralisation de l'IL-17 ne prévient pas la maladie. Il apparait que les cellules Th17 injectées sont converties en Th1 et secrètent de grande quantité d'IFNγ [139]. En 2009, A. Shapiro montre, que le traitement de souris NOD par un anticorps bloquant l'IL-17 empêche l'apparition du diabète en diminuant la péri-insulite et en augmentant la fréquence des Treg dans les pLN [140].

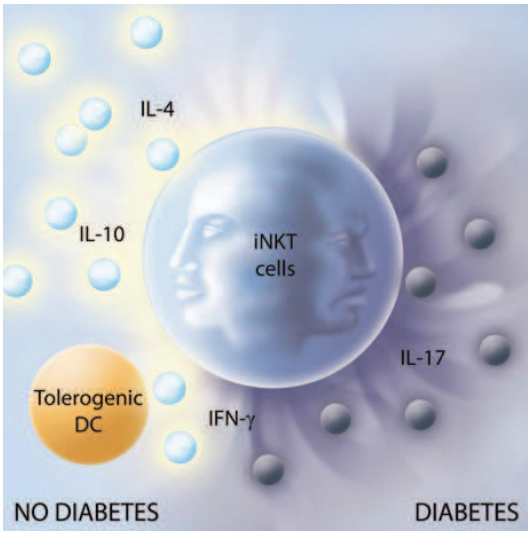
L'ensemble de ces données nous a amené à nous intéresser au rôle des lymphocytes iNKT-17 dans la pathologie du T1D.

I-1.2 Article.

Récemment, une nouvelle sous-population de cellules iNKT, qui produisent la cytokine pro-inflammatoire IL-17, a été identifiée (lymphocytes iNKT17). Cette cytokine ayant été impliquée dans plusieurs maladies auto-immunes, nous avons analysé la fréquence, le nombre absolu et le phénotype des cellules iNKT17 dans le pancréas et les organes lymphoïdes des souris NOD. Le rôle des cellules iNKT17 dans le développement du diabète a été étudié en utilisant des expériences de transfert.

Nous avons observé que dans les organes lymphoïdes, la souris NOD présente une fréquence et un nombre absolu plus élevés de lymphocytes iNKT17 par rapport aux souches de souris non auto-immunes. Les lymphocytes iNKT17 infiltrent le pancréas de la souris NOD, où ils expriment de l'ARNm codant pour l'IL-17. Les expériences de transfert adoptif des différentes sous-populations de lymphocytes iNKT ont montré, que contrairement au rôle protecteur des lymphocytes iNKT CD4+, la population de lymphocytes iNKT $CD4^-$ - qui contient les lymphocytes iNKT17- a un rôle délétère sur l'incidence du diabète. Le traitement des souris transférées avec les lymphocytes iNKT CD4- avec un anticorps bloquant l'action de l'IL-17 empêche l'aggravation de la maladie.

Cette étude révèle que les différentes sous-populations de lymphocytes iNKT jouent des rôles distincts dans la régulation du T1D. Les lymphocytes iNKT17, qui sont abondants chez la souris NOD, exacerbent le développement de la maladie.



Rôle ambivalent des lymphocytes iNKT dans le T1D.
La production d'IL-4 et d'IL-10 par les iNKT joue un rôle protecteur dans l'apparition du T1D.
Au contraire, l'IL-17 est délétère dans la maladie. Image de Martine Simoni [128].

European Journal of Immunology

NOD mice contain an elevated frequency of iNKT17 cells that exacerbate diabetes

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Invariant natural killer T (iNKT) cells are a distinct lineage of innate-like T lymphocytes and converging studies in mouse models have demonstrated the protective role of iNKT cells in the development of type 1 diabetes. Recently, a new subset of iNKT cells, producing high levels of the pro-inflammatory cytokine IL-17, has been identified (iNKT17 cells). Since this cytokine has been implicated in several autoimmune diseases, we have analyzed iNKT17 cell frequency, absolute number and phenotypes in the pancreas and lymphoid organs in non-obese diabetic (NOD) mice. The role of iNKT17 cells in the development of diabetes was investigated using transfer experiments. NOD mice exhibit a higher frequency and absolute number of iNKT17 cells in the lymphoid organs as compared with C57BL/6 mice. iNKT17 cells infiltrate the pancreas of NOD mice where they express IL-17 mRNA. Contrary to the protective role of CD4$^+$ iNKT cells, the CD4$^-$ iNKT cell population, which contains iNKT17 cells, enhances the incidence of diabetes. Treatment with a blocking anti-IL-17 antibody prevents the exacerbation of the disease. This study reveals that different iNKT cell subsets play distinct roles in the regulation of type 1 diabetes and iNKT17 cells, which are abundant in NOD mice, exacerbate diabetes development.

Key words: Autoimmunity · Diabetes · IL-17 · iNKT · NOD

Supporting Information available online

Introduction

Invariant natural killer T (iNKT) cells represent a distinct lineage of T cells that co-express a highly conserved αβ T-cell receptor TCR along with typical surface receptors for natural killer cells. The invariant TCRα chain of iNKT cells is encoded by Vα24-Jα18 gene-segments in humans and Vα14-Jα18 gene-segments in mice. The TCRβ chain is also strongly biased, encoded by Vβ11 gene-segment in humans and Vβ8.2, Vβ7 and Vβ2 gene-segments in mice. These lymphocytes recognize both self and microbial glycolipid antigens presented by the non-classical class I molecule CD1d. iNKT cells are characterized by their capacity to produce

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rapidly large amounts of both Th1 (IFN-γ, TNF-α) and Th2 (IL-4, IL-13) cytokines, which enables them to exert beneficial, as well as deleterious, effects in a variety of inflammatory or autoimmune diseases [1, 2].

Converging studies in mouse models suggest that iNKT cells can prevent the development of type 1 diabetes [3]. iNKT cells are reduced in number in diabetes-prone NOD mice [4, 5], and increasing the number of iNKT cells by adoptive transfer [6, 7] or via the introduction of a Vα14-Jα18 transgene, reduces significantly the progression of the disease [6]. A similar protection was observed after specific iNKT cell stimulation with exogenous ligands, α-galactosylceramide (α-GalCer) and its analogues [8–11]. Early reports suggested that iNKT cell protection was associated with the induction of a Th2 response to islet autoantigens [8, 10–12]. However, following studies using the transfer of anti-islet T cells showed that iNKT cells inhibit the differentiation of these auto-reactive T cells into effector cells during their priming in pancreatic lymph nodes (PLNs) [13, 14]. This regulatory role of iNKT cells could be explained by their ability to promote the recruitment of tolerogenic DCs [14, 15].

It is now well established that iNKT cells can be divided into several subpopulations using various cell surface markers, these subsets exhibiting diverse functions. According to the expression of the CD4 molecule, human iNKT cells have been shown to express a Th1 or Th0 cytokine profile [16, 17]. In the mouse, $CD4^-$ iNKT cells are more potent to promote tumor rejection [18]. Recently, a new population of $CD4^-$ $NK1.1^-$ iNKT cells producing high levels of the pro-inflammatory cytokine IL-17 together with low IL-4 and IFN-γ levels in response to several iNKT cell ligands, has been identified and named iNKT17 cells [19]. Consistent with their ability to produce IL-17 rapidly and independently of IL-6, iNKT17 cells, unlike naive T cells, were found to express constitutively IL-23R and Retinoic acid receptor – related orphan receptor γt (RORγt) [20–22].

Much of the focus on IL-17-secreting cells has been on their role in promoting organ-specific autoimmunity and chronic inflammatory conditions [23]. In the past few years, results have suggested that it was not IL-12 and Th1 cells that are required for the induction of experimental autoimmune encephalomyelitis (EAE) and collagen-induced arthritis (CIA) but rather IL-23 and Th17. EAE can be induced by the transfer of IL-17 producing autoreactive T cells and IL-17 deficient mice had reduced susceptibility to CIA and EAE. Unregulated Th17 responses or overwhelming IL-17 production from T cells and other sources is also associated with chronic inflammation in rheumatoid arthritis patients [23].

Recent studies suggest that IL-17 might also be involved in the development of type 1 diabetes. Transfer of in vitro polarized BDC2.5 Th17 cells into NOD SCID mice induced diabetes in recipient mice with similar rates of onset as transfer of Th1 cells [24–26]. However, the exact role of IL-17 in the pathogenesis of type 1 diabetes remains unclear as the neutralization of IL-17 inhibited the disease transfer in one of the studies but not in two others. Treatment with an anti-IL-17 mAb protected NOD mice against diabetes only when performed at late stage of disease development [27]. Although it is clear that Th17 cells play an important role in some autoimmune disease models, their precise role in diabetes remains to be elucidated. All these observations on the role of IL-17 and iNKT cells in autoimmune diseases led us to characterize iNKT17 cells in the NOD mouse and to investigate whether these cells play a pathogenic role in diabetes.

Results

Enhanced iNKT17 cell population in NOD mice compared with C57BL/6 mice

To investigate the role of iNKT17 cells in type 1 diabetes, we have compared the frequency and absolute number of these cells in NOD and C57BL/6 mice. C57BL/6 mice were used as the control mice, since they develop neither diabetes nor other autoimmune pathologies. iNKT17 cells were analyzed in the thymus, spleen, inguinal LNs (ILNs) and PLNs. ILNs were used as control tissue since they are enriched in iNKT17 cells [28]. IL-17 production by iNKT cells was detected after CD1d-αGalCer tetramer staining and stimulation with phorbolmyristyl acetate (PMA) and ionomycin (Fig. 1A). As previously shown in C57BL/6 mice, iNKT17 cells do not express the NK1.1 marker. These cells are also $NK1.1^-$ in NK1.1 congenic NOD mice used for this analysis (Fig. 1B). Interestingly, iNKT17 cell frequency was four to six-fold increased in NOD mice as compared with C57BL/6 mice (Fig. 1B and C). This difference was also observed in terms of absolute number (Fig. 1D). Of note, in PLNs of NOD mice, iNKT17 cells represent 13% of total iNKT cells compared with only 2% in C57BL/6 mice. The high frequency and absolute number in PLNs of NOD mice suggest that iNKT17 cells could play a role in the development of type 1 diabetes.

Enhanced expression of IL-17-associated genes by thymic iNKT cells from NOD mice

Previous studies have shown that unlike Th17 cells, iNKT17 cells are generated during thymic differentiation [19]. iNKT cell maturation can be divided in three differentiation stages; stage 1 ($CD44^-$ $NK1.1^-$), stage 2 ($CD44^+$ $NK1.1^-$ $CD4^-$ or $CD4^+$) and stage 3 ($CD44^+$ $NK1.1^+$). We have analyzed the expression of genes usually associated with the iNKT17 lineage in thymic iNKT cells. Quantitative-PCR data show that *il-17a* gene is mainly transcribed in stage 2 $CD4^-$ iNKT cells and to a lesser extent in stage 1 and stage 2 $CD4^+$ iNKT cells (Fig. 1D). In agreement with our results obtained by intracellular IL-17 staining, IL-17A mRNA level is increased (10-fold) in stage 2 $CD4^-$ iNKT cells from NOD as compared with C57BL/6 mice. Analysis of mRNA encoding RORγt, which is required for iNKT17 cell differentiation [21], revealed its high expression in the stage 2 $CD4^-$ iNKT cells and 3-fold increased in NOD mice. IL-23R is constitutively expressed by iNKT17 cells [20], and its expression is high in stage 2 $CD4^-$ iNKT cells, however, there is no significant difference between

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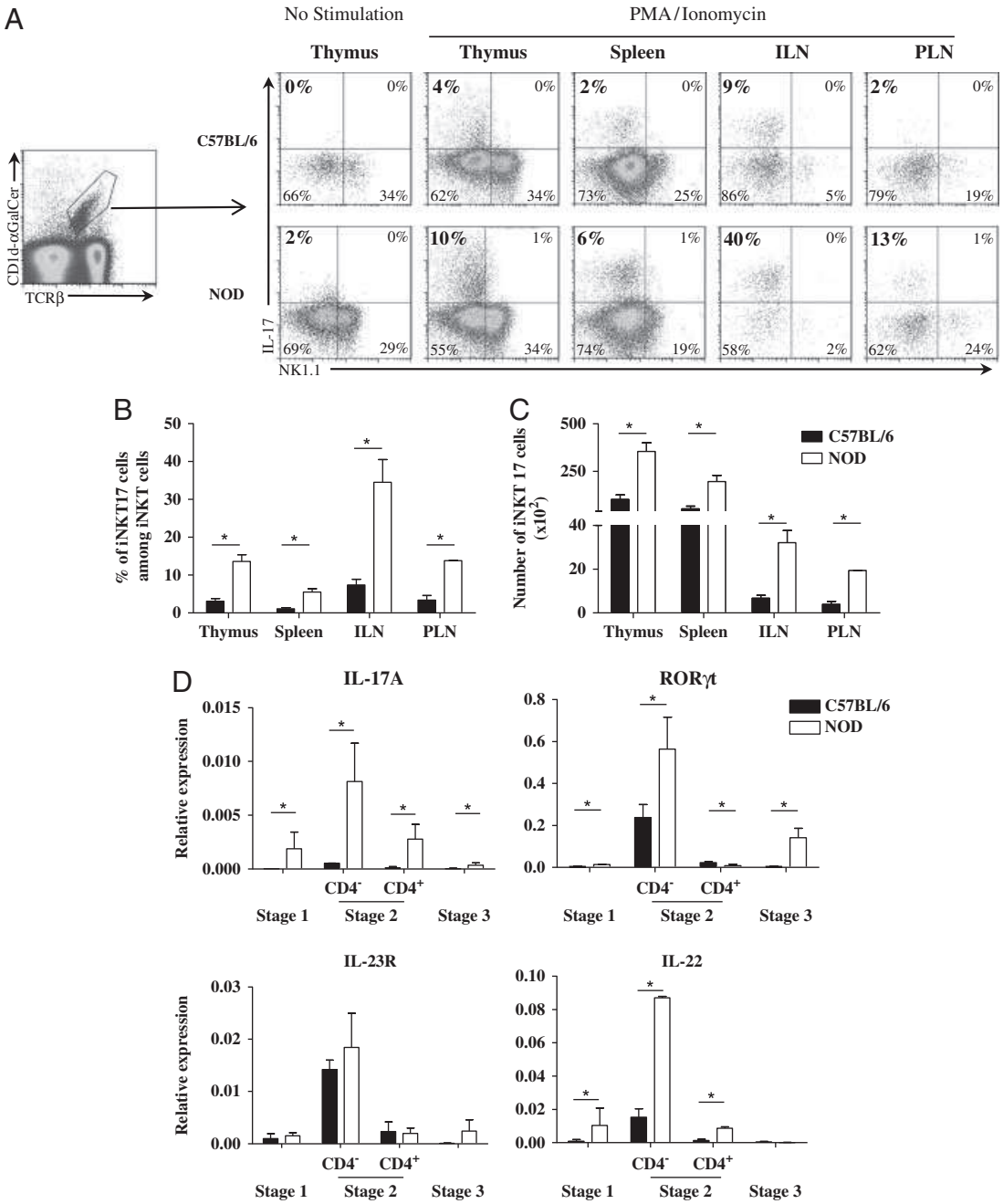

Figure 1. The size of the iNKT17 cell population is increased in NOD, as compared with C57BL/6, mice. The indicated tissues were harvested from 6 to 10-wk-old female NOD and C57BL/6 mice and cell suspensions were prepared. (A–C) Intracellular IL-17 staining of iNKT cells was performed after stimulation with PMA/ionomycin in the presence of brefeldin A for 4 h. iNKT cells were detected using CD1d-αGalCer tetramers in combination with anti-TCRβ and their subsets using anti-IL-17 and anti-NK1.1 mAbs. (A) Representative FACS profiles of IL-17 production by iNKT cells from thymus, spleen, ILNs and PLNs. (B) Frequency of IL-17-producing cells among iNKT cells and (C) absolute number of IL-17-producing iNKT cells in the indicated lymphoid organs. (D) Enhanced expression of IL-17 lineage-associated genes by thymic iNKT subsets in NOD mice. Thymic stage 1 ($CD44^-$ $NK1.1^-$), stage 2 ($CD44^+$ $NK1.1^-$ $CD4^-$ or $CD4^+$) and stage 3 ($CD44^+$ $NK1.1^+$) iNKT cells were sorted from C57BL/6 and NOD mice. The levels of IL-22, IL-17A, RORγt and IL-23R mRNA were evaluated by quantitative PCR. Data were normalized to the *gapdh* housekeeping gene. All data are from four independent experiments, each performed with cells pooled from 4 to 10 mice and are either (A) representative or (B) an analysis of all experiments (mean+SD); $^*p \leq 0.05$ between NOD and C57BL/6 mice.

NOD and C57BL/6 mice. Interestingly, stage 2 $CD4^-$ iNKT cells also expressed IL-22 mRNA and this expression is 4-fold higher in cells from NOD mice. These data showing a higher transcription of *il-17a, rorγt* and *il-22* genes in iNKT cells from NOD mice strengthen the differences in iNKT cells between this autoimmune strain and C57BL/6 mice.

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iNKT17 cells infiltrate the pancreas of NOD mice

To determine whether iNKT17 cells infiltrate the pancreas of NOD mice, we have analyzed pancreatic infiltrates from NOD and Vα14 NOD transgenic mice that express iNKT cell characteristic TCRα chain and exhibit a10 fold increased frequency and number of iNKT cells in lymphoid tissues [6] as well as in the pancreas [29]. iNKT17 cells represent 6% of all iNKT cells infiltrating the pancreas in NOD and Vα14 NOD mice (Fig. 2A). We next assessed whether this frequency varies at different stages of insulitis. At 6 wk of age NOD mice have a small infiltrate of hematopoietic cells, at 12-wk peri-insulitis is more abundant and at 20 wk many pancreatic islets are characterized by a destructive insulitis leading to diabetes onset [30]. Indeed, we observed an increased frequency of pancreatic infiltrating hematopoietic ($CD45^+$) cells with aging (Fig. 2B). Even though, iNKT17 cell frequency among iNKT cells as well as iNKT cell frequency among $CD45^+$ cells infiltrating pancreas remained stable (Fig. 2B), the number of iNKT17 cells increased with the enhanced infiltration of pancreas, meaning that they could participate in the destruction of islet cells. CCR6 and CD103 integrin expression has been described on iNKT17 cells [28] and CCR6 has been involved in the recruitment of pathogenic Th17 cells in CIA [23]. All iNKT17 cells from ILNs are $CD103^+$ and the level of CD103 expression is higher in iNKT17 cells of NOD mice as compared with C57BL/6 mice (Supporting Information Fig. 1). iNKT17 cells from ILNs are mainly $CCR6^+$, whereas in PLNs and spleen only a fraction of iNKT17 cells express CCR6 and CD103 (Supporting Information Fig. 1). The analysis of CCR6 and CD103 expression on pancreatic iNKT17 cells showed that, while 60% of iNKT17 cells expressed CD103 integrin, most of them were negative for CCR6 (Fig. 3C). These data suggest that iNKT17 cell recruitment in the pancreas is independent of CCR6, whereas CD103 could play a role in the retention of these cells.

Pancreatic iNKT17 cells express IL-17A mRNA in the absence of exogenous stimulation

To determine whether iNKT17 cells express IL-17A mRNA in the absence of exogenous stimulation such as PMA and ionomycin, iNKT cells were purified from the pancreas, PLNs and ILNs from Vα14 NOD mice. Expression of other genes usually associated with iNKT17 cells were also assessed by quantitative-PCR (Fig. 3D). IL-21 and IL-22 mRNA were barely detectable in the three organs analyzed. Interestingly, *il-17a* gene was expressed at much higher level in pancreatic iNKT cells than in iNKT cells from PLNs and ILNs (6- and 13-fold increased respectively). A similar trend was observed for *il-17f* gene. In contrast, *rorγt* and *il-23r* gene expression was not significantly different in iNKT cells from pancreas and ILNs. These data show that although iNKT17 cells are present in these three tissues, they are expressing IL-17A mRNA only in the pancreas.

Since previous studies have shown that iNKT17 cells can secrete IL-17 through TCR engagement [20], we investigated whether CD1d was required for IL-17A mRNA expression by iNKT17 cells in the pancreas (Fig. 3E). To address this question, we used Vα14 NOD mice expressing CD1d solely in the thymus ($CD1d^{pLck}$ Vα14 NOD mice) [31]. RORγt, IL-23R and IFN-γmRNA expression was similar in pancreatic iNKT cells from both types of mice. However, IL-17A mRNA expression was significantly decreased (3-fold) in iNKT cells from mice lacking peripheral CD1d expression. Altogether, our data suggest that iNKT17 cells are activated locally in the pancreas in a CD1d-dependent manner.

$CD4^-$ iNKT cells containing iNKT17 cells enhance the incidence of diabetes

To evaluate the role of iNKT17 cells in type 1 diabetes, we reconstituted immunodeficient NOD mice with different iNKT cell subsets and analyzed the induction of diabetes after transfer of anti-islet BDC2.5 T cells [32]. Since there is no specific antibody available to purify iNKT17 cells, we first determined the frequency of iNKT17 cells in different iNKT cell subpopulations divided according to CD4 and NK1.1 expression of donor cells. As shown in Fig. 3A and Supporting Information Fig. 2, iNKT17 cells are mainly present in the $CD4^-$ iNKT cell population and at a higher frequency among $NK1.1^-$ $CD4^-$ iNKT cells. Therefore, we enriched iNKT17 cells based on their lack of CD4 expression and they were found to represent around 23% of the injected $CD4^-$ iNKT cell population (Fig. 3B). Recipient NOD mice were reconstituted with $CD4^-$ or $CD4^+$ iNKT cells, which were detected in pancreas before BDC2.5 T-cell transfer (Fig. 3B). In order to detect an eventual pathogenic role of iNKT17 cells, all recipient mice were injected with a low number of BDC2.5 T cells, which induces around 30% of diabetes in control mice devoid of iNKT cells (Fig. 3C). Interestingly, in the group of mice reconstituted with $CD4^-$ iNKT cells, the incidence of diabetes was significantly ($p = 0.036$) increased and reached 70%. In contrast, reconstitution with $CD4^+$ iNKT cells significantly ($p = 0.033$) prevented the development of diabetes. Moreover, when $CD4^-$ iNKT cells were further divided according to NK1.1 expression, only $NK1.1^-$ $CD4^-$ iNKT cells containing the higher frequency of iNKT17 cells exacerbated diabetes (Fig. 3D).

Since diabetes induced by diabetogenic BDC2.5 T cells is associated with their production of IFN-γ [13], we have analyzed whether the presence of iNKT cell subsets have influenced their production of IFN-γ and IL-17. As previously described [13], in diabetic control mice devoid of iNKT cells, BDC2.5 T cells produced large amount of IFN-γ in both PLNs and pancreas (Fig. 4A). In diabetic mice reconstituted with $CD4^-$ iNKT cells, production of IFN-γ by BDC2.5 T cells was similar as in diabetic control mice and production of IL-17 remained low, less than 1%. While cytokine production by BDC2.5 T cells was similar in both groups of mice, the frequency of BDC2.5 T cells in the pancreas was increased in mice reconstituted with $CD4^-$ iNKT cells ($44 \pm 3.1\%$) compared with control mice ($32 \pm 1.4\%$). These data suggest that the enhanced incidence of diabetes in mice reconstituted with $CD4^-$

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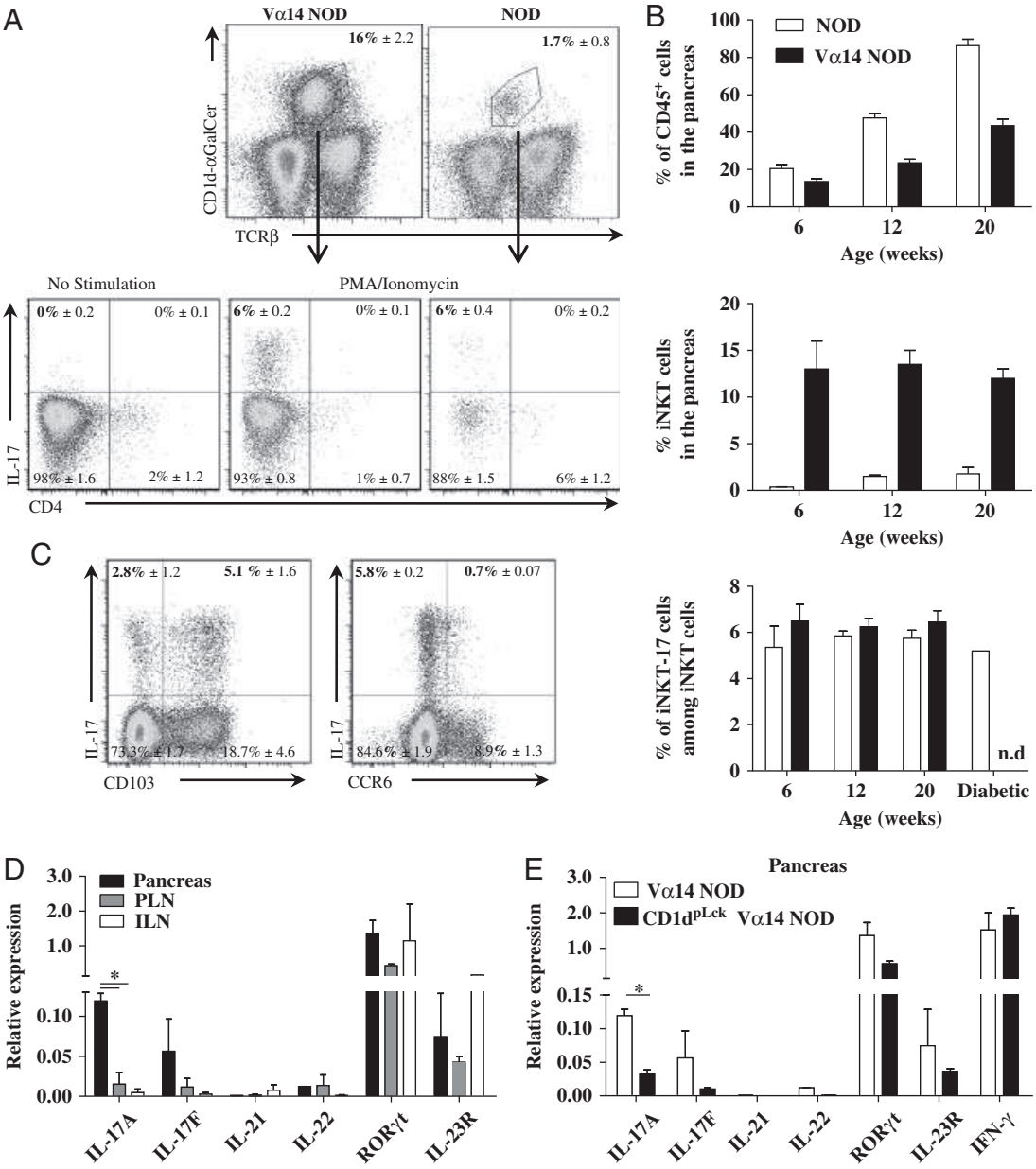

Figure 2. iNKT17 cells infiltrate the pancreas of NOD mice and express IL-17 mRNA in the absence of exogenous stimulation. (A–C) Cell suspensions from pancreatic islets of female NOD and Vα14 NOD mice were prepared and intracellular IL-17 staining of iNKT cells was performed as described in Fig. 1. iNKT cells were detected using CD1d-αGalCer tetramers in combination with anti-CD45, anti-TCRβ and their subsets using anti-IL-17, anti-CD4, anti-CD103 and anti-CCR6 mAbs. (A) Representative FACS profiles of IL-17 and CD4 expression by pancreatic iNKT cells from 12-wk-old NOD and Vα14 NOD mice. (B) Pancreatic islet cells of NOD and Vα14 NOD female mice were prepared. The frequencies of pancreatic infiltrating $CD45^+$ cells (top panel), iNKT cells (middle panel) and iNKT17 cells (bottom panel) at the indicated ages and at diabetes onset (n.d = not done) are shown. (C) Representative FACS profiles of IL-17 and CD103 or CCR6 expression by pancreatic iNKT cells from three pooled Vα14 NOD mice at 12 wk of age. All data are from four independent experiments and each experiment was performed with cells pooled from 3 to 8 mice. The data are either (A, C) representative or (B) an analysis of all experiments (mean+SD). (D, E) The levels of IL-17A, IL-17F, IL-21, IL-22, RORγt, IL-23R and IFN-γmRNA in iNKT cells purified from the (D) pancreas, PLNs and ILNs from 12-wk-old Vα14 NOD mice and from (E) the pancreas of 12 wk-old Vα14 NOD and $CD1d^{pLck}$ Vα14 NOD mice were evaluated by quantitative PCR and the data were normalized to *gapdh* housekeeping gene. (D, E) Data are mean+SD of four independent experiments are shown, each performed with cells pooled from 3 to 6 mice; $^*p \leq 0.05$ between the (D) different tissues or between (E) different mice.

iNKT cells is due to the increased frequency of diabetogenic BDC2.5 T cells. Indeed, the frequency of pathogenic BDC2.5 T cells is probably a key parameter controlling the development of diabetes, since non-diabetic mice reconstituted with $CD4^+$ iNKT cells contained only 0.9±0.2% and 12±6.4% of BDC2.5 T cells in their PLNs and pancreas, respectively. Our results highlight the pathogenic role of $CD4^-$ iNKT cells. To demonstrate the key role of IL-17, produced by iNKT17 cells, we treated mice with an anti-IL-

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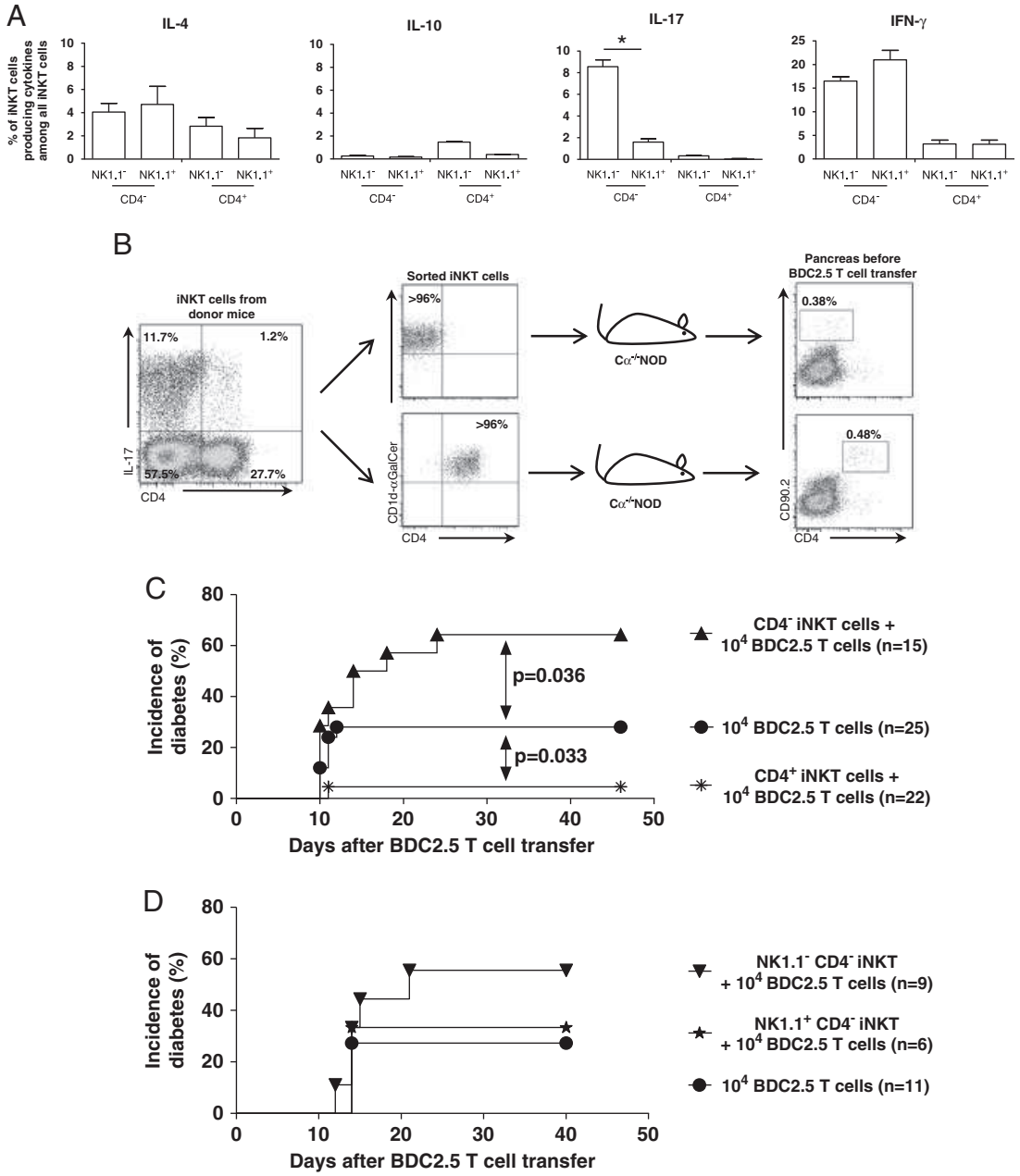

Figure 3. The CD4$^-$ iNKT cell population, which contains iNKT17 cells, enhances the incidence of diabetes. (A) Cytokine analysis by intracytoplasmic staining of the indicated iNKT cell subsets. Pooled splenocytes and mesenteric LN cells from individual Vα14 Cα$^{-/-}$ NOD donor mice were stimulated and stained as described in Fig. 1. The data correspond to the mean+SD of four independent mice. *$p \leq 0.05$ between CD4$^-$ NK1.1$^-$ and CD4$^-$ NK1.1$^+$iNKT cells for IL-17 production. The iNKT cells were detected using CD1d-αGalCer tetramers in combination with anti-TCRβ, anti-CD4 and anti-NK1.1 mAbs. (B, C) iNKT cell subset purification and cell transfer into recipient mice. (B) Intracellular staining of iNKT cells from pooled splenocytes and mesenteric LN (MLN) cells of Vα14 Cα$^{-/-}$ NOD donor mice (left panel). CD4$^-$ or CD4$^+$ iNKT cells were purified by cell-sorting and 1.5×10^6 cells were injected into 2-wk-old Cα$^{-/-}$ NOD mice (middle panel). Four wks later, the frequency of CD90.2$^+$ CD4$^-$ or CD4$^+$ iNKT cells were analyzed in the pancreas of Cα$^{-/-}$ NOD recipient mice (right panel). (C) The incidence of diabetes in recipient mice reconstituted with CD4$^-$ iNKT cells (triangles, $n = 15$), CD4$^+$ iNKT cells (asterisks, $n = 22$) or PBS (circles, $n = 25$), and then injected with 10^4 BDC2.5 T cells four wks later (day 0). (D) Similar transfer experiments to those described in (C) were performed with purified NK1.1$^-$ CD4$^-$ (inverted triangles, $n = 9$), NK1.1$^+$ CD4$^-$ (stars, $n = 6$) iNKT cell subsets or PBS controls ($n = 11$).

17 antibody. Importantly, this treatment abolished the deleterious role of CD4$^-$ iNKT cells whereas it does not alter the incidence of diabetes induced by BDC2.5 T cells alone (Fig. 4B). Altogether, our results show that CD4$^-$ iNKT cells containing iNKT17 cells exacerbate the development of diabetes in an IL-17-dependent manner.

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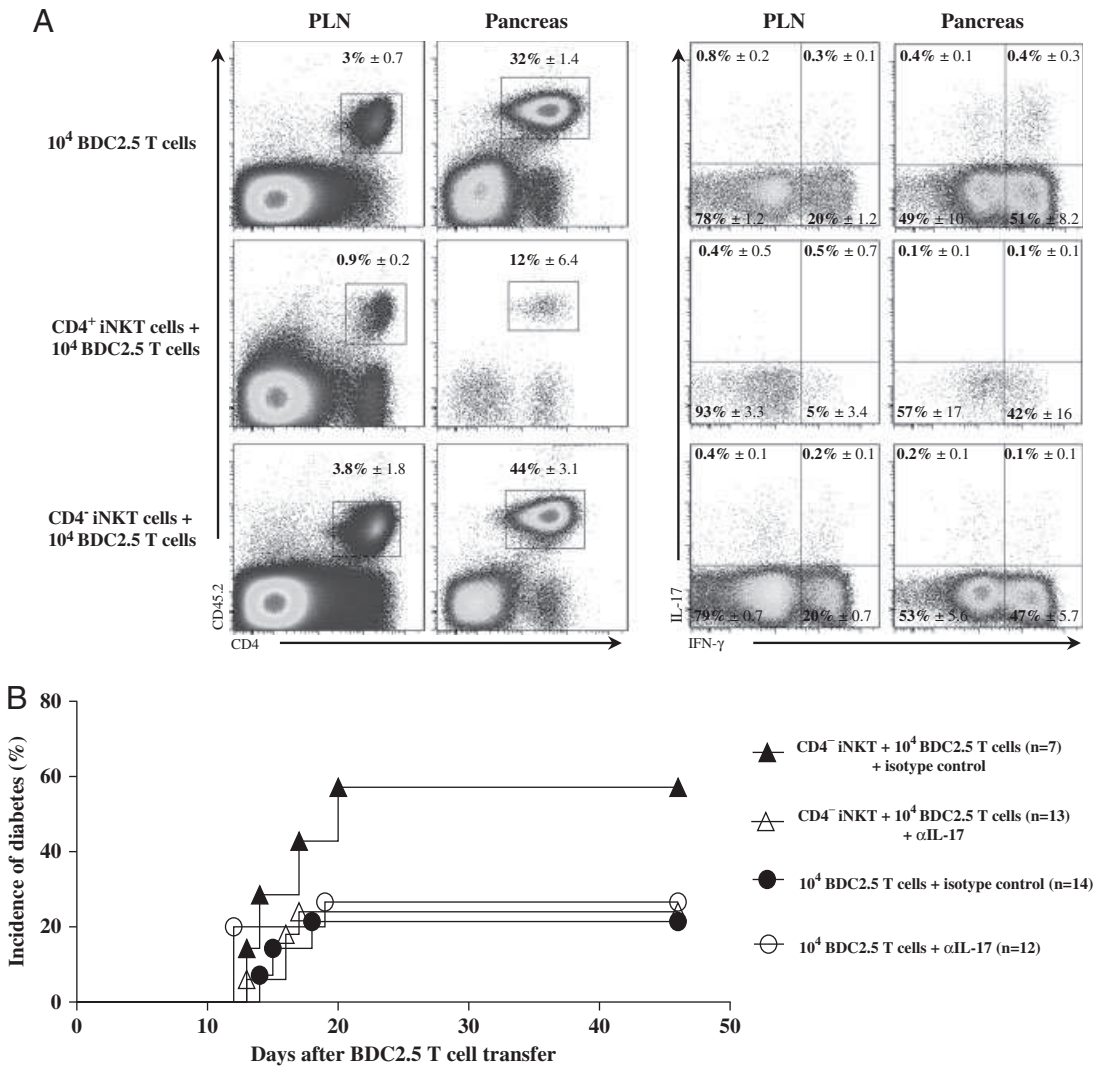

Figure 4. Analysis of diabetogenic T cells and the role of IL-17 in the exacerbation of diabetes by CD4$^-$ iNKT cells. (A) 1.5×10^6 CD4$^-$ or CD4$^+$ iNKT cells or PBS were transferred into C$\alpha^{-/-}$ NOD recipient mice, followed four wks later by injection of 10^4 BDC2.5 T cells. The frequency of CD45.2$^+$ CD4$^+$ BDC2.5 T cells (left panel) and their production of IFN-γ and IL-17 (right panel) in the PLNs and pancreas of each group of recipient mice were determined twelve days after BDC2.5 T-cell transfer after stimulation and staining as described in Fig. 1. Representative plots are shown and the values given in the plots represent the mean ± SD of two independent experiments with two pooled mice. (B) The incidence of diabetes in the C$\alpha^{-/-}$ NOD recipient mice reconstituted with CD4$^-$ iNKT cells or PBS as a control and injected with 10^4 BDC2.5 T cells four wks later. In addition, the mice were treated with anti-IL-17 antibodies (filled triangle) or isotype control (empty triangle) on days 0, 2, 4 and 6 after BDC2.5 T-cell transfer.

