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Je dédie cette thèse

A la République du Bénin et à la France !

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Liste des abbréviations

ADN : Acide désoxyribonucléique

ADE : Antibody-dependent enhancement

ARN : Acide ribonucléique

ATP: Adénosine triphosphate

CAR: Coxackievirus and adenovirus receptor

CARD : Caspase activation recruitment domain

CMH : Complexe majeur d'histocompatibilité

CVB : Coxsackievirus B

CMN : Cellules mononuclées du sang périphérique

DAF : Decay accelerating factor

DT1 : Diabète de type 1

ECP : Effet cytopathogène

GAD65 : Glutamic acid decarboxylase

GPI : glycosylphosphatidylinositol

HLA: Human leucocyte antigen

IDA: Infection dépendante d'anticorps

IFIH1: Interferon-induced helicase C domain-containing protein 1

IFN: Interféron

IRF3: Interferon regulatory factor 3

IRES: Internal ribosome entry site

MAVS : Mitochondrial antiviral signaling protein

MDA5: Melanoma Differentiation-Associated protein 5

Mi-ARN : Micro-ARN

MyD88: Myeloid differentiation primary response gene 88

NF-kB: Nuclear factor-kappa B

NLR: NOD-like receptor

NLRP3: NLR family, pyrin domain containing 3

PDC4: Programmed cell death protein 4

RLR: RIG-like receptor

TCR: T cell receptor

TLR: Toll-like receptor

TNF: Tumor necrosis factor

TRAF: TNF receptor associated factor

TRIF: TIR-domain-containing adapter-inducing interferon-β

VP: Viral protein

Liste des articles

La présente thèse est basée sur les articles énumérés ci-dessous. Les articles publiés ont été reproduits avec la permission de l'éditeur.

1. Alidjinou EK, Sané F, Engelmann I, Hober D. Serum-dependent enhancement of coxsackievirus B4-induced production of IFNα, IL-6 and TNFα by peripheral blood mononuclear cells. ***Journal of Molecular Biology***. 2013 Dec 13; 425(24): 5020-31 *(Etude n°1)*

2. Alidjinou EK, Chehadeh W, Weill J, Vantyghem MC, Stuckens C, Decoster A, Hober C, Hober D. Monocytes of Patients with Type 1 Diabetes Harbour Enterovirus RNA. ***European Journal of Clinical Investigation***. 2015;45(9):918-24 *(Etude n°2).*

3. Alidjinou EK, Sané F, Trauet J, Copin MC, Hober D. Coxsackievirus B4 Can Infect Human Peripheral Blood-Derived Macrophages. ***Viruses***. 2015; 7(11):6067-79 *(Etude n°3)*.

4. Alidjinou EK, Sané F, Bertin A, Caloone D, Hober D. Persistent infection of human pancreatic cells with Coxsackievirus B4 is cured by fluoxetine. ***Antiviral Research.*** 2015; 116:51-4 *(Etude n°4)*.

5. Alidjinou EK, Engelmann I, Bossu J, Villenet C, Figeac M, Sané F, Hober D. Persistence of *Coxsackievirus B4* in pancreatic ductal-like cells results in cellular and viral changes. **Manuscript submitted** *(Etude n°5).*

6. Alidjinou EK, Sané F, Engelmann I, Geenen V, Hober D. Enterovirus persistence as a mechanism in the pathogenesis of type 1 diabetes. ***Discovery Medicine.*** 2014; 18(100):273-82 *(Annexe n°1)*.

7. Hober D, Alidjinou EK. Enteroviral pathogenesis of type 1 diabetes: queries and answers. ***Current Opinion in Infectious Diseases.*** 2013; 26(3):263-9 *(Annexe n°2)*.

8. Alidjinou EK et Hober D. L'infection virale joue-t-elle un rôle dans la genèse du diabète de type 1 ? ***Feuillets de Biologie***. Juillet 2015; 331:5-14 *(Annexe n°3)*.

9. Alidjinou EK, Hober D. Enteroviruses and Type 1 Diabetes: Candidate Genes Linked With Innate Immune Response, Commentary. ***EBioMedicine***. 2015; 2(7):636-7 (*Annexe n°4)*.

“Knowing is not enough; we must apply.

Willing is not enough; we must do.”

—Johann Wolfgang von Goethe

Avant-propos

Le futur de la virologie est imprévisible, mais certainement très excitant. Les virus sont des êtres mystérieux situés entre le monde inerte et le monde vivant. Ce ne sont pas seulement des pathogènes responsables d'infections aiguës ou chroniques, d'épidémies ou de pandémies. Ce sont aussi de grands acteurs de la modulation de la biologie cellulaire, pouvant être impliqués dans des cancers, des troubles du développement, des maladies auto-immunes, et probablement dans beaucoup d'autres maladies chroniques, d'étiologie inconnue. Les interactions entre les virus et le système immunitaire sont assez complexes, et plusieurs pathologies sont souvent étiquetées de nature « post infection virale », résultant de manière séquellaire de la réponse élaborée par le système immunitaire au cours d'une infection virale. Si la plupart des manifestations sont non spécifiques, certaines peuvent être liées à des types particuliers de virus. Le lien de causalité est parfois difficile à mettre en évidence, car assez souvent, l'auteur du crime n'est plus sur les lieux… La compréhension des interactions virus-hôte est l'une des principales clés pour expliquer ces liens, qui parfois donnent lieu à beaucoup de débat et de contradiction !

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INTRODUCTION

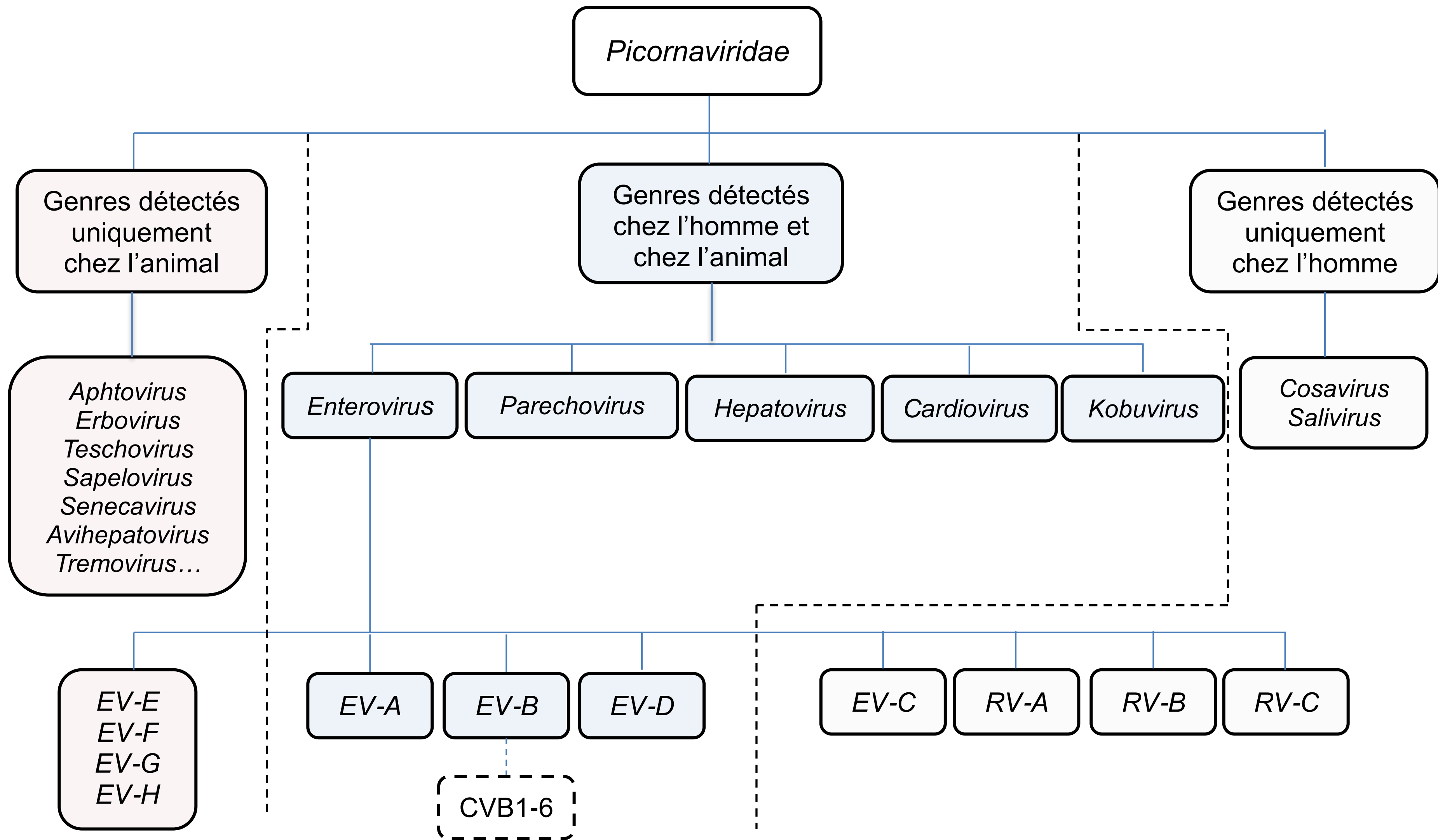
1. Entérovirus et coxsackievirus B

Les entérovirus sont des virus non enveloppés à ARN appartenant à la famille des *Picornaviridae*, une des plus anciennes et des plus diversifiées familles de virus. Historiquement, la classification des *Picornaviridae* reposait essentiellement sur une combinaison de propriétés physiques et des critères sérologiques (Tapparel et al., 2013). Les données phylogéniques représentent actuellement la référence pour la classification et permettent de regrouper ces virus en genres, espèces et types (Lauber and Gorbalenya, 2012). Ainsi, plusieurs genres impliqués aussi bien dans des pathologies humaines qu'animales sont inclus dans la famille des *Picornaviridae* (figure 1). Le genre *Enterovirus* en est le plus important avec plus de 250 virus regroupés en douze espèces dont sept peuvent causer des maladies chez l'homme (*Enterovirus A-D, Rhinovirus A-C*) (Knowles et al., 2012; Tapparel et al., 2013).

Dans le genre *Enterovirus*, les membres d'une même espèce doivent avoir une homologie de plus de 70% dans la séquence en acides aminés de la polyprotéine, plus de 60% dans la protéine P1 et plus de 70% dans les protéines non structurales 2C + 3CD. Ils partagent un nombre limité d'hôtes naturels et de récepteurs cellulaires, et les génomes ont une composition en bases G+C qui varie de moins de 2,5%. De plus, les virus d'une même espèce doivent avoir une compatibilité importante dans les processus de protéolyse, de réplication, d'encapsidation et de recombinaison génétique (Pallansch et al., 2013).

Le poliovirus, représentant le plus connu de ce genre appartient à l'espèce *Enterovirus-C*. Les coxsackievirus qui doivent leur nom au site géographique du premier isolement (Coxsackie, Etat de New York) en 1948 (Dalldorf and Sickles, 1948) étaient classés sur la base de leur pouvoir pathogène chez le souriceau. Ainsi, on pouvait distinguer les coxsackievirus A qui provoquaient une paralysie flasque avec atteinte du muscle cardiaque et squelettique, et les coxsackievirus B qui étaient associés à une paralysie spastique avec une atteinte tissulaire plus variée touchant notamment le système nerveux central, le foie, le pancréas, le muscle et la graisse brune (Crowell and Landau, 1997).

Actuellement, les coxsackievirus A se retrouvent dans les espèces *Enterovirus* A, B et C, tandis que les coxsackievirus B (1-6) sont inclus uniquement dans l'espèce *Enterovirus-B* (Tableau 1).



EV: enterovirus, RV: rhinovirus; CVB: coxsackievirus B

D'après Tapparel C et al., 2013

<u>Figure 1</u>. Place des coxsackievirus du groupe B dans la famille des *Picornaviridae*.