αGalCer treatment abolishes IL-17 production by iNKT cells

It has been well established that activation of iNKT cells by repeated αGalCer injections prevents the development of diabetes in NOD mice [8, 10, 15]. Autoimmunity prevention correlated with the ability of αGalCer to induce iNKT cell anergy and to strongly suppress their IFN-γ production while IL-4 production was less inhibited [33]. Interestingly, we have observed that αGalCer treatment suppressed not only IFN-γ by iNKT cells but also their IL-17 production whereas it does not inhibit IL-10 production (Fig. 5). This inhibition of IL-17 production could be critical in the protective role of αGalCer treatment.

Discussion

Our study reveals that NOD mice exhibit a high frequency of iNKT17 cells, which produce IL-17 in the pancreas and can exacerbate diabetes development upon cell transfer. This study suggests that IL-17 can participate in the pathology of type 1 diabetes. The role of IL-17 in autoimmune diabetes was first suggested by the low IL-17 production observed in NOD mice protected against the disease after treatment with a modified self-peptide [25]. More recent studies showed that IL-17 neutralization with specific antibodies prevents the development of diabetes in NOD mice [27]. Different immune cell populations can secrete IL-17 [34]. The role of Th17 cells in diabetes remains

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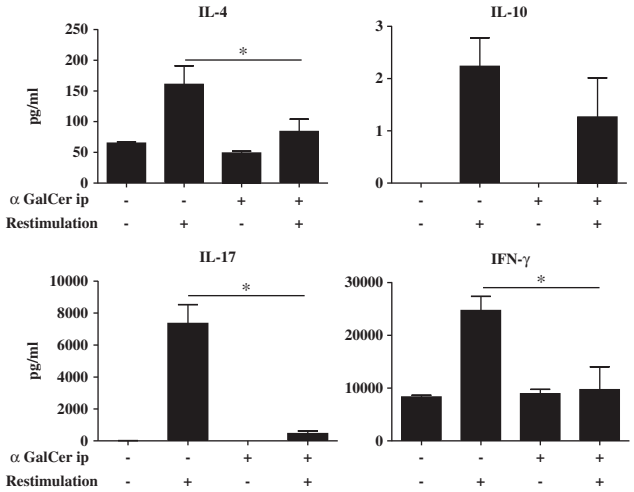

Figure 5. Repeated αGalCer injections inhibit IL-17 production by iNKT cells in NOD mice. Five wk-old NOD female mice were treated twice a wk for three wks with αGalCer (5 μg, i.p.). One wk after the last injection, the mice were sacrificed, the spleens harvested and the splenocytes from individual mice were cultured with or without 100 ng/mL of αGalCer for three days. IL-4, IL-10, IL-17 and IFN-γ levels were measured in the supernatants by ELISA as previously described [6, 19]. Data correspond to the mean+SD of four individual mice; similar data were obtained in four independent experiments. $^{*}p \leq 0.05$ between mice pretreated, or not, by αGalCer.

unclear. Indeed the induction of the disease in NOD SCID mice after transfer of in vitro polarized Th17 anti-islet T cells was abolished by anti-IL-17 treatment in one study but not in two others [25, 26]. It has been reported that IL-17-producing γδT cells do not exacerbate diabetes upon co-transfer into NOD/SCID mice [35].

iNKT17 cells represent a new subset of IL-17-producing cells [19] and we observed an increased frequency of this cell population in NOD mice as compared with non-autoimmune C57BL/6 mice. iNKT17 cells from NOD and C57BL/6 mice exhibit a similar phenotype, mainly $CD4^-$ and $NK1.1^-$. iNKT17 cells are generated in the thymus where they constitutively express IL-17 mRNA [21, 22]. The analysis of thymic iNKT cells showed higher frequency and absolute number of iNKT17 cells in NOD mice compared with C57BL/6 mice. Furthermore the analysis of the thymic stage 2 $CD4^-$ iNKT cell subset (containing iNKT17 cells) showed an enhanced expression of RORγt and IL-23R mRNA, two key molecules controlling IL-17 lineage [21]. Thus, our data suggest that the high frequency of iNKT17 cells in the peripheral tissues is subsequent to an elevated frequency of iNKT17 cells in the thymus of NOD mice, which could be due to an elevated expression of RORγt in thymic iNKT cells upon their IL-17 lineage commitment.

Not only are iNKT17 cells present at high frequency in NOD mice but more importantly, they infiltrate pancreatic islets of NOD mice. NOD pancreatic islets express the adhesion molecule E-cadherin, which interacts with the integrin CD103 [36]. Interestingly, 60% of pancreatic iNKT17 cells expressed CD103 integrin and retention of iNKT17 cells in the pancreas could be due to CD103/E-cadherin interactions as previously described for diabetogenic CD8 T cells in the context of islet allografts [37]. Moreover, CD103 can act as a co-activation molecule in human T lymphocytes [38] and could play a similar role in the activation of iNKT17 cells in the pancreas. While CCR6 is involved in the recruitment of Th17 cells in the target tissue in autoimmune CIA [39], the recruitment of iNKT17 cells in the pancreas is probably independent of CCR6 since most of them do not express this molecule. Alternatively, lack of expression of CCR6 might be due to downregulation upon entry into inflamed pancreas. Even though it has been suggested that iNKT17 cells are characterized by CCR6 and CD103 expression, the expression of these molecules by iNKT17 cells varies depending on tissues.

Since IL-17 protein is not detectable in absence of exogenous activation [19, 20], we analyzed IL-17 mRNA and other mRNAs associated with the IL-17 response. Importantly, IL-17 mRNA level was much higher in iNKT cells from the pancreatic islets than from PLNs and ILNs. No such difference in the mRNA level was observed for RORγt and IL-23R between these three tissues. Flow cytometry data showed that iNKT17 cells represent respectively 40% of iNKT cells in ILNs, 12% in PLNs and 6% in pancreas. The discrepancy between the frequency of iNKT17 cells in these three tissues and the spontaneous level of IL-17 mRNA suggests that pancreatic iNKT17 cells are locally activated in this tissue. Interestingly, IL-17, but not IFN-γ, mRNA expression by pancreatic iNKT cells was strongly decreased in mice lacking peripheral CD1d expression, demonstrating that local iNKT17 cell activation involves CD1d recognition. The residual expression of IL-17 mRNA in the absence of peripheral CD1d expression suggests that other local factors, such as IL-23 or IL-1β, could participate in the activation of iNKT17 cells [40]. IL-1β is an interesting candidate since it is present in inflamed pancreatic islets [41].

Transfer experiments of iNKT cell subsets reveal the pathogenic role of $CD4^-$ iNKT cells containing the iNKT17 cell population in the development of diabetes. Reconstitution of immunodeficient NOD mice with $CD4^-$ iNKT cells enhanced the incidence of diabetes after injection of a low dose of BDC2.5 T cells. Similar exacerbation of diabetes incidence was observed after reconstitution with the $NK1.1^-$ $CD4^-$ iNKT cell population, which exhibits a high frequency of iNKT17 cells. However, due to cell number limitations most of our experiments were performed with the whole $CD4^-$ iNKT cell population. Treatment with anti-IL-17 antibodies abolished the pathogenic role of $CD4^-$ iNKT cells suggesting that iNKT17 cells are the critical players in the exacerbation of diabetes, however, we cannot rule out that other cell types producing IL-17 are also participating. Unfortunately, we could not directly demonstrate that only iNKT17 cells were involved in the deleterious effect of $CD4^-$ iNKT cells since there is presently no specific surface marker to purify this cell population. IFN-γ is also produced by $CD4^-$ iNKT cells and this cytokine could also participate in the exacerbation of diabetes; however, no exacerbation was observed after reconstitution with $NK1.1^+$ $CD4^-$ iNKT cells producing high amounts of IFN-γ but low levels of IL-17. Of note, $CD4^-$ iNKT cells alone do not induce diabetes after transfer into immunodeficient NOD mice (data not shown). Therefore, we can propose that iNKT17 cells enhanced diabetes incidence through different mechanisms. In vitro data have shown that IL-17 synergizes with other cytokines such as IFN-γ

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and IL-1α/β to induce iNOS expression and subsequent NO production in insulinoma cells or in pancreatic islets of NOD mice [42]. Similarly in the pancreas, IL-17 produced by iNKT cells could synergize with IFN-γ secreted by BDC2.5 T cells to induce high expression of NO in β-cells resulting in their destruction. A deleterious loop could take place since β-cell death induced by NO would promote self-antigen presentation by DCs to BDC2.5 T cells. This mechanism could explain the higher frequency of BDC2.5 T cells observed in the PLNs and the pancreas of mice transferred with $CD4^-$ iNKT cells as compared with mice devoid of iNKT cells. Furthermore, it has been shown that IL-17A and IL-17F can induce CXCL10 chemokine expression in lung epithelial cells [43, 44]. Production of CXCL10 by pancreatic β-cells could contribute to the recruitment of auto reactive T cells expressing the CXCR3 chemokine receptor as previously shown in several mouse models of type 1 diabetes (T10) [45, 46]. Thus, iNKT17 cells might not be involved in the initiation of the insulitis but rather could participate in the exacerbation of -β-cell death and diabetes onset.

Our data reveal a functional dichotomy between $CD4^+$ and $CD4^-$ iNKT cell subsets in the control of diabetes development. While $CD4^-$ iNKT cells exacerbate the incidence of diabetes, $CD4^+$ iNKT cells strongly protect mice against diabetes induced by BDC2.5 T cells. Our transfer experiments demonstrate the protective role of $CD4^+$ iNKT cells as it was previously suggested in NOD mice deficient for CD38 [47]. iNKT cells represent a heterogeneous population, each subset of iNKT cells exhibiting different functions, either deleterious or beneficial toward diabetes development. Protection by iNKT cells is probably not only due to their total frequency but also to the ratio between the different iNKT cell subsets. This hypothesis is a possible explanation for the controversial role of iNKT cells in diabetic patients. In contrast to studies in NOD mice, some authors failed to detect differences in iNKT cell frequencies and IL-4 production between diabetic patients and healthy subjects [48]. Autoimmune diabetes is generally considered a Th1-type pathology, but recent reports have suggested that IL-17-producing cells are enhanced in diabetic patients and allegedly contribute to disease severity [49]. We have recently reported that human iNKT cells produce IL-17 under pro-inflammatory conditions [50]. IL-17-producing cells in T1D patients [49] express CCR6 similarly to IL-17-producing human iNKT cells [50]. Therefore, our data prompt further analysis of iNKT cell subpopulations in patients with a peculiar emphasis on determining the cytokine profile not only of circulating iNKT cells, but more relevantly of iNKT cells from tissues such as PLNs and pancreas.

Materials and methods

Mice

C57BL/6J, NOD, $C\alpha^{-/-}$ NK1.1 NOD, BDC2.5 $C\alpha^{-/-}$ NOD, Vα14 NOD, $CD1d^{pLck}$ Vα14 NOD, Vα14 $C\alpha^{-/-}$ NOD mice have already been described [6, 13, 31]. NK1.1 Vα14 $C\alpha^{-/-}$ NOD were generated for iNKT cell subset transfer experiments. NK1.1 NOD females were used for flow cytometry analysis of Fig. 1 [51]. Females were used between 6 and 20 wk of age. All experimental protocols were approved by the local ethic committee on animal experimentation.

Flow cytometry

CD1d-αGalCer tetramer staining was performed as previously described [52]. Then cells were stained at 4°C in PBS containing 5% FCS and 0.1% NaN_3. FcγR were blocked with 2.4G2 mAb. Surface staining was performed with anti-CD44 (clone IM7), anti-NK1.1 (clone PK136), anti-TCRβ (clone H57-597), anti-CD4 (clone RM4-5), anti-CD45 (clone 30F11), anti-CD90.2 (clone 30H12), anti-CD45.2 (clone 104), anti-CD103 (clone 2E7) (BD Pharmingen) and anti-CCR6 (clone 140706 – R&D). For intracellular staining, cells were stimulated for 4 h at 37°C with 10 ng/mL of PMA, 1 μg/mL of ionomycin in the presence of 10 μg/mL of brefeldin A (all from Sigma). Then cells were surface stained, fixed, permeabilized using a commercial kit (BD Pharmingen) and stained with anti-IL-17 (clone TC11-10H10), anti-IFNγ (clone XMG1.2), anti-IL-4 (clone 11B11) and anti-IL-10 (clone JES5-16E3) (BD Pharmingen). Cells were analyzed on a FACSAria (BD).

Preparation of iNKT cells and quantitative PCR

Thymic cells were expanded 5 days in the presence of 20 ng/mL of IL-7 (R&D). iNKT cells were sorted as $TCR\beta^+$ CD1d-αGalCer $tetramer^+$ cells and according to various markers CD44, NK1.1 and CD4 expression, using FACSAria. Ten thousand iNKT cells were collected in RLT buffer with 1% of β-mercaptoethanol. mRNA was isolated using RNeasy Mini Kit (Qiagen) and reverse transcripted with Superscript III (Invitrogen). Quantitative-PCR was realized with SYBR Green (Roche) and analyzed with LightCycler 480 (Roche).

Preparation of pancreatic islet cells

Pancreatic islet cells were prepared as previously described [53]. Pancreata were perfused with a solution containing collagenase P (Roche), dissected free from surrounding tissues and digested at 37°C for 10 min. Islets were then purified on a Ficoll gradient and disrupted by adding cell dissociation buffer (GIBCO) for 10 min at 37°C.

Cell purification for transfer experiments

iNKT cells from spleen and mesenteric LNs of $CD45.1^{+/+}$ $CD90.1^{+/+}$ Vα14 $C\alpha^{-/-}$ NOD mice were enriched by negative selection and

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then sorted as $CD4^-$ or $CD4^+$ CD1d-αGalCer tetramer$^+$ cells. Sorted cell purity was >96%. $CD62L^+$ BDC2.5 T cells were isolated from $CD45.2^{+/+}$ $CD90.1^{+/+}$ BDC2.5 $C\alpha^{-/-}$ NOD mice. Splenocytes were enriched in T cells by negative selection and $CD62L^+$ cells were positively selected using biotinylated anti-CD62L mAb and Streptavidin microbeads (Miltenyi Biotec). $CD62L^+$ BDC2.5 T-cell purity was >92%. Similar procedures were used for the reconstitution with $NK1.1^-$ or $NK1.1^+$ $CD4^-$ iNKT cells. Donor cells were obtained from NK1.1 Vα14 $C\alpha^{-/-}$ NOD mice.

Adoptive transfer experiments and diabetes diagnosis

At 2 wks of age, $CD45.1^{+/+}$ $CD90.1^{+/+}$ $C\alpha^{-/-}$ NOD mice were reconstituted i.v with 1.5×10^6 $CD4^-$ or $CD4^+$ iNKT cells from $CD45.1^{+/+}$ $CD90.2^{+/+}$ Vα14 $C\alpha^{-/-}$ mice. Mice were injected i.p with PK136 mAb (50 μg/mouse of on days 15, 17, 26 and with 100 μg/mouse on day 32). At 6 wks of age, recipient mice were injected i.v with 10^4 naïve $CD62L^+$ BDC2.5 T cells from $CD45.2^{+/+}$ $CD90.1^{+/+}$ BDC2.5 $C\alpha^{-/-}$ mice. Diabetes analysis was also performed in mice reconstituted with $NK1.1^-$ or $NK1.1^+$ $CD4^-$ iNKT cells. In some experiments mice were injected i.p with 200 μg of blocking anti-mouse IL-17 Ab (CA028_00511) or isotype control (101.4) on days 0, 2, 4, 6 and 8 after BDC2.5 T cell transfer (day 0). Reagents were provided by UCB Celltech. Overt diabetes was defined by two consecutive positive glucosuria tests and glycemia >200 mg/dL.

Statistical analysis

Statistical analyses were performed with the nonparametric Mann–Whitney *U* test. The log-rank test was used for the comparison of diabetes incidence.

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Conflict of interest: The authors declare no commercial or financial conflict of interest.

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Abbreviations: **α-GalCer:** α-galactosylceramide · **CIA:** collagen induced arthritis · **ILN:** inguinal LN · **iNKT:** invariant natural killer T cells · **PLN:** pancreatic LN · **RORγt:** Retinoic acid receptor-related orphan receptor γt

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Supporting Information

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NOD mice contain an elevated frequency of iNKT17 cells that exacerbate diabetes

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Figure S1

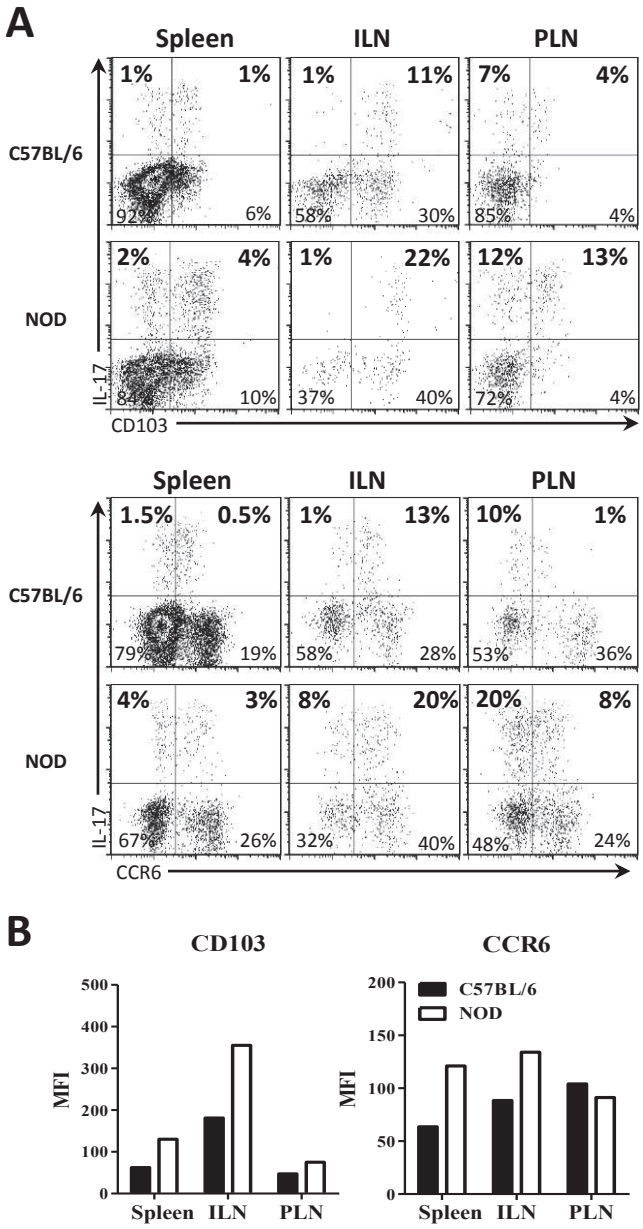

Figure S1. CCR6 and CD103 expression of iNKT17 cells from NOD and C57BL/6 mice. Organs were harvested from 6 to 10 week-old female C57BL/6 (black bars) and NOD (white bars) mice and cell suspensions were prepared. Intracellular IL-17 staining of iNKT cells was performed as described in Figure 1. iNKT cells were detected by flow cytometry using CD1d-αGalCer tetramers in combination with anti-TCRβ and their subsets using anti-IL-17, anti-CD103 and anti-CCR6 mAbs. (A) Representative FACS profiles of iNKT cells from spleen, ILN and PLN showing the expression of IL-17 and CD103 (top panel) or CCR6 (bottom panel). (B) CD103 and CCR6 MFI values of IL-17 producing iNKT cells. The data are representative of two independent experiments, each performed with 5-6 pooled mice.

Figure S2

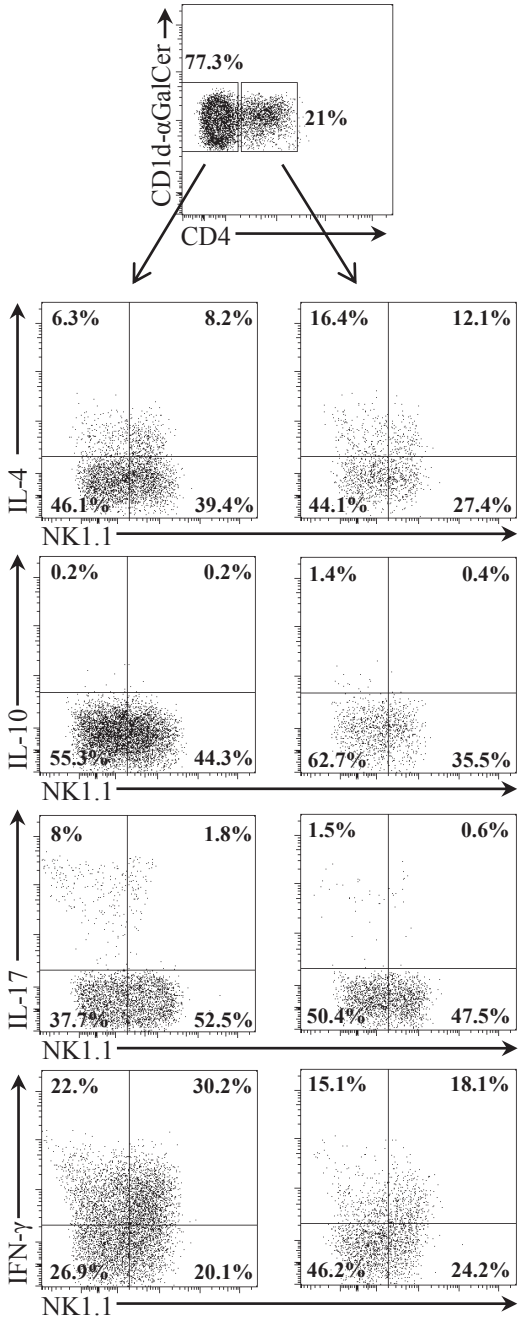

Figure S2. Characterization of iNKT cell subsets from donor mice. Cytokine analysis by intracytoplasmic staining of iNKT cell subsets. Pooled splenocytes and mesenteric LN cells from Vα14 $C\alpha^{-/-}$ NOD donor mice were stimulated and stained as described in Figure 1. iNKT cells were detected using CD1d-αGalCer tetramers in combination with anti-TCRβ, anti-CD4 and anti-NK1.1 mAbs. Cytokines were detected using anti-IL-4, anti-IL-10, anti-IL-17 and anti-IFN-γ mAbs

I-1.3 Discussion.

Notre travail montre que dans les organes lymphoïdes, la souris NOD présente une fréquence et un nombre absolu plus élevés en lymphocytes iNKT17 par rapport aux souches de souris non auto-immunes. Les lymphocytes iNKT17 infiltrent le pancréas de la souris NOD, où ils expriment de l'ARNm codant pour l'IL-17. Les expériences de transfert adoptif des différentes sous-populations de lymphocytes iNKT montrent que contrairement au rôle protecteur des lymphocytes iNKT CD4+, la population de lymphocytes iNKT CD4$^-$ - qui contient les lymphocytes iNKT17- a un rôle délétère sur l'incidence du diabète. Le traitement des souris transférées avec les lymphocytes iNKT CD4- avec un anticorps bloquant l'action de l'IL-17 empêche l'aggravation de la maladie. D'après l'ensemble de la littérature, nous proposons deux hypothèses quant aux mécanismes délétères de ces cellules dans le T1D (c.f : partie conclusion de l'article) (Figure I-1.3a).

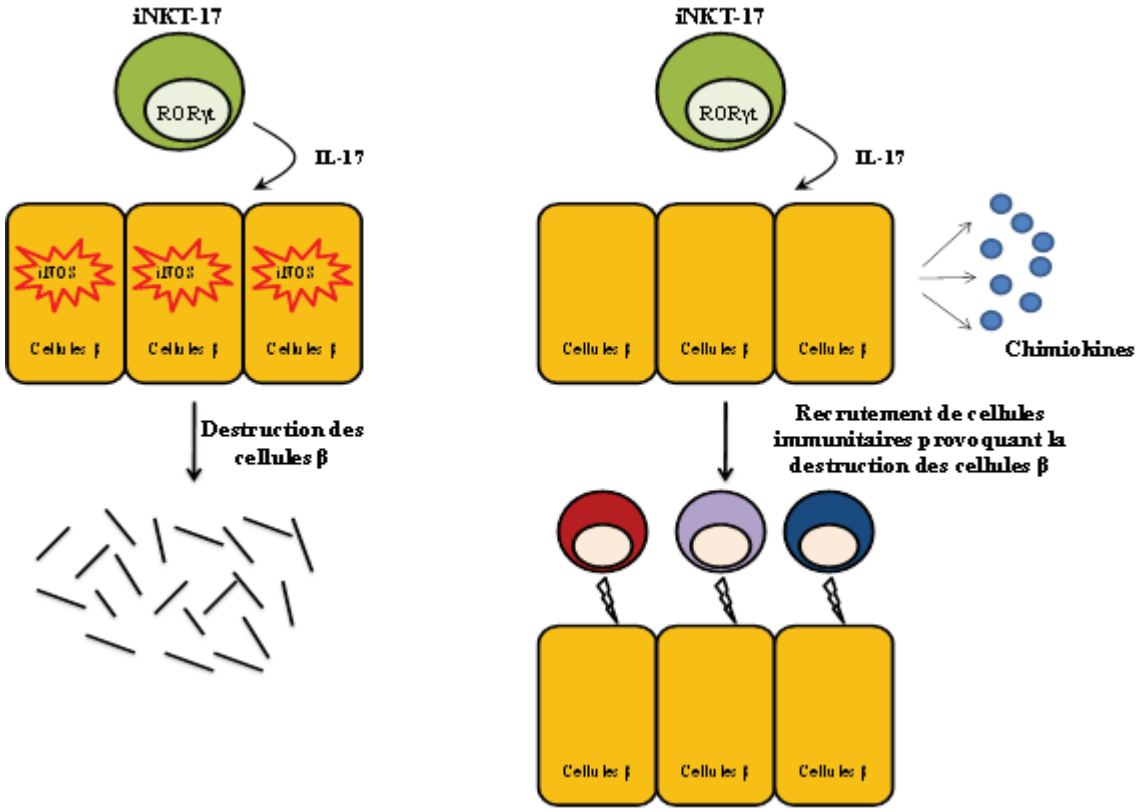

Figure I-1.3a. Hypothèse sur le mécanisme d'action délétère des iNKT17 dans le T1D. *In vitro*, l'IL-17 induit l'expression de la protéine iNOS par les cellules β. Cette protéine induit la production d'oxyde nitrique (NO) qui favorise la mort des cellules β [141] (gauche). La fixation de l'IL-17 sur son récepteur peut induire l'expression de chimiokines dont CXCL10 [142]. Nous pouvons supposer que, sur les cellules des îlots pancréatiques, l'IL-17 induit l'expression de chimiokines conduisant au recrutement de cellules immunitaires et la destruction des cellules β (lymphocytes T auto-réactifs, macrophages, lymphocytes B) (droite).

Durant ce travail, nous avons été confrontés à différents problèmes techniques :

Purification des cellules iNKT17.

Nous n'avons pas pu reconstituer les souris receveuses spécifiquement avec les cellules iNKT-17 car au moment de ces expériences, il n'existait pas de marqueur membranaire spécifique de cette population à part l'IL-23R. Dû à la reconnaissance de la partie intracellulaire d'IL-23R par l'anticorps existant, toute purification était impossible. Le marquage intra-cytoplasmique aurait tué les cellules.

Nous avons commencé à générer la souris NOD RORγt GFP. Dans cette souris, un transgène code pour la protéine fluorescente GFP sous le contrôle du promoteur de RORγt. RORγt est un facteur de transcription spécifique aux cellules produisant de l'IL-17 [143]. Par conséquent, les cellules qui produisent de l'IL-17 expriment la GFP. Il est donc aisé de purifier ces cellules par cytométrie en flux. A l'heure actuelle, nous sommes en train de stabiliser les différentes lignées obtenues par transgénèse.

Autre modèle de transfert.

Afin de confirmer nos résultats obtenus avec le modèle monoclonal de transfert adoptif (lymphocytes T BDC2.5), nous avons essayé de transférer des lymphocytes iNKT CD4+ ou CD4- (contenant les lymphocytes iNKT17) dans des souris NOD Jα18KO. Ces souris sont dépourvues de lymphocytes iNKT. Notre groupe avait fait une expérience similaire en transférant 5.10^6 lymphocytes iNKT OX40-/- dans une souris NOD avant infection par LCMV [144].

Nous avons transféré 2.10^6 lymphocytes iNKT CD4+ ou CD4- dans des souris NOD Jα18-/-. 12 semaines après le transfert des cellules, nous n'avons observé aucune différence entre les groupes sur l'incidence du T1D. Ce résultat peut s'expliquer par le fait que nous n'avons pas transféré un nombre suffisant de cellules dans les souris NOD Jα18-/- (2.10^6 contre 5.10^6). De plus, le pool de cellules iNKT exogènes diminue avec le temps après transfert [145].

Par conséquent, il faudra réitérer cette expérience en :

i) transférant plus de cellules iNKT ($\simeq 5.10^6$).

ii) faire de multiples transferts de lymphocytes iNKT afin de garder une fréquence constante de ces cellules chez la souris NOD Jα18-/-.

iii) Utiliser la souris NOD RORγt GFP afin de transférer spécifiquement des lymphocytes iNKT17.

L'IL-17 et le T1D.

Les données sur le rôle de l'IL-17 dans le T1D sont contradictoires (cf : partie conclusion de l'article). Deux groupes ont produit des souris NOD déficientes en IL-17. La première souris exprime un transgène codant pour un shRNA de l'IL-17 (ARN interférant). Par rapport à la souris NOD, cette souris NOD IL-17 *silencing* ne présente aucune différence dans l'incidence du T1D. Ce résultat suggérant l'absence de rôle de l'IL-17 dans la maladie [146]. Cependant, cette souris n'est pas totalement déficiente en IL-17. Les auteurs observent par ELISA, une faible production de cette cytokine par les lymphocytes Th17. Chez la souris NOD WT, la production d'IL-17 par les lymphocytes Th17 est de 11 000 pg/ml, 2500 pg/ml chez la souris NOD IL-17 *silencing*. Ces données montrent que la souris NOD IL-17 *silencing* n'est pas le meilleur outil pour étudier le rôle de l'IL-17 dans le T1D.

En 2013, un groupe a produit la souris NOD IL-17-/-. Cette souris présente une incidence du T1D retardée et faiblement réduite par rapport à la souris NOD WT. A 30 semaines d'âge, 80% de souris diabétiques chez la NOD WT et 70% chez la NOD IL-17-/-. Les auteurs montrent que l'insulite est réduite dans le pancréas de souris NOD IL-17-/- par rapport à la NOD WT. Les expériences de transfert adoptif de lymphocytes T CD4+ CD25- provenant des souris IL17-/-, montrent une diminution significative de l'incidence du T1D dans les souris NOD SCID receveuses par rapport au transfert de lymphocyte T WT. Ces résultats suggèrent un rôle délétère de l'IL-17 dans le T1D [147].

Le profile Th1 des lymphocytes T présents dans le pancréas des souris NOD suggère que l'IFNγ a un rôle délétère dans l'apparition du T1D. De manière inattendu, la souris NOD IFNγR-/- ne présente aucune différence d'incidence du T1D par rapport à la souris NOD WT [148]. Suggérant ainsi l'absence de rôle de l'IFNγ dans le T1D.

Les chercheurs ayant produit la souris NOD IL-17-/- ont aussi généré la souris NOD IL-17-/- IFNγR-/-. Cette souris double KO est partiellement protégée du T1D (30% de souris diabétiques à 30 semaines d'âge contre 80% chez la NOD WT et 70% chez la NOD IL-17-/-) [147]. Cette protection semble être associée à une augmentation de la fréquence en lymphocyte Treg. Cependant, les auteurs n'ont pas identifié le(s) mécanisme(s) délétère(s) de l'IL-17 dans le T1D.

En conclusion, les résultats indiquent que l'IL-17 participe au développement de l'insulite dans le T1D. L'IFNγ et l'IL-17 ont une action synergique qui contribue au développement de la maladie chez la souris NOD.

Les lymphocytes iNKT17 chez les patients T1D.

Chez l'homme, après stimulation par PMA/Ionomycine, il ne semble pas que les lymphocytes iNKT produisent de l'IL-17 (observations non publiées par notre groupe). Cependant, les expériences *in vitro* montrent qu'en présence d'IL-1β, IL-23 et TGFβ, les lymphocytes iNKT secrètent de l'IL-17 [149]. Il semble que la capacité à produire de l'IL-17 par les iNKT humains soit dépendante de l'environnement pro-inflammatoire. Il serait intéressant d'étudier la production de cette cytokine part les lymphocytes iNKT présents dans les pLN des patients T1D.

Chapitre 2 Rôle des pDC, neutrophiles et lymphocytes B-1a

I-2.1a Les cellules dendritiques plasmacytoïdes dans le T1D[j].

Les cellules dendritiques plasmacytoïdes (pDC).

En 1958, Lennert *et al* ont identifié un type cellulaire ayant une morphologie comparable aux plasmocytes, mais n'exprimant pas de marqueur spécifique de ces cellules [150]. Ces cellules étant présentes dans les zones T des organes lymphoïdes, elles furent appelées cellules plasmacytoïdes T. En 1978, Trincheri *et al* ont mis en évidence dans le sang humain, une population cellulaire ayant la caractéristique de produire de grande quantité d'IFNα et de n'exprimer aucun marqueur connu des lymphocytes T, B ou NK [151]. En 1994, O'Doherty *et al* ont décrit dans le sang humain, une sous-population de cellules dendritiques exprimant très faiblement les molécules du CMH II. Cette population cellulaire présentait la caractéristique d'acquérir une morphologie de cellules dendritiques et d'exprimer fortement les molécules du CMH II après activation [152]. En 1997, Grouard *et al* ont montré que cette sous-population de cellules dendritiques correspondait aux cellules plasmacytoïdes T. Le terme cellule dendritique plasmacytoïde (pDC) fut adopté (Figure I-2.1) [153]. En 1999, Siegle *et al* ont démontré de façon définitive que les cellules produisant de fortes quantités d'IFNα et les pDC étaient la même population de cellules[154].

En dépit de certaines différences, les pDC sont conservées entre l'homme et la souris. Leur fréquence est faible (0.3%-0.5% des cellules du sang périphérique), elles se développent dans la moëlle osseuse et résident dans les organes lymphoïdes et le sang. Différentes molécules permettent leur identification telle que Siglec-H et Bst2 (chez la souris) ou BDCA-2 et CD123 (chez l'homme).

Les pDC ont la caractéristique d'être un important médiateur de l'immunité antivirale. La reconnaissance des virus par ces cellules fait intervenir principalement deux récepteurs : les TLR7 et TLR9. Ces deux récepteurs reconnaissent respectivement l'ARN simple brin et l'ADN CpG non méthylé. L'activation de ces cellules n'est pas uniquement dépendante de ces récepteurs. Le récepteur à l'IL-3 (IL-3R), CD40, Siglec-H et OX40 activent ces cellules.

[j] Cette partie ne prend pas en compte les publications parues après notre article. Voir discussion

Mais, contrairement aux TLR7/9, l'activation par ces autres récepteurs semble favoriser leur maturation en APC et non leur production d'IFNα [155].

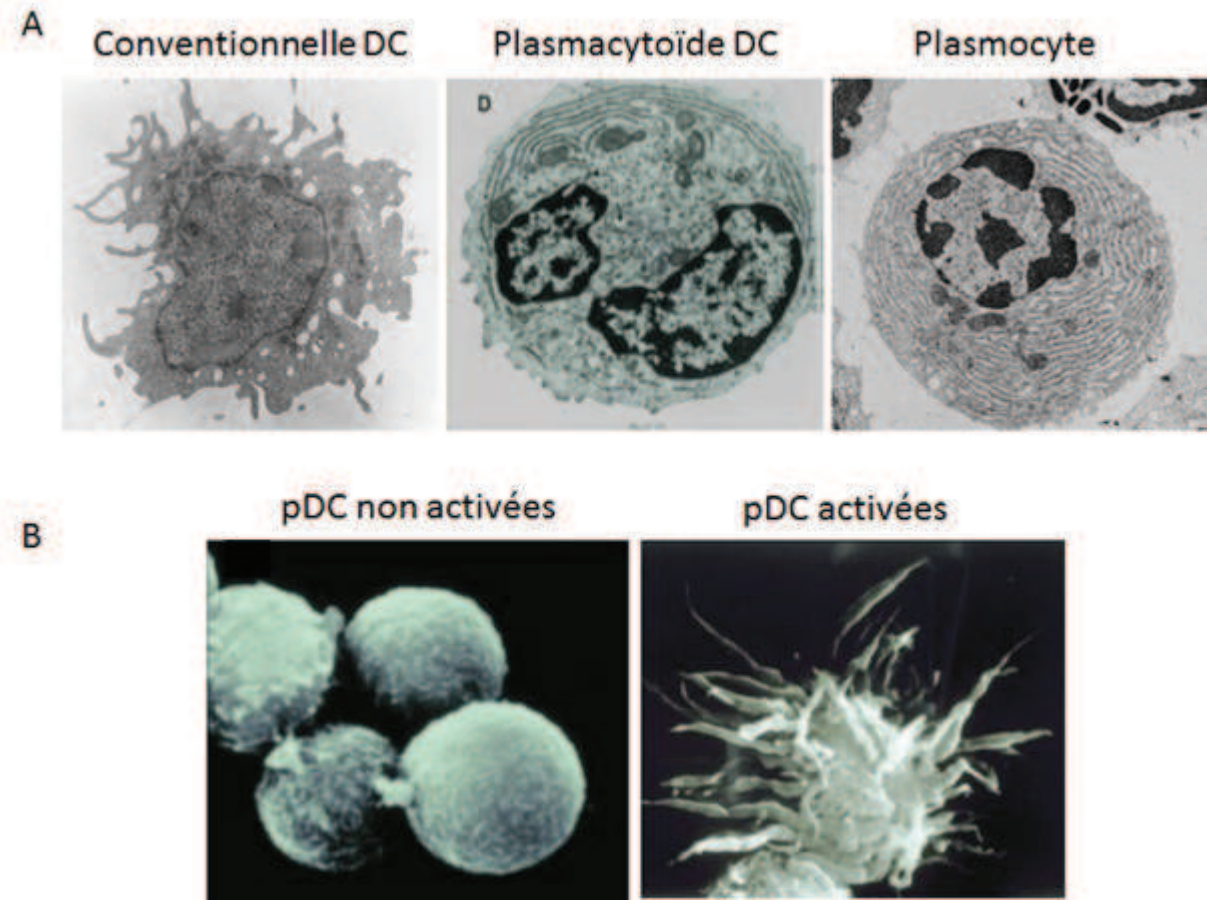

Figure I-2.1 (A) Caractérisation morphologique des pDC. Contrairement aux cellules dendritiques conventionnelles (gauche), les cellules dendritiques plasmacytoïdes non activées (centre) présentent une morphologie comparable aux plasmocytes (droite). Photographie en MET. Modifiée d'après Siegal *et al*. [154]
(B) Morphologie des pDC avant et après activation. Les pDC non activées ont une morphologie comparable aux lymphocytes (gauche). Après activation, elles acquièrent une morphologie de cellules dendritiques conventionnelles qui leur permet de présenter efficacement les antigènes aux lymphocytes T (droite).
Photographie en SEM. Modifié d'après Colonna *et al* [156].