Tableau 1. Classification des entérovirus humains

Espèces d'entérovirus	Types
Enterovirus A	coxsackievirus A2–8, 10, 12, 14, 16. enterovirus A 71, 76, 89–92, 114, 119–121.
Enterovirus B	coxsackievirus A-9, B1-6. echovirus 1–9, 11–21, 24–27, 29–33. enterovirus B 69, 73–75, 77–88, 93, 97–101, 106, 107, 110–113.
Enterovirus C	coxsackievirus A1, 11, 13, 17, 19–22, 24. poliovirus 1–3. enterovirus C 95, 96, 99, 102, 104, 105, 109, 116–118.
Enterovirus D	enterovirus D 68, 70, 94, 111, 120.
Rhinovirus A	rhinovirus A1, 2, 7–13, 15, 16, 18–25, 28–34, 36, 38–41, 43, 45–47, 49–51, 53–68, 71, 73–78, 80–82, 85, 88–90, 94, 96, 100–109.
Rhinovirus B	rhinovirus B3-6, 14, 17, 26, 27, 35, 37, 42, 48, 52, 69, 70, 72, 79, 83, 84, 86, 91–93, 97, 99, 100–106.
Rhinovirus C	rhinovirus C1-55.

D'après www.picornaviridae.com

2. Transmission, tropisme et virulence des CVB

Les entérovirus sont des pathogènes ubiquitaires et constituent une cause importante d'infection virale chaque année dans le monde, surtout chez les enfants de moins de dix ans. Pour les entérovirus humains, l'homme est le seul réservoir de virus, les enfants étant le vecteur principal de diffusion et les facteurs favorisant la dissémination sont des conditions socioéconomiques précaires et une promiscuité importante entre les membres d'une même famille ou d'une collectivité (Racaniello, 2013).

Les entérovirus sont relativement résistants dans l'environnement. Dans les pays développés, les infections évoluent souvent sur un mode épidémique durant les périodes estivo-automnales, alors qu'elles sont endémiques dans les pays en développement (Antona D, 2004).
La transmission se fait le plus souvent par voie oro-fécale, bien que les aérosols semblent permettre au virus d'atteindre l'épithélium de l'arbre respiratoire supérieur (transmission aérienne pulmonaire), ou l'épithélium conjonctival (transmission oculaire ; généralement auto-inoculation par les mains contaminées) (Racaniello, 2013). Après la contamination, la réplication primaire survient au niveau de l'épithélium gastro-intestinal et respiratoire, et une virémie peut être observée, même dans les formes asymptomatiques, permettant la dissémination du virus aux organes cibles (Racaniello, 2013).
Les entérovirus peuvent infecter une large gamme de tissus, et certains types peuvent avoir des cibles préférentielles. Les tissus cibles les plus importants pour les CVB sont le pancréas, le myocarde et le système nerveux central (Tracy and Gauntt, 2008). La susceptibilité des tissus à l'infection est certes souvent corrélée avec l'expression du récepteur du virus (Freimuth et al., 2008); cependant certains tissus comme le foie avec une expression significative du récepteur ne sont pas une cible fréquente de l'infection (Wessely et al., 2001). Le tropisme semble aussi lié à des déterminants génomiques (Harvala et al., 2002, 2005). Ainsi en ce qui concerne les Coxsackievirus B par exemple, des travaux ont associé le cardiotropisme de CVB3 (Cameron-Wilson et al., 1998; Knowlton et al., 1996) et le pancréatropisme de CVB4 (Caggana et al., 1993; Kang et al., 1994; Ramsingh and Collins, 1995; Yin et al., 2002) à des déterminants en relation notamment avec des protéines structurales.
Même si la majorité des infections est asymptomatique ou paucisymptomatique, les entérovirus peuvent être responsables de syndromes infectieux variés, parfois graves pouvant engager le pronostic vital. Plusieurs manifestations sont communes aux entérovirus, mais d'autres sont attribuées plus fréquemment à des sérotypes particuliers. Les CVB ont été incriminés dans des infections aigues incluant des manifestations neurologiques, des éruptions cutanéo-muqueuses, des atteintes respiratoires hautes ou basses, des atteintes cardiaques, musculaires, pancréatiques ou digestives. Des infections systémiques notamment chez le nouveau-né, ou chez des sujets immunodéprimés ont été également décrites (Pallansch et al., 2013). Le tableau 2 présente les principales pathologies pour lesquelles une infection par les CVB peut être incriminée.

Tableau 2. Pathologies associées à l'infection par les CVB

Atteintes et manifestations cliniques	
Infections asymptomatiques	
Syndrome fébrile non spécifique	
Infections néonatales	
Infections du système nerveux central	Méningite aseptique, encéphalite
	Myélite, paralysie
Atteintes cardiaques	Péricardites
	Myocardite aiguë et chronique
	Cardiomyopathie dilatée
Atteintes cutanéo-muqueuses	Éruption maculo-papuleuse, rash cutané
	Syndrome pied-main-bouche
Infections respiratoires	Rhinopharyngite
	Bronchiolite, pneumonie
Atteintes musculaires	Maladie de Bornholm
	Myopathie aigues ou chroniques
Atteintes digestives	Hépatite
	Pancréatite
	Diabète de type 1

D'après Pallansch et al. (Pallansch et al., 2013)

3. Biologie structurale et moléculaire des CVB

Structure

Les CVB et les entérovirus en général sont des petits virus (environ 30 nm de diamètre), non enveloppés, à capside icosaédrique (symétrie cubique). La capside des entérovirus est formée de 60 exemplaires de capsomères, chacun étant constitué de quatre polypeptides (VP1-VP4). Les protéines structurales VP1-VP4 proviennent du précurseur P1. Selon les données de cristallographie, les protéines VP1-VP3 se trouvent à la surface de la capside alors que VP4 est une protéine interne (Racaniello, 2013).

La surface de la capside virale est caractérisée par la présence de dépressions dont une, d'une profondeur de 2,5 nm entourant les axes de symétrie d'ordre 5, et est appelée « canyon ». Cette dépression forme une structure tridimensionnelle impliquée dans les mécanismes d'attachement au récepteur cellulaire. A l'intérieur de VP1 et juste en-dessous

du fond du canyon, se trouve une poche hydrophobe qui renferme un acide gras, présent chez la plupart des entérovirus. Cette poche contribue à la stabilité du virus. Cette poche hydrophobique représente également le site de liaison de certaines molécules anti-picornavirus comme les composés WIN dont le dérivé le plus connu est le picovir (pléconaril). Ces composés déplacent le lipide présent dans la poche hydrophobe, bloquant ainsi la décapsidation (Racaniello, 2013).

Génome et protéines virales

Le génome viral est constitué d'une molécule d'ARN simple brin, de polarité positive (ARN infectieux) d'environ 7500 bases. Il comporte un cadre de lecture unique, flanqué aux extrémités de deux régions non codantes (NC). Chez les CVB, la région 5'NC est d'environ 750 bases et est liée de façon covalente à une protéine appelée VPg de 22 à 24 acides aminés. Cette région contient des séquences qui contrôlent la réplication du génome et la traduction. Elle contient le site interne d'entrée du ribosome (IRES) qui dirige la traduction de l'ARN messager par la liaison du ribosome. De plus, cette région contient de nombreux résidus conservés, et est fréquemment utilisée comme cible pour des techniques de RT-PCR (Oberste, 2008; Racaniello, 2013). La région 3'NC comporte environ 100 nucléotides et possède une queue poly (A). Elle est le site d'initiation de la synthèse de l'ARN négatif intermédiaire indispensable dans la réplication du virus (Oberste, 2008).

La traduction de l'ARN viral donne une polyprotéine d'environ 2200 acides aminés, au sein de laquelle on peut distinguer 3 régions fonctionnelles : la région P1 qui est à l'origine des 4 protéines de capside (VP1-VP4) et les régions P2 et P3 qui donnent naissance aux protéines non structurales impliquées dans la protéolyse et dans la réplication du génome viral.

La séquence des événements se présente brièvement comme suit. Un clivage co-traductionnel très précoce par la protéase virale 2A ($2A^{pro}$) permet de libérer le précurseur P1 de l'extrémité N-terminale de la polyprotéine. La protéine P1 est ultérieurement clivée par la protéase virale 3C ($3C^{pro}$) pour produire les protéines de capside VP1 et VP3 et une protéine de capside immature VP0 qui est finalement clivée en VP4 et VP2. La région P2 est d'abord clivée en $2A^{pro}$ et 2BC, et ce dernier est ensuite à l'origine de 2B et 2C. Quant à la région P3, elle est clivée en 3AB et 3CD qui donneront d'une part 3A et $3B^{VPg}$ et d'autre part $3C^{pro}$ et $3D^{pol}$ (Racaniello, 2013).

La région de la capside est la plus variable de la polyprotéine, mais il existe également des motifs conservés. La plus grande variation s'observe au niveau de la protéine VP1. Dans l'espèce EV-B, les protéines de capside notamment VP1-VP3 sont monophylétiques. Les évènements de recombinaison sont donc très rares au sein de la capside (Oberste, 2008).

La protéine 2A comme décrite ci-dessus est une cystéine protéase qui intervient dans le clivage de la polyprotéine, et contribue également à l'inhibition de la synthèse des protéines

de l’hôte. La protéine 2B joue un rôle dans la réplication en participant à la formation des vésicules de réplication au niveau des membranaires intracellulaires. La protéine 2C possède des motifs assez conservés de liaison à l’ARN, d’activité ATPasique, et des motifs riches en cystéine. Les protéines 2C et 2BC participent aussi à la formation des vésicules intracellulaires. La protéine 3AB s’associe aux membranes intracellulaires au cours de la réplication, et 3B (Vpg) est la protéine d’initiation de la synthèse de l’ARN viral. La protéine 3C ou 3CD est une sérine protéine qui joue un rôle dans plusieurs processus de protéolyse au cours de la réplication. Quant à 3D, elle est l’ARN polymérase ARN-dépendante, enzyme majeure dans la réplication du virus (Oberste, 2008; Racaniello, 2013; Sean and Semler, 2008). Les protéines 2A et 3C participent également à la modulation de plusieurs fonctions protéiques notamment les réponses de l’immunité innée (Lei et al., 2016).
La figure 2 montre schématiquement le génome et l’organisation structurale des entérovirus.

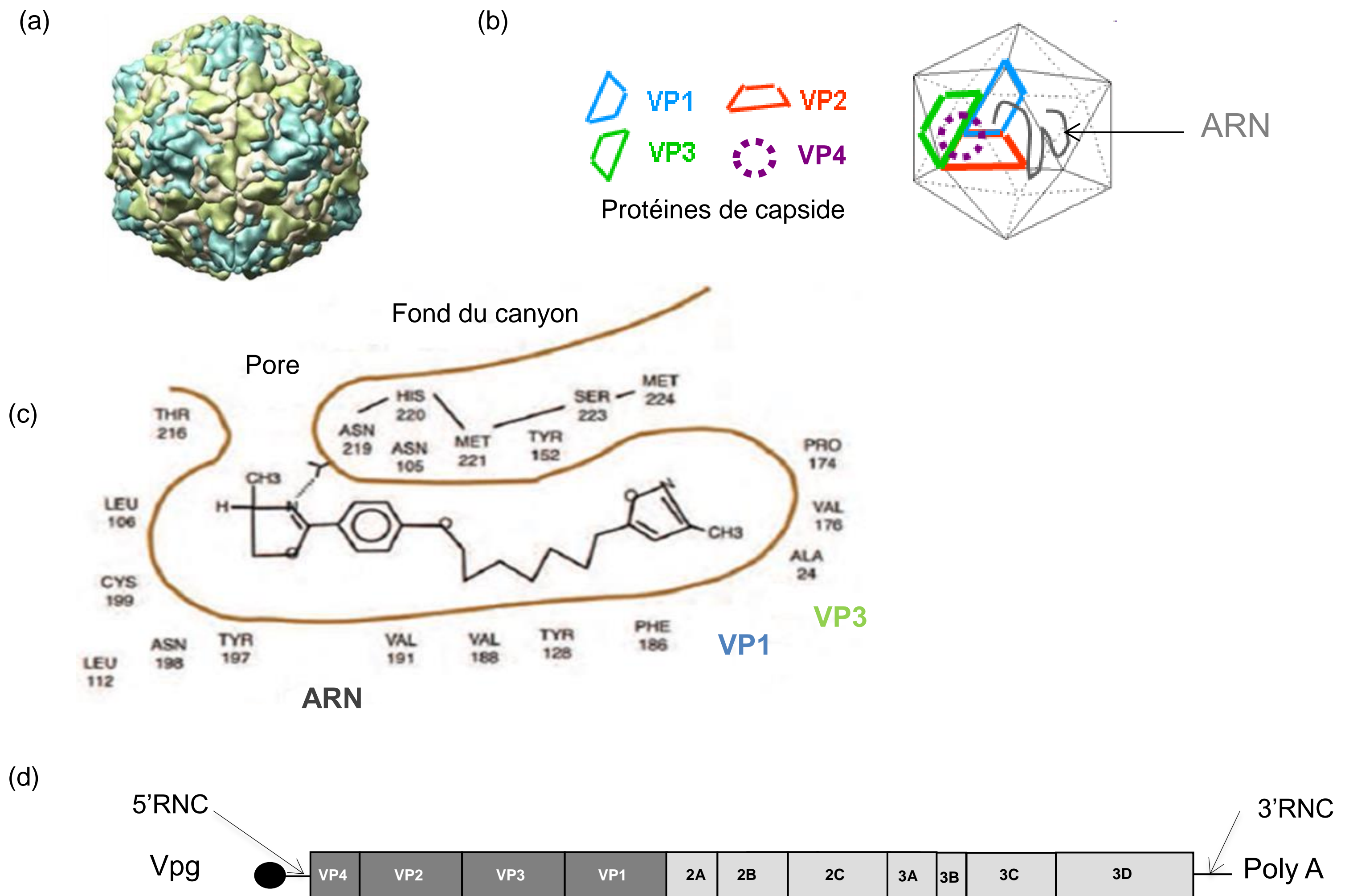


Figure 2. Représentation schématique de la structure et du génome des entérovirus

(a) Structure de la capside de CVB3 obtenue par diffraction de rayons X avec une résolution de 3.50 Å (Muckelbauer et al., 1995); (b) La capside de symétrie icosaédrique est formé de 60 capsomères comprenant chacun 4 protéines structurales (VP1, VP2, VP3 et VP4). Selon de données de cristallographie, la protéine VP4 est située à l'intérieur de la capside, et est représentée ici en pointillés; (c) la poche hydrophobique au sein de la capside des entérovirus : la présence du composé WIN 52084 y est figurée à la place du lipide (Rocaniello et al, 2013) ; (d) le génome à ARN simple brin positif de 7400 bases environ comporte un seul cadre de lecture flanqué aux extrémités de deux régions non codantes (RNC). La 5' RNC comporte une région interagissant avec la petite sous-unité du ribosome (IRES) et la 3' RNC comporte une queue poly A. Le génome est traduit en une polyprotéine qui après clivage donne les 4 protéines structurales (en gris sombre) et 7 protéines non structurales (en gris clair). A l'extrémité de la 5' RNC, se trouve la protéine Vpg issue du gène 3B.

4. Entrée et réplication des CVB

Récepteur et autres molécules d'attachement

- ***Le récepteur CAR (Coxsackie and Adenovirus Receptor)***

Le récepteur CAR, commun aux coxsackievirus et aux adénovirus a été caractérisé en 1997 conjointement par trois équipes (Bergelson et al., 1997; Carson et al., 1997; Tomko et al., 1997). Le gène codant pour la protéine CAR est désigné CXADR et est situé chez l'homme sur le chromosome 21q11.1 (Hattori et al., 2000). Plusieurs pseudogènes ont été également décrits sur les chromosomes 13, 18 et 21 (Bowles et al., 1999). Le gène CXADR humain comporte 8 exons (Excoffon et al., 2010) et s'étend sur 54000 nucléotides (Hattori et al., 2000). L'expression de CXADR donne lieu à plusieurs variants (épissages alternatifs) avec chez l'homme, deux isoformes membranaires CAR1 (hCAR1, exons I-VII) et CAR2 (hCAR2, exons I-VIII) qui diffèrent dans leur domaine intracellulaire, et trois isoformes solubles (hCAR2/7, hCAR3/7 et hCAR4/7) (Dörner et al., 2004; Thoelen et al., 2001).

Le cadre de lecture de hCAR1, le principal isoforme code pour une protéine de 365 acides aminés (aa), avec un peptide signal (19 aa), un domaine extracellulaire (218 aa), un domaine transmembranaire (21aa), et une queue cytoplasmique (107 aa) (Excoffon et al., 2010; Loustalot et al., 2016). La partie extracellulaire comporte deux domaines Ig-like (D1 et D2), ce qui a conduit à la classification de la protéine CAR dans la superfamille des immunoglobulines (Chrétien et al., 1998; Du Pasquier et al., 1999). Le domaine distal D1 est un domaine variable (V-Ig) (van Raaij et al., 2000) alors que le domaine proximal D2 est constant (C-Ig) (Freimuth et al., 2008). Le poids moléculaire théorique de la protéine CAR (hCAR1) est de 38 kDa (Freimuth et al., 2008).

La protéine CAR est exprimée dans plusieurs tissus. Les études dans le modèle murin ont montré que l'expression est plus importante chez l'embryon, comparée à celle de la souris adulte. Ainsi CAR est fortement exprimé dans le système nerveux (Honda et al., 2000; Tomko et al., 2000), mais aussi au niveau des cellules épithéliales du foie de l'embryon, les poumons, le cœur, les yeux, le tractus digestif, le pancréas, les reins, les glandes sous-mandibulaires (Raschperger et al., 2006). L'expression diminue à la naissance dans la plupart de ces tissus (Raschperger et al., 2006). Dans le pancréas, l'expression de CAR, n'est pas clairement élucidée. Les données existantes montrent soit une expression isolée au niveau des cellules canalaires (Raschperger et al., 2006), des acini pancréatiques (Mena et al., 2000), des îlots (Myers et al., 2004) ou encore à la fois au niveau des cellules acineuses et des ilôts (Drescher et al., 2004; Kanno et al., 2006).

Chez l'homme, CAR est faiblement exprimé dans le cœur adulte, mais l'expression augmente au cours des processus inflammatoires et de la cicatrisation, de même que chez les sujets avec une cardiomyopathie dilatée (Fechner et al., 2003; Noutsias et al., 2001).

Dans le pancréas humain, l'expression de CAR a été mise en évidence au niveau des îlots chez le sujet diabétique ou prédisposé (Oikarinen et al., 2008; Spagnuolo et al., 2013). Des anticorps anti-CAR peuvent inhiber l'infection des îlots in vitro (Ylipaasto et al., 2004). L'isoforme présent dans le tissu pancréatique n'a pas été clairement caractérisée (Dörner et al., 2004).

Dans les cellules à polarité, la molécule CAR est préférentiellement exprimée à la partie baso-latérale des jonctions serrées, et donc difficilement accessible au virus notamment à partir du pôle apical. Comme d'autres protéines des jonctions serrées, des mouvements sont probables sous l'effet de stimulations (Freimuth et al., 2008). De plus, CAR peut être exprimé à la face apicale de certaines cellules épithéliales, au moins dans certains tissus comme la prostate (Bao et al., 2005; Rauen et al., 2002).

En dehors du rôle de récepteur pour les CVB et les adénovirus, la protéine CAR est impliquée dans plusieurs fonctions cellulaires comme la médiation d'adhésion cellulaire pour les cellules épithéliales (interactions homophyliques CAR/CAR), la signalisation cellulaire, l'homéostasie cellulaire ou encore la réponse immune (Freimuth et al., 2008; Loustalot et al., 2016). Les reconstructions du complexe CAR-CVB3 montrent que les boucles distales et la surface latérale de D1 interagissent avec CVB3 (He et al., 2001). Cette interaction est cohérente avec le modèle d'interaction du canyon des picornavirus avec des récepteurs de type Immunoglobuline (Freimuth et al., 2008).

- **Autres molécules d'attachement**

Plusieurs molécules alternatives ont été suggérées comme facteur d'attachement des CVB aux cellules, parmi lesquelles la molécule DAF (decay accelerating factor ou CD55), la nucléoline ou les protéoglycanes héparane sulfate (Bergelson et al., 1995; de Verdugo et al., 1995; Zautner et al., 2003, 2006). La plus étudiée de ces molécules de liaison reste la molécule DAF qui est une protéine de membrane ancrée au glycolipide GPI. Les données de la littérature suggèrent que les CVB peuvent être classés en trois phénotypes en fonction de l'utilisation de DAF (Freimuth et al., 2008). Ainsi on peut distinguer les CVB qui ne se lient pas à DAF (qui incluent notamment la plupart des CVB2, CVB4 et CVB6), et ceux qui se lient à DAF mais qui nécessitent la présence de CAR (la plupart des souches CVB1, CVB3 et CVB5). Ces deux phénotypes ont été observés avec des isolats cliniques, mais ils peuvent être influencés par le type cellulaire utilisé pour la multiplication du virus (Reagan et al., 1984; Schmidtke et al., 2000). Le troisième phénotype est représenté par des virus qui ont été soumis à de nombreux passages sur des cellules déficientes en CAR. Ces souches sont capables d'infecter les cellules en utilisant uniquement DAF, mais gardent l'aptitude à utiliser CAR lorsque celle-ci est disponible (Goodfellow et al., 2005; Spiller et al., 2002). Néanmoins,

le mécanisme d'entrée en l'absence de CAR n'est pas encore clairement élucidé, car DAF ne serait pas impliqué dans l'internalisation (Marjomäki et al., 2015).
Il a été montré que dans les cellules polarisées, l'attachement initial à DAF permettait un accès plus aisé de CVB au récepteur CAR, qui est séquestré au niveau des jonctions serrées (Coyne and Bergelson, 2006).

Mécanismes d'entrée

Le changement conformationnel de CVB et la décapsidation débutent dès l'interaction virus/récepteur. L'interaction des CVB avec CAR conduit à la formation de particules A (altered particules) caractérisée par une exposition partielle de l'ARN viral et la libération de la protéine VP4. De même, la partie N-terminale de VP1 qui est normalement à l'intérieur, est exposée en surface et participe à la formation d'un pore membranaire dans la cellule, probablement avec un rôle de VP4. L'internalisation du virus reste cependant indispensable avant la libération du génome viral (Patel et al., 2009). Le mécanisme d'internalisation varie selon le type cellulaire. L'entrée du virus dans les cellules à polarité comme la ligne cellulaire Caco-2 est dépendante de la cavéoline, mais pas de la dynamine et de la clathrine et présente plusieurs caractéristiques de la macropinocytose. CAR n'est pas internalisé avec le virus (Coyne and Bergelson, 2006; Coyne et al., 2007).
Dans le cas des cellules sans polarité comme les cellules HeLa, l'entrée du virus requiert la dynamine et les radeaux lipidiques. Par contre, la clathrine, la cavéoline ou encore l'acidification endosomale ne sont pas indispensables à l'infection (Patel et al., 2009). Des données plus récentes dans des cellules trophoblastiques à polarité montrent que l'entrée des CVB est indépendante de la dynamine, de la clathrine, de la cavéoline, mais nécessite des radeaux lipidiques intacts (Delorme-Axford et al., 2013).
Un autre mécanisme d'entrée est possible pour les cellules exprimant le récepteur Fc gamma (FcγR) à leur surface. Les CVB peuvent en effet entrer dans ces cellules en présence d'anticorps non neutralisants qui vont se lier au virus et au FcγR (Sauter and Hober, 2009). C'est le phénomène de facilitation dépendante d'anticorps de l'infection ou d'infection dépendante d'anticorps («antibody-dependent enhancement (ADE)» en anglais) décrit pour plusieurs microorganismes (Halstead et al., 2010), et détaillé dans le paragraphe suivant. Enfin, l'infection via des récepteurs n'est probablement pas l'unique moyen pour ces virus d'entrer dans les cellules. Des cellules phagocytaires comme les macrophages ou les cellules dendritiques peuvent ingérer des cellules en cours d'apoptose contenant du virus (Kemball et al., 2010).