Après infection virale, les pDC sont les premières cellules à sécréter de l'IFNα. Elles possèdent aussi la caractéristique de produire de l'IL-12 et du TNFα [157]. De plus, les pDC contribuent à l'activation des cellules NK [158] et des plasmocytes [159]. Des expériences *in vitro* montrent que les pDC possèdent la capacité de capturer et de présenter des antigènes via les molécules du CMH I ou CMH II [160]. La capture des antigènes par les pDC fait intervenir différents récepteurs impliqués dans l'endocytose tel que Siglec-H, FcγRII ou BDCA-2 [161]. Une fois activées, elles acquièrent la capacité de présenter efficacement les antigènes par la molécule du CMH II et vont ainsi pouvoir activer les lymphocytes T CD4

(Figure I-2.1b) [157]. Ces cellules peuvent aussi *cross*-présenter des antigènes exogènes aux lymphocytes T CD8 après activation [162]. Cette capacité de présentation antigénique des pDC influence la réponse T. In vitro, l'activation des pDC par les TLR et la capture d'antigène via BST-2 induit une réponse T de type Th1 [163]. Les pDC peuvent ainsi induire une réponse de type Th17 par les lymphocytes T [161, 164, 165]. L'induction d'un biais Th17 par les pDC semble faire intervenir la présence du TGFβ [166, 167]. Enfin, ces cellules peuvent induire l'expansion des lymphocytes Treg [168].

Les pDC jouent un rôle dans la tolérance immunitaire. La tolérance est un mécanisme qui permet de prévenir l'activation délétère du système immunitaire par des antigènes du soi (i.e. l'insuline) ou des antigènes ne présentant aucun danger pour l'organisme (i.e. le gluten). Les pDC semblent être un acteur clé de la tolérance orale. L'utilisation de modèles animaux met en évidence que ces cellules induisent l'anergie et la suppression des lymphocytes T CD8 réactifs contre les antigènes alimentaires dans le foie et les mLN. De plus, les pDC activent la fonction suppressive des lymphocytes Treg dans les organes lymphoïdes secondaires [169, 170]. Les pDC ont aussi un impact sur la tolérance centrale. Ces cellules peuvent migrer dans le thymus et présenter des antigènes du soi aux lymphocytes T. *In vitro*, ce mécanisme contribue à la suppression de lymphocytes T auto-réactifs mais aussi au développement de lymphocytes nTreg [171, 172].

Cependant, la fonction tolérogène des pDC peut s'avérer délétère pour l'organisme. L'utilisation de modèles animaux de cancers montre que les pDC favorisent la fonction immunosuppressive de lymphocytes Treg par l'expression d'IDO et l'interaction ICOS/ICOS-L. Cela a pour effet d'inhiber la réponse immunitaire anti-cancéreuse et ainsi favoriser la progression de la maladie [173, 174].

Les pDC sont aussi impliquées dans différentes pathologies auto-immunes et auto-inflammatoires. Suivant la pathologie, ces cellules ont un rôle délétère (i.e. lupus, psoriasis) ou protecteur (i.e EAE) [161].

L'utilisation de modèle murin du psoriasis a montré que les pDC infiltrent les lésions de la peau durant la phase précoce de la maladie [175]. Ces cellules produisent de l'IFNα et sont activées par les TLR7/9 [176]. Les traitements des souris avec un anticorps bloquant l'action de l'IFNα ou déplétant les pDC, ont montré une inhibition du développement de la pathologie [175]. Confirmant ainsi un rôle délétère de ces cellules dans la phase initiale du psoriasis. La présence de ces cellules ainsi que la production d'IFNα sont observées au niveau des lésions cutanées des patients psoriasiques [175, 177].

A l'inverse, dans le modèle animal de la sclérose en plaque (EAE), la déplétion des pDC conduit à une exacerbation de la maladie [178]. L'utilisation de souris transgéniques n'exprimant pas la molécule du CMH II sur les pDC, a mis en évidence que les pDC présentent les auto-antigènes aux lymphocytes T CD4. Ce mécanisme aboutit à l'expansion de lymphocytes Treg [161, 168].

Les pDC dans le T1D.

La souris NOD possède une fréquence plus élevée de pDC que la souche de souris non auto-immune C57Bl/6. De plus, les pDC de la souris NOD produisent de plus grande quantité d'IFNα [179]. Bien que l'ensemble des publications montrent que les pDC peuvent infiltrer les îlots pancréatiques de la souris NOD, la fonction des pDC dans le T1D reste controversée. La déplétion des pDC chez les jeunes souris NOD (par l'anticorps mPDCA-1) réduit et retarde l'apparition du diabète. Les auteurs montrent que le rôle délétère des pDC est dû à leur production d'IFNα. Cette molécule favorise la maturation des cDC et induit l'activation des lymphocytes T auto-réactifs [180, 181]. De plus, le traitement des souris NOD avec la molécule Flt-3, qui induit l'expansion des cDC et pDC, augmente l'incidence du T1D. Suggérant ainsi un rôle délétère des pDC [182]. A l'opposé, il est montré, via l'utilisation de transfert adoptif de cellules T diabétogène BDC2.5, que la déplétion des pDC (chez la souris CD11c-DTR) accélérait le développement du T1D. A la différence des premières études, les auteurs déplètent les pDC chez des souris de 10 semaines d'âge. Dans cette étude, le rôle protecteur des pDC est dû à la production d'IDO par ces cellules. L'IDO est une enzyme ayant une fonction immunosuppressive. Il est supposé par les auteurs que cette molécule inhibe les lymphocytes T auto-réactifs (Figure I-2.1a) [183].

Notre groupe s'est intéressé au rôle des pDC dans le T1D lors des infections virales. Après infection virale (par LCMV chez la souris transgénique Ins-NP), les cellules iNKT contribuent au recrutement des pDC dans le pancréas. Les pDC contrôlent la réplication virale et préviennent l'inflammation et le développement du T1D. Ce processus est dépendant de l'interaction OX40-OX40L entre les pDC et iNKT [144]. De plus, les cellules iNKT dans les pLN, produisent de l'IL-10 qui induit la sécrétion de TGFβ par les pDC. Ces pDC tolérogènes favorisent la conversion des lymphocytes T CD4+ naïfs en lymphocyte Treg, qui inhibent le développement du T1D [184].

Chez l'homme, les données divergent sur la fréquence des pDC dans le sang des patients T1D par rapport aux sujets contrôles [185, 186]. Cependant, il a été montré une augmentation de la fréquence en pDC au moment de l'apparition du T1D [187]. Ces cellules ont la capacité de présenter les antigènes issus des cellules β plus efficacement que les cDC en présence d'auto-anticorps. Par conséquent, les pDC ont une tendance accrue à activer les lymphocytes T auto-réactifs. *In vitro*, aucune différence dans la production d'IFNα par ces cellules n'a été observée entre les patients diabétiques et individus contrôles [187].

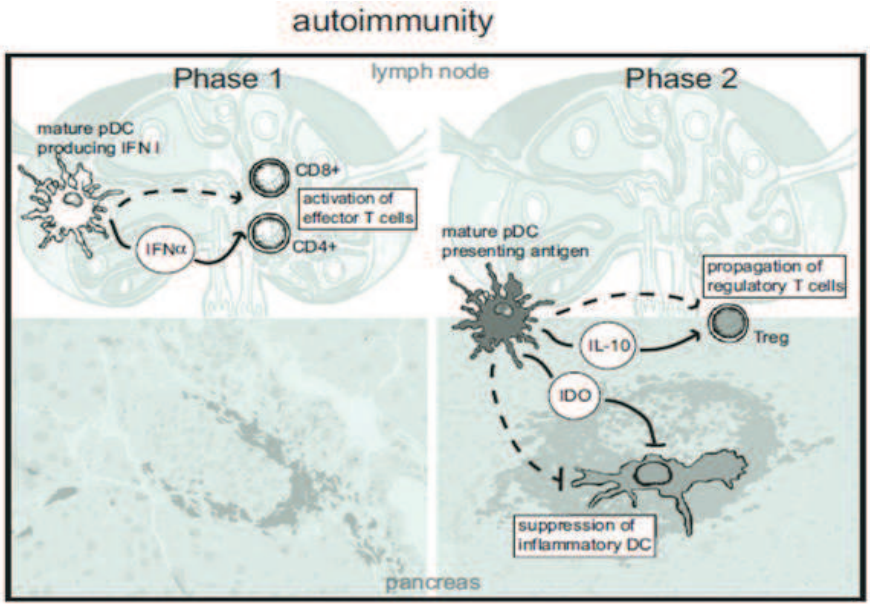

Figure I-2.1a. Hypothèse sur le rôle des pDC dans le T1D en 2009. Dans la phase initiale du développement du T1D (Phase 1), l'activation des pDC conduit à leur maturation et à la production d'IFNα. Cette cytokine va contribuer à l'activation des lymphocytes T auto-réactifs CD4 et CD8. Ces lymphocytes vont contribuer au développement du T1D. Dans la phase effectrice (Phase 2), l'activation des pDC conduit ces cellules à produire de l'IDO. Cette molécule supprime l'action pro-inflammatoire des cDC dans le pancréas. L'IL-10 produit par les pDC pourrait induire l'apparition de Treg dans le pancréas (non démontré) D'après Nikolic *et al* 2009 [188]

I-2.1b Les lymphocytes B-1 dans le T1D.

Les lymphocytes B.

Les lymphocytes B sont des cellules du système immunitaire ayant la caractéristique spécifique de sécréter des anticorps. La lettre B est l'abréviation pour "Bursa fabricii". C'est dans cet organe, présent uniquement chez l'oiseau, que ces cellules sont générées. Chez les mammifères, ces cellules sont générées dans la moelle osseuse[k]. Les lymphocytes B sont divisés en sous-populations de cellules ayant chacune des caractéristiques propres.

- Les lymphocytes B folliculaires représentent la sous-population la plus importante. Ils font partie de l'immunité adaptative, leur activation est dépendante des lymphocytes T. Ces cellules produisent des anticorps ayant une très forte affinité pour l'antigène.
- Les lymphocytes MZB (Marginal Zone B cell) sont présents presque exclusivement dans la zone marginale de la rate. Leur activation est indépendante des lymphocytes T. Ces cellules produisent des anticorps de faible affinité contre les antigènes bactériens [189].
-Les lymphocytes Breg (ou B10) sont caractérisés par leur sécrétion d'IL-10, une cytokine anti-inflammatoire [190].
- Les lymphocytes B-1 sont divisés en deux sous-populations : B-1a et B-1b. Ils ont la particularité principale de sécréter des anticorps "naturels". Ces cellules produisent continuellement des anticorps de faible affinité contre des antigènes microbiens. Cette production est indépendante de la présence d'agent infectieux [190].

Les lymphocytes B-1 dans le T1D.

Les lymphocytes B-1 ont été décrits pour la première fois en 1983. Ces cellules furent identifiées comme une sous-population de lymphocytes B exprimant le marqueur CD5 et sécrétant spontanément des IgM [191]. Les lymphocytes B-1 représentent la sous-population de lymphocytes B la plus représentée dans la cavité péritonéale et pleurale (20-30% des lymphocytes) [192]. Les lymphocytes B-1 se divisent en deux sous-populations : les lymphocytes B-1a et B-1b. La différence phénotypique entre ces deux types cellulaires est l'expression du marqueur CD5 par les cellules B-1a [193]. L'étude de ces deux populations montre que les lymphocytes B-1a sont impliqués dans la production d'anticorps alors que les lymphocytes B-1b semblent jouer un rôle dans la réponse mémoire B-1 [194, 195].

[k] A l'exception du lapin chez lequel ces cellules sont générées dans *l'appendix-sacculusrotundus*.

Les lymphocytes B-1a ont la caractéristique première de secréter des IgM. Ces cellules sont aussi capable de faire un switch isotopique afin de produire des IgA et des IgG dans certaines conditions [196-199].

La fonction première des lymphocytes B-1a est de produire des anticorps contre les pathogènes. Cependant, chez l'homme et la souris, les lymphocytes B-1 peuvent secréter des auto-anticorps contre différentes molécules dont l'ADN [200, 201]. Il est observé une augmentation du pool de ces cellules dans différents modèles de la maladie auto-immune du lupus [191, 202]. Les lymphocytes B-1a auto-réactifs sont recrutés dans les organes cibles (i.e. reins) et produisent des auto-anticorps, dont des anticorps anti-ADN, qui contribuent localement à la pathologie du lupus [198, 199]. Ce profil auto-immun des lymphocytes B-1a n'est pas détecté chez les souris non auto-immunes (i.e. C57Bl/6) [199].
Dans le T1D, différentes études ont essayé de déterminer la contribution des lymphocytes B-1. La souris NOD btk-/- est protégée du diabète. Cette souris présente une interruption du développement des lymphocytes B-1 suggérant ainsi leur rôle délétère dans la maladie [203]. Les lymphocytes B-1a sont présents dans le pancréas des souris NOD [204]. La déplétion de ces cellules entre 3 et 6 semaines d'âge, par des lavages du péritoine, conduit à une réduction de l'incidence du T1D (à 24 semaines, 70% des souris du groupe contrôle sont diabétiques contre 11% dans le groupe traité). Les auteurs observent une diminution de la fréquence des lymphocytes B dans le pancréas des souris traitées, ainsi qu'une diminution de la quantité d'auto-anticorps contre l'insuline. Les auteurs suggèrent que les lymphocytes B-1a orchestrent la réponse auto-immune des lymphocytes B-2 [204].
La présence des lymphocytes B-1a dans le pancréas est confirmée par une seconde étude. Via l'utilisation d'une souris transgénique qui développe le T1D (D011 RIP-mOVA[l]), les auteurs montrent que les lymphocytes B-1a sont indispensables pour le recrutement des lymphocytes T dans le pancréas. Ces cellules influencent l'expression de molécules d'adhésion, notamment VCAM, qui permettent l'entrée des lymphocytes T dans le pancréas [205].

[l] La souris BALB/c D011 RIP-mOVA est une souris double transgénique. Le premier transgène code pour un TCR spécifique de l'ovalbumine. Le second transgène code pour l'ovalbumine sous le contrôle du promoteur de l'insuline. Par conséquent, Les lymphocytes T anti-ovalbumine détruisent les cellules β du pancréas.

I-2.1c Les neutrophiles.

Les neutrophiles.

A la fin du XIXème siècle, Paul Ehrlich mis au point une technique de coloration des cellules sanguines. Ces expériences mirent en évidence l'hétérogénéité des cellules constituant le sang. Il observa que certaines cellules étaient marquées par les colorants basiques (les basophiles), d'autres par l'éosine (les éosinophiles) et certaines cellules ne fixaient aucun colorant (les neutrophiles).

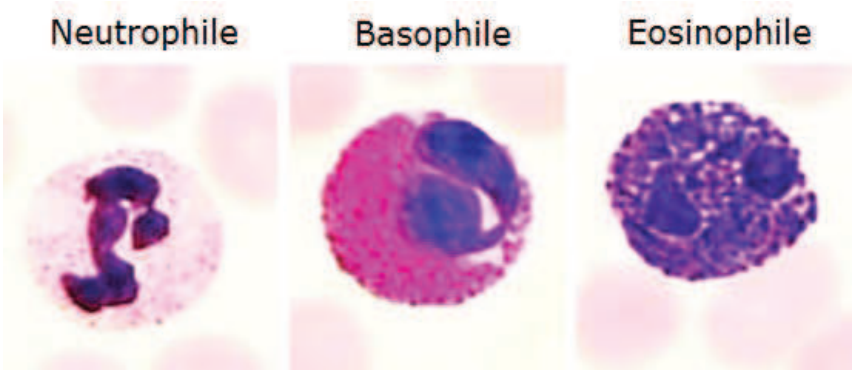

Figure I-2.1c Caractéristique phénotypique d'un neutrophile, basophile, et éosinophile.
Coloration H&E. Source: www.scientificpsychic.com/mind/whitecells.html

La fonction antimicrobienne des neutrophiles fut évoquée pour la première fois par Elie Metchnikoff en 1893. Celui-ci observa qu'une blessure conduisait au recrutement de cellules. Il supposa que ces cellules participaient à la digestion des microbes au niveau de la lésion [206].

Les patients souffrant d'un défaut numérique de neutrophiles présentent une susceptibilité accrue aux infections. Les neutrophiles représentent 50-70% des cellules dans le sang (20% chez la souris). Les neutrophiles sont les premières cellules à être recrutées au niveau des lésions. Ces cellules vont " phagocyter " les microorganismes afin de protéger l'organisme contre une infection. Ce processus est maintenant appelé NETosis (Neutrophils Extracellular Traps). La NETosis est une forme de mort cellulaire spécifique des neutrophiles. Les NET sont composés d'ADN et d'histones. Ces molécules s'associent aux protéines contenues dans les granules des neutrophiles. Ces protéines ont des fonctions de reconnaissances des pathogènes (i.e. PTX3), des fonctions antimicrobiennes (i.e. CRAMP) et des fonctions de remodelage des tissus (i.e. MMP9) [206, 207] (Figure I-2.1c).

Durant l'inflammation, les neutrophiles interagissent aussi avec les cellules du système immunitaire. Lors d'infection par *Toxoplasmagondii*, l'activation des neutrophiles favorise la maturation des cDC [208]. Ces cDC vont induire la prolifération et la polarisation des

lymphocytes T vers un profile Th1 [209, 210]. Les neutrophiles peuvent aussi interagir avec les cellules NK via l'interaction ICAM3-CD18/CD11d. Ce contact cellulaire aboutit à la production d'IFNγ par les cellules NK [211].

Les neutrophiles interagissent aussi avec l'immunité adaptative. Une fois activées, ils peuvent recruter les lymphocytes T et B au niveau du site d'inflammation par la production de chimiokines (i.e. CXCL9, CXCL10, CCL20) [212].

L'activation des neutrophiles est dépendante de la reconnaissance de microorganismes par ces cellules. Cependant, il a été observé dans certaines maladies auto-immunes que les neutrophiles pouvaient s'activer en l'absence d'infection et ainsi causer des dommages dans les tissus.

Les neutrophiles dans l'auto-immunité.

- La vascularite des petits vaisseaux (SVV) est une maladie impliquant l'inflammation des vaisseaux sanguins, due à la présence de cellules immunitaires. Cette maladie se caractérise par la présence d'auto-anticorps contre les neutrophiles (ANCA). Ces auto-anticorps activent les neutrophiles et induisent leur NETosis. L'importance de la NETosis dans le développement de la SVV reste à déterminer [213]
- Les modèles animaux de l'arthrite montrent la présence de neutrophiles au niveau des articulations. Ces cellules sont activées par les auto-anticorps présents dans les articulations inflammées. En réponse, les neutrophiles produisent de l'IL-1β qui stimule les cellules synoviales. Ces cellules vont à leur tour produire des chimiokines qui attirent de nouveaux neutrophiles [214].
- Le Lupus ou Systemic lupus erythemosus (SLE) est une maladie auto-immune dont l'étiologie n'a pas été encore identifiée. Cependant, les études menées chez l'homme ont identifié la présence de complexes immuns dans le sang des patients lupiques. Ces complexes sont composés d'auto-anticorps spécifiques à l'ADN et aux ribosomes, ainsi que des molécules spécifiques des NET tel que LL37 ou HNPs (des peptides antimicrobiens). De façon surprenante la présence de ces protéines autour de l'ADN a pour conséquence de protéger l'ADN contre la dégradation par les DNAse. Ces complexes (ADN-anticorps-peptides antimicrobiens) sont endocytés par les pDC via leur récepteur Fc. Dans les endosomes des pDC, ces complexes se dissocient. L'ADN va alors se fixer sur les récepteurs TLR9 et induire l'activation des pDC. Cette activation via le TLR9 conduit les pDC à secréter de grande quantité d'IFNα. En retour, l'IFNα induit la NETosis par les neutrophiles.

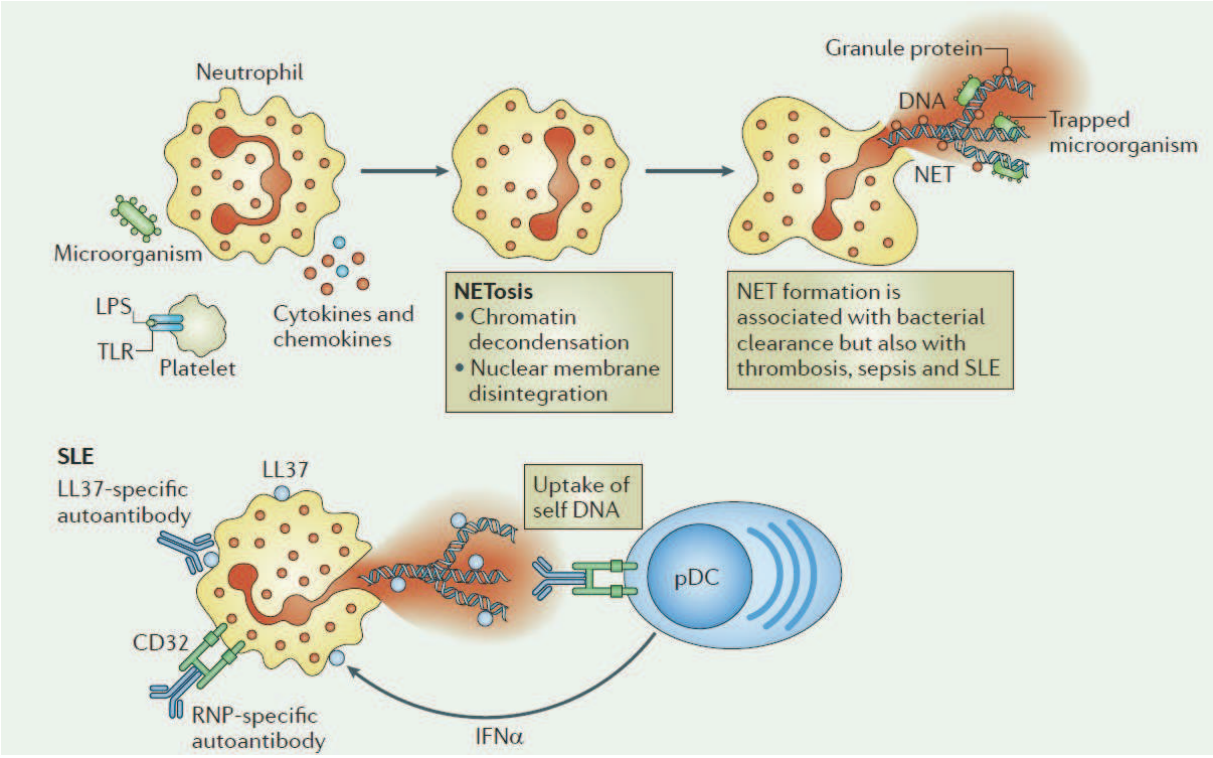

Figure I-2.1c. Mécanismes d'induction de la NETosis chez les neutrophiles. La reconnaissance des microorganismes par le neutrophile induit la formation de NET qui se fixent et détruisent les microorganismes (en haut). Dans la maladie auto-immune du lupus (SLE), ce sont les auto-anticorps qui induisent la formation de NET. Ces NET (composés d'ADN) activent les pDC via le TLR9. En réponse, ces cellules sécrètent de grandes quantités d'IFNα qui à son tour active la NETosis des neutrophiles (en bas). Figure d'après Montovani *et al* [207]

I-2.1d L'interféron alpha.

L'interféron (IFN) de type I est le principal médiateur de l'immunité innée contre les infections virales. Les interférons de type I comprennent différentes molécules dont l'interféron alpha (IFNα) et l'interféron bêta (IFNβ). La fixation de l'IFN de type 1 sur son récepteur aboutit à la transcription de nombreux IFN-stimulated genes (ISG). Les ISG ont la spécificité de limiter la propagation des virus en bloquant la réplication virale ou en induisant l'apoptose des cellules infectées. De plus, L'IFN de type I va moduler l'activation du système immunitaire inné et adaptatif [215].

L'IFN de type I peut induire la production de cytokines (i.e. IL-12, IL-15, IL-18) par les cDC ainsi que la différenciation des monocytes en cDC [216, 217]. L'IFN de type I favorise la cross-présentation d'antigènes exogènes aux lymphocytes T CD8 par les cDC [218]. La stimulation des cellules NK par l'IFN de type I, en association avec l'IL-6, induit la différenciation des lymphocytes B en plasmocytes [159]. L'IFN de type I peut aussi activer les neutrophiles (voir partie I-2.1.c).

Chez l'homme, la présence d'IFN de type I est détectée chez les patients lupiques (voir partie I-2.1.c) mais aussi dans d'autres pathologies auto-immunes telles que : le syndrome de Sjögren's (destruction des glandes salivaires) [219, 220], la sclérodermie systémique (destruction du tissu conjonctif) et un sous-groupe de patients atteints d'arthrite rhumatoïde (destruction des articulations) [221].

Aucune étude n'a mis en évidence la présence d'IFN de type I dans le sang des patients T1D. Cependant, la présence d'IFNα a été détectée dans le pancréas de patients T1D [222, 223]. De plus, le traitement de patients leucémiques ou infectés par le virus de l'hépatite C avec l'IFNα a été associé à l'apparition du T1D [224-227].

Chez des souris non auto-immunes, la surexpression de l'IFNα par les cellules β induit l'apparition du T1D (souris RIP-IFNα) [228]. Chez la souris NOD, l'expression de gènes induit par l'IFNα est observée dans les lymphocytes T des pLN à 4 semaines d'âge. A 3 semaines d'âge, les pDC présentes dans les pLN secrètent de l'IFNα. Le traitement des souris NOD, entre 2 et 3 semaines d'âge, avec un anticorps bloquant le récepteur à l'IFNα (IFNAR1) réduit l'incidence du T1D [180, 181].

I-2.1e La mort des cellules β.

Chez la souris NOD, les lymphocytes T auto-réactifs sont présents dès la naissance. Cependant, l'apparition des premiers signes d'insulite est observée entre 2 et 3 semaines d'âge [229]. Différentes études de transferts adoptifs montrent que les lymphocytes T auto-réactifs (T CD4+ BDC2.5 [30, 229] ou T CD8+ 8.3 [230]) ne sont pas activés dans les pLN avant l'âge de 2 semaines.

Quel événement conduit à l'activation du système immunitaire à cette période ?

Il est montré que le mécanisme qui va initier l'activation des lymphocytes T auto-réactifs est la mort physiologique des cellules β [30, 229-231]. Les modèles mathématiques prédisent une période de réorganisation de la structure du pancréas chez les rongeurs durant le développement. Cette réorganisation impliquerait une mort d'environ 60% des cellules β du pancréas [232]. Ce phénomène de réorganisation du tissu après la naissance n'est pas spécifique au pancréas, il est observé dans les reins [233] et au niveau du nerf optique [234]. Chez le rongeur, la vague d'apoptose des cellules β se situe entre 10 et 17 jours après la naissance, juste avant l'activation du système immunitaire [28, 232, 235, 236]. Cette mort des cellules β est aussi observée dans le pancréas humain [29].

En 2007, M. Lee *et al* ont montré que le TLR2 détecte les cellules β apoptotiques et contribue à l'initiation du T1D. Les auteurs montrent que la souris NOD TLR2-/- a une incidence du T1D réduite. La vague d'apoptose des cellules β entre 2 et 3 semaines d'âge, active les APC par le TLR2. Ces cellules activées induisent l'activation des lymphocytes auto-réactifs en présentant les antigènes dérivés des cellules β apoptotiques. Le ligand de TLR2 n'a pas encore été identifié [231].

L'ensemble de ces observations (présence de pDC dans le pancréas, rôle délétère de l'IFNα, apoptose des cellules β) nous ont amené à nous intéresser aux mécanismes d'activation des pDC dans le T1D et leur rôle dans l'initiation de la maladie.

I-2.2 Article.

Le T1D est une maladie auto-immune caractérisée par la destruction des cellules β du pancréas par les lymphocytes T auto-réactifs. Cependant, nous avons montré que les cellules de l'immunité innée : lymphocytes B-1a, neutrophiles et cellules dendritiques plasmacytoïdes (pDC) jouent un rôle important dans l'initiation de cette maladie.

L'IFN de type I produit par les pDC avait été précédemment impliqué dans l'initiation du diabète chez la souris NOD. Conformément à ces observations, nous avons constaté un pic d'expression d'IFNα à 3 semaines d'âge dans le pancréas de ces souris. De façon intéressante, cette production d'IFNα n'est pas observée chez les souches de souris non auto-immunes. Le niveau élevé d'IFNα coïncide avec le nombre élevé de pDC dans les îlots pancréatiques. Nous avons observé que les pDC produisent de l'IFNα dans les îlots pancréatiques et activent la réponse T diabétogène après une stimulation via la voie Myd88-TLR7/9. De façon similaire aux observations faites précédemment, la déplétion des pDC entre 2 et 3 semaines d'âge retarde le développement et diminue l'incidence du diabète.

Les analyses histologiques et par cytométrie de flux, montrent la présence des lymphocytes B-1a dans le pancréas des jeunes souris NOD. Ces cellules produisent des anticorps spécifiques à l'ADN double-brin (dsDNA). La déplétion de ces cellules par des lavages du péritoine, entre 1 et 3 semaines d'âge, diminue la production d'IFNα par les pDC, inhibe la réponse T diabétogène et diminue l'incidence du diabète chez la souris NOD. Il s'avère que les anticorps anti-dsDNA sécrétés par les lymphocytes B-1a induisent la production d'IFNα par les pDC. Nous avons aussi identifié des neutrophiles dans le pancréas des jeunes souris NOD. La déplétion de ces cellules, réduit la production d'IFNα par les pDC et inhibe la réponse T diabétogène. Les expériences *in-vitro* montrent que les anticorps anti-dsDNA sécrétés par les lymphocytes B-1a se lient aux récepteurs Fc des neutrophiles et induisent l'expression de CRAMP par ces cellules. La molécule CRAMP et les anticorps anti-dsDNA se complexent et activent les pDC à produire de l'IFNα via le TLR9.

L'élément déclenchant l'activation de ces cellules semble être la mort physiologique des cellules β chez la jeune souris NOD. L'administration d'un traitement inhibant la mort physiologique des cellules β empêche l'activation de ces cellules et la production d'IFNα.

nature medicine

Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes

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Type 1 diabetes develops over many years and is characterized ultimately by the destruction of insulin-producing pancreatic beta cells by autoreactive T cells. Nonetheless, the role of innate cells in the initiation of this disease remains poorly understood. Here, we show that in young female nonobese diabetic mice, physiological beta cell death induces the recruitment and activation of B-1a cells, neutrophils and plasmacytoid dendritic cells (pDCs) to the pancreas. Activated B-1a cells secrete IgGs specific for double-stranded DNA. IgGs activate neutrophils to release DNA-binding cathelicidin-related antimicrobial peptide (CRAMP), which binds self DNA. Then, self DNA, DNA-specific IgG and CRAMP peptide activate pDCs through the Toll-like receptor 9–myeloid differentiation factor 88 pathway, leading to interferon-α production in pancreatic islets. We further demonstrate through the use of depleting treatments that B-1a cells, neutrophils and IFN-α–producing pDCs are required for the initiation of the diabetogenic T cell response and type 1 diabetes development. These findings reveal that an innate immune cell crosstalk takes place in the pancreas of young NOD mice and leads to the initiation of T1D.

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T cells infiltrate the pancreas and target insulin-producing beta cells during type 1 diabetes (T1D) development[1]. However, T cells represent only one piece of the puzzle of the multiple immune cells implicated in beta cell loss[2]. Plasmacytoid dendritic cells (pDCs) are central mediators of antiviral immunity through their ability to produce large amounts of interferon-α (IFN-α) and IFN-β[3]. In addition to their antiviral role, pDCs also promote various autoimmune diseases such as psoriasis and systemic lupus erythematosus (SLE)[4,5]. Regarding autoimmune diabetes, a pathogenic role for IFN-α and pDCs has been proposed, as IFN-α treatment of patients with viral infections or with leukemia has been shown to be associated with increased incidence of T1D[6,7]. Additionally, IFN-α–producing pDCs have been detected in the blood of patients with T1D at the time of diagnosis[8]. Furthermore, genetic analysis supports a diabetogenic role for IFN-α–induced genes in prediabetic children[9]. In mice, transgenic non–autoimmune-prone mice expressing IFN-α in beta cells develop autoimmune diabetes[10]. Subsequently, it was shown that IFN-β accelerates the onset of the disease in nonobese diabetic (NOD) mice and breaks self tolerance to beta cell antigens in nonobese resistant mice[11]. This potential role of IFN-α and IFN-β and pDCs in T1D and in other autoimmune diseases prompted us to investigate the functions of pDCs in the initiation of diabetes. It has been found that during the first postnatal weeks, waves of physiological beta cell death occur in rodents[12–14], pigs[15] and humans[16]. Given that dead cell clearance is defective in NOD mice[17], we speculate that the accumulation of beta cell debris (for example, self DNA) can activate pDCs in the pancreas.

RESULTS

Innate cells infiltrate the pancreas of young NOD mice

We initially focused on characterizing immune cell infiltration in the pancreas of NOD female mice starting at 2 weeks of age (**Fig. 1a**,**b**). As early as 2 weeks of age, various innate immune cells such as neutrophils ($Ly6G^+CD11b^+$) and pDCs ($m927^+CD11c^{med}$) and also B cells ($CD19^+$) infiltrated the islets. The presence of neutrophils and pDCs was only transient, reaching a maximum at 3 and 4 weeks of age, respectively. Notably, the recruitment of these innate cells was specific to the NOD mice, as we did not observe such cells in the islets of C57BL/6 or BALB/c mice (**Supplementary Fig. 1a**,**b**).

Histological analysis confirmed the presence of pDCs ($B220^+CD11c^+$), conventional DCs (cDCs) ($B220^-CD11c^+$), B cells ($B220^+CD11c^-$) and neutrophils ($NIMP\text{-}R14^+$) surrounding or inside the NOD islets at 3 but not at 6 weeks of age (**Fig. 1c**,**d** and **Supplementary Fig. 2**). Together, these data reveal that innate cells infiltrate the pancreatic islets of young NOD mice, which suggests they have a role in T1D initiation.

Pancreatic IFN-α–secreting pDCs are crucial for T1D initiation

We analyzed IFN-α production in the islets of NOD mice because this cytokine is associated with activated pDCs and autoimmunity[3]. IFN-α was detected in the islets only at 3 weeks of age (**Fig. 2a**). To determine the association of IFN-α with T1D development, we analyzed the mRNA expression of IFN-α, IFN-α–induced gene products (ISG15, IRF7, IFIT1 and IFIT3) and proinflammatory gene products

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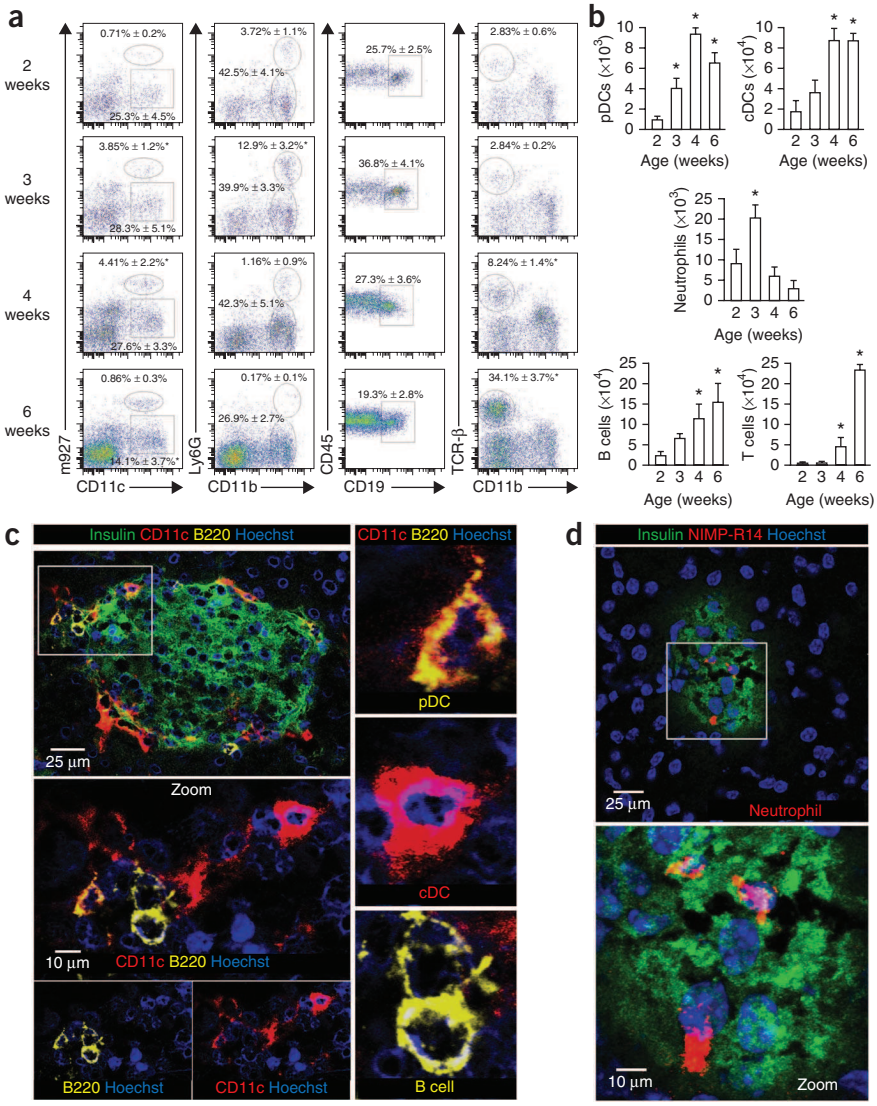

Figure 1 Innate immune cells infiltrate the pancreas of NOD mice in the first postnatal weeks. (**a**,**b**) FACS analysis of CD45⁺ infiltrating cells from pancreatic islets collected from 2 to 6 weeks. Frequencies of pDCs (m927⁺CD11c⁺), cDCs (m927⁻CD11c⁺), neutrophils (Ly6G⁺CD11b⁺), B cells (CD19⁺) and T cells (TCRβ⁺) are shown in each quadrant. Frequencies of positive cells among CD45⁺ cells are represented in **a** and absolute number in **b**. Data are mean values ± s.e.m. and are representative of four independent experiments with four pooled mice for each group. $^*P < 0.05$ for each group compared to 2-week-old group. (**c**,**d**) Immunohistological analysis of DCs (**c**) and neutrophils (**d**) from the pancreas of 3-week-old NOD mice. Zoomed-in boxed area in **c** shows cDCs (CD11c⁺) in red, B cells (B220⁺) in yellow and pDCs (CD11c⁺B220⁺) in orange and in **d** shows neutrophils (NIMPR14⁺) in red. Pancreatic sections were prepared as described in the Online Methods. Representative data from nine pancreases with ten sections for each pancreas from three independent experiments are shown. TCR-β, T cell receptor-β.