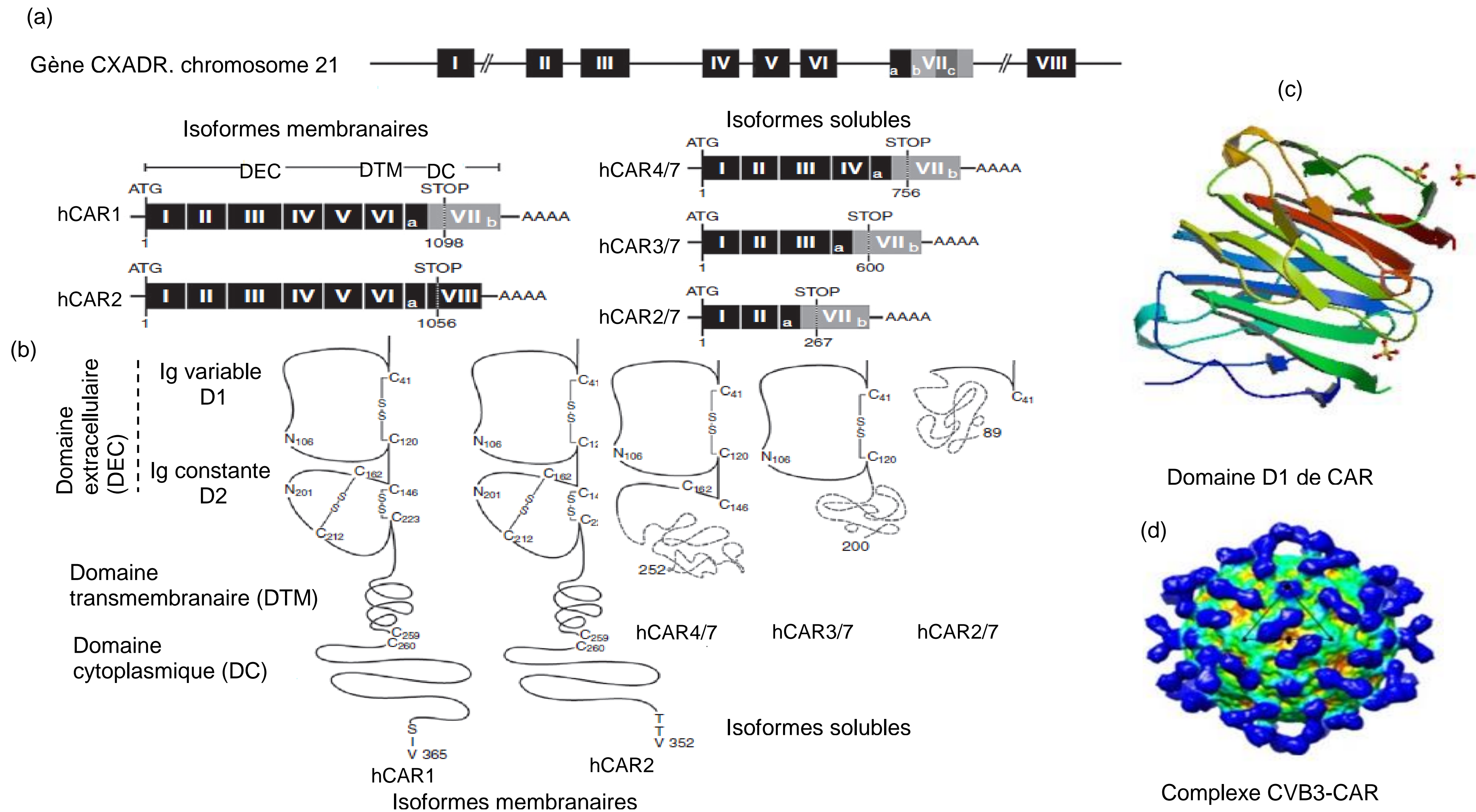


Figure 3. CAR humain : gène, transcrits et protéines

Plusieurs transcrits sont possibles à partir du gène CXADR (a), donnant deux isoformes membranaires et trois isoformes solubles (b) (Loustalot et al., 2016). Le domaine D1 a une structure dimérique (c) (van Raaij et al., 2000), et est le principal site d'interaction avec avec CVB (d) (Organtini et al., 2014).

CVB et infection dépendante d'anticorps

Les virus initient l'infection en reconnaissant et en se liant à la cellule cible via l'interaction entre des protéines des particules virales et des récepteurs spécifiques et/ou des corécepteurs situés à la surface la cellule cible. Des anticorps «neutralisants» spécifiques des protéines virales de surface peuvent inhiber cette étape d'interaction. En revanche, dans certaines circonstances, des anticorps peuvent permettre une entrée plus efficace du virus dans la cellule cible potentialisant ainsi l'infection virale (Sauter and Hober, 2009; Takada and Kawaoka, 2003). Ce phénomène d'infection dépendante d'anticorps (IDA) a été décrit depuis les années 1960 (Hawkes and Lafferty, 1967).

Plusieurs mécanismes d'infection dépendante d'anticorps ont été proposés. Le principal mécanisme décrit est celui dépendant des FcγR. Selon ce concept, le complexe virus-anticorps peut se lier aux cellules dotées de FcγR comme c'est le cas des monocytes, des macrophages, des lymphocytes B et des granulocytes, à travers l'interaction entre le Fc des anticorps et le FcγR, fournissant ainsi un pont solide entre le virus et la cellule cible (Sauter and Hober, 2009). D'autres mécanismes de l'IDA ont été ensuite décrits, et impliquent notamment les molécules du complément. En effet la liaison de C1 au Fc des anticorps peut initier la voie classique du complément avec activation de C3 qui peut se lier de façon covalente aux anticorps ou à la surface des particules virales. Le complexe qui en résulte va interagir avec le récepteur du complément (CR) à la surface cellulaire et faciliter l'entrée du virus. Alternativement, le C1q peut servir directement de lien via son récepteur cellulaire (C1qR) (Takada and Kawaoka, 2003).

Pour les CVB, la facilitation est dépendante des FcγR; mais elle ne permet pas au virus de s'affranchir complètement de son récepteur spécifique, la molécule CAR (Sauter et Hober, 2009). En effet, des travaux antérieurs ont montré que la facilitation de l'infection à CVB des monocytes nécessite à la fois la molécule CAR et des FcγR à leur surface (Hober et al., 2001).

L'IDA semble dépendre de nombreux facteurs incluant notamment le virus, l'interaction entre l'anticorps et la cellule hôte mais également la spécificité (épitope), la classe et la concentration de l'anticorps (Takada and Kawaoka, 2003). En effet, le répertoire des anticorps produits au cours d'une infection virale varie en fonction du virus, de l'état immunologique de l'hôte et du stade de l'infection. Les caractéristiques de ces anticorps dépendent essentiellement de leurs épitopes. Un anticorps neutralisant doit inhiber une protéine clé du virus. Lorsque la conformation du complexe virus-anticorps permet l'interaction de la portion Fc de l'anticorps avec le FcγR ou le complément, on peut observer un phénomène de facilitation (Sauter and Hober, 2009). Des études précédentes ont rapporté que les anticorps impliqués dans la facilitation de l'infection à CVB sont des IgG dirigées contre la protéine VP4 de la capside virale alors que les anticorps neutralisants sont

dirigés contre la protéine VP1 (Chehadeh et al., 2005; Sauter et al., 2008). Il a été également décrit qu'un anticorps neutralisant pour un virus peut être facilitant pour un autre sous-type du même virus ou un autre virus, démontrant ainsi qu'un épitope associé à la neutralisation d'un virus peut être associé à la facilitation d'un autre (Halstead et al., 2010). De même la concentration de l'anticorps est importante. Ainsi, il est possible que des anticorps neutralisants, à des concentrations subneutralisantes, facilitent l'infection in-vitro. En effet, en présence de fortes concentrations de l'anticorps, la plupart des sites fonctionnels des protéines virales sont bloqués, inhibant l'entrée du virus. Par contre, si la portion Fc de l'anticorps neutralisant est libre pour se lier au FcγR cellulaire, il peut y avoir une facilitation de l'infection dans des conditions subneutralisantes où des protéines virales inoccupées vont médier l'entrée (Takada and Kawaoka, 2003). Ainsi la facilitation de l'infection à CVB a été observée avec du plasma fortement dilué, mais n'était pas détectée avec des dilutions moindres de plasma (Hober et al., 2001, 2002).

Cycle de réplication

Après l'internalisation, le génome viral libéré sert d'ARN messager qui est traduit par la cellule hôte en protéines virales nécessaires pour la réplication et la production de nouvelles particules virales. La traduction de l'ARN viral se fait selon un mécanisme cap-indépendant. La voie classique cap-dépendante de traduction des ARN messagers cellulaires implique la reconnaissance de l'extrémité 5' et le recrutement de la sous-unité ribosomale 40S à l'ARN, grâce à son interaction avec eIF3 qui se lie à eIF4G. Ce dernier fait partie du complexe eIF4F qui contient aussi eIF4A et eIF4E, la protéine qui se lie à l'extrémité 5'. La traduction de l'ARNm viral repose sur une voie alternative. En effet, la sous-unité 40S du ribosome peut se lier directement à l'ARN ou peut être recrutée à la séquence IRES, via les protéines d'initiation de la traduction. Dans les cellules infectées par la plupart des picornavirus, la protéine eIF4G est clivée, entraînant ainsi l'inactivation de la traduction de la plupart des ARNm cellulaires. Néanmoins, la sous-unité 40S du ribosome est recrutée à l'IRES grâce à l'interaction de la protéine eIF3 lié au domaine C-terminal de la protéine eIF4G qui se lie directement à l'IRES.

La réplication de l'ARN viral se déroule en 2 stades : (i) la synthèse d'un brin négatif complémentaire à partir de l'ARN génomique parental, (ii) la synthèse de nouveaux brins positifs qui servent à la fois pour la traduction et pour la formation de nouveaux virions. Ces événements ont lieu à la surface de petites vésicules intracellulaires. En effet l'infection entraîne la prolifération et le réarrangement des membranes intracellulaires dans les cellules infectées. Le réticulum endoplasmique et l'appareil de Golgi sont détruits dans ce processus et le cytoplasme est rempli de vésicules à double membrane (Racaniello, 2013).

Une fois que la production de protéines est suffisante, l'encapsidation commence. La protéine VPg serait impliquée dans l'encapsidation. L'encapsidation de l'ARN est associée à la maturation de VP0 en VP2 et VP4. Il est généralement admis que la libération des nouveaux virions se fait par lyse cellulaire. Cependant des voies alternatives existent car les entérovirus peuvent établir des infections persistantes dans les cellules. Le temps nécessaire pour un cycle de réplication varie entre 5 et 10h et dépend de plusieurs facteurs tels que le type viral, la cellule hôte, la température, le pH ou la dose infectieuse (Racaniello, 2013).

La figure 4 présente les principales étapes de la réplication des CVB.

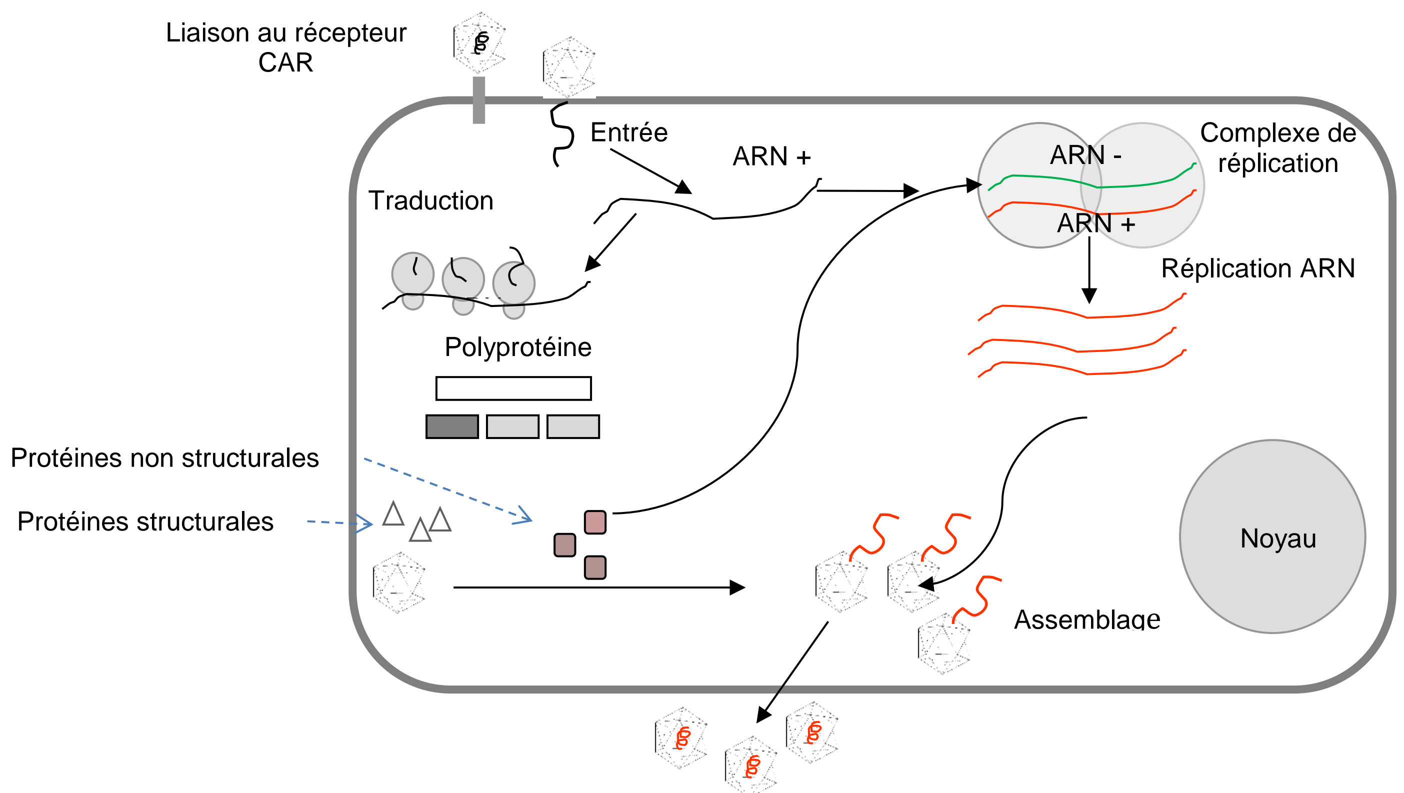


Figure 4. Représentation schématique du cycle de réplication des CVB

Les principales étapes de la réplication sont la liaison au récepteur, l'entrée et la décapsidation, la traduction de l'ARN viral en polyprotéine qui donne après clivage les protéines structurales et non structurales, la synthèse de brins d'ARN négatif qui servent de support à la synthèse de nouveaux brins d'ARN viral, l'assemblage et la libération des nouveaux virions.

5. Interaction des CVB avec l'immunité innée et mécanismes d'échappement

Plusieurs types de récepteurs de l'immunité innée sont impliqués dans la réponse aux virus notamment les récepteurs Toll-like (TLR), RIG-I like (RLR) et NOD-like (NLR). L'activation de ces récepteurs entraîne la production de cytokines, chimiokines et autres protéines ayant pour but, d'une part de contenir directement l'infection virale et d'autre part d'activer l'immunité adaptative (Kemball et al., 2010). Le rôle des TLR et des RLR dans la réponse aux CVB est bien connu. Le TLR3, récepteur endosomal, reconnaissant des molécules d'ARN double-brin produites au cours de la réplication virale, joue un rôle central dans la réponse au CVB. L'infection par CVB4 de souris déficientes en TLR3 entraîne une réduction de la production de médiateurs pro-inflammatoires ainsi qu'une augmentation de la réplication virale avec des lésions tissulaires plus importantes (Richer et al., 2009). Des observations similaires ont été obtenues au cours de l'infection par CVB3 (Abston et al., 2013; Negishi et al., 2008). Le rôle d'autres TLR situés au sein des endosomes comme le TLR7 et le TLR8 a été également décrit au cours de l'infection à CVB. Ils sont notamment impliqués dans la reconnaissance de l'ARN viral simple brin (Takeuchi and Akira, 2009; Triantafilou et al., 2005; Xagorari and Chlichlia, 2008). Le TLR4, récepteur exprimé à la surface cellulaire et habituellement activé par le LPS bactérien, serait impliqué dans la production de cytokines, induite par CVB4 dans des cellules pancréatiques (Triantafilou and Triantafilou, 2004).

Après leur reconnaissance, les TLR recrutent des adaptateurs comme MyD88 et TRIF pour la transmission de signaux. En réponse à l'ARN double-brin, la signalisation TLR3 est activée par l'adaptateur TRIF qui recrute TRAF3. Ce dernier active des kinases qui médient la phosporylation des facteurs transcriptionnels IRF3/7. Ces facteurs pénètrent dans le noyau et stimulent la production d'interférons de type 1 (IFN I). La signalisation TLR3 médie également l'activation de NF-kB via l'adaptateur TRIF, qui peut aussi pour cela interagir directement avec TRAF6 (Jiang et al., 2004; Kawai and Akira, 2006; Takeda and Akira, 2005). Les signalisations TLR7 et 8 reposent aussi sur les adaptateurs MyD88 et TRAF6, et aboutissent à la phosphorylation de IRF3/7 et à l'activation de NF-kB (Muroi and Tanamoto, 2008).

Parmi les RLR, deux senseurs jouent un rôle dans la reconnaissance de l'ARN viral et sont situés dans le cytosol. Il s'agit de RIG-I et surtout de MDA5 (melanoma differentiation-associated gene 5). RIG-I reconnaît l'ARN double brin court et l'ARN simple brin comportant un groupement triphosphate en 5', alors que MDA5 détecte surtout l'ARN double brin long (Hornung et al., 2006; Kato et al., 2006). Le rôle de MDA5 au cours de l'infection par les CVB a été rapporté in vivo. En effet, l'infection par CVB3 de souris MDA5KO s'accompagne d'une

réduction importante de la production d'IFN I (Hühn et al., 2010; Wang et al., 2010), et d'une augmentation tout au moins transitoire du titre viral (Hühn et al., 2010). RIG-I et MDA5 comportent deux domaines CARD (caspase activation recruitment domain) qui sont importants pour l'activation des IFN I, via une interaction avec l'adaptateur MAVS (mitochondrial antiviral signalingprotein). MAVS sert d'échafaudage pour l'interaction avec TRAF3 et les kinases, et l'activation de IRF3/7, conduisant à la production d'IFN I (Takeuchi and Akira, 2009; Yan and Chen, 2012).

Les NLRs représentent la troisième catégorie de senseurs de l'immunité innée. Ils sont intracellulaires et certains NLR peuvent être assemblés en structures plus larges appelées inflammasomes dont le plus connu reste NLRP3 (NLR, pyrin domain containing 3). La signalisation NLRP3 conduit à l'activation de la caspase-1 qui aboutit à une production de cytokines pro-inflammatoires notamment IL-1β et IL-18 (Keller et al., 2008; Lu et al., 2014). Le lien entre NLRs et infection à entérovirus a été très peu étudié. Récemment, il a été rapporté que l'inflammasome NLRP3 joue un rôle important dans la pathogenèse de CVB3 (Wang et al., 2014).

Par ailleurs, les entérovirus interagissent également avec la voie de l'autophagie. Cette interaction a été clairement démontrée pour le poliovirus, avec le rôle notamment des protéines 2BC et 3A (Suhy et al., 2000). Elle a été également évoquée pour les CVB. Le blocage de l'autophagie entraîne une réduction significative du titre viral de CVB3 dans les cellules HeLa et les cellules neuronales primaires (3 fois et 30 fois respectivement) (Wong et al., 2008; Yoon et al., 2008). Ces virus entraveraient la maturation de l'autophagosome, probablement après la fusion endosomale et avant l'interaction avec les TLRs (Kemball et al., 2010).

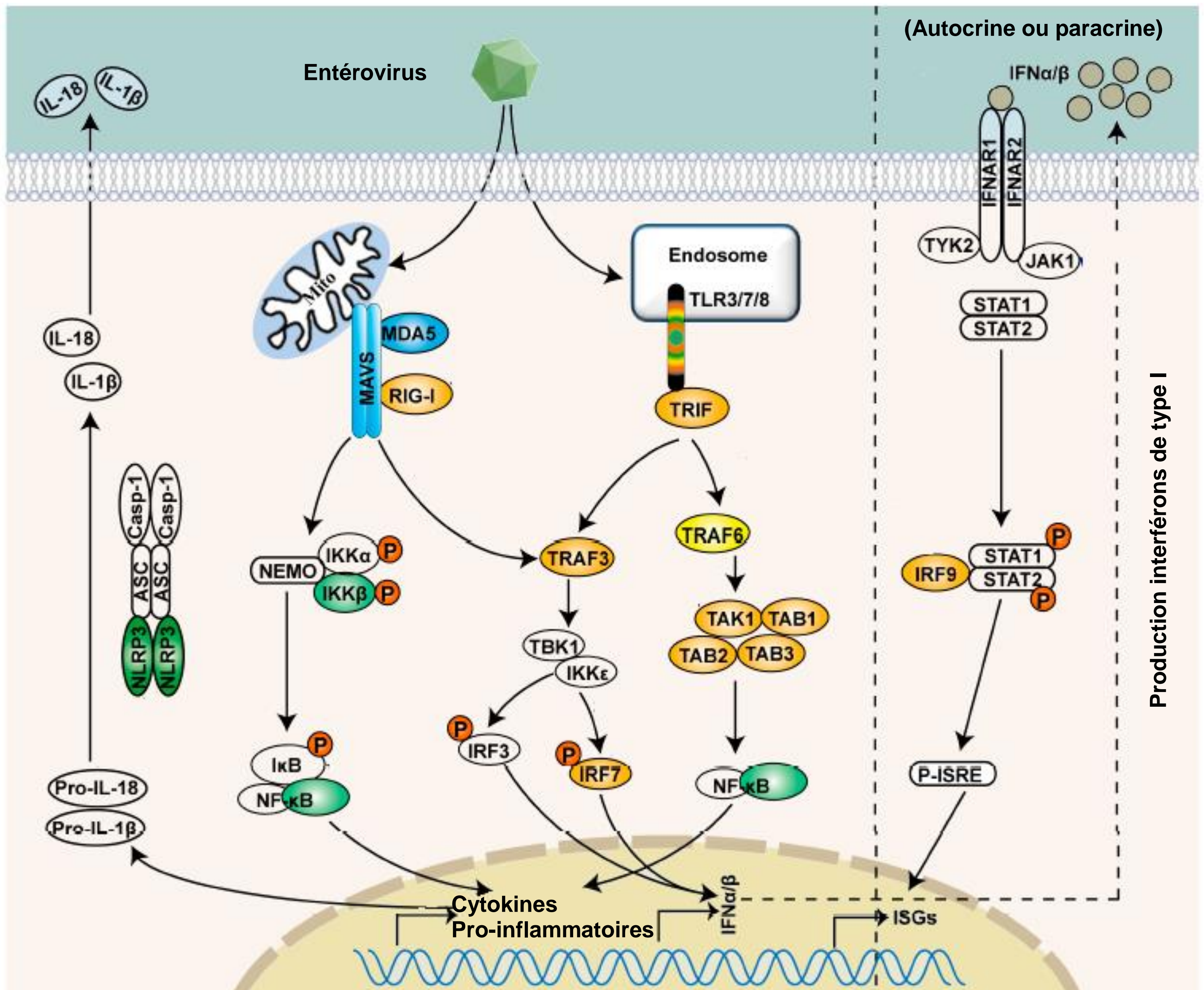


Figure 5. Réponse de l'immunité innée aux entérovirus

Les principaux senseurs, les voies de signalisation et les réponses induites sont représentés.