(interleukin-1β, IFN-γ and CXCL10) in the islets of NOD mice at various ages. Expression of IFN-α and IFN-α–induced gene products reached a maximum at 3 and 4 weeks of age, respectively (**Supplementary Fig. 3**). Proinflammatory gene expression increased continually between 4 and 8 weeks of age in the islets of NOD mice (**Supplementary Fig. 3**). mRNA expression of IFN-α and IFN-α–induced gene products was not observed in nonautoimmune C57BL/6 or BALB/c mice (**Supplementary Figs. 3** and **4**). Together, these data reveal that the IFN-α signature in young mice was associated with the autoimmune-prone NOD genetic background. We next focused on pDCs and their putative role in T1D initiation. FACS analysis showed that pDCs isolated from the islets of NOD mice produced IFN-α, which peaked at 3 and declined at 6 weeks of age (**Fig. 2b**). Accordingly, the expression of IFN-α–induced genes in 4-week-old NOD mice was abolished after pDC depletion using m927 monoclonal antibody (mAb) against the BST2 antigen between 2 and 3 weeks of age (**Fig. 2c** and **Supplementary Fig. 5**). To assess the diabetogenic role of pDCs, we evaluated the autoreactive CD8⁺ T cell response against the beta cell antigen islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{206–214}$ in 8-week-old NOD mice that were depleted of pDCs or that had been treated with Siglec-H–specific mAb to prevent IFN-α production by pDCs (**Supplementary Fig. 6**). Both treatments administered between 2 and 3 weeks of age led to a dramatic reduction in the frequency of NRP-V7-tetramer⁺ CD8⁺ T cells specific for $IGRP_{206–214}$ within the pancreatic lymph nodes (PLN) and to a strong decrease in $IGRP_{206–214}$-reactive IFN-γ⁺ CD8⁺ T cells within the islets of 8-week-old NOD mice (**Fig. 2d** and **Supplementary Fig. 7**). Accordingly, pDC depletion in 2-week-old NOD mice prevented the development of T1D up to 30 weeks of age (**Fig. 2e**). Together, these data demonstrate that pancreatic pDCs produce IFN-α in young NOD mice and subsequently induce autoimmune diabetes. To determine whether Toll-like receptor (TLR) pathways were necessary for IFN-α expression in the islets of NOD mice, we used *Myd88*$^{-/-}$ NOD mice, which lack the ability to signal through TLRs (excluding TLR3). We failed to detect IFN-α production in the islets from 3-week-old *Myd88*$^{-/-}$ NOD mice (**Supplementary Fig. 8a**). Accordingly, the increase in IFN-α–induced gene expression observed in NOD mice was also absent in the islets of 4-week-old *Myd88*$^{-/-}$ NOD mice (**Supplementary Fig. 8b**). To determine the TLR involved, we treated 2-week-old NOD mice with IRS_{954}, an antagonist of TLR7 and TLR9 pathways. Such treatment decreased the expression of IFN-α–induced genes in the islets of 4-week-old NOD mice (**Supplementary Fig. 8b**). Finally, treatment of 2-week-old NOD mice with IRS_{954} prevented the development of T1D, whereas CpG_{1585}, a TLR9 agonist, increased the incidence of T1D, confirming the role of TLR7 and TLR9 and subsequent IFN-α production in the initiation of T1D in young NOD mice (**Fig. 2e**).

B-1a cells activate pDCs in the pancreas

During psoriasis and lupus development, self DNA released from dying keratinocytes forms immune complexes with IgGs specific for double-stranded DNA (dsDNA), which then activate pDCs via TLR9 (ref. 4). Thus, we investigated whether this mechanism of pDC activation takes place in T1D. First, we detected dsDNA-specific IgGs

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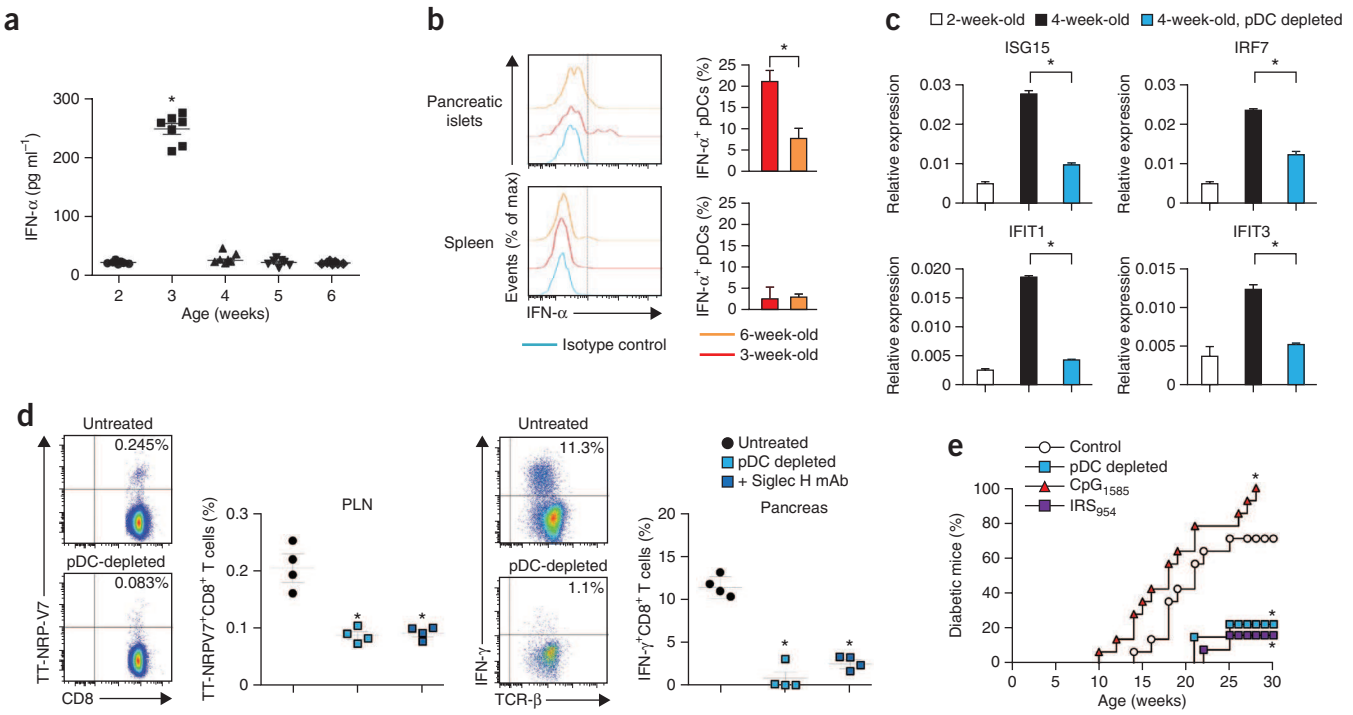

Figure 2 Pancreatic pDCs express IFN-α and are required for T1D development in NOD mice. (**a**) IFN-α production in the islets of NOD mice from 2 to 6 weeks of age, measured after 36-h culture. Data represent seven independent mice for each group from four independent experiments. $*P < 0.05$ for each group compared to 2-week-old group. (**b**) FACS analysis of IFN-α by pDCs from islets of 3- or 6-week-old NOD mice. Frequency (left) and absolute number (right) of IFN-α+ cells among pDCs in pancreatic islets (top) and the spleen (bottom). Data are mean values ± s.e.m. of four independent experiments with four pooled mice for each group. $*P < 0.05$ for 3-week-old group compared to 6-week-old group. (**c**) mRNA expression of IFN-α–induced genes as assessed by quantitative PCR in the islets of NOD mice treated with depleting m927 mAb or isotype control. Data are mean values ± s.e.m. of three independent experiments with four independent mice for each group. $*P < 0.05$. (**d**) Analysis of CD8+ T cells specific for $IGRP_{206–214}$ in 8-week-old NOD mice after pDC depletion or blockade of IFN-α–producing-pDCs at 2–3 weeks of age. Left, frequency of NRP-V7 tetramer-specific cells among the CD8+ T cell population from PLN. Right, frequency of IFN-γ+ cells among the CD8+ T cell population after re-stimulation with $IGRP_{206–214}$ peptide. Representative dot plots are shown, and values in the graph correspond to four independent mice for each group from two independent experiments. $*P < 0.05$ for treated group compared untreated group. (**e**) Incidence of diabetes in NOD mice after pDC depletion or TLR9 targeting. Mice were treated with m927 mAb, isotype control, IRS_{954} or CpG_{1585} at 2 weeks of age over 2 weeks. $*P < 0.05$ for each treated group compared to control group ($n = 12$ mice per group).

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at a low concentration in the sera of NOD, BALB/c and C57BL/6 mice and at a high concentration in serum from NZB/W F1 lupus-prone mice used as a positive control (**Fig. 3a**). Conversely, in the islets, we observed a higher amount of dsDNA-specific IgGs from NOD mice as compared to BALB/c or C57BL/6 mice (**Fig. 3b**). We also observed a higher amount of total IgG in islets from NOD mice compared to BALB/c or C57BL/6 mice (**Supplementary Fig. 9**). Among B cell populations, the $CD5^+CD19^+CD1d^{med}$ B-1a cells may produce high titers of dsDNA-specific IgGs[18]. Accordingly, we observed a large population of B-1a cells in the islets of NOD mice as early as 2 weeks of age (**Fig. 3c**). Their frequency among the total B cell population in the islets declined with the age (**Fig. 3d** and **Supplementary Fig. 10**).

Next, we performed peritoneal lavages between 1 to 3 weeks of age to deplete B-1a cells from pancreatic islets of NOD mice as previously described[19] (**Supplementary Fig. 11**). B-1a cell depletion largely reduced the production of dsDNA-specific IgGs in the islets of 3-week-old NOD mice (**Fig. 3b**). Additionally, pancreatic B-1a cells sorted from 3-week-old NOD mice produced dsDNA-specific IgGs after 7 d of culture, contrary to pancreatic B-2 cells (**Fig. 3e**). B-1a cell depletion also strongly reduced the frequency of IFN-α–secreting pDCs in the islets of 4-week-old NOD mice (**Fig. 3f**) and inhibited the diabetogenic T cell response within the PLN and islets of 8-week-old NOD mice (**Fig. 3g**). Accordingly, B-1a cell depletion in 2-week-old NOD mice prevented T1D development (**Fig. 3h**). Collectively, these data unveil a crucial role of pancreatic B-1a cells in the activation of pDCs and T1D initiation.

Pancreatic neutrophils activate pDCs and initiate T1D

Our analysis of the pancreatic infiltrate of 3-week-old NOD mice revealed the presence of neutrophils (**Fig. 1**). This group of innate cells is a key player in the clearance of extracellular pathogens[20]. In individuals with SLE, neutrophils secrete DNA-binding antimicrobial peptides (LL-37 in humans and CRAMP in mice) that bind immune complexes and potentiate their stimulatory effect on IFN-α–secreting pDCs[21,22]. We first observed that neutrophil depletion between 2 and 3 weeks of age using the NIMP-R14 mAb against Ly6G antigen (**Supplementary Fig. 12**) attenuated the expression of IFN-α–induced genes in the pancreas of 4-week-old NOD mice (**Fig. 4a**). Injection of CRAMP in 4-week-old NOD mice increased the expression of IFN-α–induced genes in the islets 24 h later (**Fig. 4a**). Moreover, coinciding with the presence of neutrophils, peak CRAMP mRNA expression was detected at 3 weeks of age (**Fig. 4b**), and neutrophil depletion markedly reduced this expression (**Fig. 4c**). FACS analysis confirmed that pancreatic neutrophils were the source of CRAMP in young NOD mice (**Fig. 4d**). No CRAMP expression was

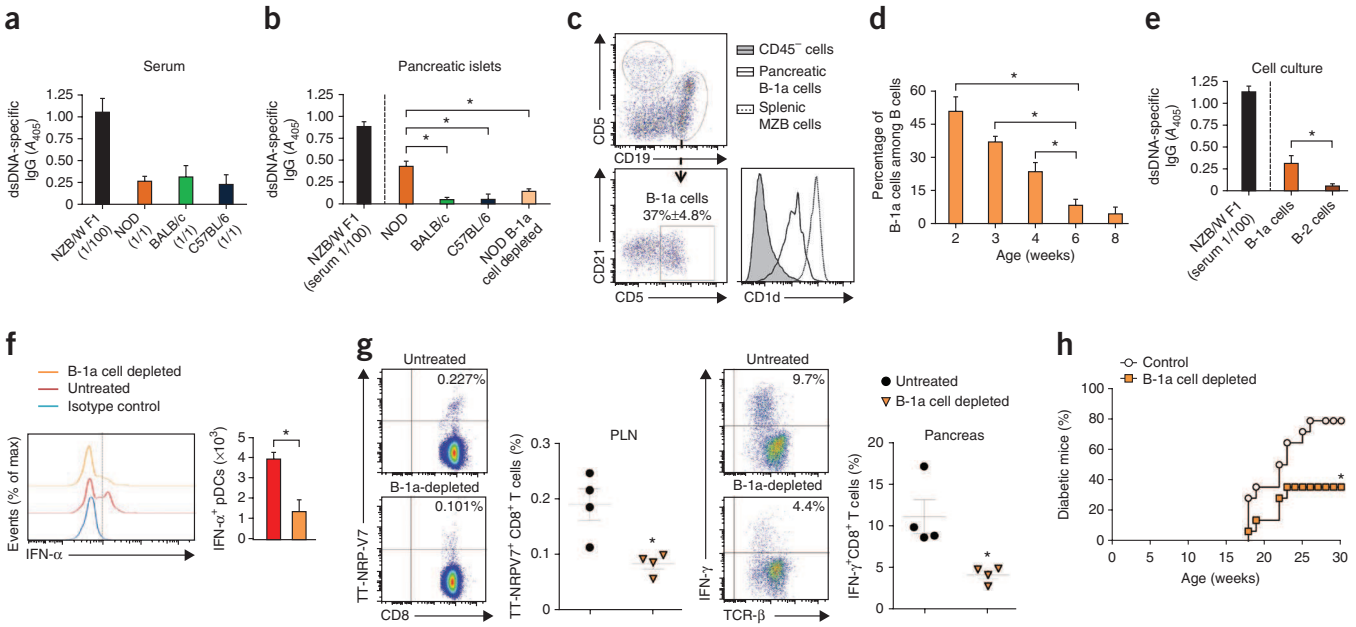

Figure 3 B-1a cells activate pDCs in the pancreas and participate in the initiation of T1D. (**a**,**b**) dsDNA-specific IgG production in the serum (**a**) or in the islet supernatants (**b**) from 3-month-old NZB/W F1 mice or NOD, C57BL/6 and BALB/c mice at 3 weeks of age. In some conditions, B-1a cells were depleted in NOD mice as described in the Online Methods. Values are obtained by ELISA and are expressed as mean absorbance values at 405 nm with corrected cutoff values. Data are mean values ± s.e.m. of two independent experiments with three independent mice for each group. $*P < 0.05$. (**c**) FACS analysis of B cell populations in the islets of NOD mice at 3 weeks of age. Frequency of B-1a cells ($CD19^+CD5^+CD21^-$) among $CD45^+$ cells are represented, and representative expression of CD1d among $CD45^+CD19^+$ cells is shown. (**d**) FACS analysis of B-1a cells in the islets of NOD mice at various ages. Frequencies of B-1a cells among $CD45^+CD19^+$ cells are represented. Data are mean values ± s.e.m. of four independent experiments with four pooled mice for each group. $*P < 0.05$. (**e**) dsDNA-specific IgG production in B-1a and B-2 cell cultures from 3-week-old NOD. Data are mean values ± s.e.m. of two independent experiments with three independent mice for each group. $*P < 0.05$. (**f**) FACS analysis of IFN-α secretion by pDCs after B-1a cell depletion in NOD mice at 3 weeks of age. Right, absolute number of IFN-α^+ cells among pDCs. Data are mean values ± s.e.m. of four independent experiments with four pooled mice for each group. $*P < 0.05$, treated group compared to untreated group. (**g**) Analysis of $CD8^+$ T cells specific for $IGRP_{206-214}$ in 8-week-old NOD mice after B-1a cell depletion at 2–3 weeks of age. Left, frequency of NRP-V7 tetramer-specific cells among $CD8^+$ T cell population from PLN. Right, frequency of IFN-γ^+ cells among $CD8^+$ T cell population after re-stimulation with $IGRP_{206-214}$ peptide. Representative dot plots are shown, and values in the graphs correspond to four independent mice for each group from two independent experiments. $*P < 0.05$ for treated group compared untreated group. (**h**) Incidence of diabetes in NOD mice after B-1a cell depletion in NOD mice. Mice were treated between 7 d and 21 d of age. $*P < 0.05$ for treated group compared to control group ($n = 12$ mice per group).

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observed in other $CD45^+$ or in $CD45^-$ cells in the pancreatic islets (**Supplementary Fig. 13**). Histological analysis confirmed the presence of CRAMP-secreting neutrophils inside the islets of 3-week-old NOD mice (**Fig. 4e**). Pancreatic neutrophils appeared to release neutrophil extracellular traps (NETs), which are typically associated with CRAMP release and activation of IFN-α–secreting pDCs in patients with SLE[21,22].

We confirmed that *ex vivo*–isolated pancreatic neutrophils produced NETs containing DNA by staining with a Ly6G-specific antibody and SYTOX-Green, and these NETs were associated with CRAMP peptide (**Fig. 4f** and **Supplementary Fig. 14**). Neutrophil depletion at 2 weeks of age reduced the frequency of IFN-α–secreting pDCs in the islets of treated NOD mice (**Fig. 5a**) and dampened the diabetogenic T cell response within the PLN and islets of 8-week-old NOD mice (**Fig. 5b**). Accordingly, neutrophil depletion in 2-week-old NOD mice inhibited T1D development at later ages (**Fig. 5c**). Together, these data support a role for CRAMP-secreting neutrophils in the activation of pancreatic pDCs and T1D initiation.

Neutrophils and B-1a cells cooperate to activate pDCs

We next investigated the molecular interplay between innate cells in the islets of young NOD mice. Neutrophils are activated via various pathways including Fcγ receptors (FcγRs), TLRs or both. First, we excluded the TLR pathway because CRAMP mRNA expression in the islets was unaltered in *Myd88*$^{-/-}$ NOD mice as compared to WT NOD mice (**Fig. 4c**). As IgG-secreting B-1a cells have been shown to activate neutrophils via FcγRs[20], we performed *in vitro* cultures with neutrophils isolated from bone marrow and activated with phorbol-12-myristate-13-acetate (PMA), immune complexes (dsDNA plus commercial dsDNA-specific IgGs) or B-1a immune complexes (dsDNA plus pancreatic B-1a cell–conditioned medium), and measured CRAMP expression on the surface of neutrophils (**Fig. 5d**). As expected, PMA (used as positive control) induced expression of CRAMP. Immune complexes and B-1a immune complexes also induced CRAMP expression, which was blocked by the addition of a mix of FcγRII/III-specific and FcγRIV-specif mAbs, revealing that the neutrophils were activated through FcγRs (**Fig. 5d**). Notably, our data obtained with severe combined immunodeficiency NOD mice, which are devoid of T and B cells, demonstrated the requirement of B cells in the activation of pancreatic neutrophils (**Supplementary Fig. 15**). These data strongly suggest that in the pancreatic islets of NOD mice, CRAMP-producing neutrophils are activated by IgG-secreting B-1a cells via FcγRs.

To determine whether both neutrophils and B-1a cells are required for activation of pDCs, we cultured splenic pDCs with pancreatic B-1a cell–conditioned medium, supernatant of bone marrow–isolated

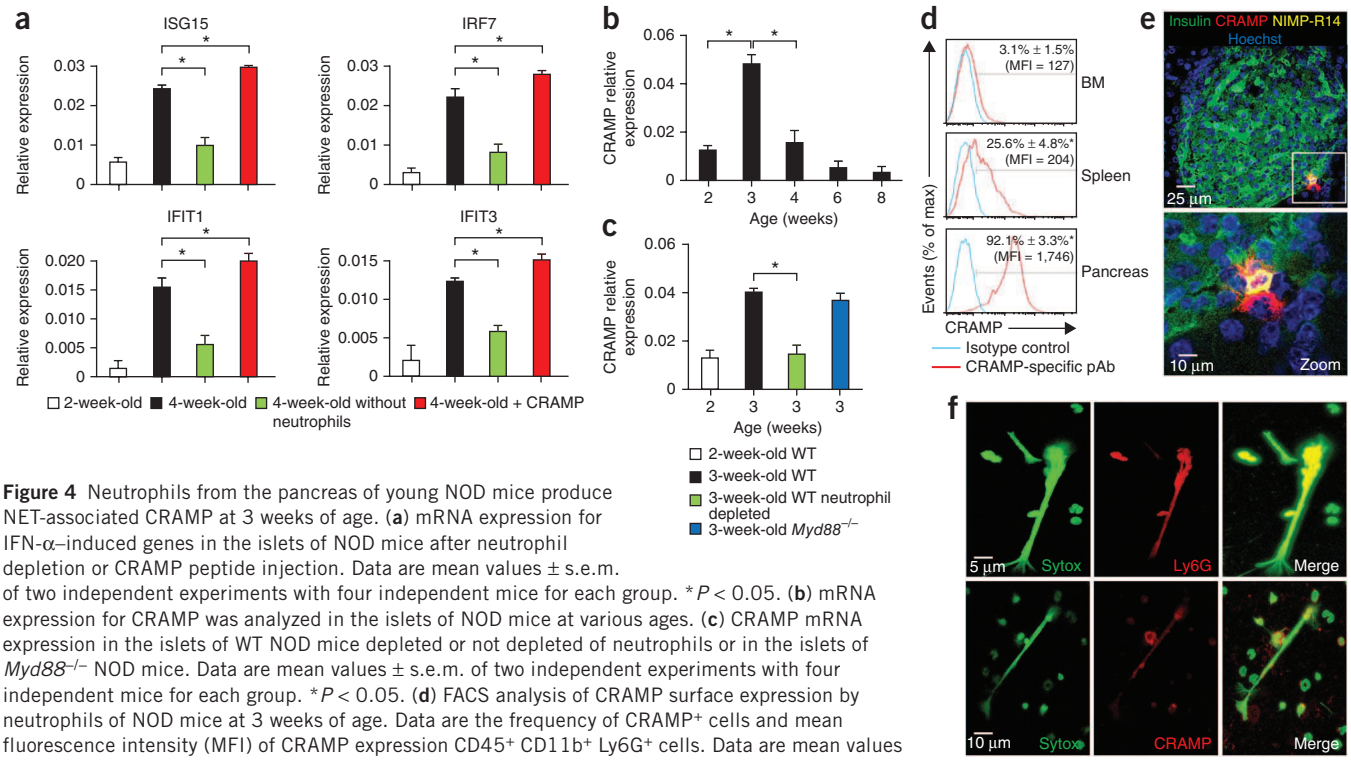

Figure 4 Neutrophils from the pancreas of young NOD mice produce NET-associated CRAMP at 3 weeks of age. (**a**) mRNA expression for IFN-α–induced genes in the islets of NOD mice after neutrophil depletion or CRAMP peptide injection. Data are mean values ± s.e.m. of two independent experiments with four independent mice for each group. $^*P < 0.05$. (**b**) mRNA expression for CRAMP was analyzed in the islets of NOD mice at various ages. (**c**) CRAMP mRNA expression in the islets of WT NOD mice depleted or not depleted of neutrophils or in the islets of *Myd88*$^{-/-}$ NOD mice. Data are mean values ± s.e.m. of two independent experiments with four independent mice for each group. $^*P < 0.05$. (**d**) FACS analysis of CRAMP surface expression by neutrophils of NOD mice at 3 weeks of age. Data are the frequency of CRAMP$^+$ cells and mean fluorescence intensity (MFI) of CRAMP expression CD45$^+$ CD11b$^+$ Ly6G$^+$ cells. Data are mean values ± s.e.m. of four independent experiments with four pooled mice for each group. $^*P < 0.05$ for each group compared to bone marrow (BM) group. (**e**) Immunohistological analysis of pancreatic neutrophils in 3-week-old NOD mice. Zoomed-in boxed area shows NETs released by the neutrophil. Representative data from six pancreases with ten sections for each pancreas from three independent experiments are shown. (**f**) The ability of neutrophils to form CRAMP-associated NETs *ex vivo* when isolated from islets of NOD at 3 weeks of age was determined by immunostaining. Representative data from five fields of cells from the islets of eight pooled mice from three independent experiments are shown.

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neutrophils preactivated with B-1a immune complexes, or both. As a positive control of pDC activation, CpG_{1585} induced IFN-α production in pDCs, which was inhibited by IRS_{954} (**Fig. 5e**). Soluble IC induced moderate production of IFN-α, which was potentiated by the addition of CRAMP as previously described[21]. B-1a cell–conditioned medium also induced moderate IFN-α production that was potentiated by the addition of neutrophil-conditioned medium (**Fig. 5e**). This strong IFN-α production induced by the addition of both supernatants was inhibited by IRS954 (**Fig. 5e**). These data demonstrate that B-1a cells secreting dsDNA-specific IgG and CRAMP-secreting neutrophils cooperate to stimulate IFN-α production by pDCs through TLR9.

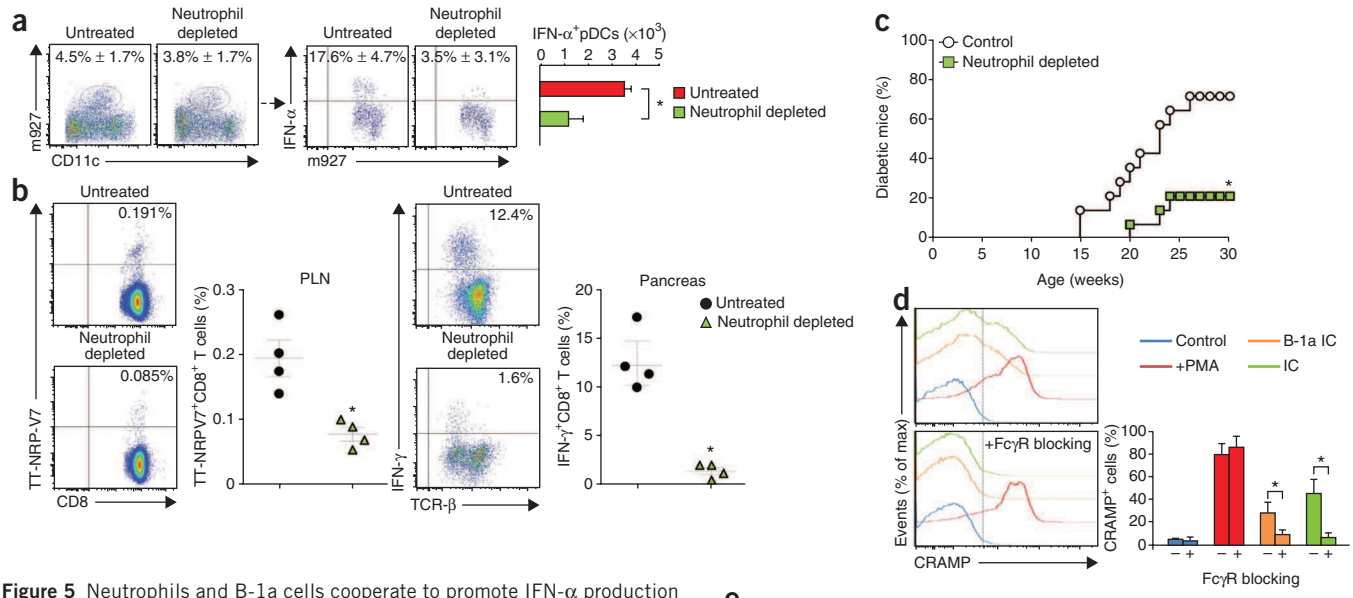
Beta cell death initiates the innate cell activation cascade

Our data revealed that an innate cell crosstalk takes place in the pancreas of young NOD mice and leads to the initiation of T1D. However, the initial event that triggers this crosstalk remained unclear. We investigated the putative role of physiological beta cell death that occurs spontaneously in young NOD mice during organogenesis and after weaning[13]. To mimic this event, we induced beta cell death by injecting streptozotocin (STZ). On day 1 after STZ injection in 6-week-old NOD mice, we observed an increase in the expression of IFN-α–induced genes in the islets (**Fig. 6a**). This increase was dependent on pDCs, as revealed by m927 depleting mAb treatment (**Fig. 6b**). STZ treatment failed to induce expression of IFN-α–induced genes and recruitment of pDCs, neutrophils or B-1a cells in the islets of non–autoimmune-prone C57BL/6 and BALB/c mice (**Supplementary Figs. 16** and **17a**).

We next demonstrated that STZ treatment at 6 weeks of age induced the production of dsDNA-specific IgGs and increased the frequency of CRAMP-expressing neutrophils in the islets of NOD mice 24 h later (**Fig. 6c**,**d** and **Supplementary Fig. 17b**). This increased production of dsDNA-specific IgGs and the recruitment of CRAMP$^+$ neutrophils in the islets induced by STZ were similar to that which we observed in 3-week-old NOD mice, suggesting that beta cell death could be required for the activation of pancreatic pDCs in young NOD mice. To test this hypothesis, we treated 7-d-old NOD mice with a single injection of Z-VAD, a pan-caspase inhibitor. This treatment abrogated dsDNA-specific IgG secretion, CRAMP-production by neutrophils and IFN-α–induced gene expression in islets of 4-week-old NOD mice (**Fig. 6e–g**) and prevented T1D development up to 30 weeks of age (**Fig. 6h**). Altogether, our study demonstrated that in young NOD mice, physiological beta cell death initiates the activation of innate immune cells, which leads to the local production of IFN-α, the development of diabetogenic T cell responses and autoimmune diabetes.

DISCUSSION

Despite increased knowledge of the pathogenesis of T1D, the early stages of disease pathogenesis remain poorly defined. Here, we demonstrate that in young NOD mice, when physiological beta cell death occurs, innate immune cell crosstalk takes place in the pancreas that is crucial for T1D development (**Supplementary Fig. 18**). We propose that beta cell debris (that is, self DNA) form immune complexes with dsDNA-specific IgGs secreted by B-1a cells. Neutrophils produce DNA-binding peptide that potentiates these immune complexes, inducing IFN-α secretion by pancreatic pDCs through TLR9.



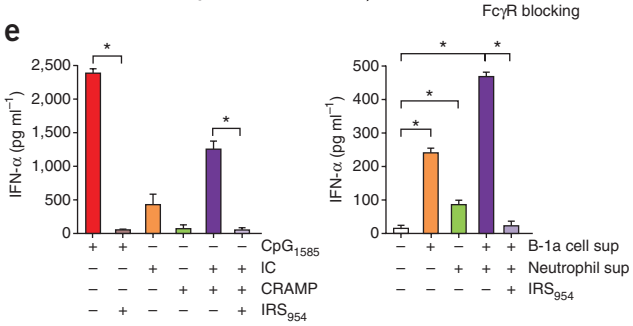


Figure 5 Neutrophils and B-1a cells cooperate to promote IFN-α production by pDCs. (**a**) FACS analysis of IFN-α secretion by pDCs after neutrophil depletion in NOD mice at 3 weeks of age. Frequency of pDCs (left), frequency (middle) and absolute number of IFN-α+ cells among pDCs (right) are represented. Data are mean values ± s.e.m. of four independent experiments with four pooled mice for each group. $*P < 0.05$, treated group compared to untreated group. (**b**) Analysis of CD8+ T cells specific for $IGRP_{206–214}$ in 8-week-old NOD mice after neutrophil depletion at 2–3 weeks of age. Left, frequency of NRP-V7 tetramer-specific cells among CD8+ T cell population from PLN. Right, frequency of IFN-γ+ cells among CD8+ T cell population after restimulation with $IGRP_{206–214}$ peptide. Representative dot plots are shown, and values in the graphs correspond to four independent mice for each group from two independent experiments. $*P < 0.05$ for treated group compared untreated group. (**c**) Incidence of diabetes in NOD mice after neutrophil depletion in NOD mice. Mice were treated with NIMP-R14 mAb or isotype control between 7 and 21 d of age. $*P < 0.05$ for treated group compared to control group ($n = 12$ mice per group). (**d**) FACS analysis of surface expression of CRAMP by neutrophils after *in vitro* culture. Bone marrow neutrophils were added to PMA, immobilized immune complexes (IC) or B-1a IC and cultured for 20 min. In some conditions, neutrophils were preincubated with FcγR-blocking mAbs. Data are mean values ± s.e.m. of four independent experiments. $*P < 0.05$, each group compared to control group. (**e**) IFN-α production evaluated by ELISA in *in vitro* pDC culture in the presence of neutrophil or B-1a cell–conditioned medium. pDCs were isolated from the spleen of 3-week-old NOD mice and were incubated with CpG_{1585}, soluble IC or CRAMP (left) or cultured with neutrophil-conditioned medium and/or B-1a cell–conditioned medium (right), all in the presence or absence of TLR7 and TLR9 inhibitor (IRS_{954}). Data are mean values ± s.e.m. of three independent experiments with four pooled mice for each group. $*P < 0.05$.

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This cytokine creates an inflammatory milieu favorable for the diabetogenic adaptive response and autoimmune diabetes.

Aside from the key role of IFN-α–secreting pDCs in antiviral defense, a growing body of evidence argues for a pathogenic role for them in several autoimmune diseases such as psoriasis and lupus[23]. In T1D, differing results are found in the literature concerning the role of IFN-α and pDCs in disease pathogenesis. The expansion of IFN-α–producing pDCs has been documented in patients with T1D around the time of diagnosis[8], and IFN-α treatment of patients with hepatitis infection or with leukemia has been shown to induce diabetes development[6,7,24]. Recent studies revealed that NOD mice harbor high IFN-α levels in PLN before the onset of diabetes[25] and that pDC depletion prevents T1D development in these mice[26]. NOD mice harbor substantially more splenic pDCs in comparison to C57BL/6 mice, and NOD pDCs produce more IFN-α after *in vitro* stimulation than C57BL/6 pDCs[27]. Paradoxically, older studies showed that IFN-α treatment reduces diabetes incidence in NOD mice or Biobreeding (BB) rats, a strain of rats that spontaneously develop autoimmune diabetes[28–31]. However, treatment with an inducer of IFN-α and IFN-β production such as poly(I:C) can prevent[32,33] or accelerate[34–36] the disease depending on the dose, time, duration and route of administration (oral, intraperitoneal or subcutaneous). The proinflammatory role of type I IFNs has been extensively documented; IFN-α and IFN-β induce cDC maturation, activate immunoglobulin-secreting B cells, enhance CRAMP expression by neutrophils and boost effector T cell responses[37,38]. Type I IFNs also directly affect pancreatic beta cells by inducing cytokine and chemokine secretion and major histocompatibility complex class I expression, enhancing their susceptibility to attack from diabetogenic T cells[39]. The protective role of IFN-α treatment remains unclear; one hypothesis is that type I IFN can boost the activity of regulatory T cells[40–43].

It has been also reported in some diabetes models that pDCs can exert disease-protective effects against the development of T1D[44,45]. However, the precise mechanism leading to T1D prevention and the direct role of pDCs were not precisely addressed. In our previous studies[46,47], we demonstrated that during viral infection of 6-week-old NOD mice, pDCs can inhibit T1D development by two complementary pathways but at different times and locations. Indeed, 1–2 d after infection, pDCs transiently produced IFN-α in the pancreas to dampen viral replication, avoiding tissue damage, and then migrate to the PLN

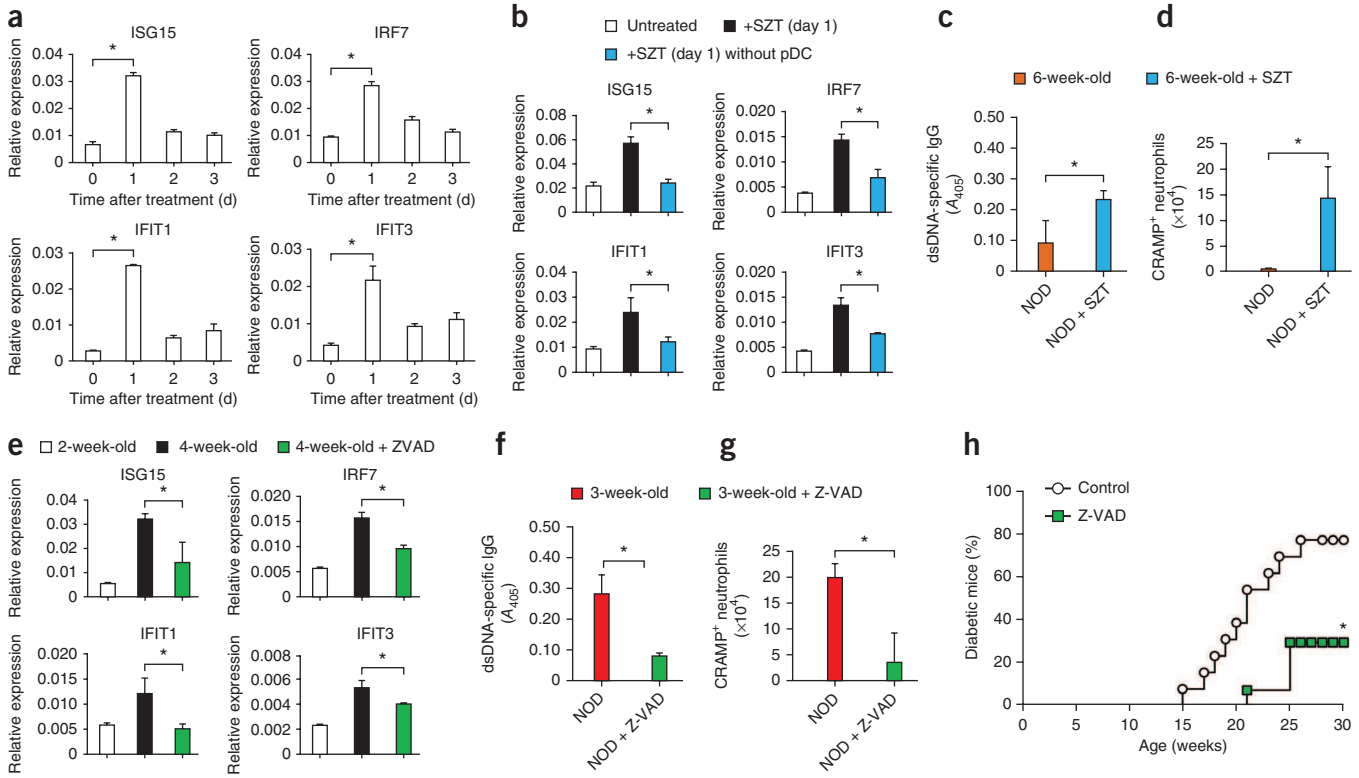

Figure 6 Initial beta cell death is required to induce innate cell activation and T1D development. (**a**,**b**) mRNA expression for IFN-α–induced genes in islets from 6-week-old NOD mice after injection of STZ. In some conditions (**b**), pDCs were depleted 1 d before the injection of STZ. Data are mean values ± s.e.m. of two independent experiments with four independent mice for each group. $*P < 0.05$. (**c**) DNA-specific IgG production in the islet supernatants from 6-week-old NOD mice 12 h after STZ injection. Data are mean values ± s.e.m. of two independent experiments with two independent mice for each group. $*P < 0.05$. (**d**) FACS analysis of CRAMP expression by pancreatic neutrophils from 6-week-old NOD mice 12 h after STZ injection. Absolute number of CRAMP+ cells among CD45+CD11b+Ly6G+ cells is represented. Data are mean values ± s.e.m. of four independent experiments with two pooled mice for each group. $*P < 0.05$. (**e**) mRNA expression for IFN-α–induced genes in islets of 2- or 4-week-old NOD mice after Z-VAD treatment . Data are mean values ± s.e.m. of two independent experiments with four independent mice for each group. $*P < 0.05$. (**f**) DNA-specific IgG production in the islet supernatants from 3-week-old NOD mice treated or not with Z-VAD. Data are mean values ± s.e.m. of two independent experiments with two independent mice for each group. $*P < 0.05$. (**g**) FACS analysis of CRAMP expression by pancreatic neutrophils from 3-week-old NOD mice treated or not with Z-VAD. Absolute number of CRAMP+ cells among CD45+CD11b+Ly6G+ cells is represented. Data are mean values ± s.e.m. of four independent experiments with two pooled mice for each group. $*P < 0.05$. (**h**) Incidence of diabetes in NOD mice after blockade of apoptosis by Z-VAD treatment. Mice were treated with Z-VAD at 7 d of age. $*P < 0.05$ for treated group compared to control group ($n = 12$ mice per group).