Adapté de Lei X et al., 2016

Tout l'arsenal décrit ci-dessus montre que l'immunité innée joue un rôle central dans le contrôle de l'infection par les entérovirus. Pour survivre et se répliquer, ces virus doivent donc développer des mécanismes d'échappement notamment aux réponses IFN. Nous évoquons ici brièvement les stratégies décrites dans la littérature en insistant sur les observations faites pour les CVB. La revue par Lei et al. (Lei et al., 2016) présente de façon plus détaillée ces mécanismes d'évasion. Les entérovirus peuvent altérer ou contourner de différentes manières les réponses de l'immunité innée. L'inhibition de la synthèse des protéines de l'hôte, l'interférence avec les récepteurs ou les signalisations qui en découlent, l'altération de la signalisation induite par les IFN I, ou d'autres voies sont les stratégies les plus fréquemment décrites (Lei et al., 2016). La protéase 2A de CVB3 peut cliver directement MDA5 ou cibler l'adaptateur MAVS (Feng et al., 2014). La protéase 3C de CVB3 peut également cliver MAVS surexprimé in vitro (Lei et al., 2016; Mukherjee et al., 2011; Wang et al., 2013) ou encore l'adaptateur TRIF, entravant ainsi la signalisation TLR3 (Mukherjee et al., 2011). De même, au cours des stades précoces de l'infection, des granules de stress avec des propriétés antivirales peuvent se former, et sont plus tard clivées par la protéase 3C (Reineke and Lloyd, 2015; White et al., 2007). Par ailleurs, l'échappement des CVB peut également être médié par les microARNs (Lei et al., 2016) et sera abordée dans le paragraphe ci-après.

6. Coxsackievirus B et microARNs

Les microARNs (miARNs) sont des petits ARNs non codants (environ 22 nucléotides) découverts récemment et qui agissent par interférence au niveau des ARNs messagers (Bartel, 2009; Ha and Kim, 2014; Hammond, 2015; Park and Shin, 2014). Plus d'un millier de miARNs ont été identifiés à ce jour, et sont impliqués dans la régulation d'environ 30% des gènes chez l'animal et l'homme (Bartel, 2009; Lewis et al., 2005). La biogenèse des miARNs est initiée dans le noyau cellulaire par l'ARN polymérase II ou III. Le transcrit miARN ou pri-miARN est clivé par la RNase III Drosha en précurseur du miARN ou pré-miARN. Le précurseur est transporté dans le cytoplasme et sous l'action de la RNase III Dicer, des duplexes miARN-miARN seront générés (Bernstein et al., 2001; Borchert et al., 2006; Cullen, 2004; Grishok et al., 2001; Hutvágner et al., 2001). Le miARN mature s'associe à une protéine Ago pour former un complexe RISC (de l'anglais « RNA induced silencing complex ») grâce auquel le miARN peut dégrader des ARNs messagers spécifiques, en ciblant des sites complémentaires de ces ARNm spécifiques, notamment dans la région 3' non codante (Hammond et al., 2000; Standart and Jackson, 2007; Wu et al., 2006). Néanmoins, certains miARNs peuvent cibler parfois la région 5' non codante des ARNm (Duursma et al., 2008; Grey et al., 2010; Lee et al., 2009; Rigoutsos, 2009). Les miARNs

peuvent également agir au niveau de l'étape post-transcriptionnelle, et régulent par ce mécanisme plusieurs fonctions biologiques comme la prolifération, la différenciation, l'apoptose et les interactions hôte-pathogène (Umbach and Cullen, 2009; Winter et al., 2009) Des virus à ADN sont capables de coder pour des miARNs qui sont capables de réguler l'expression génique virale et cellulaire et contribuer ainsi à la virulence virale (Gottwein et al., 2007; Pfeffer et al., 2004; Umbach and Cullen, 2009). Mais le plus souvent, ce sont des miARNs cellulaires qui sont induits par l'infection virale, et qui jouent un rôle dans la pathogenèse du virus, ou dans la réponse de l'hôte (Jopling et al., 2005; Triboulet et al., 2007).

Le rôle de plusieurs miARNs a été décrit au cours de l'infection par les entérovirus. Nous décrivons brièvement ici les principales observations rapportées dans la littérature pour les coxsackievirus du groupe B. Pour un exposé plus complet sur les entérovirus et les miARNs, le lecteur est invité à se reporter à la revue générale publiée par Ho et al (Ho et al., 2016).

Les miARNs induits par les entérovirus peuvent interférer avec la réponse immunitaire et contribuer à l'échappement du virus. Ainsi, il a démontré que des miARNs tels que miR-155 et miR-148a, induits par l'infection à CVB3, interfèrent avec la réponse immune notamment la voie NFkB (Bao and Lin, 2014).

Des miARNs induits par les entérovirus peuvent être impliqués dans le processus d'apoptose. L'expression de miR-21 est réduite au cours de la myocardite à CVB3. Le rôle anti-apoptotique de ce miARN au cours de la myocardite virale a été démontré ; sa cible étant PDCD4 (Programmed Cell Death 4).

Les miARNs peuvent également interférer avec des molécules de la membrane cellulaire et moduler la signalisation qui en découle. Une étude a rapporté que miR-21 induit au cours de l'infection à CVB3, augmente la dégradation de la démine en ciblant l'enzyme YOD1 (Ye et al., 2014). Quant à miR-1, il cible la protéine GPJ1 (Gap junction protein alpha 1) (Xu et al., 2012). CVB3 induit également miR-126 qui joue un rôle important dans le lien entre ERK1/2 et les voies de signalisation WNT/bêta-catenine, et favorise la propagation de CVB3 (Ye et al., 2013).

Par ailleurs des miARNs cellulaires peuvent cibler des séquences virales et moduler la réplication virale. Ainsi par exemple, il a été rapporté que miR-342-5p cible la région codant pour la protéine 2C de CVB3 et inhibe la réplication virale (Wang et al., 2012), alors que la réplication virale est plutôt favorisée par l'expression de miR-10a qui cible directement la séquence du génome de CVB3 codant pour la protéine 3D (Tong et al., 2013).

7. CVB, inflammation et auto-immunité

Dès l'entrée et la réplication des CVB dans la cellule hôte, l'interaction avec les senseurs de l'immunité innée s'accompagne d'une réponse inflammatoire. L'activation de facteurs transcriptionnels tels que IRF3/7 conduit à la production d'interférons de type I, alors que celle de NFkB entraîne la synthèse de plusieurs cytokines, chimiokines et autres molécules impliquées dans la réponse de l'hôte (Buskiewicz et al., 2012; Yajima and Knowlton, 2009). Le but initial de cette réponse inflammatoire induite est d'éliminer le virus, mais elle peut également dans certains cas contribuer à la pathogénie de l'infection virale (Fairweather and Rose, 2005).

Le pouvoir inflammatoire des CVB a été démontré in vitro. Il a été montré que l'infection des monocytes par CVB3 induit la production de cytokines proinflammatoires tels que TNFα, d'IL-1b et d'IL-6 (Henke et al., 1992; Vreugdenhil et al., 2000). Il en est de même pour les cellules endothéliales (Conaldi et al., 1997; Zanone et al., 2003) et les fibroblastes du myocarde (Heim et al., 2000), les cellules INS-1 (Nair et al., 2010), ou les cellules Panc-1 (Triantafilou and Triantafilou, 2004).

La production d'IFNα a été retrouvée dans les cellules bêta au cours de l'infection persistante à CVB4 (Chehadeh et al., 2000). Le virus peut induire également des quantités élevés d'IFNα dans les monocytes, en présence d'anticorps facilitateurs (Hober et al., 2001).

Les cytokines et les chimiokines sont importantes pour l'amplification et la propagation de la réponse inflammatoire notamment par le recrutement de cellules de l'immunité, permettant l'initiation des réponses adaptatives. Cet environnement cytokinique peut jouer un rôle dans l'initiation de processus auto-immuns.

L'idée d'un lien entre auto-immunité et infection virale n'est pas récente et a parfois fait l'objet de controverses. En ce qui concerne les CVB, ils sont particulièrement incriminés dans l'auto-immunité associé à deux pathologies que sont la myocardite chronique et le diabète de type 1 (Eringsmark Regnéll and Lernmark, 2013; Reddy et al., 2013).

Une hypothèse majeure est que l'infection par des agents infectieux cytopathiques comme les CVB entraîne des lésions tissulaires, avec pour conséquence la libération d'antigènes cellulaires séquestrés ou présents à très faible concentration avant l'infection. De même, il est envisageable que les clones de lymphocytes T auto-réactifs vis-à-vis de ces antigènes aient pu échapper au phénomène de tolérance centrale, étant donné que ces antigènes étaient peu ou pas représentés dans l'épithélium thymique (Massilamany et al., 2016). Ainsi à la suite d'une infection, ces lymphocytes T peuvent rencontrer les auto-antigènes présentés de façon efficace par des cellules de l'immunité innée dans un environnement inflammatoire, où l'expression de molécules de co-stimulation et la présence de cytokines permettent leur prolifération. Du fait de la faible affinité probable de ces lymphocytes T, le

processus auto-immun sera progressif et évoluera sur une longue période avant le développement de la maladie (Getts et al., 2013). Dans ce scénario il s'agit donc d'une « activation en passant » de l'auto-immunité par le virus à travers l'induction d'une inflammation. Plusieurs données chez l'animal supportent que l'activation de MDA5, un récepteur de l'immunité innée utilisé par les CVB, puisse directement contribuer au développement de l'auto-immunité. Ainsi, certaines mutations entraînant un gain de fonction de MDA5, sont associées à une auto-immunité de type lupus-like chez la souris (Funabiki et al., 2014). De même une production chronique de taux élevés d'IFN I aboutit à une situation pathologique similaire (Sozzani et al., 2010). Par ailleurs, les souris transgéniques qui expriment plusieurs copies du gène IFIH1 (codant pour MDA5), sont plus sujettes à l'auto-immunité (Crampton et al., 2012). Ces données sur modèle animal sont confortées par l'apparition de maladies auto-immunes chez des patients ayant des mutations naturelles d'hyperactivité sur le gène IFIH1. De tels patients présentent également une affinité forte de MDA5 pour l'ARN ainsi qu'une signature IFN (Rice et al., 2014).

Une autre théorie évoquée pour l'auto-immunité induite par des virus est le mimétisme moléculaire. Elle repose sur l'existence d'épitopes peptidiques similaires entre le virus et des antigènes du soi. Quelques exemples ont été décrits pour les CVB. Des auto-anticorps dirigés contre la myosine cardiaque ont été observés au cours de l'infection à CVB3 chez des souris développant une myocardite, et l'administration passive de ces auto-anticorps peut transférer la maladie (Liao et al., 1995; Neu et al., 1987). De même l'infection de souris par CVB3 génère des lymphocytes T dont le transfert entraîne une myocardite chez des souris naïves (Gangaplara et al., 2012). Des résultats non concordants ont été rapportés concernant l'hypothèse d'un mimétisme moléculaire entre la glutamate décarboxylase et une séquence peptidique de la protéine 2C des CVB (Atkinson et al., 1994; Schloot et al., 2001).

L'hypothèse d'un rôle du mimétisme moléculaire dans l'induction de l'auto-immunité reste assez fragile à cause de la flexibilité dans la reconnaissance des épitopes par les lymphocytes T. En effet, l'existence d'un ou de quelques acides aminés communs peut être suffisant pour entraîner des réactions croisées dans la reconnaissance par les lymphocytes T (Evavold et al., 1995; Massilamany et al., 2016).

Un mécanisme alternatif pour l'induction de l'auto-immunité par les CVB pourrait être la double expression de TCR par les cellules T. En effet, il a été rapporté qu'environ 30% des lymphocytes T chez l'homme et 15% chez la souris expriment des TCR avec deux différents réarrangements Vα et donc deux spécificités antigéniques (Cusick et al., 2012).

La figure 6 décrit les divers mécanismes possibles de l'induction de l'auto-immunité par les picornavirus.

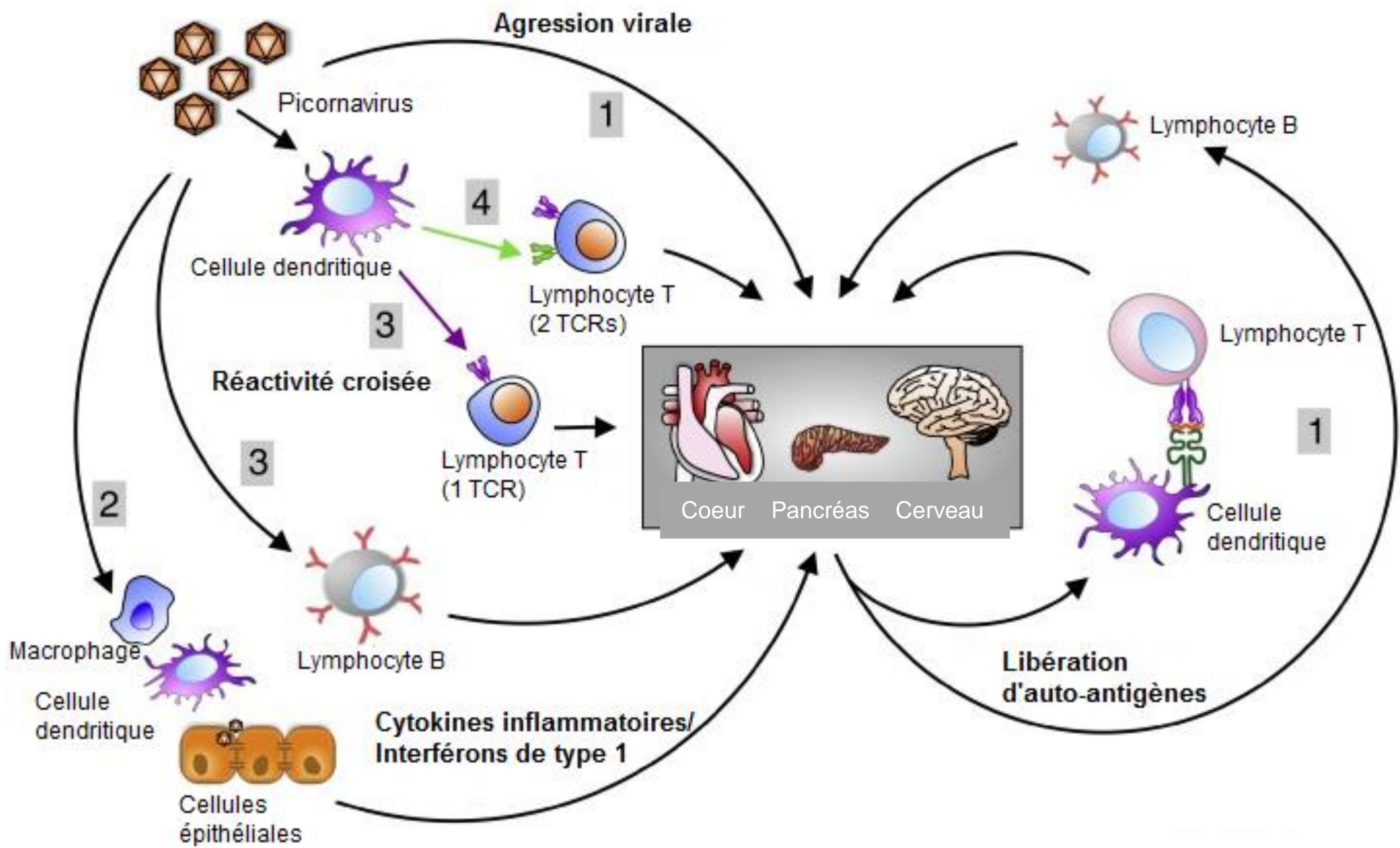


Figure 6. Mécanismes possibles de l'auto-immunité induite par les picornavirus

Après l'infection, les picorvavirus peuvent directement agresser les organes cibles, entraînant une libération d'auto-antigènes y compris ceux situés à l'intérieur des cellules. Ces antigènes sont présentés par les cellules dendritiques aux lymphocytes T auto-réactifs. Les lymphocytes B auto-réactifs peuvent également être stimulés par ces antigènes et produire des auto-anticorps (1). L'agression peut être due également à la production excessive de cytokines pro-inflammatoires et d'interférons de type 1 par les cellules de l'immunité innée notamment les macrophages et les cellules dendritiques (2). L'infection par des virus comportant des séquences similaires à des auto-antigènes peut induire des réponses T et B croisées (mimétisme moléculaire) (3). De façon alternative, certains lymphocytes pourraient avoir un double TCR (un spécifique pour le virus, et l'autre pour un auto-antigène) (4).

Adapté de Massilamany et al. (Massilamany et al., 2016)

8. CVB et persistance

Les CVB sont des virus cytolytiques, et cette propriété est à la base de la plupart des manifestations aiguës chez l'hôte. Cette lyse massive peut être aisément mise en évidence in vitro par l'apparition d'un effet cytopathique (ECP) dans plusieurs systèmes cellulaires. Néanmoins ces virus peuvent également établir une persistance dans plusieurs tissus cibles et dans les cellules in vitro, avec peu ou pas d'ECP visible (Frisk, 2001; Pinkert et al., 2011). La persistance virale a été associée à la pathogenèse des maladies chroniques dans lesquelles le rôle des CVB a été évoqué comme la cardiomyopathie dilatée, le diabète de type 1 ou la myopathie inflammatoire chronique (Chapman and Kim, 2008; Jaïdane and Hober, 2008a; Jaïdane et al., 2010; Tam et al., 2003). Le lien entre ces affections et la persistance des CVB n'est pas encore entièrement élucidé, mais il est admis que la pathogenèse est liée moins à une réplication virale active, qu'à une présence prolongée des composants viraux et leur interaction avec l'immunité innée. Ce scénario est compatible avec l'activation du processus auto-immun évoqué dans ces affections. In vitro, deux types majeurs de persistance virale ont été décrits : (i) la persistance de type stable « steady-state » dans laquelle toutes les cellules sont infectées, sans réplication lytique, et (ii) la persistance de type porteur « carrier-state » caractérisée par l'infection d'une proportion des cellules, avec une production de nouvelles particules virales (Frisk, 2001). Les données de la littérature supportent une infection persistante du deuxième type pour les CVB. La persistance virale résulte d'une coévolution de la cellule hôte et du virus, notamment à travers une adaptation de la résistance de la cellule et de la virulence de la souche virale (Heim et al., 1992, 1995; Pinkert et al., 2011). Les facteurs viraux ont été étudiés au cours de la persistance des CVB. Les résultats les plus souvent retrouvés concernent des altérations du génome viral. Il peut s'agir de mutations entraînant des substitutions d'acides aminés, qui résultent en une liaison plus faible avec le récepteur du virus (Schmidtke et al., 2000). De même il a été retrouvé fréquemment une délétion dans la région 5' non codante qui est connue pour jouer un rôle important dans la réplication du virus. Cette délétion a été décrite in vivo dans le myocarde humain et murin ainsi que dans des modèles cellulaires (Chapman et al., 2008; Kim et al., 2005, 2008). La même observation a été rapportée dans un modèle murin de persistance de CVB dans le pancréas (Tracy et al., 2014). Un autre mécanisme implique la formation et la persistance d'une forme atypique et stable d'ARN double brin, décrite notamment dans les fibres musculaires (Tam and Messner, 1999). Parmi les facteurs cellulaires, le cycle cellulaire et l'état d'activation de la cellule ont été décrits comme pouvant jouer un rôle important (Feuer and Whitton, 2008; Feuer et al., 2002, 2004). Par ailleurs, la diminution de l'expression du récepteur CAR a été retrouvée au cours de la persistance et

s’accompagne d’une diminution de la réplication virale et de la lyse cellulaire (Fechner et al., 2007; Pinkert et al., 2011; Werk et al., 2005).
Notre revue générale présentée en annexe 1, décrit plus en détail la persistance des entérovirus et ses conséquences (Alidjinou et al., 2014).

9. CVB et diabète de type 1

Le diabète de type 1 (DT1) est une maladie chronique, de nature auto-immune due à un défaut de production d’insuline, consécutive à une destruction ou une altération de fonction sélective des cellules bêta du pancréas. La prédisposition au DT1 est influencée par des facteurs génétiques et les gènes les plus importants prédisposant au DT1 sont situés au niveau des loci HLA-DR et DQ (Hober and Sauter, 2010). Cependant cette susceptibilité génétique est insuffisante, et des facteurs environnementaux dont notamment les virus sont nécessaires pour le développement de la maladie (Knip and Simell, 2012).
Divers virus humains ou animaux peuvent provoquer expérimentalement une altération des cellules béta des îlots de pancréas productrices d’insuline et induire l’apparition d’auto-anticorps et d’un DT1 auto-immun. Chez l’homme, de nombreuses données épidémiologiques, clinico-biologiques et expérimentales supportent fortement l’implication des entérovirus et notamment des CVB dans la pathogenèse du DT1 (Hober et al., 2013).
La suspicion du lien entre EV et DT1 remonte aux années 1960 lorsque Gamble et al. (Gamble et al., 1969) ont rapporté une fréquence plus élevée d’anticorps neutralisants anti-CVB4 chez les sujets DT1 que chez les sujets contrôles. Mais la principale observation historique ayant accéléré les travaux sur le rôle des EV dans le DT1 a été faite par Yoon et al. qui isolèrent en 1979 un coxsackievirus B4 (CVB4) à partir du pancréas d’un enfant décédé d’une acidocétose diabétique. L’inoculation de cet isolat à des souris s’accompagna de l’apparition d’un diabète auto-immun (Yoon et al., 1979). Un cas similaire impliquant coxsackievirus B5 a été décrit un an plus tard (Champsaur et al., 1980).
L’un des principaux arguments qui supporte fortement un lien entre les entérovirus et le DT1 est la prévalence des marqueurs d’infection à EV plus élevée chez les patients que chez les sujets sains. Les fréquences rapportées varient largement dans la littérature, et dépendent de la technique utilisée et de la nature du prélèvement. Néanmoins, la majorité des données disponibles convergent vers une détection plus fréquente chez les patients DT1 de composants viraux, ARN et protéines, à l’aide de techniques moléculaires ou immunologiques. Une méta-analyse (environ 4500 patients) reprenant la plupart des travaux a confirmé une association statistiquement significative entre l'infection à EV, principalement à CVB, et l'auto-immunité associée au DT1 (Odds ratio: 3.7) et le développement de la maladie (Odds ratio: 9,8) (Yeung et al., 2011).

La plupart des études de détection des composants viraux et notamment l'ARN viral, ont été réalisées à partir du sang périphérique qui est plus facile d'accès (Yeung et al., 2011). Néanmoins quelques travaux ont été conduits à partir de pancréas (nécropsies ou rarement biopsies) qui est le principal site de la maladie (Krogvold et al., 2014; Richardson et al., 2009).