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and produce transforming growth factor-β, which induces regulatory T cells at later time points. However, to be efficient, these mechanisms of protection required the stimulation of invariant natural killer T cells at the time of infection. Altogether, these studies support the notion that pDCs could harbor both pathogenic and protective functions during autoimmune diabetes development, depending on the course of the disease, the infectious context and the localization of the cells.

B cells are proposed to be involved in T1D pathogenesis as self antigen–presenting cells, but there is little evidence for a diabetogenic role for autoantibodies[48]. Our data revealed that the innate-like CD5+ B-1a cells have an as yet undescribed role in T1D initiation through the production of dsDNA-specific IgGs and the activation of neutrophils and pDCs in the pancreas. A previous publication showed that peritoneal B-1a cells participate in T1D development in NOD mice, but their mechanism of action remained unknown[19]. Notably, the number of circulating CD5+ B cells is higher in children with a very recent onset of T1D, as compared with patients with long-term disease or controls[49]. An altered B cell receptor signaling threshold has also been observed in patients with T1D as compared to healthy controls[50]. High frequencies of CD5+ B cells have been reported in patients with other autoimmune diseases, such as Sjögren's disease[51] and rheumatoid arthritis[52]. Similarly, NOD mice harbor an elevated frequency of self-reactive B cells compared to C57BL/6 and BALB/c mice[53].

The role of neutrophils in autoimmunity has been described in small-vessel vasculitis, SLE, psoriasis and a mouse model of skin inflammation[21,22,54,55]. In these studies, neutrophils act by producing the DNA-binding peptides LL-37 and CRAMP, which form complexes with self DNA and dsDNA-specific IgGs, activating IFN-α production by pDCs. DNA-binding peptides are released by neutrophils during NETosis[20]. Our study demonstrates that neutrophils release NET-associated CRAMP in pancreatic islets, which promotes diabetes initiation in NOD mice.

We show that spontaneous or STZ-induced beta cell death is required for the activation of IFN-α–secreting pDCs probably by releasing self DNA essential to the formation of immune complexes, as shown in other diseases[56]. Previous studies in NOD mice provided evidence that beta cell death naturally occurring during the first postnatal weeks initiates the activation of cDCs and the priming of autoreactive T cells[14]. Notably, these waves of physiological beta cell death also occur in pigs[15] and humans[16]. Beta cell debris could accumulate

in the pancreas of NOD mice because of a defect in phagocytic clearance in this genetic background[17]. Previous data indicate that proinflammatory responses and autoimmune disorders can be elicited by defective clearance of apoptotic cells[57]. During infancy, the immune system may be provoked with a physiological massive rebuilding of the pancreatic islets via programmed cell death and may initiate the diabetogenic process in an autoimmune genetic background[58].

Finally, the interplay between B-1a cells, neutrophils and pDCs seems to be a common feature of autoimmune diseases. This new aspect of the pathogenesis of autoimmune diseases is likely to open new therapeutic avenues directed toward the targeting of innate immune cells. One of the major needs is to set up more efficient tools that allow the detection of these primary diabetogenic events. Approaches focused on pDCs would be more selective and tolerable than targeting of the IFN-α response, which can disturb antiviral responses.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.D. initiated and led the whole project, coordinated with different investigators, designed and performed experiments, analyzed the data and wrote the manuscript; Y.S. designed and performed experiments, analyzed the data and wrote the manuscript; L.F. did confocal microscopy experiments, analyzed the data and wrote the manuscript; L.B. provided technical assistance; B.A. and F.B. provided intellectual input and key reagents for neutrophil and pDC analysis; and A.L. was responsible for project planning, data analysis, discussion and writing.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice and treatments. Female BALB/c, C57BL/6, NOD and *Myd88*$^{-/-}$ NOD[59] mice were bred and housed in specific pathogen–free conditions. For pDC and neutrophil depletion, WT NOD mice were injected i.p. on days 14 and 21 after birth, respectively, with 500 μg of depleting m927 mAb per mouse (from M. Colonna) or 250 μg of depleting NIMP-R14 mAb per mouse (from S. Mecheri). For inhibition of IFN-α production by pDCs, WT NOD mice were injected i.p. on days 14 and 21 after birth, with 200 μg of anti–Siglec-H mAb in 200 μL of PBS (ebio440c, eBioscience). The same treatment was performed with respective isotype control. Inhibition of TLR7 and TLR9 was performed using 1 μg per mouse of IRS_{954} injected i.p. on days 14 and 21 after birth. TLR9 activation was performed by intravenous injection with 100 μg per mouse of CpG_{1585} (Invivogen) on day 14 after birth. For B-1a cell depletion we used a protocol previously described with some modifications[19,60]. Briefly, 1-week-old mice were treated with i.p. injection of 500 μL water every 2 d over 14 d; PBS was injected as negative control. Apoptosis inhibition was performed using Z-VAD (Sigma) as previously described[14]. Mice were injected i.p. with 100 μL per mouse of Z-VAD at 10 μM on days 14 and 21 after birth. Beta cell death was induced by one i.p. injection with 1.6 mg of STZ (Sigma) per mouse. The synthetic mouse cathelicidin peptide CRAMP (Innovagen) was injected i.p. in 4-week-old mice, 200 μg per mouse. This study was approved by the local ethics committee on animal experimentation of Paris Descartes University (CEEA34.JD.046.12).

Preparation of B-1a cell–conditioned medium. B-1a cells (5×10^3 in 100 μL) sorted as $CD5^+CD19^+$ cells from pancreatic islets of ten pooled 3-week-old NOD mice were incubated in RPMI complete medium, and cell-free supernatant was recovered after 7 d and stored at −80 °C before addition to neutrophil or pDC *in vitro* cultures.

Preparation of plate-bound immune complexes. Immobilized immune complexes were prepared as follows. Briefly, immune complex–covered surfaces were prepared by incubating 96-well Maxisorp F96 (Nunc International) ELISA plates with 20 μg mL^{-1} genomic dsDNA (Promega) in PBS for 12 h at 37 °C, followed by blocking with 10% BSA in PBS for 1 h and a further 1-h incubation with anti-dsDNA IgG2b mAb (DNA11-M, Gentaur) at 10 μg/mL. Alternatively, to form B-1a immune complexes, anti-dsDNA IgG2b mAbs were replaced by pancreatic B-1a cell–conditioned medium. Parallel wells prepared without the addition of anti-dsDNA IgGs served as controls.

Neutrophil stimulation. Neutrophil activation by immobilized immune complexes (with anti-dsDNA IgG2b mAb) or B-1a immune complexes was achieved by plating the cells on the immune complex–coated surfaces without any additional stimulus. Neutrophils (2×10^5 in 100 μL) sorted as $Ly6G^+$ $CD11b^+$ cells from bone marrow of 3-week-old NOD mice were incubated in complete RPMI. In some conditions, neutrophils were preincubated during 1 h at 37 °C with a mix of blocking anti-FcγRII/III (clone 24G2, BD) and anti-FcγRIV mAbs (clone 9E9, from J. Ravetch) at 10 μg mL^{-1}. Neutrophils were incubated for 20 min for evaluation of CRAMP expression by FACS or for 6 h to generate neutrophil-conditioned medium to stimulate pDCs *in vitro*.

***In vitro* pDC activation by neutrophils and B-1a cells.** Purified splenic pDCs from 3-week-old NOD mice (1×10^5, Miltenyi kit) were stimulated in 150 μL complete RPMI with neutrophil-conditioned medium (1:3 vol/vol) and/or B-1a cell-conditioned medium (1:3 vol/vol) in the presence or absence of a TLR7/9 inhibitor (IRS_{954} 1 μg mL^{-1}). As a positive control, pDCs were incubated with CpG_{1585} (5 μg mL^{-1}), soluble immune complexes (3 μg/mL dsDNA + 1 μg mL^{-1} anti-dsDNA IgGs, incubated 30 min at room temperature) or CRAMP (50 μg mL^{-1}). After 36 h of culture, supernatants were recovered and IFN-α production was measured by ELISA (PBL).

Diabetes diagnosis. Mice were tested every day for diabetes. Overt diabetes was defined as two positive urine glucose tests, confirmed by a blood sugar level of >200 mg dL^{-1}. The Glukotest kit was purchased from Roche.

Preparation of pancreatic islets. Mice were killed and pancreata were perfused with 3 mL of a solution of collagenase P (1.5 mg mL^{-1}, Roche), then dissected free from surrounding tissues. Pancreata were then digested at 37 °C for 10 min. Digestion was stopped by adding HBSS–5% FCS followed by extensive washes. Islets were then purified on a discontinuous Ficoll gradient and disrupted adding 1 mL of cell dissociation buffer (GIBCO) for 10 min at 37 °C. After another wash, cells were resuspended, counted and used.

Flow cytometry. Cell suspensions were prepared from various tissues and were stained at 4 °C in PBS containing 2% FCS and 0.5% EDTA after FcγRII/III blocking. Surface staining was performed with antibodies all from BD Pharmingen or eBioscience (anti-CD11c (clone N418), anti-CD11b (clone M1/70), anti-Ly6G (clone 1A8), anti-CD45 (clone 30-F11), anti-CD19 (clone 1D3), anti-TCRβ (clone H57-597), anti-CD8 (clone 53-6.7), anti–IFN-γ (clone XMG1.2), anti-CD5 (clone 53-7.3), anti-CD21 (clone 8D9), anti-CD1d (clone 1B1)) except m927 mAb and NIMP-R14 mAb, which were conjugated in our laboratory and were used at the concentration of 1 μg mL^{-1}. For CRAMP surface staining, cells were stained sequentially with rabbit anti-CRAMP pAb (from B. Agerberth, 1 μg mL^{-1}) and anti-rabbit-PE pAb (12-4739-81, eBiosciences, 0.5 μg mL^{-1}). For intracellular IFN-α staining, the cell suspension was incubated 4 h at 37 °C with Brefeldin A. After fixation and permeabilization (BD Fix&Perm), cells were first stained with anti–IFN-α (clone RMMA-1, 10 μg mL^{-1} PBL); then the surface staining was performed. For NRP-V7 tetramer (from the National Institutes of Health tetramer core facility) staining, cells were stained with tetramers for 45 min at room temperature followed by surface staining for 15 min at 4 °C. Dead cells were excluded using Fixable Viability Dye staining (eBioscience). Stained cells were analyzed and/or sorted on a FACS Aria flow cytometer (BD Biosciences).

Histology. Pancreases from NOD mice were collected, embedded in tissue-freezing medium (Jung) and stored at −20 °C. Tissues were cut into 5-μm sections in a cryostat (Leica). Frozen sections were fixed in cold acetone. Staining with primary antibodies was performed for 1 h with the following antibodies: anti-Ly6G (clone NIMP-R14, from S. Mecheri, 1 μg mL^{-1}), anti-B220 (clone RA3-6B2, from BD, 1 μg mL^{-1}) and anti-CD11c-PE (clone N418, from eBiosciences, 1 μg mL^{-1}) mAbs and insulin (A0564, Dako, 1 μg mL^{-1}) or CRAMP (from B. Agerberth, 1 μg mL^{-1}) pAbs. After washing, second-step reagents were applied: anti–rat biotin (A10517, Invitrogen, 1 μg mL^{-1}) or anti–guinea pig A488 (A11073, Invitrogen, 1 μg mL^{-1}) pAbs. If necessary, a third-step reagent was applied: streptavidin-PE or streptavidin-APC (eBioscience, 1 μg mL^{-1}). Nuclei were stained with Hoechst (H1399, Invitrogen, 5 μg mL^{-1}). Controls with isotype control staining were negative (data not shown).

Microscopy. NET production by neutrophils was assessed as follows. Briefly, pancreatic or bone marrow neutrophils were seeded on poly-L-lysine–coated glass (Polysine, Kindler GmhH) at a concentration of 10^6 cells per mL for 1 h at 37 °C in RPMI with 2% FCS. Then, cells were stained with anti–Ly-6G–conjugated antibody (clone NIMP-R14) for 10 min on ice and immediately fixed in 2% paraformaldehyde and counterstained for DNA with SYTOX Green (Invitrogen, 100 nM). In some experiments, after fixation, neutrophils were further stained with anti-CRAMP pAb (from B. Agerberth, 1 μg mL^{-1}) and then counterstained for DNA with SYTOX Green. Slides were analyzed using a Leica TCS SP5 AOBS confocal microscope.

Quantitative PCR. Cells were collected in lysis buffer (RLT, Qiagen) buffer with 1% of β-mercaptoethanol. mRNA was isolated using RNeasy Mini Kit (Qiagen) and reverse transcribed with Superscript III (Invitrogen). Quantitative PCR was performed with SYBR Green (Roche) and analyzed with a LightCycler 480 (Roche). Data were normalized to *Gapdh* housekeeping gene.

Detection of IFN-α or anti-dsDNA IgGs in the pancreatic islets. Pancreatic islets were recovered by handpicking under a polarized microscope without any prior density separation. Fifty islets from the same mouse were then cultured for 48 h in 100 μL of RPMI complete medium. Supernatants were used for IFN-α-quantification by ELISA (PBL) or for anti-dsDNA IgG quantification by ELISA (Calbiochem).

Statistical analyses. 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 non-parametric Mann-Whitney *U*-test. Reported values are means ± s.d. as indicated. P values < 0.05 were considered statistically significant. All data were analyzed using GraphPad Prism v5 software.

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Crosstalk between neutrophils, B-1a cells and plasmacytoid dendritic cells initiates autoimmune diabetes.

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Condensed title: innate immune cell interplays initiate T1D.

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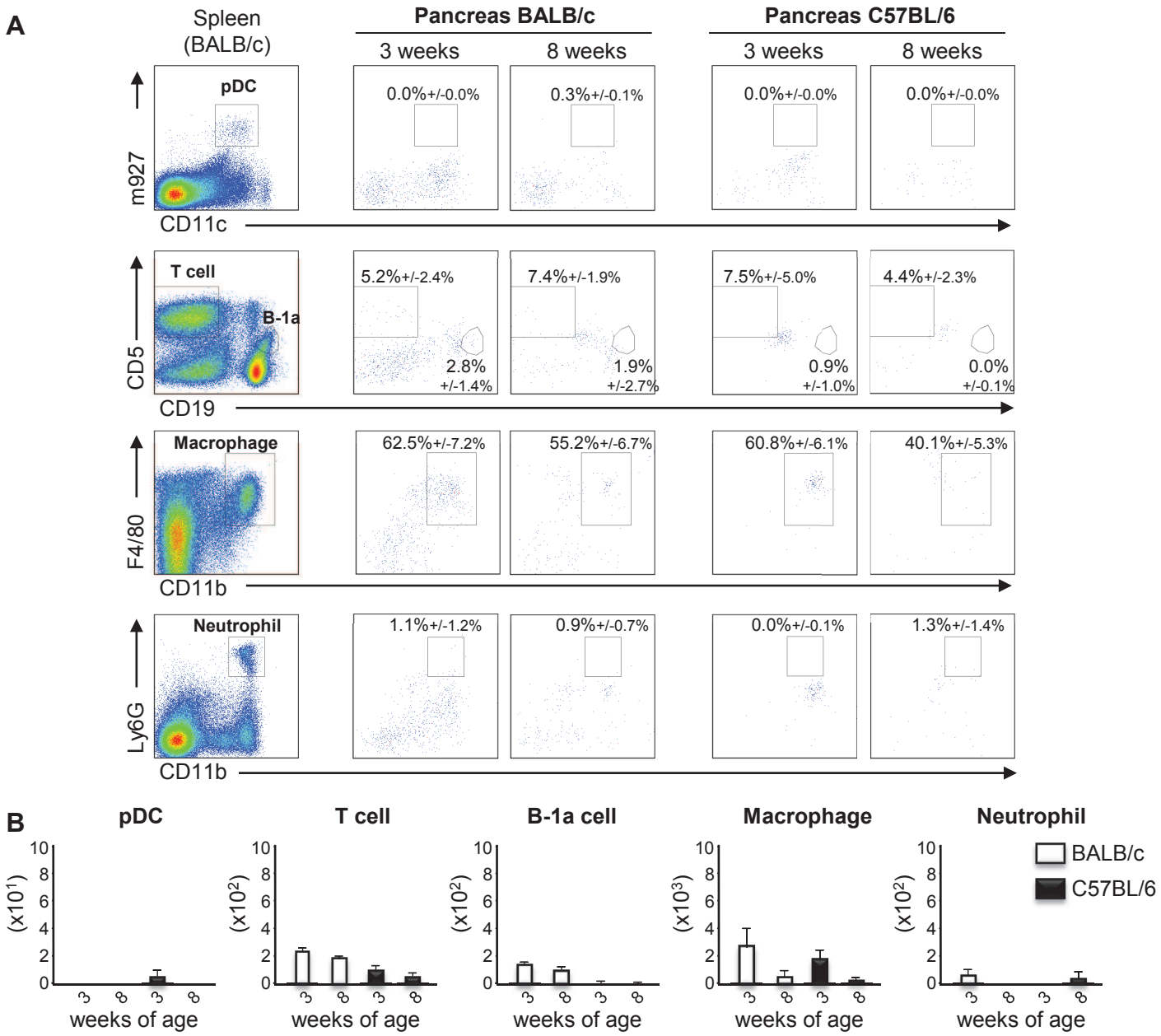


Figure S1. The recruitment of pDCs and neutrophils in the pancreatic islets at 3 weeks of age is specific to the NOD genetic background. (**a-b**) Cells were recovered from pancreatic islets or the spleen of 3-week-old or 8-week-old C57BL/6 or BALB/c mice and stained with anti-CD45, anti-CD11c, m927, anti-CD5, anti-CD19, F4/80, anti-CD11b and anti-Ly6G mAbs. (**a-b**) Data represent the frequency (**a**) or absolute number (**b**) among $CD45^+$ cells. Data are representative of 2 independents experiments with eight pooled mice.

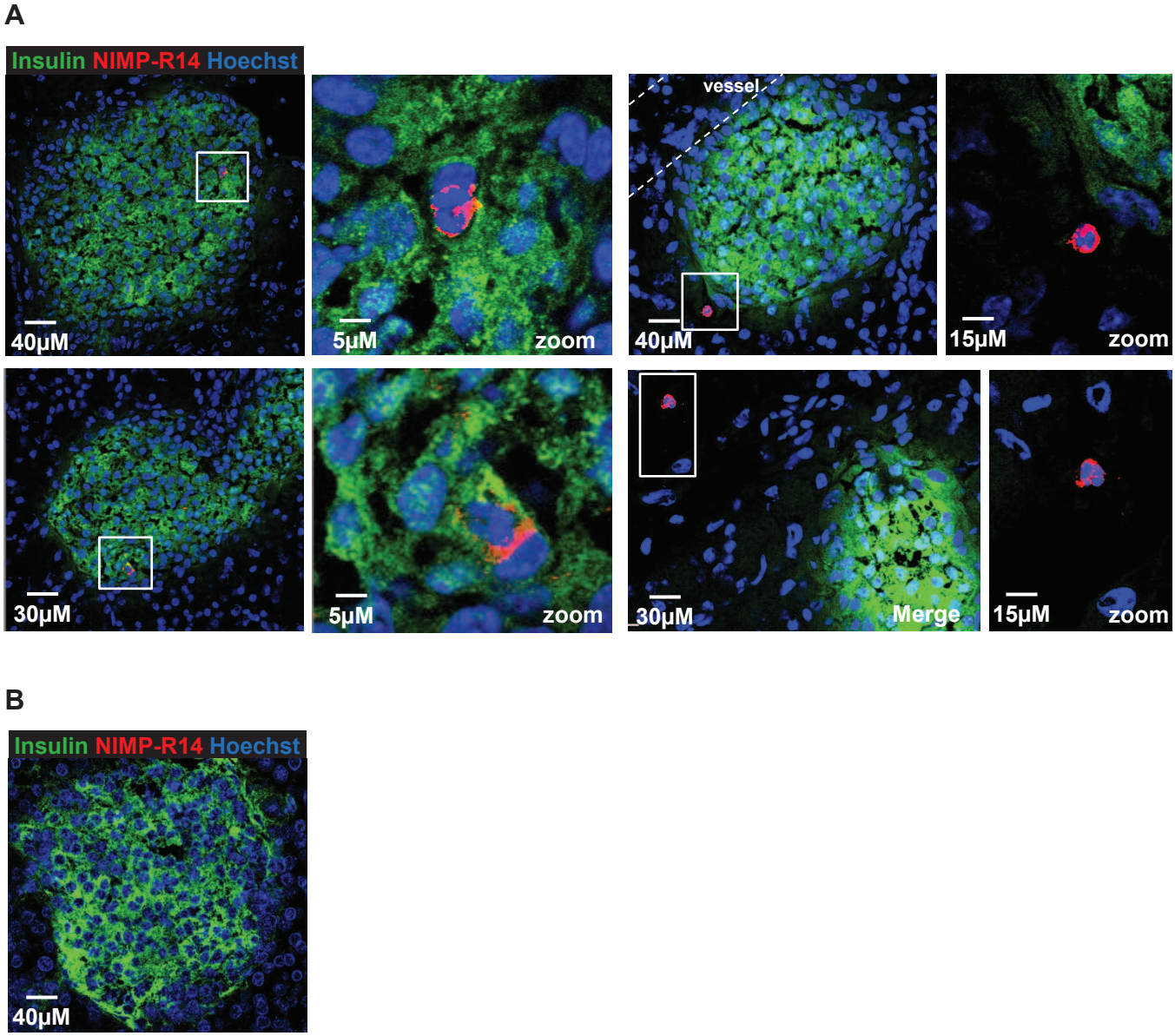


Figure S2. Neutrophils infiltrate the pancreatic islets of 3-week-old NOD mice. Immunofluorescence analysis of neutrophils (NIMP-R14+ cells) and insulin in the pancreatic islets of 3-week- (**a**) or 6-week- (**b**) old NOD mice. The pancreas was isolated in OCT and frozen in liquid nitrogen. Consecutive 5 μM sections were cut and mounted on slides and then fixed in cold acetone. Immunofluorescence were performed using polyclonal guinea pig anti-insulin antibody at a 1:100 dilution (DakoCytomation) as the primary antibody, neutrophils were stained using the NIMP-R14 mAb (1 μg/mL). Representative data from 9 pancreases with 10 sections for each pancreas from three independent experiments are shown.

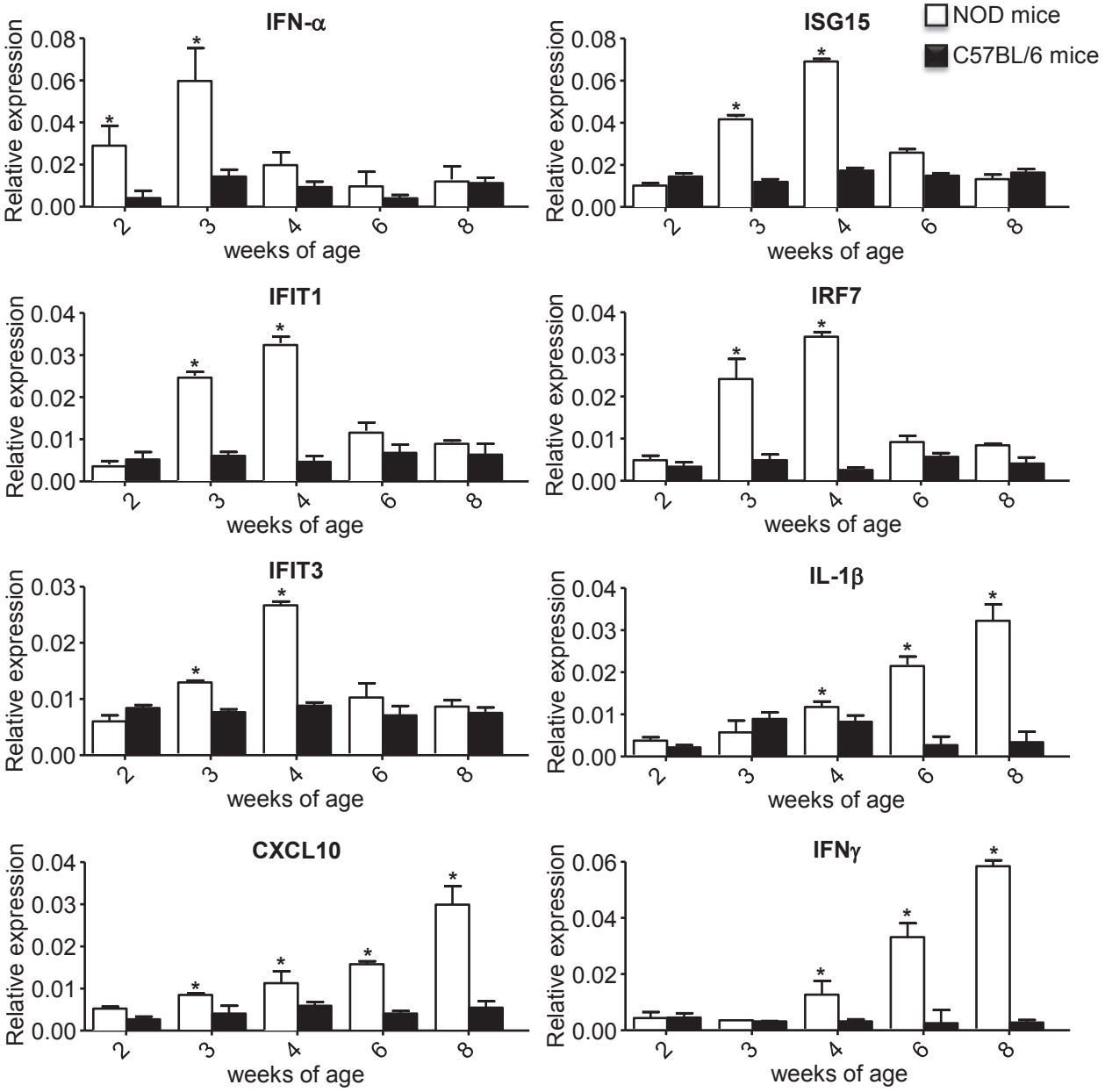


Figure S3. mRNA expression of IFNα-induced genes in the pancreatic islets. Islets were recovered from female C57BL/6 mice or NOD mice at various ages. Pancreas were prepared as described in Methods. Quantitative-PCR was performed for the expression of IFNα-induced genes. Data were normalized to *gapdh* housekeeping gene. Data are mean values +/- s.e.m. of two independent experiments with four independent mice for each group. *: $P < 0.05$.

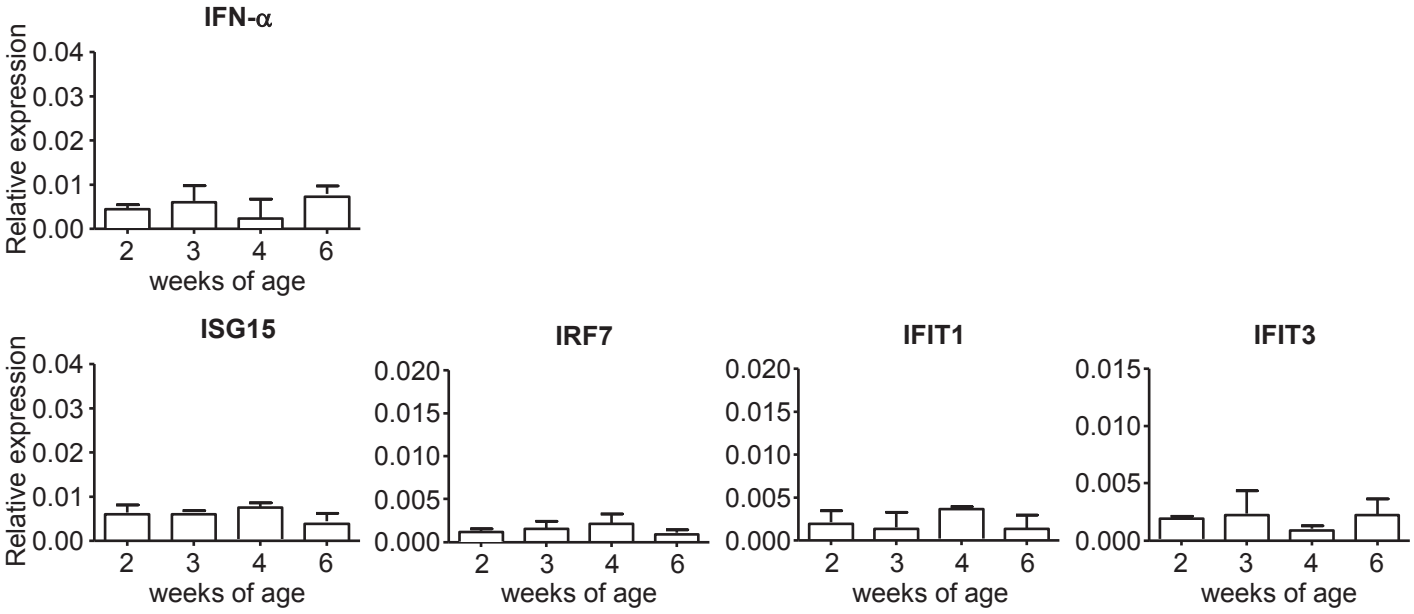
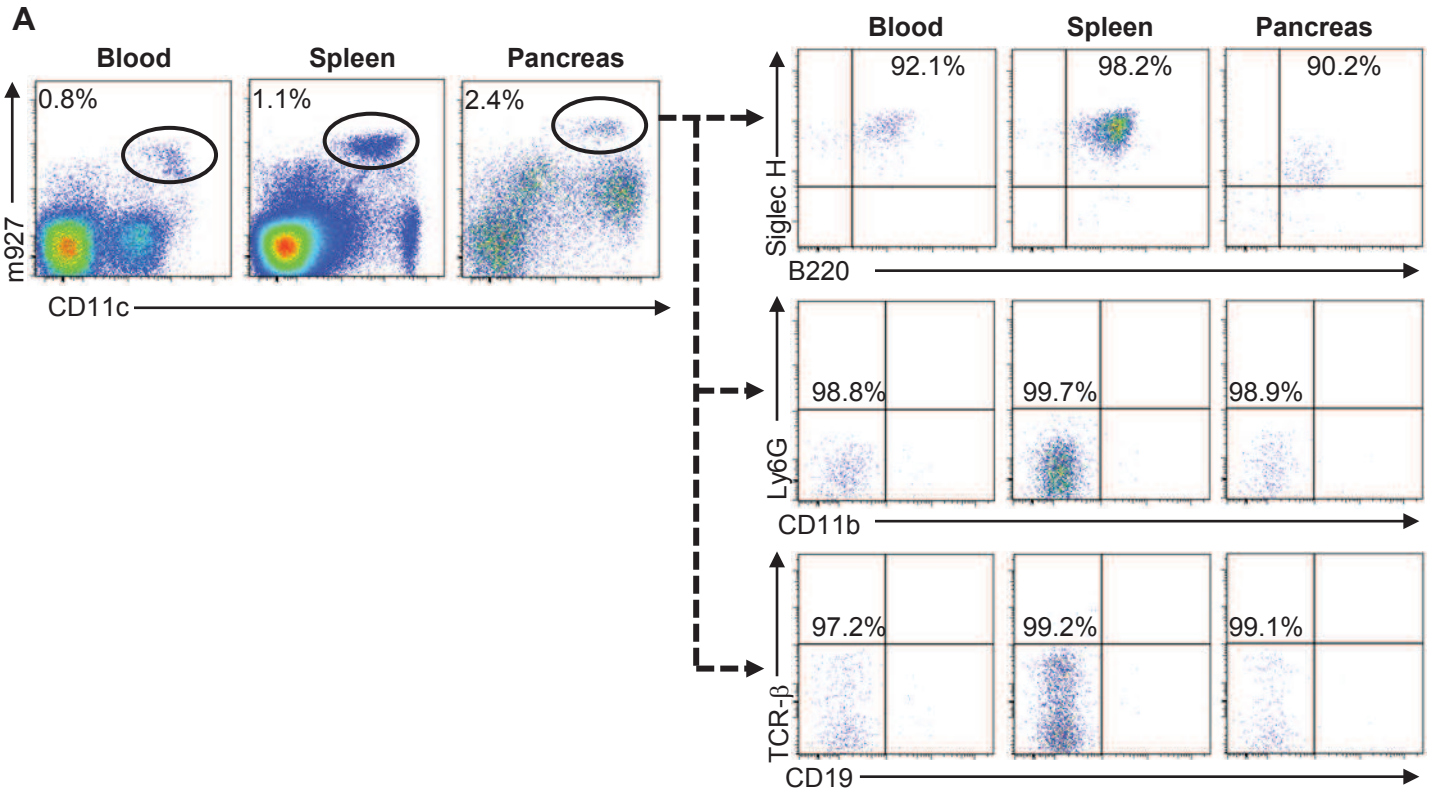
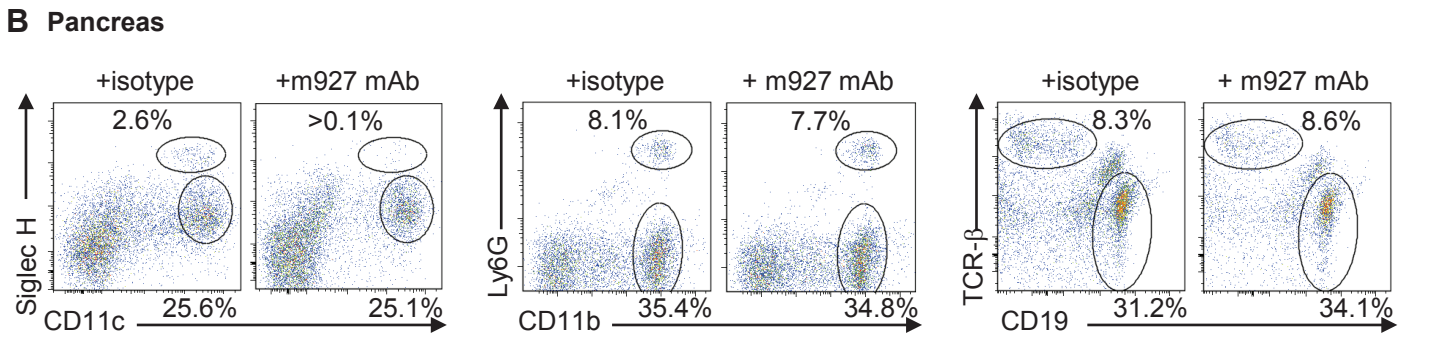


Figure S4. mRNA expression of IFNα-induced genes in the pancreatic islets. Islets were recovered from female BALB/c mice at various ages. Pancreas were prepared as described in fig S1. Quantitative-PCR was performed for the expression of IFNα-induced genes. Data were normalized to *gapdh* housekeeping gene. Data are mean values +/- s.e.m. of two independent experiments with four independent mice for each group. *: $P < 0.05$.





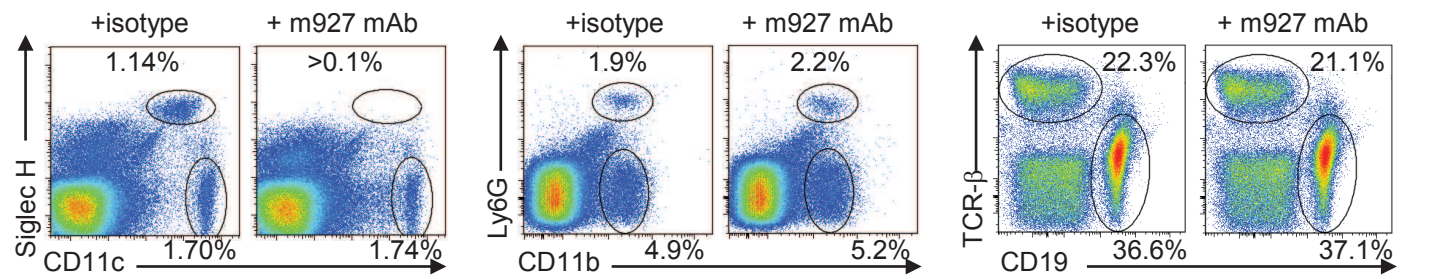


Figure S5. Efficacy and specificity of m927 mAb treatment on pDCdepletion in NOD mice. (a) Cells from the blood, the spleen and the pancreas of 3-week-old NOD mice were recovered and stained with m927, Siglec-H, anti-Ly6G, anti-CD11b, anti-CD11c, anti-CD19, anti-TCR-β and anti-CD45 mAbs. **(b-c)** NOD mice were treated at 2 weeks of age during 2 weeks with 2 injections in the week of m927 mAb or isotype control (500 µg, i.p.). Cells were recovered 2 days after the last treatment from the pancreas (b) and the spleen (c) and surface stained with Siglec-H, anti-CD11c, anti-CD11b, anti-CD19, anti-Ly6G, anti-TCR-β and anti-CD45 mAbs. Data indicate the frequency among $CD45^+$ cells. Data are representative of three independent experiments with two pooled mice for each group.

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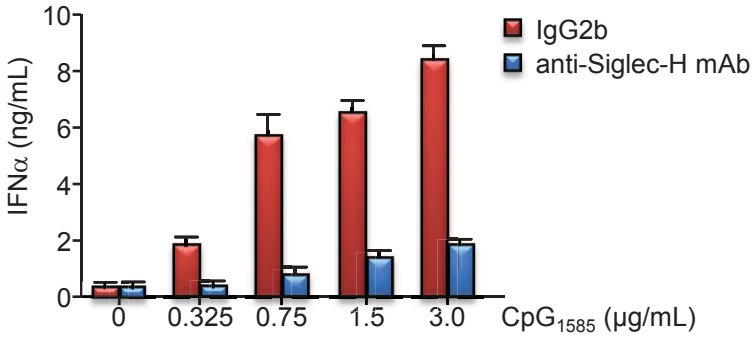

Figure S6. Blockade of IFNα production by pDCs after anti-Siglec-H treatment. BMpDCs were cultured for 24 hours in medium alone or with varying concentrations of CpG-ODN$_{1585}$. Cells (4x10^5/100 µL) were cultured on 96-well plates coated with anti-Siglec-H mAb (ebio440c, 5 µg/mL, ebioscience) or control rat IgG2b. Culture supernatants were collected and tested for IFNα by ELISA. Data are mean values +/- s.e.m. of two independent experiments.

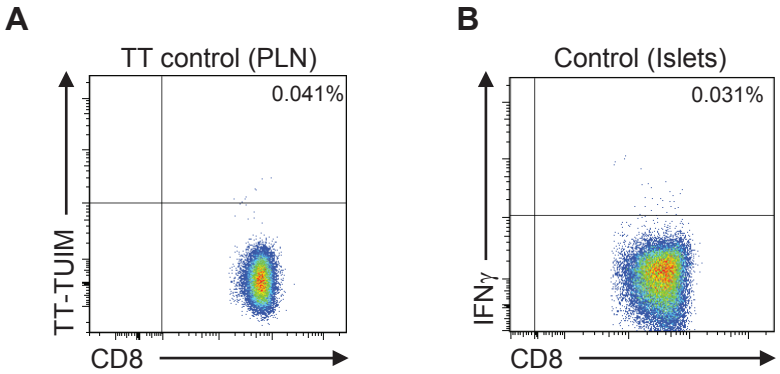

Figure S7. Control staining for IGRP$_{206-214}$ specific CD8$^+$ T cell response. (**a**) Cells from the pancreatic lymph nodes of 8-week-old NOD mice were stained with TUM-tetramer and then surface stained with anti-TCRβ and anti-CD8 mAbs. Values correspond to the frequency of tetramer-specific cells among the CD8$^+$ T cell gate. (**b**) Pancreatic islets were harvested and cultured for 8 days in complete RPMI medium with rIL-2. Cells were then stimulated for 5 h with peptide diluent (DMSO) and stained with anti-TCRβ and anti-CD8 mAbs and then IFNγ mAb. Values correspond to the frequency of IFNγ$^+$ cells among the CD8$^+$ T cell gate.

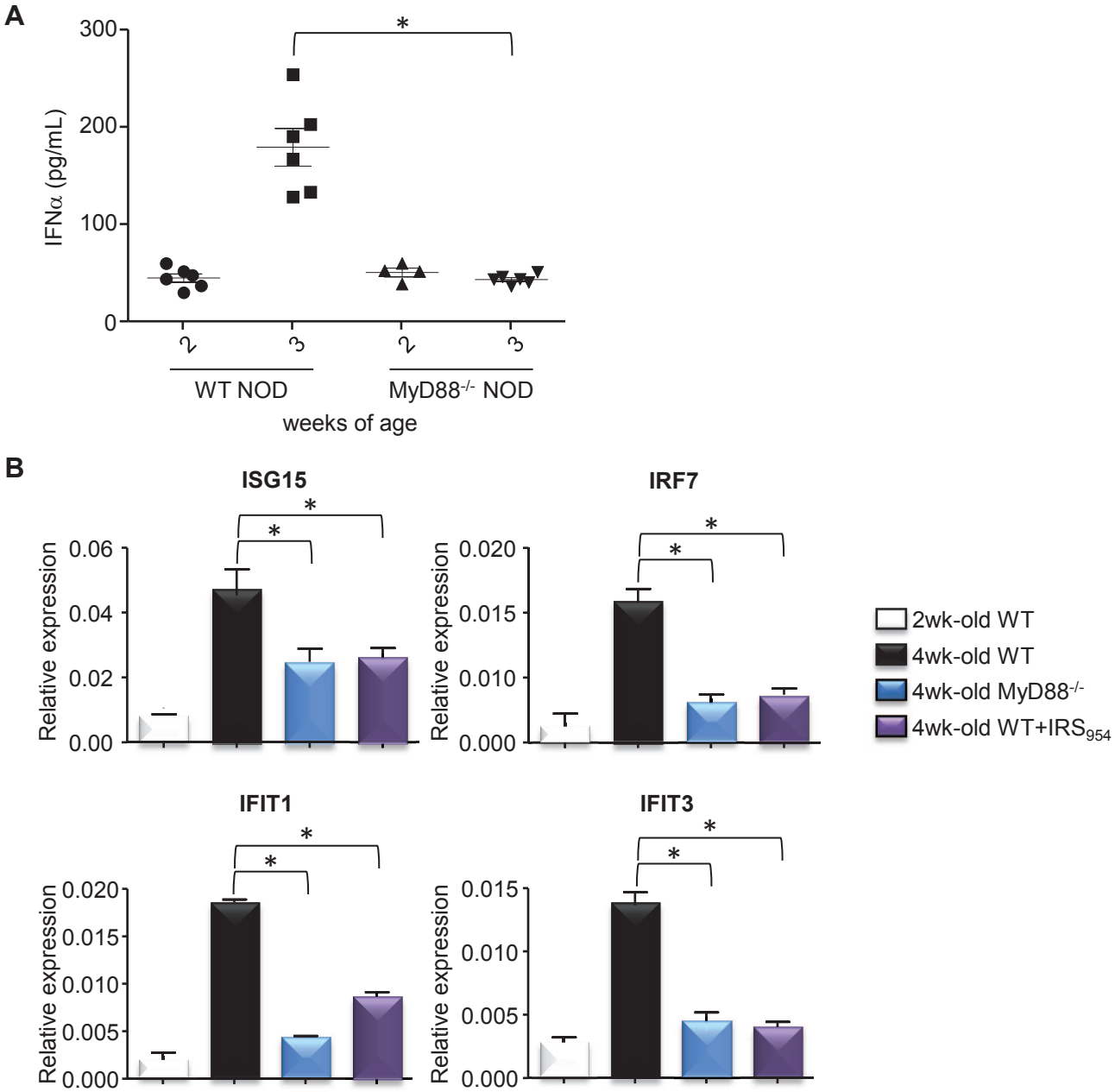

Figure S8. IFNα production in the pancreas is dependent on TLR7/9 pathway. (**a**) IFNα production in the pancreatic islets of WT or MyD88$^{-/-}$ NOD mice at 2 and 3 weeks of age. Pancreatic islets were handpicked and cultured overnight. IFNα secretion was measured by ELISA in the culture supernatant. Data are mean values +/- s.e.m. of two independent experiments with three independent mice for each group. *: $P < 0.05$. (**b**) Pancreatic islets of WT or MyD88$^{-/-}$ NOD mice treated or not with IRS_{954} were recovered at 2 and 4 weeks of age and mRNA expression for IFNα-induced genes were analyzed by quantitative PCR. Data are mean values +/- s.e.m. of two independent experiments with four independent mice for each group. *: $P < 0.05$.

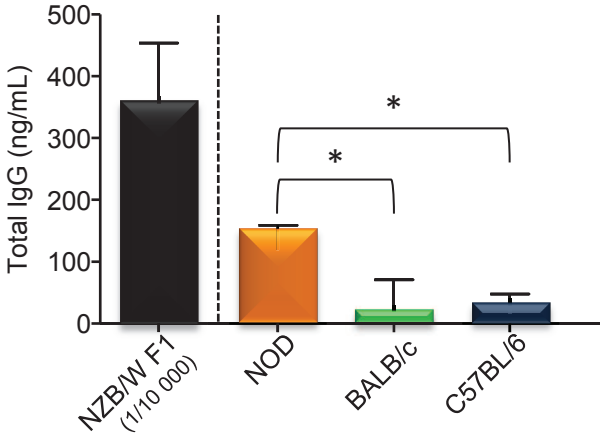

Figure S9. Production of IgG in the pancreatic islets. Pancreatic islets of 3-week-old-NOD, BALB/c or C57BL/6 mice were cultured for 48h in RPMI complete medium. As positive control, the serum of 3-month-old-NZB/W F1 mice was used diluted 1/10 000 in RPMI. The secretion of IgG was measured by ELISA. Data are mean values +/- s.e.m. of four independent experiments with three independent mice for each group. *: $P < 0.05$.

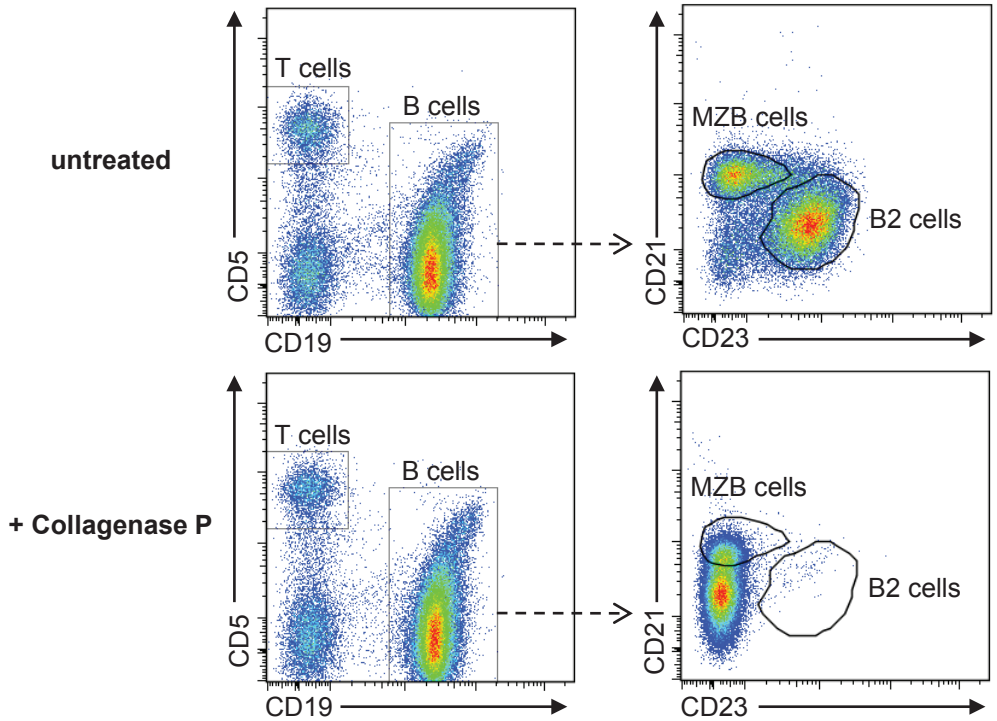

Figure S10. Collagenase P treatment alters B cell markers. Splenocytes of NOD mice were incubated 5 min at 37°C in a solution containing collagenase P (1 mg/ml) or in PBS. Surface staining was performed with anti-CD45 (clone 30-F11), anti-CD19 (clone 1D3), anti-CD5 (clone 53-7.3), anti-CD21/CD35 (clone 4E3) and anti-CD23 (clone B3B4). Data are representative of two independent experiments performed with 2 independent mice and 2 independent batches of collagenase P.

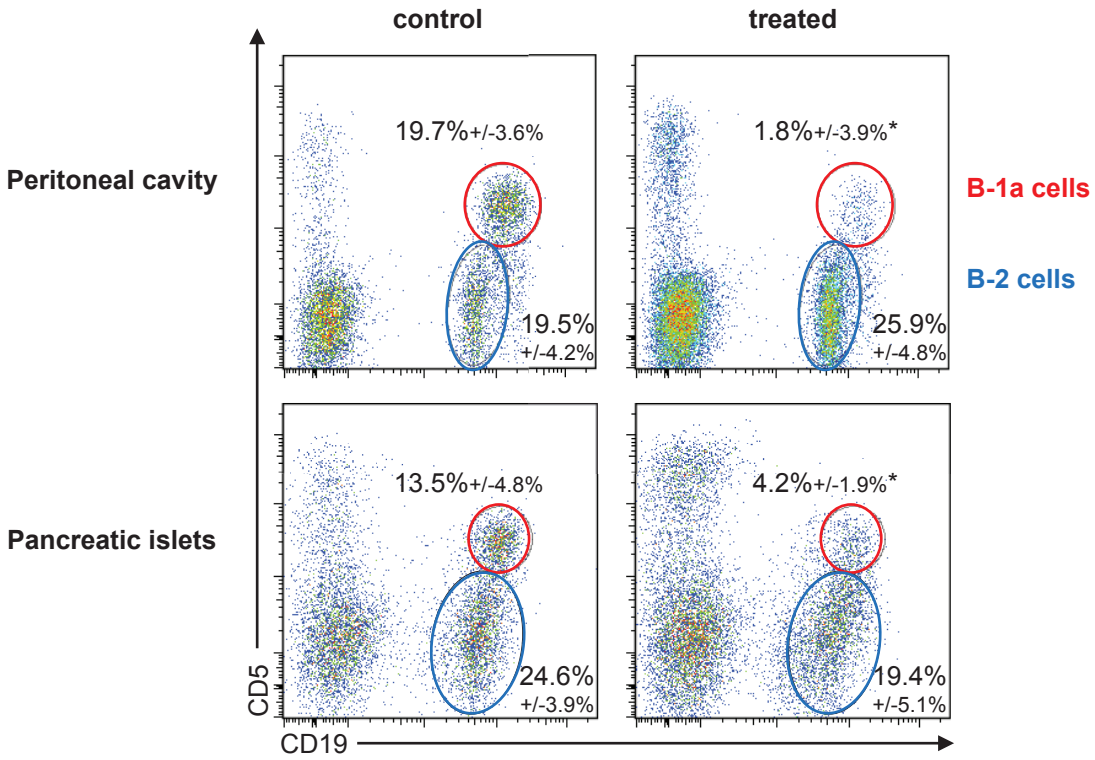

Figure S11. Efficacy and specificity of B-1a cell depletion in the pancreatic islets and peritoneal cavity of NOD mice at 3 weeks of age. 1-week-old NOD mice were treated with intraperitoneal injection of 500 µL of water (treated) or PBS (control) every two days during 14 days. Data are the frequency of cells among $CD45^+$ cells recovered 2 days after the last treatment after staining of cells from pancreatic islets and peritoneal cavity with anti-CD45, anti-CD19 and anti-CD5 mAbs. Data are mean values +/- s.e.m. of four independent experiments with four pooled mice for each group. *: $P < 0.05$ for treated group compared to untreated group.

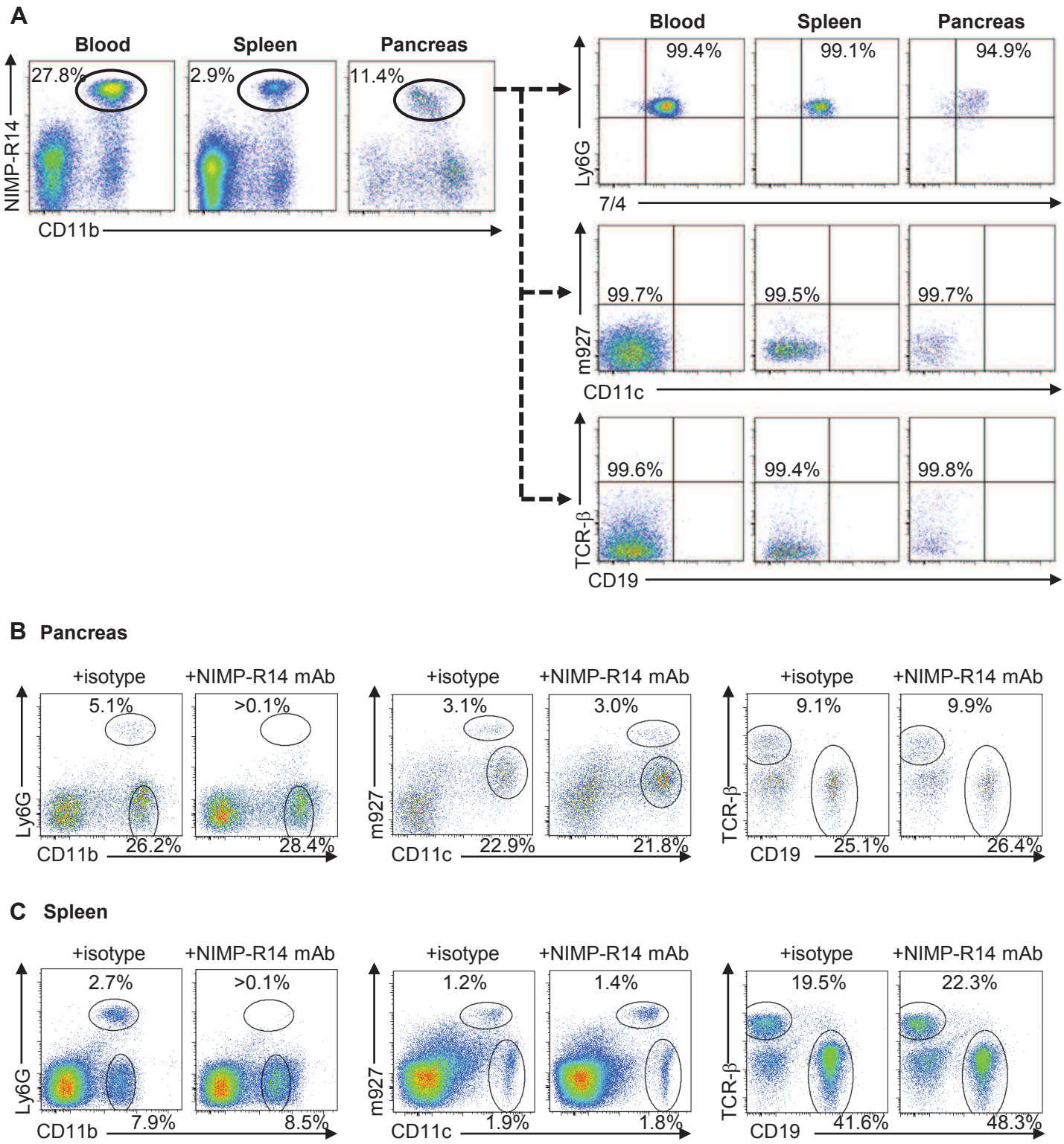

Figure S12. Efficacy and specificity of NIMP-R14 mAb treatment on neutrophil depletion in NOD mice. (a) Cells from the blood, the spleen and the pancreas of 3-week-old NOD mice were recovered and stained with NIMP-R14, anti-Ly6G, anti-7/4, anti-CD11b, m927, anti-CD11c, anti-CD19, anti-TCR-β and anti-CD45 mAbs. **(b-c)** NOD mice were treated at 2 weeks of age during 2 weeks with 2 injections in the week of NIMP-R14 mAb or isotype control (250 µg, i.p.). Cells were recovered 2 days after the last treatment from pancreas (b) and spleen (c) and surface stained with anti-Ly6G, anti-CD11b, m927, anti-CD11c, anti-CD19, anti-TCR-β and anti-CD45 mAbs. Data indicate the frequency among CD45^{+} cells. Data are representative of three independent experiments with two pooled mice for each group.

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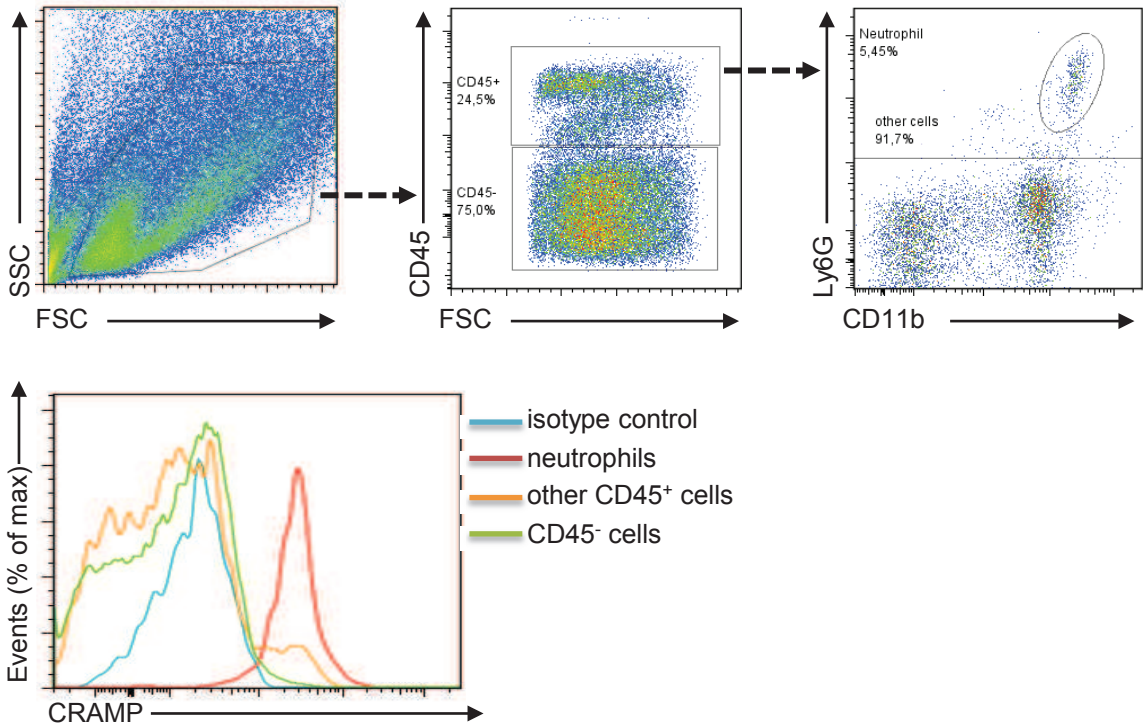

Figure S13. Surface CRAMP expression is detected only in the CD45+ CD11b+ Ly6G+ cells in the pancreatic islets of 3-weeks old NOD mice. Cells were recovered from pancreatic islets of 3-week-old NOD mice and stained with anti-CD45, anti-CD11b and anti-Ly6G mAbs, and then with anti-CRAMP pAb and anti-rabbit mAb. Data represent the frequency of cells among $CD45^+$ cells. Data are representative of three independent experiments with four pooled mice.

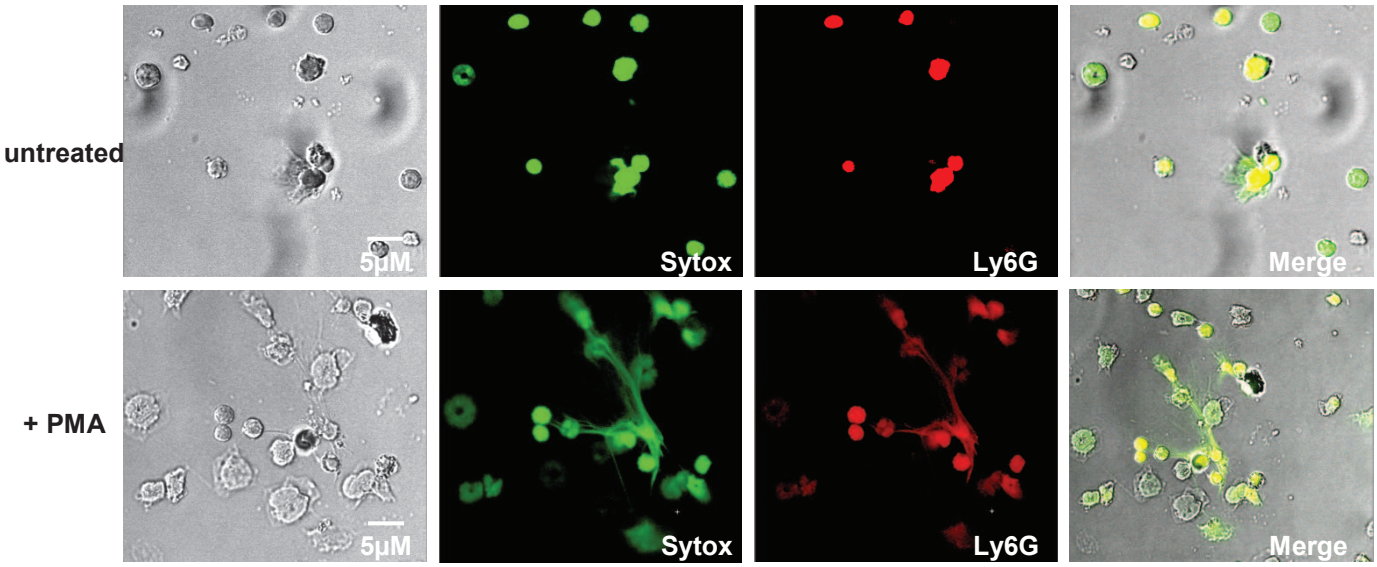

Figure S14. Neutrophils from bone marrow form NETs after PMA stimulation. $2x10^5$ cells from bone marrow were seeded on coated glass (0.001% polylysine; Sigma-Aldrich) for 4h at 37°C in RPMI-10%-FCS. Then, cells were fixed in 2% paraformaldehyde and stained with anti-Ly6G-conjugated mAb for 30 min on ice and counterstained for DNA with SYTOX® green (Invitrogen). Representative data from the bone marrow of 4 independent mice are shown.

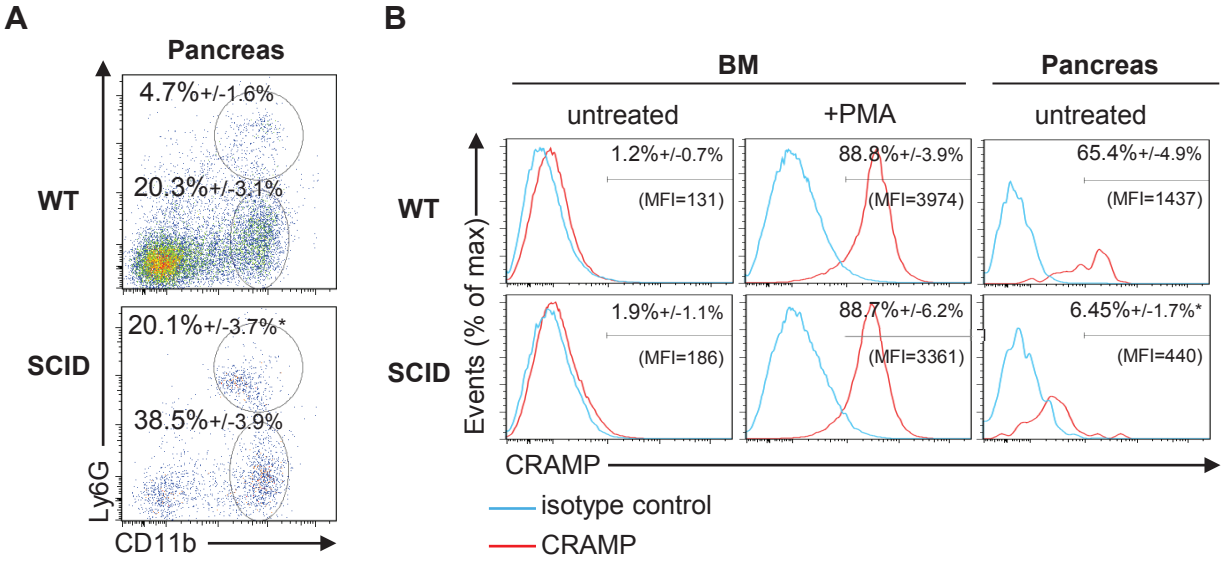

Figure S15. The activation but not the recruitment of neutrophils are affected in the pancreas of NOD SCID mice. (**a**) Cells were recovered from pancreatic islets of 3-week-old NOD mice and stained with anti-CD45, anti-CD11b and anti-Ly6G mAbs. Data represent the frequency of cells among $CD45^+$ cells. (**b**) Cells were recovered from bone marrow (BM) or pancreatic islets from NOD mice and stained with anti-CD45, anti-CD11b and anti-Ly6G mAbs, and then with anti-CRAMP pAb and anti-rabbit mAb. In some conditions cells were stimulated for 20 min with PMA (50nM) before staining. Data are mean values +/- s.e.m. of three independent experiments with four pooled mice for each group. *: $P < 0.05$ for SCID group compared to WT group.

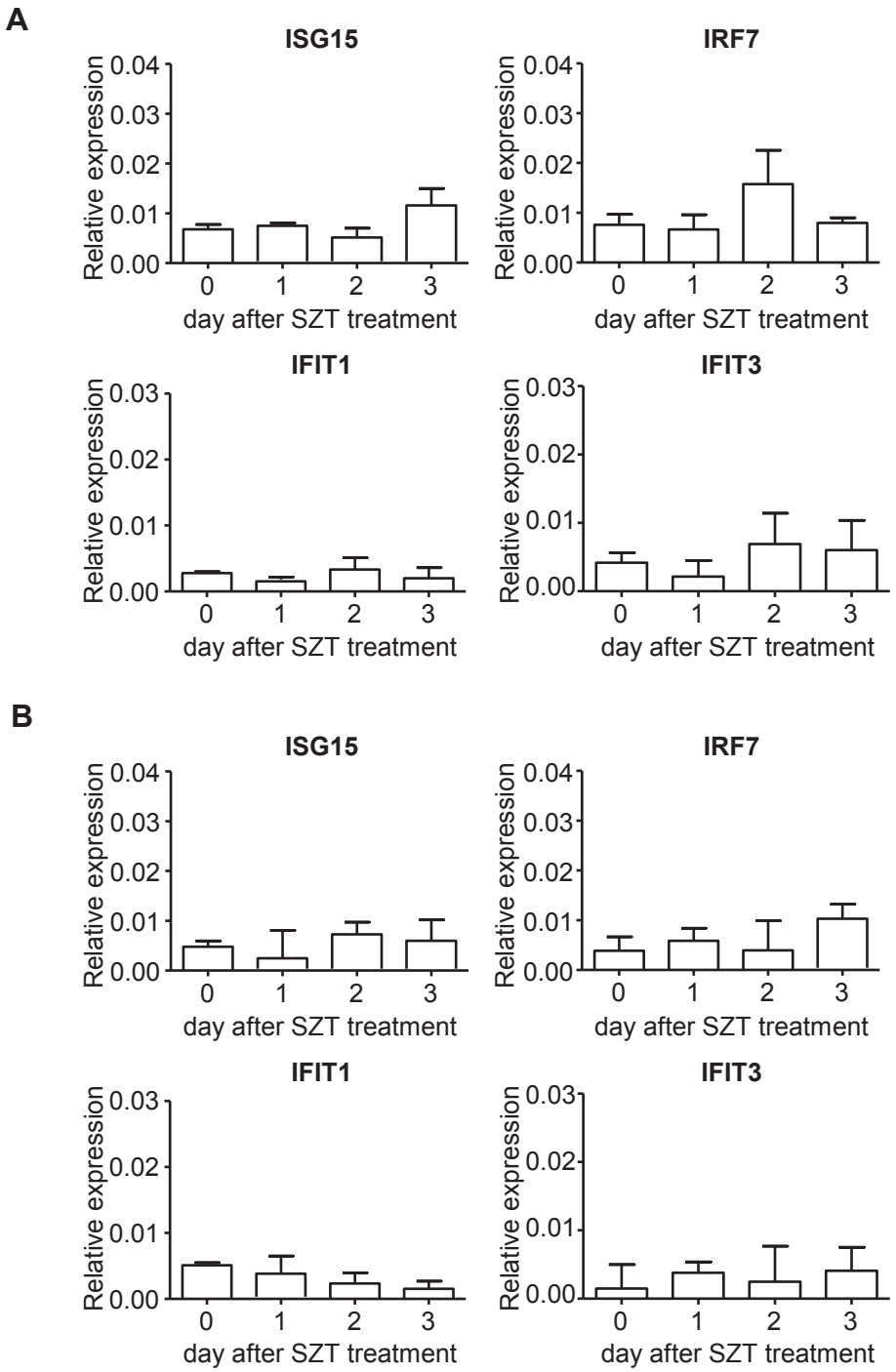

Figure S16. mRNA expression in pancreatic islets of C57BL/6 (a) or BALB/c (b) females (6-week-old) after streptozotocin injection. Pancreatic islets were prepared as described in fig S1. Quantitative-PCR on pancreatic islets was performed for the expression of IFNα-induced genes. Data were normalized to *gapdh* housekeeping gene. Data are mean values +/- s.e.m. of three independent experiments with three independent mice for each group.

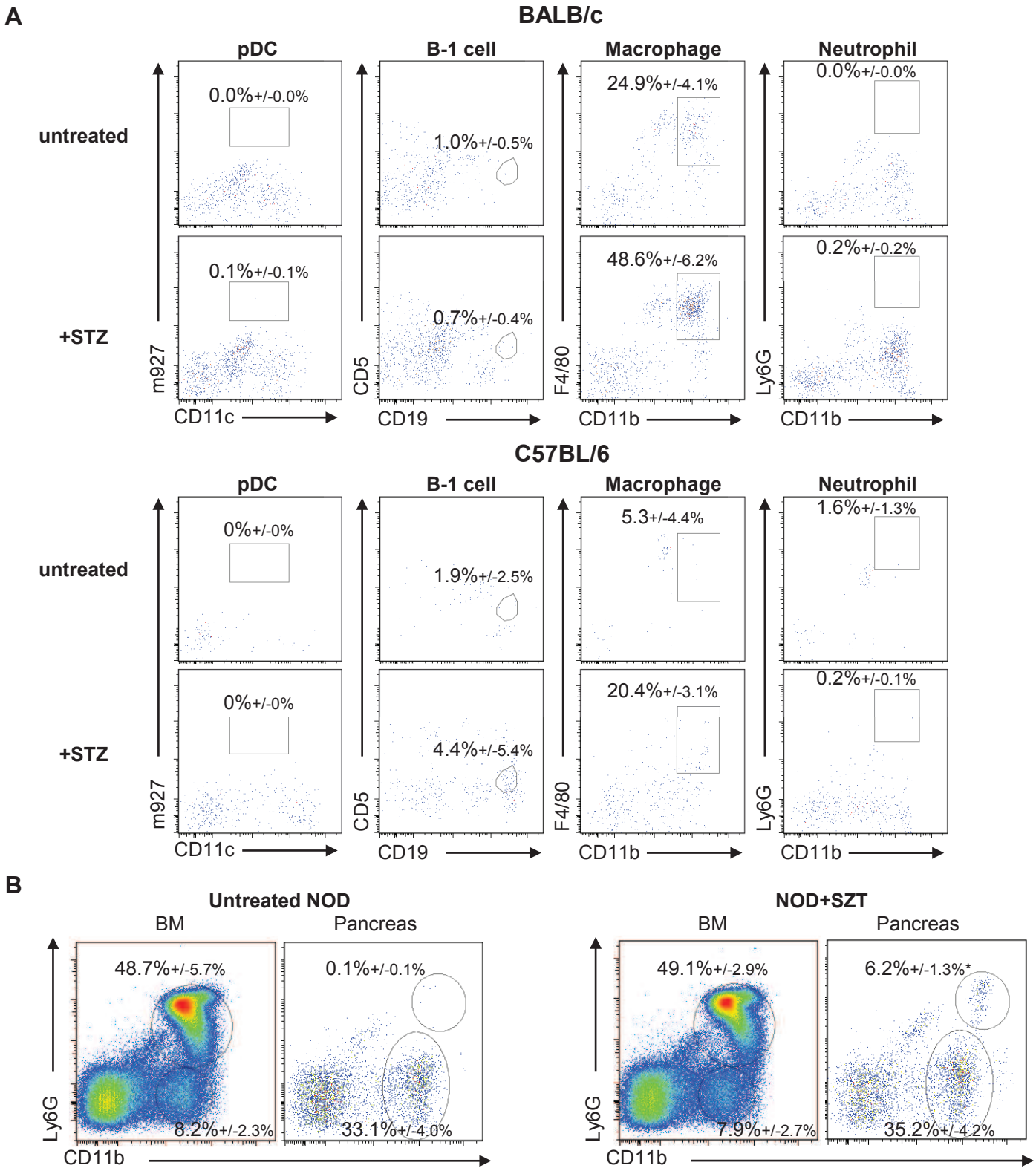

Figure S17. Streptozotocin treatment induced the recruitment of neutrophils specifically in the pancreas of NOD mice. Cells were recovered from pancreatic islets of 6-wk-old C57BL/6 and BALB/c (**a**) or from pancreatic islets or bone marrow of 6-wk-old NOD (**b**) mice treated 12h before with SZT (1.6 mg/mouse) and stained with anti-CD45, anti-CD11c, m927, anti-CD5, anti-CD19, F4/80, anti-CD11b and anti-Ly6G mAbs. Data represent the frequency of cells among $CD45^+$ cells. Data are mean values +/- s.e.m. of two independent experiments with six (**a**) or two (**b**) pooled mice for each group. *: $P < 0.05$ for treated group compared to untreated group.

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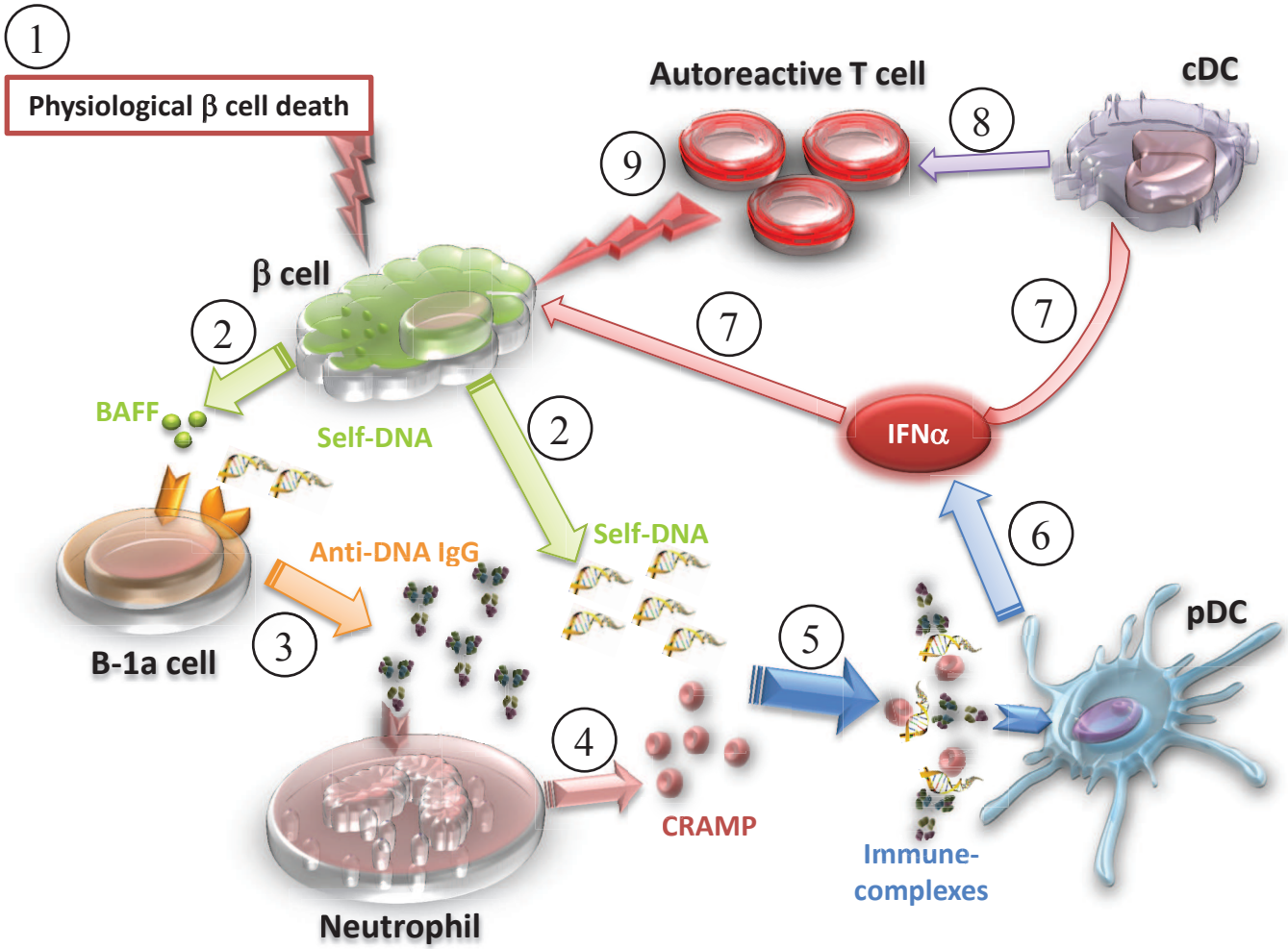

Figure S18. A schematic view of the innate immune cell crosstalk in the pancreas of NOD mice that initiates autoimmune diabetes. In young female NOD mice, when physiological β-cell death occurs, an innate immune cell dialog takes place in the pancreas that is critical for T1D development. Physiological β-cell death occurring at 2-3 weeks of age (1), induces the release of self-DNA and cytokines/chemokines (2) that recruit B-1a cells, neutrophils and pDCs in the pancreas. Activated B-1a cells produce anti-DNA IgGs (3). IgGs activate neutrophils to secrete CRAMP peptide that binds to self-DNA (4). Then, self-DNA, anti-DNA IgG and CRAMP peptide form immune-complexes (5) that are endocyted by pDC cells. Endocyted immune-complexes induce pDC activation through the TLR9-Myd88 pathways, leading to IFNα production in the pancreatic islets (6). Then, IFNα induces cDC maturation, and also impact directly on pancreatic β cells by inducing the secretion of cytokines/chemokines (7). Activated cDC would then migrate in the pancreatic lymph nodes and present islet antigens to naïve autoreactive T cells (8). This pleiotropic effect on the effector and target cells allows dramatic destruction of β cells and the development of autoimmune diabetes (9).

I-2.2 Discussion.

Nous avons observé chez des souris NOD que la mort physiologique des cellules β conduit à l'activation des cellules de l'immunité innée : les neutrophiles, les lymphocytes B-1a et les cellules dendritiques plasmacytoïdes (pDC). La coopération entre ces cellules conduit à l'activation des pDC qui produisent de l'IFNα. Cette cytokine active les lymphocytes T auto-réactifs qui vont détruire les cellules β du pancréas (cf : discussion article) (Figure I-2.2).

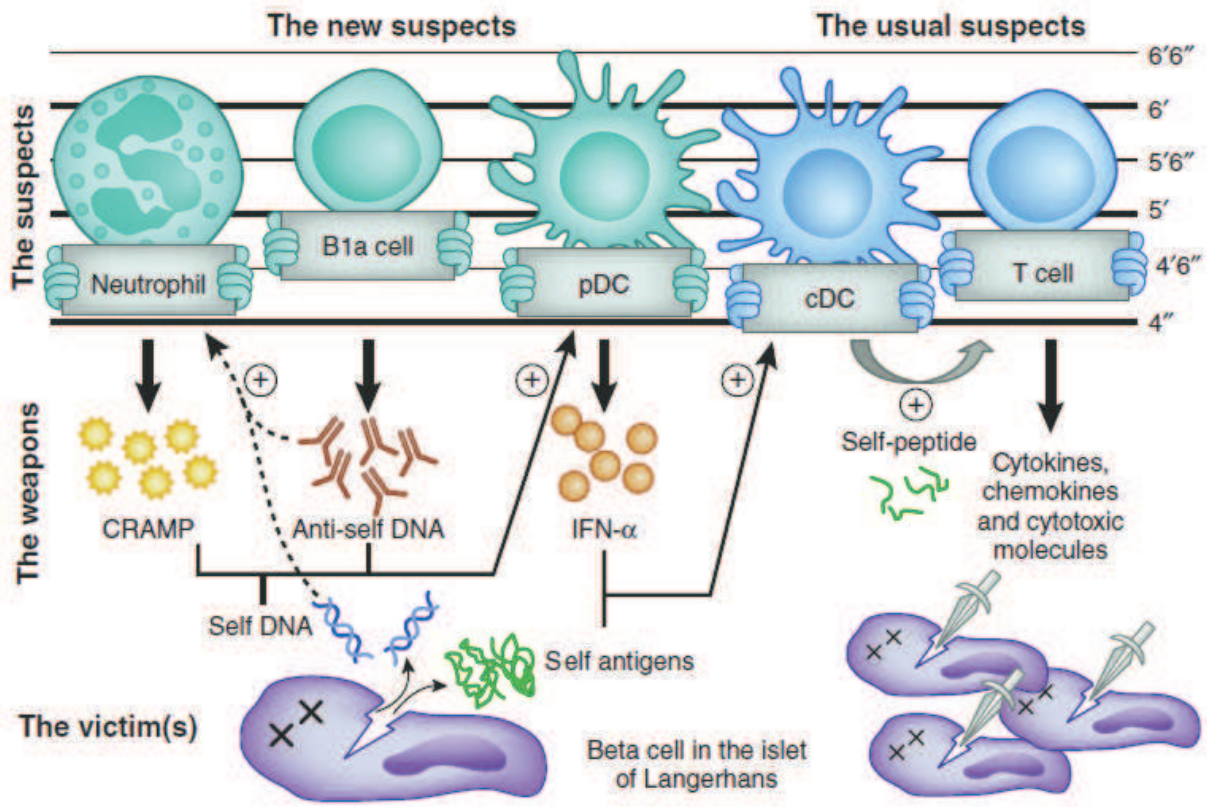

Figure I-2.2. Mécanisme proposé pour l'initiation du T1D chez la souris NOD
Figure d'après Creuzo [237].

La mort des cellules β: phénomène initiateur du T1D ?

Notre travail montre que l'inhibition de la mort des cellules β, par le traitement des souris NOD avec la molécule anti-apoptotique ZVAD à 2-3 semaines d'âge, réduit l'incidence du T1D. Deux autres groupes ont montré que le traitement des souris NOD avec la molécule ZVAD, inhibe la prolifération des lymphocytes T auto-réactifs dans les pLN [30, 231]. Ce résultat est confirmé par l'utilisation de la souris déficiente en caspase 12 (protéine pro-apoptotique). Cette souris présente un défaut de prolifération des lymphocytes T auto-réactifs à 3 semaines d'âge dans les pLN [30]. Cependant, l'utilisation de la souris NOD RIP-Bcl2[m],

[m] La souris NOD RIP-Bcl2 exprime spécifiquement la molécule anti-apoptotique Bcl2 dans les cellules β.

qui présente une apoptose réduite des cellules β, ne montre pas de défaut d'activation des lymphocytes T auto-réactifs dans les pLN à trois semaines [238]. Il est à noter que cette souris développe des tumeurs au niveau du pancréas [238]. L'influence de ces tumeurs sur le système immunitaire reste à étudier.

La streptozotocine[n] (STZ) est une molécule induisant la mort des cellules β. Nos données montrent que le traitement des souris NOD à 6 semaines d'âge avec une injection de STZ (80mg/kg) augmente le processus pro-inflammatoire. L'utilisation du même protocole conduit à une prolifération des lymphocytes T auto-réactifs chez les souriceaux NOD de 6 jours [30] et 9 jours [230, 231]. A 12 semaines, ce traitement provoque une augmentation de l'incidence du T1D [239].

Cependant, le traitement des souris NOD à 4 semaines d'âge avec une injection de STZ (40 ou 60mg/kg) induit une diminution de l'incidence du T1D. Ces doses de STZ induisent une apoptose limitée des cellules β. Cette protection est associée à l'induction de cellules régulatrices qui inhibent la réponse des lymphocytes T auto-réactifs [240].

L'ensemble de ces résultats montrent des rôles opposés de l'apoptose des cellules β sur le T1D. Le rôle immunogène ou tolérogène de la STZ semble être dépendant de l'âge auquel le traitement est effectué et de la concentration de STZ injectée.

Sachant que cette vague d'apoptose se produit dans toutes les souches de souris (i.e. NOD, C57Bl/6), pourquoi est-il observé une activation du système immunitaire uniquement dans la souche auto-immune NOD?

La comparaison entre souches de rongeurs a montré un défaut de phagocytose des corps apoptotiques par les macrophages des rongeurs développant un T1D (NOD et BB rats) [236, 241]. Ce résultat suggère que le défaut fonctionnel des macrophages conduit à une accumulation de débris cellulaires après la vague d'apoptose des cellules β dans le pancréas. La souris NOD présente une prédisposition génétique à l'apparition de maladie auto-immune (i.e. CMH). Cet excès d'auto-antigènes, associé au fond génétique de la souris, conduit à l'activation de lymphocytes auto-réactifs puis au T1D [30].

En conclusion, la vague d'apoptose des cellules β contribue à l'initiation du T1D. Cependant, nous ne pouvons exclure que d'autres mécanismes interviennent dans l'initiation de la maladie (i.e. microbiome).

[n] La STZ pénètre dans les cellules β via le récepteur Glut2.

Les neutrophiles dans le T1D chez l'homme.

En juin 2013, le groupe de M. Battaglia a publié un article montrant qu'une réduction du nombre de neutrophiles dans le sang précède l'apparition du T1D chez l'homme [242]. Les auteurs ont analysé la fréquence de ces cellules dans le sang de patients pré-diabétiques (présence d'auto-anticorps), récemment diabétiques ou diabétiques depuis plusieurs années. La neutropénie est spécifique des patients pré-diabétiques et récemment diabétiques. Cette diminution du nombre de neutrophiles n'est pas observée chez les patients T2D. De plus, les neutrophiles sont observés dans le pancréas des patients T1D mais pas chez les patients T2D ou individus contrôles [242]. Ce résultat confirme en partie nos données sur la présence de neutrophiles dans le pancréas lors du T1D. De nouvelles études permettront de définir la fonction des neutrophiles dans le T1D humain.

Les pDC et l'IFNα dans le T1D.

Grâce à la souris NOD Myd88-/- et aux antagonistes des TLR7/9, nous observons que l'activation des pDC est dépendante de la voie TLR7/9-Myd88. Ce résultat confirme les précédentes données obtenues par le groupe de JP Dutz, qui a observé une diminution de l'incidence du T1D dans les souris TLR9-/-. Cette souris, se caractérise par une diminution de la fréquence en pDC et en lymphocytes T auto-réactifs, ainsi qu'une diminution de l'expression d'IFNα dans les pLN par rapport à la souris NOD WT [243]. De la même manière, le traitement des jeunes souris NOD avec un antagoniste du TLR7 réduit l'expression d'IFNα et la fréquence des lymphocytes T auto-réactifs [244].

En janvier 2013, le groupe de M. Versnel a étudié la cinétique d'apparition dans le pancréas des souris NOD entre 4 et 20 semaines d'âge [245]. Comme nos données le montrent, la présence des pDC est observée à partir de 4 semaines d'âge dans le pancréas et est spécifique à la souris NOD. La fréquence de ces cellules augmente jusqu'à 10 semaines puis reste stable dans le pancréas. L'expression des récepteurs aux chimiokines CXCR3 et CXCR4 par les pDC ainsi que l'expression des chimiokines CXCL9, CXCL10 et CXCL12 par le pancréas pourraient induire le recrutement de ces cellules. Comme proposé précédemment [183], les pDC présentes dans le pancréas des souris NOD expriment la molécule IDO (co-localisation des marqueurs Siglec-H et IDO). Cependant, ces cellules n'expriment que très faiblement la molécule inhibitrice PD-L1 à leur surface. Cette étude ne montre pas de données fonctionnelles sur la fonction immunosuppressive des pDC sur les lymphocytes T dans le T1D. A l'inverse de nos observations, les auteurs ne détectent pas d'IFNα (protéine et

ARNm) dans le pancréas de la souris NOD. Nos expériences ont été faites sur des îlots pancréatiques purifiés et non sur le pancréas total comme l'ont fait les auteurs. Cette différence de protocole pourrait expliquer ces résultats contradictoires.

En avril 2013, le groupe de E. Unanue a caractérisé les premiers évènements se produisant dans les îlots pancréatiques de la souris NOD au niveau transcriptionnel. De manière similaire à nos observations, les auteurs montrent l'expression d'ARNm codant pour l'IFNα dans le pancréas. Cette expression précède l'activation des lymphocytes T [246].

Conclusion

L'ensemble de ce travail montre que l'immunité innée est un acteur important de la pathologie du T1D. Cette action fait intervenir différents types cellulaires et différentes molécules.

L'immunité innée intervient à différents stades de la maladie. Ainsi, les lymphocytes B-1a, les neutrophiles et les pDC sont impliqués dans l'initiation de la pathologie. Ces cellules vont contribuer à l'action du système adaptif.

A l'opposé, les lymphocytes iNKT jouent un rôle dans le développement du T1D. Ces cellules peuvent avoir un effet protecteur via l'induction de DC tolérogènes, ou un effet délétère par la production d'IL-17. De plus, bien que les pDC jouent un rôle délétère dans l'initiation de la maladie, ces cellules semblent avoir un effet protecteur à un stade plus tardif.

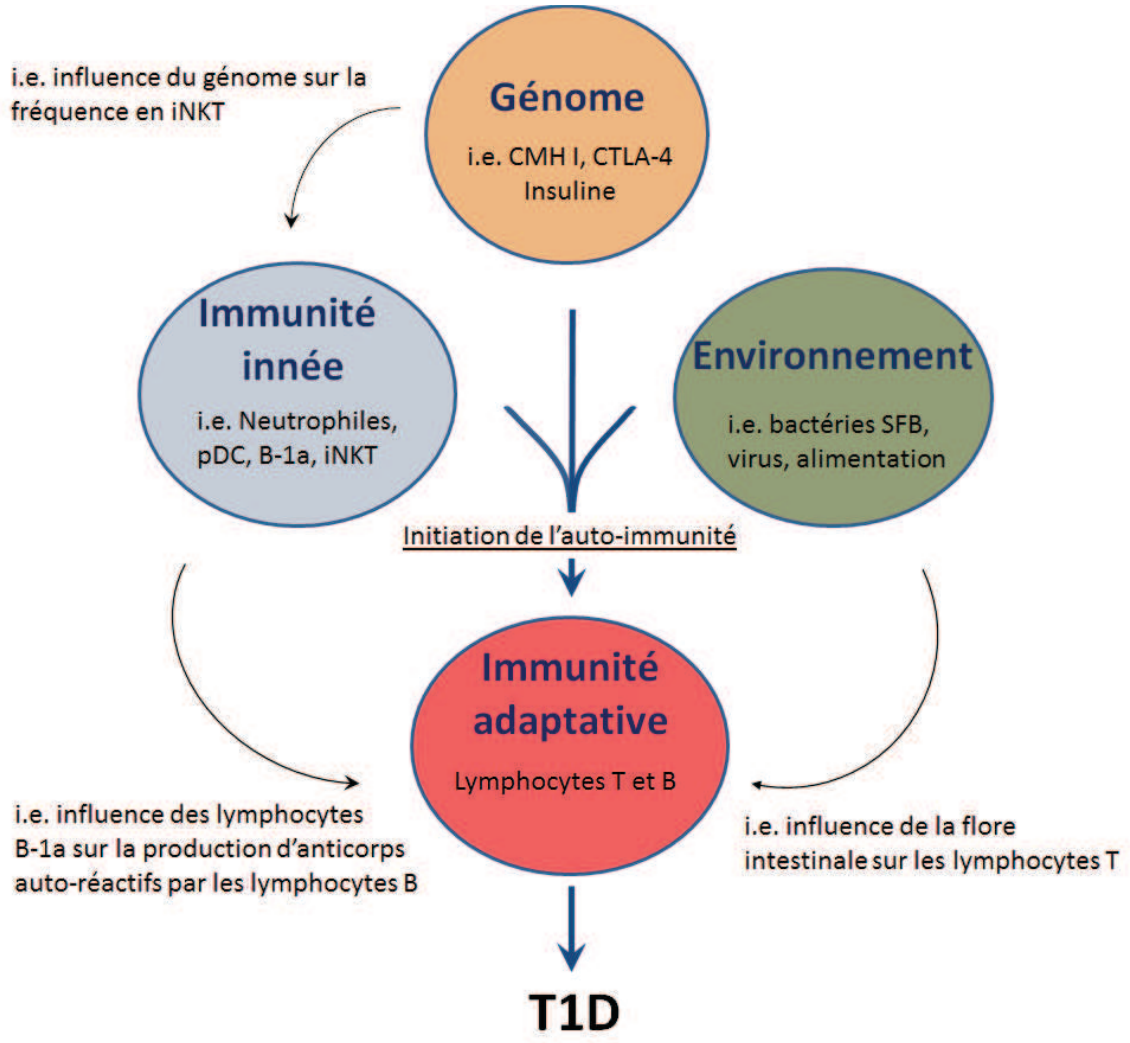

Figure. Facteurs influençant l'apparition du T1D.

Ce nouvel aspect de la pathologie du T1D est susceptible d'ouvrir de nouvelles voies thérapeutiques dirigées vers le ciblage des cellules immunitaires innées.

Toutefois, il serait important de mettre en place des outils plus efficaces qui permettront la détection de ces événements (i.e. activation des pDC, des neutrophiles) chez les sujets à risque.

Seconde partie

Introduction Le diabète de type 2

Chapitre 3 Les lymphocytes MAIT

Introduction Le diabète de type 2

II-0.a Caractéristique du diabète de type 2.

En 1956, Jean Vague a mis en évidence le lien entre l'obésité et l'apparition du diabète et ses complications [247]. En 1960, fut mis au point le premier test de détection de l'insuline [248]. Cette nouvelle technique a permis de mettre en évidence une hyper insulinémie chez les patients diabétiques. La résistance à l'insuline fut rapportée comme étant une caractéristique des patients diabétiques de type 2 en 1970 [249]. Une autre caractéristique mise en évidence chez les patients diabétiques est la diminution de la masse des cellules β [250]. Les études épidémiologiques ont montré que les facteurs génétiques et environnementaux influencent le développement du T2D. L'apparition du T2D est étroitement liée à l'indice de masse corporelle (BMI) d'un l'individu. Ainsi, 80% des diabétiques de type 2 ont une BMI supérieure à 30. Une personne ayant une BMI supérieure à 35 aura 42 fois plus de risques de développer le T2D que quelqu'un avec une BMI inférieure à 23 [251]. Les autres facteurs environnementaux favorisant le T2D sont, l'inactivité physique [252], l'âge [253], l'origine ethnique [254] et l'histoire familiale du T2D [255].

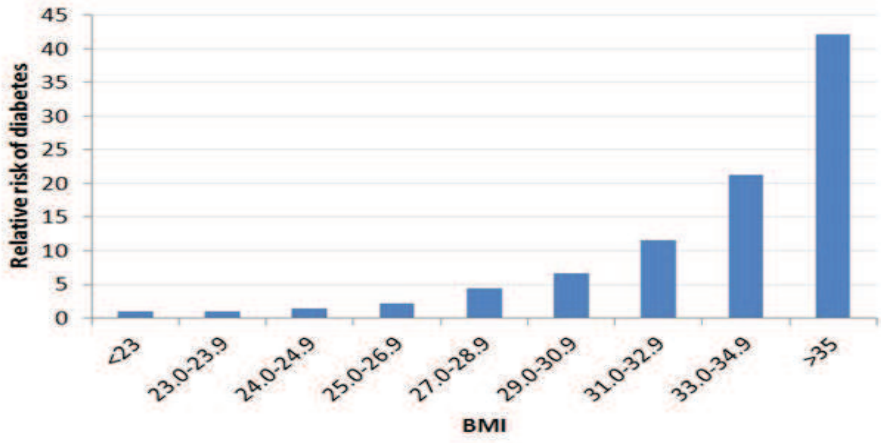

Figure II-0.a. Risque relatif de développement du T2D en fonction de la BMI d'une personne. D'après Chan *et al* [251].

Les études de cohortes ont montré une association entre le génome et l'apparition du T2D. Les études sur les jumeaux ont montré une concordance de 70% dans l'apparition du T2D chez les jumeaux monozygotes et de 20-30% chez les jumeaux dizygotes [256]. Les études GWAS ont permis de déterminer que plus de 65 variants génétiques étaient associés avec un

risque de développement du T2D. Cependant, une grande partie de ces variants sont présents dans des régions non codantes pour des protéines [257]. De manière inattendue, les analyses génétiques montrent que la majorité des gènes impliqués dans le T2D ne sont pas associés à l'insulino-résitance mais à la fonction des cellules β. Ces gènes influencent la sécrétion de l'insuline [258].

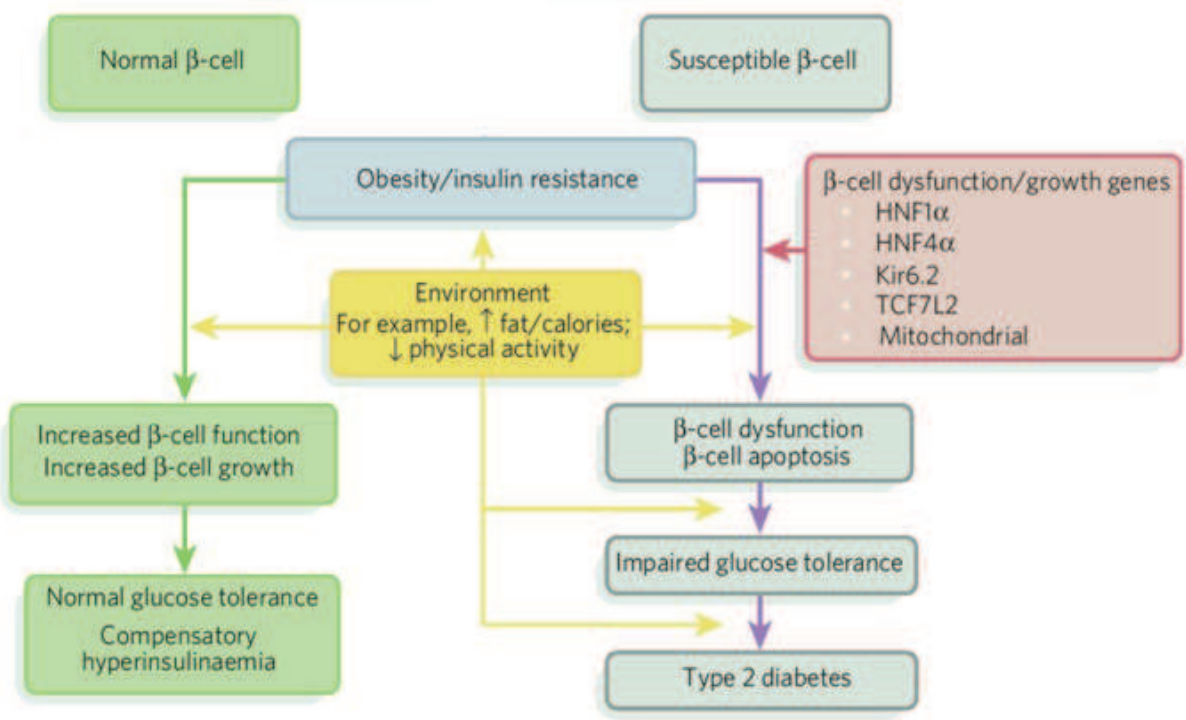

Figure II-0.a1. Modèle d'apparition du T2D chez l'homme. Les facteurs environnementaux (i.e. régime alimentaire, faible activité physique…) favorisent l'apparition du surpoids/l'obésité puis de l'insulino-résitance. Afin de contrer cette résistance à l'insuline, les cellules β augmentent leur sécrétion d'insuline. En présence de prédisposition génétique (en rose), les cellules β n'arrivent pas à contrebalancer l'effet de l'insulino-résitance. L'hyperglycémie chronique conduit au T2D. Au contraire, en l'absence de facteurs de risques génétiques (en vert), Les cellules β augmentent leur sécrétion d'insuline et ainsi compensent l'hyperglycémie. Figure modifiée d'après Khan *et al* [259].

II-0.b Le diabète de type 2 : une maladie auto-inflammatoire ?

A partir des années 90, des cytokines pro-inflammatoires ont été misent en évidence dans le sang des patients atteints du T2D (i.e. protéine de la phase aigüe, TNF, IL-6) [260]. Dans le sérum des patients, il a été observé une augmentation d'IL-1β, d'IL-6 et IL-1Rα juste avant l'apparition du T2D. La présence de ces cytokines dans le sérum traduit l'activation du système immunitaire chez les patients T2D. La présence du TNF fut aussi bien observée dans le sang que dans le tissu adipeux des patients T2D. Le TNF participe à l'induction de la résistance à l'insuline [261]. Les premières études ont identifié les adipocytes comme source du TNF [262]. Cette notion fut révisée en 2003, après la découverte de macrophages dans le tissu adipeux [263]. Ainsi, il fut démontré que ces cellules étaient recrutées dans le tissu adipeux et avaient un phénotype pro-inflammatoire (ou macrophages M1) et produisaient du TNF [264]. Ce phénotype pro-inflammatoire est spécifique des patients obèses/diabétiques. Chez les patients ayant un poids normal, les macrophages présents dans le tissu adipeux ont un phénotype anti-inflammatoire (Macrophage M2) [265]. Ces observations furent la preuve d'une activation du système immunitaire chez les patients T2D (et plus généralement obèses).
Les macrophages sont les cellules immunitaires les plus représentées dans le tissu adipeux. Parmi les autres populations, il a été observé des cellules de l'immunité innée : mastocytes, neutrophiles, NKT et de l'immunité adaptative : lymphocytes B et lymphocytes T.
Chez la souris, les mastocytes favorisent l'apparition du T2D via la production des cytokines IL-6 et IFNγ [266]. Cependant, le rôle délétère de l'IL-6 dans le T2D reste controversé [267, 268]. Les neutrophiles aussi jouent un rôle délétère dans le T2D. Il semble que la production d'élastase par ces cellules dans le foie conduise à l'insulino-résitance [269].
Le rôle des cellules iNKT dans le T2D et l'insulino-résitance reste très controversé. L'analyse des souris déficientes en iNKT (Jα18-/-) montre que les cellules iNKT peuvent avoir un rôle protecteur [270], aucun effet [271], une faible exacerbation [272] ou délétère [273] sur l'insulino-résitance.
L'utilisation de modèles animaux a montré que contrairement aux lymphocytes T CD4 et Treg, les lymphocytes T CD8 contribuent à l'insulino-résitance via la production de cytokines [274, 275]. Dans le tissu adipeux, les Treg produisent de l'IL-10. Cette cytokine anti-inflammatoire inhiberait localement l'inflammation. Les lymphocytes Treg représentent 50% des lymphocytes T CD4 dans le tissu adipeux chez la souris normale. Cette fréquence tend à diminuer avec l'augmentation du poids [274]. Par conséquent, la diminution en lymphocytes

Treg pourrait expliquer le recrutement et l'action délétère de cellules immunitaires dans le tissu adipeux des souris obèses.

Les lymphocytes B ont la capacité d'activer les lymphocytes T, qui à leur tour produisent des cytokines pro-inflammatoires conduisant à l'insulino-résitance [276]. De plus, ces cellules produisent des auto-anticorps favorisant la résistance à l'insuline. Le mécanisme induisant cette insulino-résitance reste inconnu [277].

Il est a noté que sur l'ensemble des études portant sur l'implication du système immunitaire adaptatif (lymphocytes T et B), le(s) antigène(s) reconnu(s) par les lymphocytes T ou B n'ont pas été identifiés.

Le tissu adipeux n'est pas le seul organe dans lequel il y a un recrutement du système immunitaire. Les modèles animaux ainsi que les patients T2D présentent une inflammation dans les îlots pancréatiques [278, 279]. Il a été proposé que la production d'IL-1β par les macrophages pourrait induire la mort des cellules β du pancréas [280].

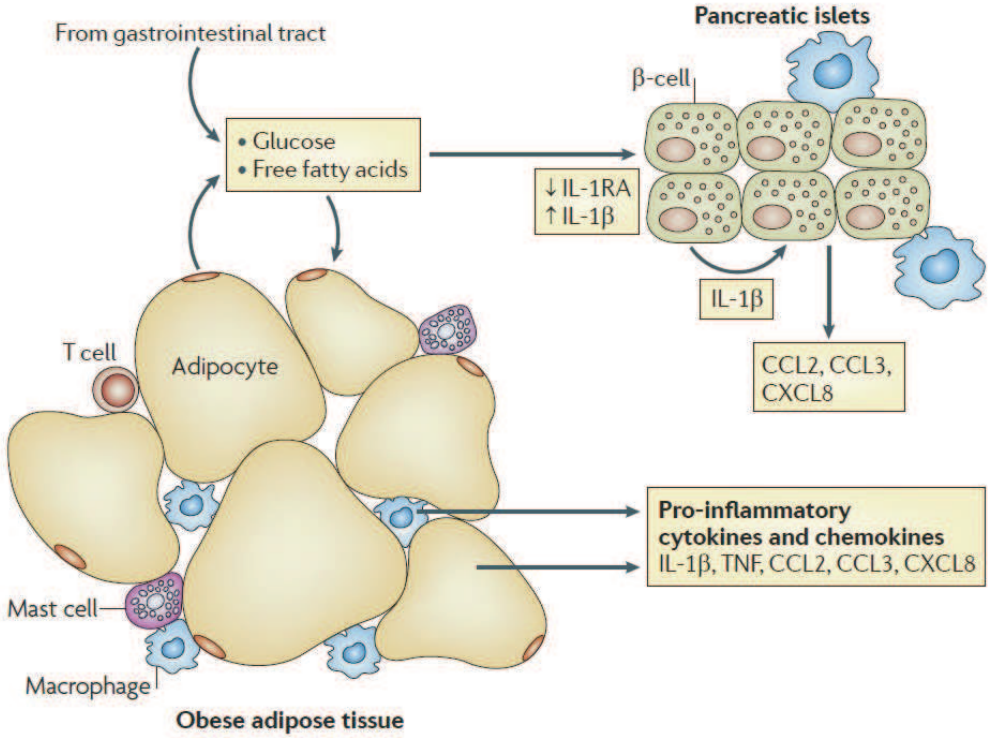

Figure II-0.b. Hypothèse sur le développement de l'inflammation dans le T2D. Un niveau excessif de nutriments (glucose et acides gras) induirait un stress et une insulino-résitance sur les différents tissus (i.e. tissus adipeux, îlots pancréatiques). Ce stress conduirait à la production locale de cytokines (IL-1β, TNF) et de chimiokines (CXCL8, CCL2, CCL3). A leur tour, les cellules immunitaires recrutées contribueraient à augmenter l'inflammation et l'insulino-résitance. Figure d'après Donath *et al* 2011[15]

II-0.c Rôle de la flore intestinale dans le diabète de type 2.

Un lien très fort existe entre surpoids et T2D (Figure II-0.1a). En 2006, une étude pionnière a montré que la composition bactérienne de la flore intestinale des souris obèses était différente de celle des souris non-obèses. La flore bactérienne des souris obèses a une plus grande capacité à récupérer l'énergie des nutriments. De ce fait, pour une même quantité de nutriments ingérés, l'apport énergétique pour l'organisme est plus important chez les souris obèses [64]. Ces données furent confirmées chez l'homme [281]. L'utilisation de modèles animaux a permis de mettre en évidence que l'inflammation locale induite par la flore intestinale influençait la tolérance au glucose et la résistance à l'insuline chez les souris [282, 283]. La flore intestinale modifie aussi la production d'hormones par l'intestin. Parmi ces hormones, GLP-1 et GIP induisent la production d'insuline par les cellules β [284].

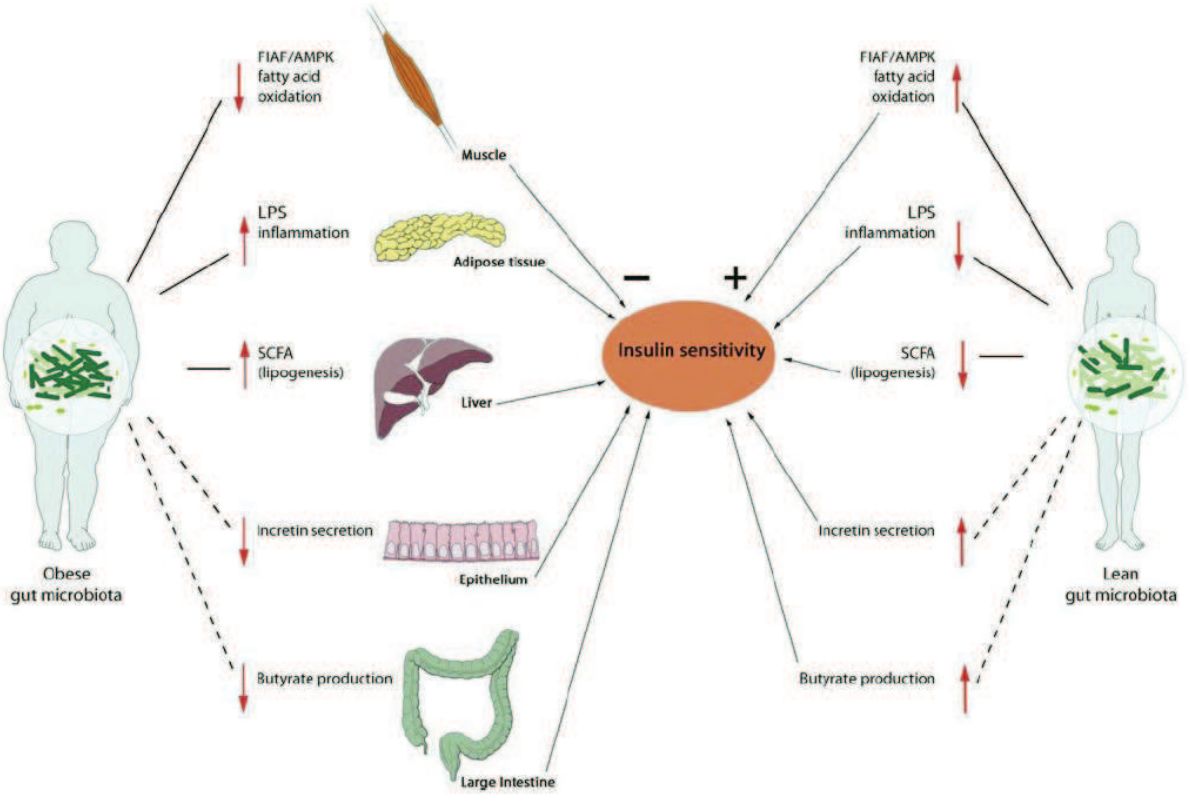

Figure II-0.c. Hypothèse sur les mécanismes liant la flore intestinale à la résistance à l'insuline. Figure d'après Vrieze *et al* 2010[284].

En 2012 et 2013, deux études se sont intéressées à la composition des différentes espèces bactériennes présentes dans la flore intestinale de patients T2D et contrôles [285, 286]. Ces deux études portant sur un nombre important de patients (345 et 145) ont mis en évidence une différence significative dans la composition bactérienne chez les patients T2D. Parmi ces différences, il est observé une diminution des bactéries produisant le butyrate (*Roseburia/E.*

rectale et *F. prausnitzii*). Le butyrate posséde la caractéristique d'être une molécule anti-inflammatoire, qui augmenterait la sensibilité à l'insuline [283]. Une réduction des bactéries produisant des gènes impliqués dans le métabolisme de la riboflavine (ou vitamine B2) est aussi observé.

En 2012, un des métabolites de la riboblovine a été identifié comme étant un ligand des lymphocytes MAIT [287, 288]. Cette observation met en lumière le lien possible entre le T2D et les lymphocytes MAIT.

Chapitre 3 Rôle des lymphocytes MAIT

II-3.1 Les lymphocytes MAIT.

En 1999 une population de lymphocytes T exprimant un TCRα invariant et restreint à la molécule non-conventionnelle du CMH de class Ib (ou MR1) a été caractérisée [289]. Cette chaine α invariante du TCR (Vα19-Jα33 chez la souris et Vα7.2-Jα33 chez l'homme) s'associe avec un nombre limité de chaine β (Vβ6, Vβ8 chez la souris et Vβ2, Vβ13 et Vβ22 chez l'homme). Ce TCR reconnait spécifiquement les antigènes présentés par la molécule MR1.

Le développement thymique des lymphocytes MAIT est différent de celui des lymphocytes T conventionels. Ceux-ci sont sélectionnés par les cellules épithéliales thymiques alors que les lymphocytes MAIT sont sélectionnés par des thymocytes $CD4^+$ $CD8^+$ (ce processus de sélection est similaire pour les cellules NKT) [290]. Cependant, le ligand endogène permettant la sélection des lymphocytes MAIT reste inconnu. Les lymphocytes MAIT expriment un programme de différenciation thymique qui leur est spécifique. C'est la reconnaissance de la molécule MR1 par le TCR, qui induirait ce programme spécifique [291]. Cette différence d'expression génétique se traduit par l'expression de récepteurs aux chimiokines (CCR6, CXCR6, CXCR9), de récepteurs aux cytokines (IL-12R, IL-18R, IL-23R) et la capacité de produire différentes cytokines (IL-17, IL-22, IFNγ, TNFα) [292].

Différemment des cellules NKT, les lymphocytes MAIT ont un phénotype naïf dans le thymus. Ces cellules acquièrent rapidement un phénotype mémoire après la naissance. Les infections virales ne semblent pas participer à l'activation directe de ces cellules [293]. Les lymphocytes MAIT sont absents chez les souris Germ-free[o] suggérant une forte relation entre ces cellules et la flore microbienne intestinale [294]. La reconstitution de ces souris avec certaines espèces de bactéries induit l'expansion des lymphocytes MAIT [293, 294]. Ainsi, la colonisation du tube digestif par la flore bactérienne aboutit à l'expansion, l'activation et l'acquisition d'un phénotype mémoire par ces cellules. Des expériences *in vitro* montrent que l'activation des lymphocytes MAIT est MR1 dépendante et que les ligands bactériens activent

[o]Germ free: souris dépourvues de flore intestinale

ces cellules. Cependant, certaines espèces de bactéries sont incapables d'activer les lymphocytes MAIT (i.e. *streptocci, listeriaIn, E.feacalis*) [295]. En 2012 et 2013, deux articles portant sur l'étude cristallographique de la molécule MR1 ont montré que celle-ci présentait un produit de dégradation de la vitamine B2 [287, 288]. La caractéristique commune des bactéries pouvant activer les lymphocytes MAIT *in vivo* est leur capacité à métaboliser la vitamine B2.

Cette molécule de petite taille peut se fixer directement sur la molécule MR1. De plus sa petite taille lui permettrait de passer à travers les parois de l'épithélium intestinal. D'autres mécanismes (signaux cytokiniques, autres composants bactériens…) contribuent-ils à l'activation des lymphocytes MAIT.

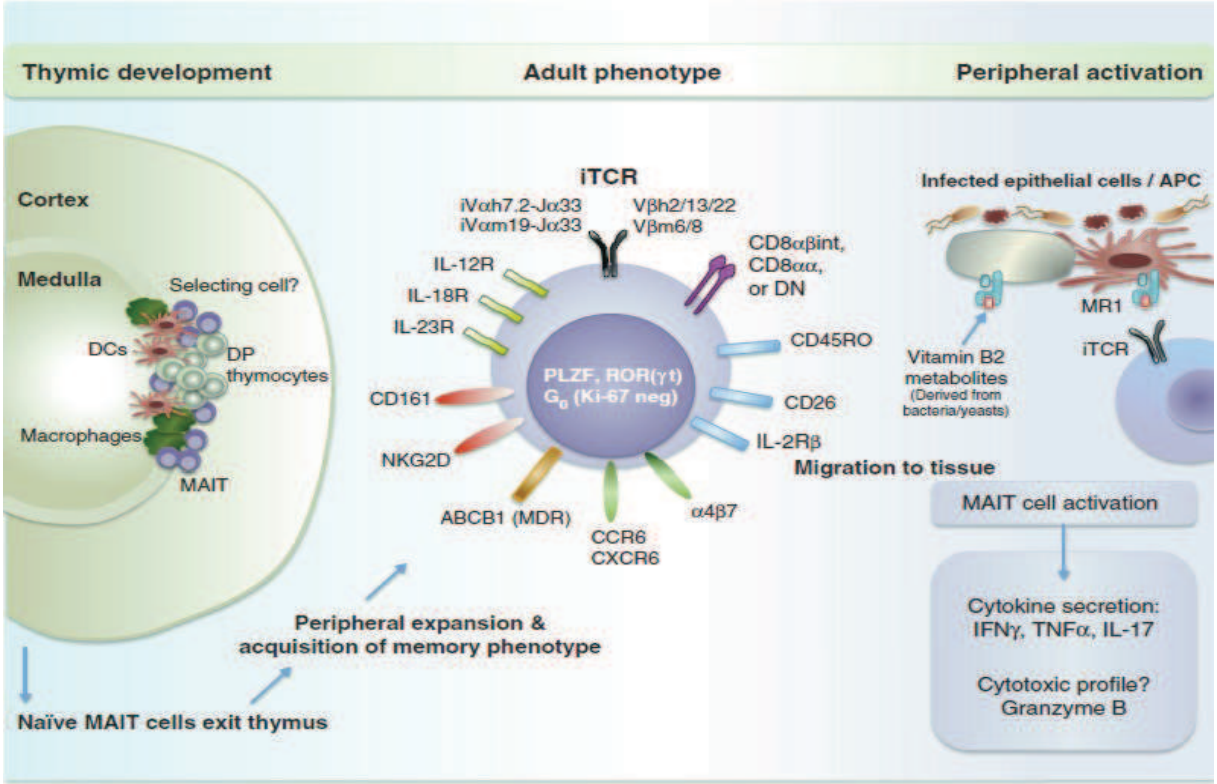

Figure II-3.1. Développement, phénotype et activation des lymphocytes MAIT.
Figure d'après Le Bourhis *et al* 2013[292].

Dans le sang périphérique, les lymphocytes MAIT représentent 1-10% des lymphocytes T, dans le foie, ces cellules peuvent représenter jusqu'à 20-40% des lymphocytes T et 3-10% dans l'intestin [296]. Le rôle antimicrobien des lymphocytes MAIT est confirmé dans différents modèles d'infection bactérienne (*Escherichia coli*, *Mycobacteria*, *Klebsiella*). L'absence de ces cellules conduit à une augmentation du nombre de bactéries dans l'organisme lors de l'infection bactérienne. Le mécanisme antibactérien induit par ces cellules reste inconnu [293].

Les lymphocytes MAIT peuvent aussi jouer un rôle dans les pathologies non-microbiennes. Dans la sclérose en plaque, une maladie auto-immune, il a été observé la présence de ces cellules dans les lésions au niveau du cerveau [297]. La modification de leur fréquence dans le sang périphérique des patients reste controversée [298, 299]. L'étude de cette maladie chez la souris montre que ces cellules pourraient avoir un rôle protecteur [300]. Les mécanismes induisant l'activation de ces cellules dans un contexte auto-immunitaire reste inconnu. Cependant, la composition de la flore bactérienne intestinale influence le développement de cette maladie [301]. Il est donc possible que les lymphocytes MAIT soient activés par la flore dans un contexte d'auto-immunité.
Les lymphocytes MAIT semblent aussi impliqués dans d'autres maladies auto-immunes telles que l'IBD[p]. Il est observé une diminution de leur fréquence dans le sang des patients [292].

L'ensemble de ces observations, spécificité antibactérienne des lymphocytes MAIT, rôle du système immunitaire et de la flore intestinale dans le T2D, nous ont amené à nous intéresser au rôle des lymphocytes MAIT dans le T2D.

[p] IBD: Inflamatory bowel desease

II-3.2 Résultats.

II-3.2a Réduction de la fréquence en lymphocytes MAIT chez les patients T2D.

Chez l'homme, les lymphocytes MAIT expriment à leur surface le marqueur CD161 et la chaine α du TCR Vα7.2. A l'aide de ces marqueurs, nous avons analysé la fréquence de ces cellules dans le sang des patients T2D. Ne connaissant pas le lien entre obésité et lymphocytes MAIT, et sachant que l'obésité est étroitement liée à l'apparition du T2D, nous avons divisé notre cohorte de patients en deux groupes : Patients non obèses (BMI < 30) et patients obèses (BMI > 30) (Figure II-3.2a).

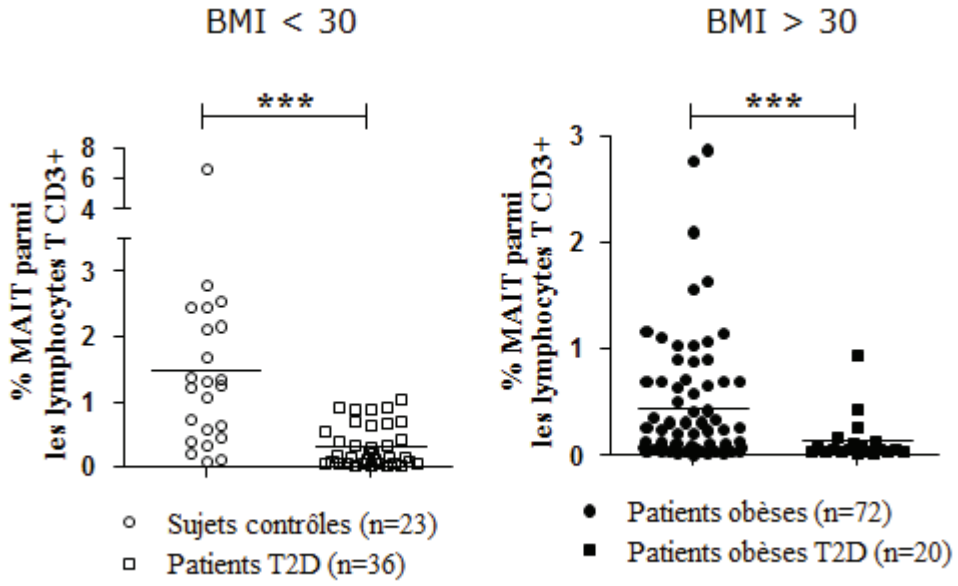

Figure II-3.2a. Fréquence en lymphocytes MAIT dans le sang des patients T2D. La fréquence des lymphocytes MAIT (Vα7.2+ CD161+ CD3+) a été analysée par FACS. Pour le groupe des patients non obèses (BMI <30), la moyenne de BMI des patients contrôles est de 23.3 et 25.9 pour les patients T2D. Pour le groupe des patients obèses (BMI >30), la moyenne de BMI des patients contrôles est de 46 et 44.7 pour les patients T2D.
*** p<0.001 (Test Mann-whitney). Expériences réalisées par Karine Pingris.

Dans chacun des deux groupes, nous observons une diminution significative de la fréquence en lymphocytes MAIT dans le sang des patients T2D par rapport aux patients contrôles.

La comparaison entre les deux groupes montre que les patients obèses (contrôles et T2D) ont une fréquence en lymphocytes MAIT plus faible que les patients non obèses. De plus, indépendamment de la BMI du patient, la fréquence en lymphocytes MAIT est diminuée dans le sang des patients T2D.

II-3.2b Les lymphocytes MAIT chez la souris.

Contrairement à l'homme ou l'anticorps anti-TCR Vα7.2 permet d'identifier spécifiquement les lymphocytes MAIT, aucun outil similaire existe chez la souris. Nous avons donc utilisé des souris transgéniques. Dans la souris C57Bl/6 Vα19, l'ensemble des lymphocytes expriment la chaine α du TCR Vα19 spécifique des lymphocytes MAIT. Cette chaine va s'associer avec les différentes chaines β du TCR. Lorsque la chaine Vα19 s'associera avec la chaine β Vβ6 ou Vβ8, le lymphocyte T aura une forte probabilité d'être un lymphocyte MAIT restreint à la molécule MR1. Si la chaine Vα19 s'associe à une autre chaine β (i.e. Vβ1), la cellule sera un lymphocyte T conventionnel. Par conséquent, la souris C57Bl/6 Vα19 se caractérise par une fréquence augmentée en lymphocyte MAIT par rapport à la souris C57Bl/6 WT [293].

Il existe différents modèles animaux du T2D (i.e. souris ob/ob ou db/db, rat Zucker [302]). Afin de pouvoir utiliser nos souris C57Bl/6 Vα19, nous avons opté pour un modèle induit : le HFD (High Fat Diet). Les souris sont soumises à un régime alimentaire riche en graisse. Ce régime conduit à l'apparition d'une résistance à l'insuline. Comme chez l'homme, la présence de cellules immunitaires (macrophages, lymphocytes T) est observée dans le tissu adipeux de ces souris.

Les lymphocytes MAIT influencent la prise de poids.

L'analyse de la prise de poids en régime HFD montre que les souris C57Bl/6 Vα19+/- sont moins grosses que les souris contrôles. Ce résultat indique que les lymphocytes MAIT influencent la prise de poids chez les souris.

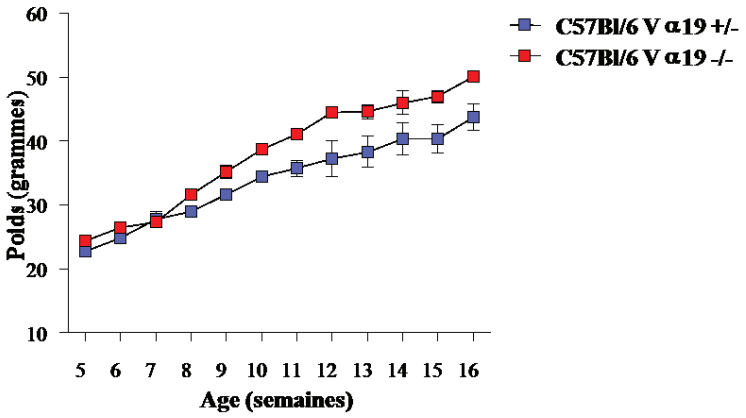

Figure II-3.2b. Prise de poids des souris C57Bl/6 Vα19+/- ou -/- en régime HFD. A partir de 5 semaines d'âge, les deux groupes de souris sont mis en régime HFD. Le poids des souris est mesuré chaque semaine. n=2 souris/groupe.

Les lymphocytes MAIT modifient la fonction des cellules β.

Le test de tolérance au glucose (GTT) permet de mesurer la faculté de régulation du glucose par l'organisme. Du glucose est injecté par voie orale aux souris et la glycémie est mesurée à intervalles réguliers. Si la souris a un problème de régulation dans la glycémie, son taux de glucose restera élevé par rapport à une souris n'ayant pas de problème (Figure II-3.2c).

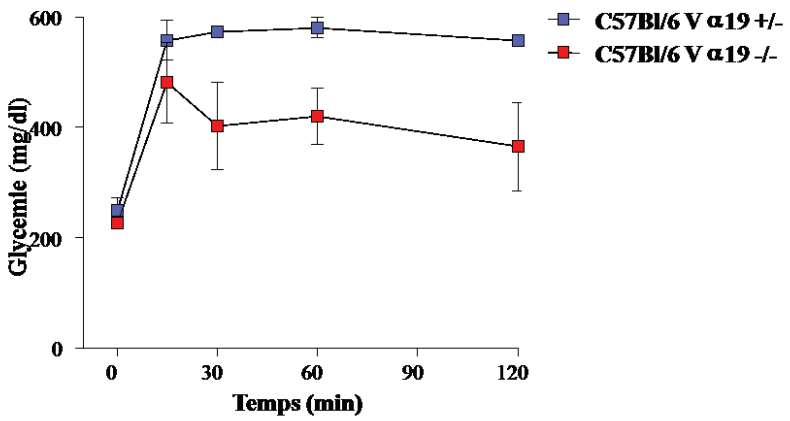

Figure II-3.2c. Test de tolérance au glucose. Le GTT est pratiqué 6 semaines après le début du régime HFD. A t=0, du glucose (2g/kg) est injecté par voie orale à la souris. n=2 souris/groupe.

Le GTT montre que les souris Vα19 +/- ont une intolérance au glucose. A T=0, les deux groupes de souris ont une glycémie similaire (environ 230 mg/dl). Soixante minutes après l'injection, la glycémie des souris Vα19 +/- est à 600 mg/dl alors que celle des souris contrôles est à 400 mg/dl. A la fin du test, la glycémie des souris Vα19+/- est toujours vers 600 mg/dl alors que chez les souris contrôles celle-ci est à 350mg/dl.

Cette intolérance au glucose des souris Vα19 +/- peut être la conséquence d'une insulino-résitance et/ou d'un défaut fonctionnel des cellules β. Afin de déterminer quel mécanisme est responsable de l'intolérance au glucose des souris Vα19+/-, nous avons pratiqué un test de tolérance à l'insuline et un dosage de l'insuline dans le sang.

Le test de tolérance à l'insuline (ITT) permet de mesurer l'insulino-résitance. De l'insuline est injectée dans la souris et la glycémie est mesurée à intervalles réguliers. Si la souris est insulino-résitante, l'action de l'insuline est moindre. Donc la glycémie chutera moins vite par rapport à une souris non insulino-résistante (Figure II-3.2d).

La comparaison entre les deux souches de souris montre des profils similaires durant l'ITT. Par conséquent, les lymphocytes MAIT n'induisent pas d'insulino-résitance.

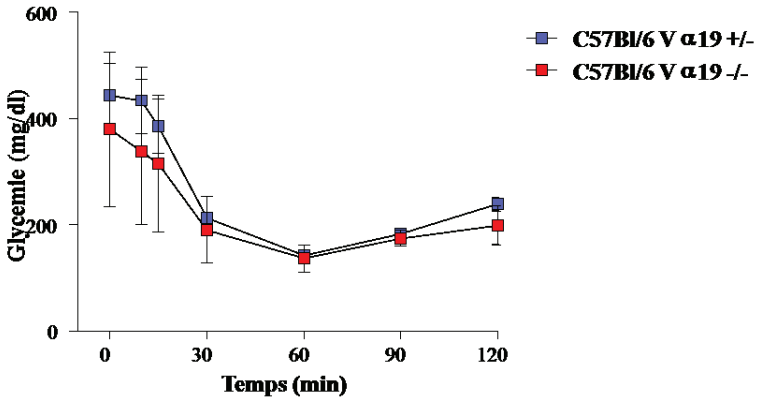

Figure II-3.2d. Test de tolérance à l'insuline. L'ITT est pratiqué 6 semaines après le début du régime HFD. A t=0, de l'insuline (0.5U/kg) est injectée en i.p à la souris. n=2 souris/groupe.

Un défaut de fonction des cellules β, peut se caractériser par une insulinémie faible. Nous avons mesuré la concentration en insuline dans le sang après injection de glucose à la souris.

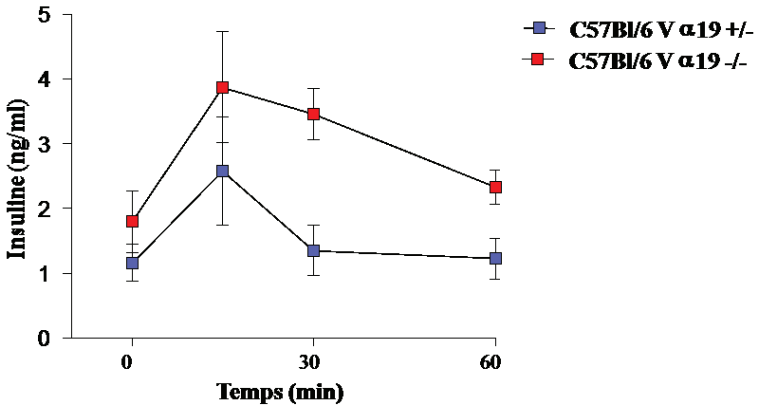

Figure II-3.2e. Variation de la concentration plasmique en insuline après injection de glucose. La mesure de l'insulinémie se pratique durant le GTT. Après injection du glucose à la souris (2g/kg), du sang est prélevé à intervalles réguliers afin d'analyser la concentration en insuline par ELISA. n=2 souris/groupe.

L'insulinémie révèle que les souris Vα19+/- ont une concentration en insuline dans le sang plus faible que les souris contrôles. Quinze minutes après l'injection du glucose, nous observons un pic de production d'insuline dans les deux groupes. Pour les souris contrôles, ce pic atteint la valeur de 4 ng/ml et 2.5 ng/ml pour les souris Vα19+/-. Une heure après injection, l'insulinémie est toujours plus élevée chez les souris contrôles (2.3ng/ml) que chez les souris Vα19 +/- (1.2ng/ml).

Par conséquent, la souris Vα19+/- présente une intolérance au glucose due à un défaut de sécrétion et/ou production d'insuline par les cellules β. La quantification de la masse des cellules β du pancréas, nous permettra de déterminer si les souris Vα19 +/- ont un défaut de

production d'insuline. Si aucun défaut de production d'insuline n'est observé, l'analyse de la production d'insuline par des îlots pancréatiques de souris contrôles ou Vα19+/- mis en culture, nous permettra de mettre en évidence un défaut de sécrétion d'insuline.

L'action des lymphocytes MAIT sur la fonction des cellules β est indirecte.

L'influence des lymphocytes MAIT peut être directe ou indirecte sur la fonction des cellules β. Nous avons analysé par immunomarquage et cytométrie de flux la présence de cellules immunitaires dans le pancréas des souris Vα19+/- et contrôles. Par cytomérie en flux, nous n'observons pas d'infiltrat de cellules immunitaires dans les îlots pancréatiques. L'immunomarquage confirme ce résultat. Des cellules immunitaires sont observées dans le tissu exocrine et proche des îlots pancréatiques. Aucune cellule immunitaire n'est observée dans desîlot pancréatique ou collé à ceux (Figure II-3.2f). Ces résultats suggèrent que les cellules immunitaires, et les lymphocytes MAIT en particulier, n'ont pas un effet direct sur les cellules β.

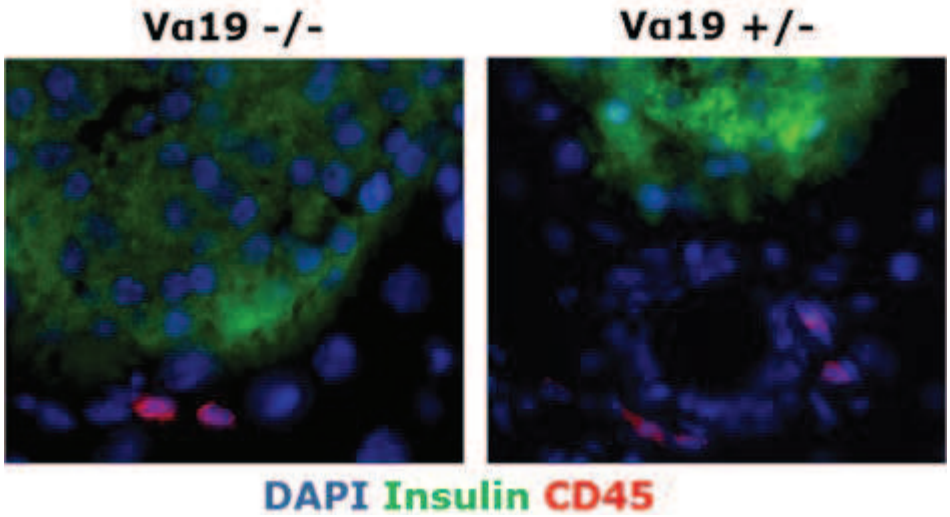

Figure II-3.2f. Immunomarquage du pancréas de souris C57Bl/6 Va19-/- **ou Va19+/-**Après 6 semaines de régime HFD, le pancréas des souris a été prélevé puis coupé en frozen-section (5µm). Les noyaux sont marqués en DAPI (bleu), les cellules β avec l'insuline (Vert) et les cellules immunitaires avec le marqueur CD45 (rouge).

II-3.3 Discussion.

Chez la souris, l'augmentation du nombre de lymphocytes MAIT (souris Vα19 +/-) se traduit par une intolérance au glucose. Cette intolérance au glucose est la conséquence d'un défaut fonctionnel des cellules β. Ce défaut peut toucher la production et/ou la sécrétion d'insuline. L'analyse de la masse des cellules β et la capacité de sécrétion de l'insuline par ces cellules, nous permettront de trancher entre ces deux hypothèses.

Les cellules immunitaires ne sont pas présentes dans le pancréas lors du régime HFD. Nous pouvons donc supposer que les lymphocytes MAIT ont un rôle indirect sur la fonction des cellules β. Afin d'expliquer le rôle de ces cellules, nous avons développé l'hypothèse suivante :

Le développement des lymphocytes MAIT est étroitement associé avec la flore intestinale [294]. Dans la souris Vα19+/-, cette flore induit l'expansion et l'activation des lymphocytes MAIT. Ces cellules présentes en grand nombre dans la souris Vα19 +/- modulent le système immunitaire mucosal via la production de cytokines (i.e. IL-17, IL-22, IFNγ). En réponse, le système immunitaire mucosal influence la composition de la flore intestinale par la production de cytokines, anticorps et molécules anti-microbiennes. Le changement dans la composition de la flore intestinale pourrait permettre l'émergence de bactéries influençant la production d'hormones par l'intestin telles que GLP-1 ou GIP [284]. Ces hormones ont la caractéristique de réguler la production d'insuline par les cellules β. De la même manière, la flore intestinale influence l'obésité [64]. La différence de poids entre les souris contrôles et Vα19 +/- pourrait être due à une différence de flore intestinale entre ces souris.

Afin de confirmer ces hypothèses, nous analyserons la composition de la flore intestinale des souris contrôles et Vα19 +/- par séquençage de l'ADN bactérien.

Actuellement, nous ne disposons pas d'outil permettant d'identifier les lymphocytes MAIT. Nous ne pouvons donc pas étudier la fonction de ces cellules *in vivo* (fréquence, profils cytokiniques). Nous avons généré la souris C57Bl/6 Vα19 Vβ6. Cette souris exprime deux transgènes codant pour la chaine α et β du TCR spécifique des lymphocytes MAIT. Cette souris contient de nombreux lymphocytes MAIT. Grâce à des expériences de transferts adoptifs, nous pourrons suivre ces cellules dans l'organisme (via les marqueurs congéniques CD45.1 et CD45.2) et ainsi analyser la présence de ces cellules dans l'intestin et déterminer leur phénotype.

De plus, nous sommes actuellement en train de générer la souris C57Bl/6 MR1-/-. La molécule MR1 permet la sélection thymique des lymphocytes MAIT. Par conséquent, la

souris C57Bl/6 MR1-/- se caractérise par une absence de lymphocytes MAIT. Cette souris nous permettra de confirmer les observations faites sur la souris C57bl/6 Vα19 +/- et ainsi de confirmer le rôle des lymphocytes MAIT sur la fonction des cellules β.

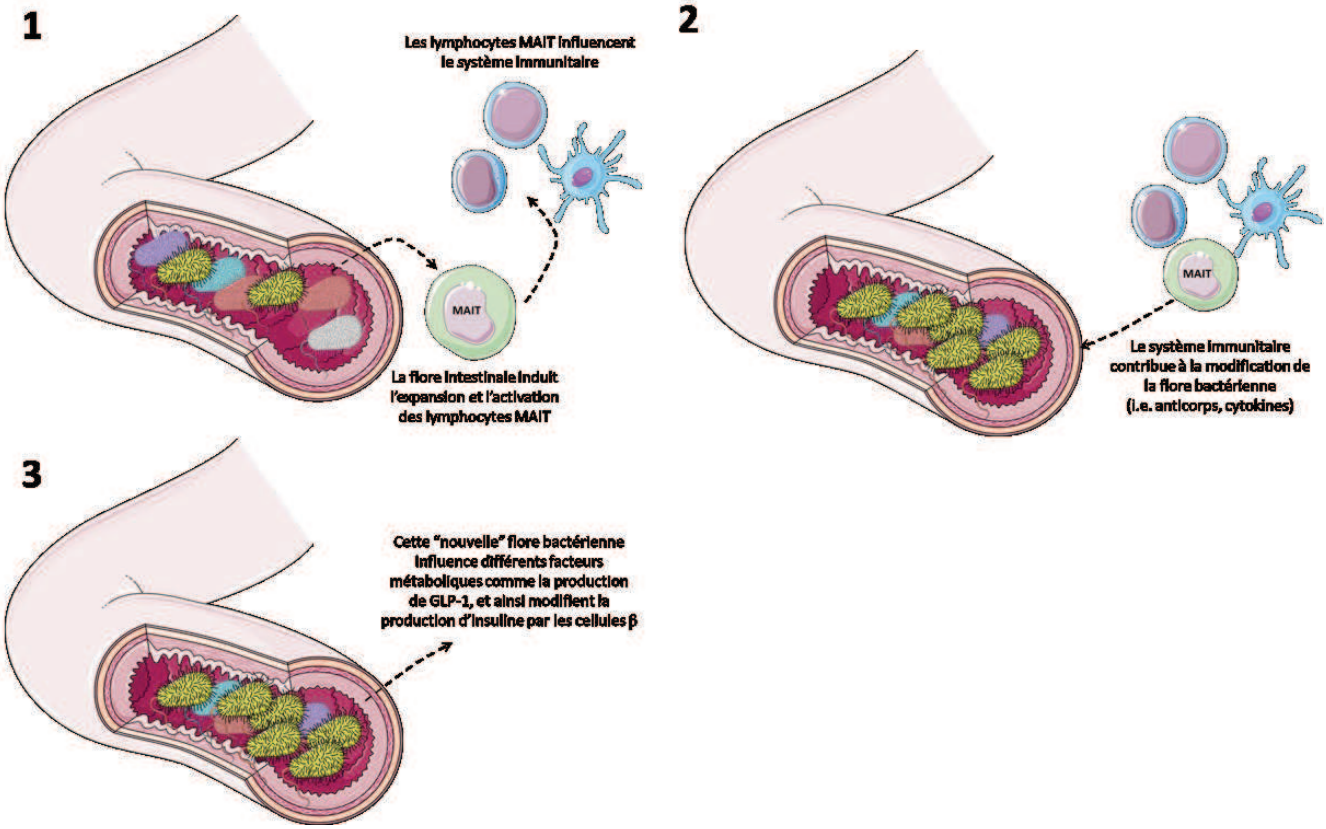

Figure II.3.3. Hypothèse sur le rôle des lymphocytes MAIT dans le T2D.

Notre travail montre que les patients T2D ont un défaut numérique en lymphocytes MAIT dans le sang. Chez les patients T2D, il est observé une réduction des bactéries produisant des gènes impliqués dans le métabolisme de la riboflavine (le ligand des lymphocytes MAIT). Nous pouvons supposé que la diminution de la fréquence en lymphocytes MAIT est la conséquence de la diminution du nombre de bactéries impliquées dans le métabolisme de la riboflavine. Afin de confirmer ces observations, nous analyserons la fréquence et le phénotype de ces cellules dans l'intestin des patients T2D et contrôles.

En conclusion, un défaut numérique de lymphocytes MAIT est observé chez les patients T2D. Les premières données chez la souris montrent que ces cellules influencent indirectement la fonction des cellules β du pancréas. Nos prochaines expériences nous permettrons de confirmer ou d'infirmer nos hypothèses sur le lien entre la flore bactérienne, les lymphocytes MAIT et le T2D.

Revues

Revue 1 :
L'immunité innée dans le diabète de type 1

Revue 2 :
Manipulation thérapeutique des cellules NKT dans l'auto-immunité : Sommes-nous proche de la réalité ?

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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-1β (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-1β, 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 insulitis stage.

iNKT cells

iNKT cells are non-conventional αβ T cells that are restricted by the non-polymorphic CD1d molecule presenting glycolipids. These cells express an invariant TCRα chain (Vα14-Jα18 in mice and Vα24-Jα18 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 Vα14-Jα18 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 various 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.

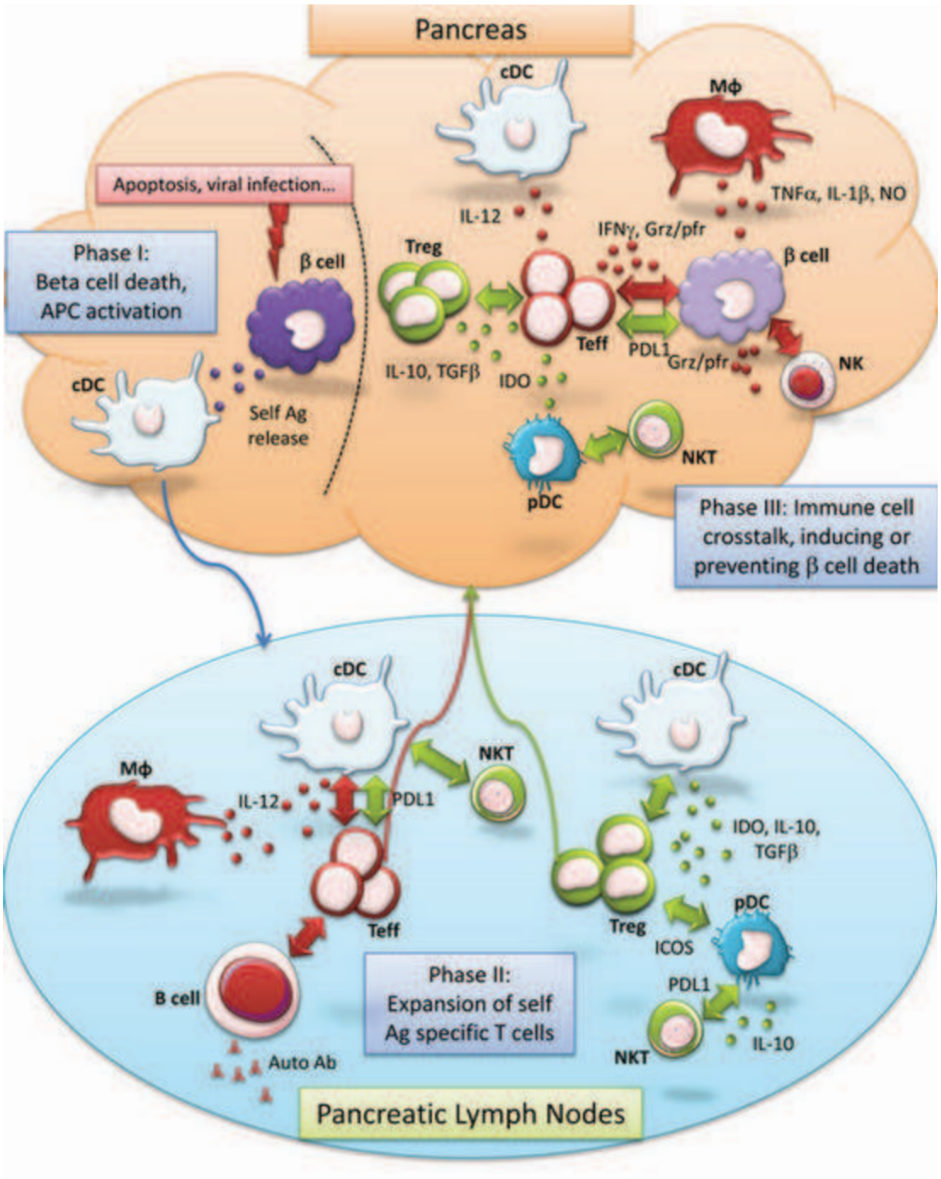

Figure 1. Interactions between innate and adaptive immune cell types during the development of type 1 diabetes. In the pancreas, conventional dendritic cells (cDCs) initiate T1D by capturing and processing β-cell antigens released after β-cell death. This process can be a consequence of a physiological 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-β. iNKT cells can promote the recruitment and the tolerogenic functions of cDCs and pDCs. Lastly, β-cells themselves can prevent their destruction by inhibiting diabetogenic T cells via PD-L1/PD-1 pathway. The dual functions of innate immune cells can promote or inhibit the development of T1D. Abbreviations: Ag, antigen; APC, antigen presenting cell; cDC, conventional dendritic cell; ICOS, inducible T cell co-stimulator; IDO, indoleamine 2,3-dioxygenase; IFN-γ, interferon γ; IL-, interleukin-; Grz/pfr, granzyme/perforin; Mf, macrophage; NK, natural killer cell, NKT, natural killer T cell; NO, nitric oxide; pDC, plasmacytoid dendritic cell; PD-L1, programmed cell death ligand 1; Teff, effector T cell; TGF-β, tumor growth factor β; TNF-α, tumor necrosis factor α; Treg, regulatory T cell.

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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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

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), Vα14-Jα18 in mice and Vα24-Jα18 in humans, and are associated with a limited set of TCR-β chains (Vβ2, 7 or 8·3 in mice and Vβ11 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 $NK1{\cdot}1^-$ [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 Vα10-Jα50, 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 Vα3·2-Jα7/9 or Vα1-Jα7/9 in mice), a limited TCR-β chain (such as Vβ8 in mice) [14], and are

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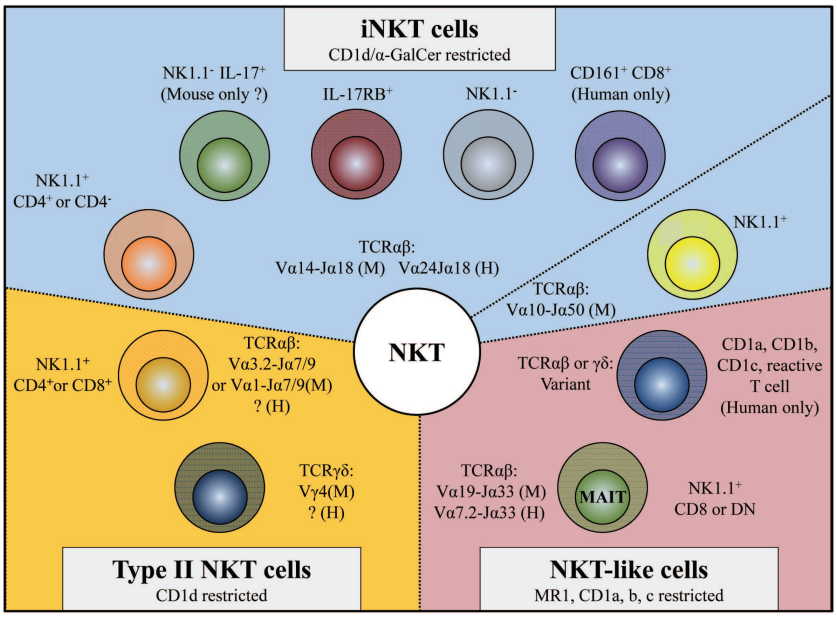

Fig. 1. Natural killer (NK T) cell populations. NK T cells can be divided into three groups: invariant NK (iNK) T (blue background), type II NK T (yellow background) and NK T-like cells (pink background). Each group is composed of distinct subsets.

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 γδ T cells, expressing TCR Vγ4 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 (Vα19-Jα33 in mice and Vα7·2-Jα33 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 αβ TCR [27] or γδ 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,

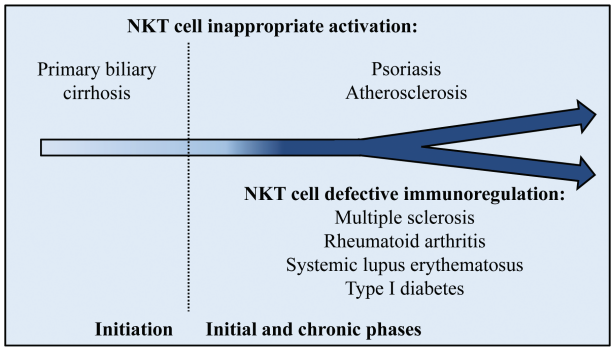

Fig. 2. Implication of natural killer (NK) T cells in human autoimmune diseases. The development of autoimmune diseases (blue arrow) can be divided into an initial and chronic phase. In primary biliary cirrhosis, invariant NK (iNK) T cells play a key role in the initial phase, whereas in other autoimmune 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).

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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 (Jα18-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 (Vα19 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 Vα19 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) F_1 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) F_1 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) F_1 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 Jα18-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 default 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 (Jα18-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 FcγRIII in an antibody-induced arthritis

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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
Multiple sclerosis	Inhibit autoreactive T cells by IL-4 or IL-10 secretion Infiltrate lesions	Defect of DN subset in blood Not present in lesions	Infiltrate lesions Protective role		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)F_1 mice Induce autoantibody production Hyperactive Disease amelioration in CD1d-deficient mice	Low frequency (CD4, DN) in blood of patients Regulate autoantibody production				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 atherosclerosic 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.

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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 Vα14-Jα18 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 $V\alpha24^+$ 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 Vα3·2-Vβ9 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 Vα19-Jα33 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,

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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–αGalCer 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

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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 anergy 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. Vα24 PCR, TCR^+ $NK1{\cdot}1^+$ 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

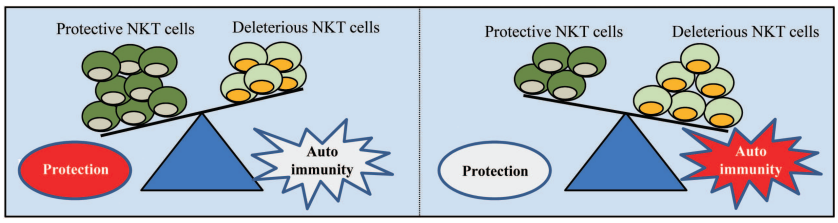

Fig. 3. Hypothesis regarding the effect of various natural killer (NK) T subsets in autoimmunity. NK T cell subsets exert different roles in autoimmune diseases. For example, in non-obese diabetic (NOD) mice developing type 1 diabetes $CD4^+$ invariant NK (iNK) T cells are protective, whereas iNK T17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

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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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