Plusieurs mécanismes ont été proposés pour expliquer l'implication des EV dans le DT1 notamment : (i) le mimétisme moléculaire évoqué devant l'homologie de séquence entre la protéine entérovirale conservée 2C et le glutamate décarboxylase (GAD65), un auto-antigène des cellules béta ; (ii) l'activation «en passant» (bystander activation) de lymphocytes T autoréactifs préexistants grâce à des phénomènes inflammatoires iii) la persistance virale (Jaïdane and Hober, 2008b). Le scénario le plus plausible combine les deux dernières hypothèses, et repose sur l'induction de l'auto-immunité, à travers l'activation de processus inflammatoires par le virus. Une infection à entérovirus persistante non cytolytique est capable d'induire une inflammation locale au niveau des îlots (production d'interféron α et d'autres cytokines pro-inflammatoires), et une hyperexpression des molécules du CMH de classe I. Cette inflammation pourrait s'accompagner d'une présentation d'antigènes des cellules bêta et de leur destruction par des lymphocytes T CD8 cytotoxiques auto-réactifs (Hober and Sauter, 2010). Il a été démontré que de tels lymphocytes T, spécifiques des cellules bêta sont préexistants chez des sujets génétiquement prédisposés (Coppieters et al., 2012). Les modalités de l'interaction entre le virus et le système immunitaire notamment l'immunité innée jouent un rôle important dans la pathogenèse. Ainsi une réponse initiale très forte pourrait prévenir une infection productive efficace chez l'hôte et une dissémination du virus vers le pancréas. Par contre une réponse modérée ou faible permettrait au virus d'atteindre cet organe cible, voire d'y persister et initier une réponse inflammatoire notamment via l'activation des senseurs de l'immunité innée notamment les TLR (Lind et al., 2012). Par ailleurs, l'interaction entre le virus et le système immunitaire semble également dépendre de facteurs génétiques. Certains polymorphismes du gène Interferon-Induced Helicase 1 (IFIH1) ont été associés de façon significative à un risque élevé de diabète de type 1. Ce gène code pour la protéine MDA5, un récepteur intra-cytoplasmique responsable d'une activation de l'inflammation en réponse aux entérovirus et notamment aux CVB qui sont les sérotypes les plus incriminés dans le DT1 (Nejentsev et al., 2009). Récemment, le rôle de l'activation de l'immunité innée dans l'initiation de l'auto-immunité anti-îlot a été conforté par la mise en évidence d'une signature transcriptionnelle interféron de type 1 dans les cellules du sang périphérique avant l'apparition des auto-anticorps chez les sujets prédisposés (Ferreira et al., 2014; Kallionpää et al., 2014). Le lien entre les entérovirus et le diabète de type 1 est largement détaillé dans les annexes 2 et 3 (Alidjinou and Hober, 2016; Hober and Alidjinou, 2013).

OBJECTIF DU TRAVAIL DE THESE

Ce travail est une contribution qui vise une meilleure compréhension des interactions entre CVB4 et les cellules cibles, dans la perspective d'une implication du virus dans la pathogenèse du diabète de type 1 (DT1). Le rôle suspecté des entérovirus et notamment des CVB dans cette pathologie n'est pas lié à une infection lytique au niveau des cellules bêta du pancréas. Il relève plutôt des modalités de l'interaction entre le virus et les cellules pancréatiques, ainsi que les cellules de l'immunité innée. La figure ci-dessous présente schématiquement les principaux mécanismes potentiellement en jeu.

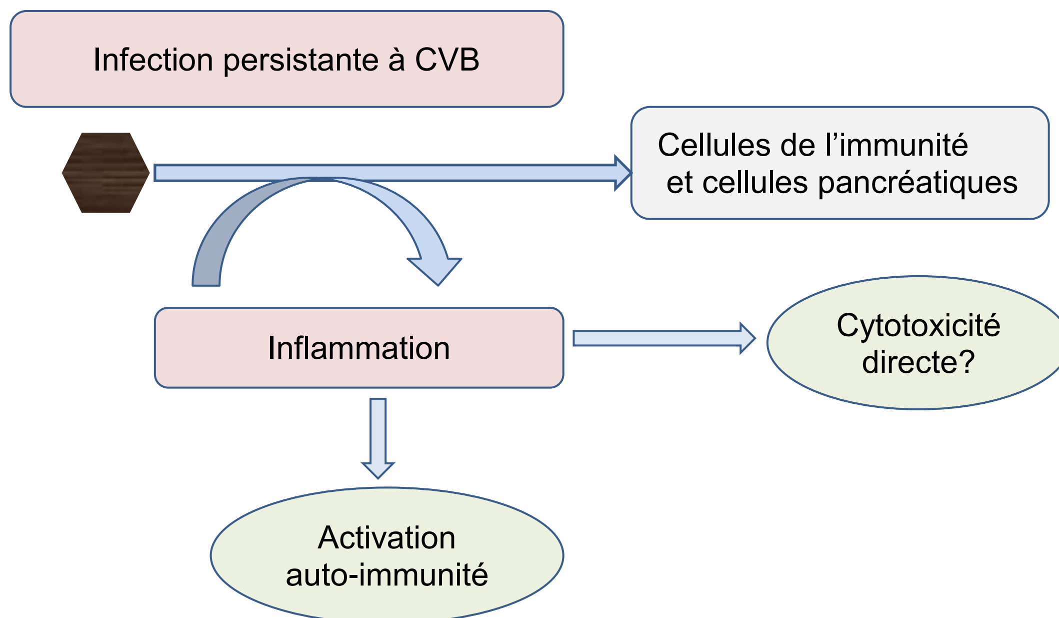


Figure 7. Hypothèse physiopathologique du diabète de type 1 auto-immun.

Le scénario physiopathologique actuellement admis combine chez des sujets génétiquement prédisposés, une persistance du virus, probablement dans plusieurs types cellulaires, et une activation de processus inflammatoires qui contribuent à un environnement favorable à l'activation de l'auto-immunité.

L'objectif global est donc d'étudier l'inflammation induite par CVB4 ainsi que sa persistance dans les cellules cibles.

- ***Objectif spécifique n°1 :*** *Décrire l'inflammation induite par CVB4 dans un modèle de cellules mononucléées (CMN) du sang périphérique.*

Les entérovirus ont été en effet détectés fréquemment dans le sang périphérique des patients diabétiques, notamment dans les CMN du sang périphérique. Les modalités de l'infection de ces cellules ainsi que leur réponse à l'infection peuvent conditionner l'issue de l'infection et notamment sa dissémination ou non aux autres organes cibles.

- ***Objectif spécifique n°2 :*** *Rechercher la persistance de CVB dans les monocytes/ macrophages humains et l'association ou non d'une inflammation à cette persistance*

Les monocytes sont un réservoir potentiel pour le virus et peuvent servir de véhicule pour le transport du virus vers des organes cibles. Les macrophages peuvent résulter de la différentiation des monocytes au niveau tissulaire. Ce sont des cellules clés de l'immunité innée qui jouent un rôle prépondérant de la réponse locale aux pathogènes.

- ***Objectif spécifique n°3 :*** *Décrire la persistance de CVB dans des cellules pancréatiques et identifier les facteurs cellulaires et viraux impliqués dans cette persistance.*

La persistance des CVB est possible dans plusieurs types cellulaires. Notre but est de rechercher dans un modèle de persistance du virus dans des cellules pancréatiques, les modifications cellulaires (niveau d'expression de récepteurs, expression de microARNs cellulaires) et viraux (degré de virulence, mutations) qui permettent l'équilibre hôte-pathogène au cours de la persistance virale.

TRAVAUX REALISES

ETUDE N°1 : Facilitation par le sérum humain de la production de cytokines inflammatoires, induite par coxsackievirus B4 dans des cellules mononucléées du sang périphérique.

Travail publié dans Journal of Molecular Biology

Alidjinou EK, Sané F, Engelmann I, Hober D. Serum-dependent enhancement of coxsackievirus B4-induced production of IFNα, IL-6 and TNFα by peripheral blood mononuclear cells. ***Journal of Molecular Biology***. 2013; 425(24): 5020-31

Résumé.

Objectif

Les interactions entre coxsackievirus B4 (CVB4) et les cellules mononucléées (CMN) du sang périphérique ont été peu étudiées. Il a été montré que l'incubation préalable de CVB4 avec du sérum dilué ou des IgG anti-CVB4 non neutralisants, avant l'inoculation aux CMN, entraîne la production d'interféron alpha (IFNα), alors que l'inoculation du virus seul entraîne très peu ou pas d'IFNα. La production d'autres cytokines et le rôle du sérum ont été étudiés dans ce travail.

Méthodes

Des CMN provenant de donneurs sains ont été inoculées avec du CVB4 infectieux ou inactivé, préalablement incubé ou non avec des dilutions de sérum humain ou d'IgG polyvalentes présentant une activité anti-CVB4. Les cytokines ont été quantifiées dans les surnageants de culture de CMN par technique ELISA. L'infection a été évaluée dans les cellules par RT-PCR quantitative.

Principaux résultats

L'inoculation de CVB4 aux CMN n'a pas induit de production d'IFNα, quel que soit le temps d'incubation, contrairement à *Herpes simplex virus*. Par contre, une quantité importante d'IL-6, et des taux significatifs de TNFα, d'IL-12 et d'IFNγ étaient détectés dans les surnageants de culture. La production d'IL-10 était faible. La production d'IL-6 et de TNFα était spécifique et dose-dépendante. L'inactivation du virus par les UV ou la chaleur, de même que l'inhibition de l'infectivité par du pléconaril ou le blocage du TLR4 n'ont pas supprimé la production de ces cytokines.

L'incubation préalable de CVB4 avec des dilutions de sérum humain, avant l'inoculation aux CMN, a entraîné la production de taux élevés d'IFNα, ainsi qu'une amplification de la production de l'IL-6 et du TNFα. Des résultats similaires étaient observés avec des IgG polyvalentes diluées.

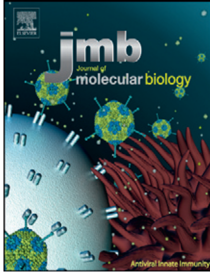
La facilitation de la production des cytokines n'était possible qu'avec du virus infectieux. Cette facilitation était associée à la détection d'un taux très élevé d'ARN viral intracellulaire, alors que l'ARN viral était peu détectable quand les CMN étaient inoculées avec du CVB4.

Conclusion

Dans les cultures de CMN, CVB4 infectieux ou non peut induire la production de cytokines pro-inflammatoires, alors que l'IFNα n'est pas détectable. Par contre le virus infectieux en présence de sérum ou d'IgG immunes peut stimuler la production d'IFNα et potentialiser la

production des autres cytokines. En conclusion, la facilitation de la production de cytokines est liée à l'infection des cellules. Cependant les particules non-infectieuses participent à la réponse inflammatoire au virus.

Article



Serum-Dependent Enhancement of *Coxsackievirus* B4-Induced Production of IFNα, IL-6 and TNFα by Peripheral Blood Mononuclear Cells

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Abstract

Only a few reports have been published on the interactions between *Coxsackievirus* B4 (CVB4) and human peripheral blood mononuclear cells (PBMC) but have not been extensively documented. Human serum containing non-neutralizing anti-CVB4 antibodies increased CVB4-induced synthesis of IFNα by PBMC. In this study, we determined if CVB4 and human serum have the ability to activate inflammatory cytokines in addition to IFNα in PBMC cultures. PBMC from healthy donors were inoculated with infectious, inactivated CVB4 or with CVB4 incubated with dilutions of human serum or polyvalent IgG with anti-CVB4 activity. Levels of IFNα, TNFα, IL-6, IL-12, IFNγ and IL-10 in the cell-free supernatants of PBMC cultures were measured using ELISA. Infection was assessed by real-time PCR. PBMC inoculated with CVB4 produced inflammatory cytokines but not IFNα. When CVB4 was incubated with serum or IgG, IFNα was detected in the culture supernatants, and high concentrations of TNFα and IL-6 were measured. The concentrations of TNFα and IL-6 were not reduced in cultures inoculated with inactivated CVB4, whereas the IgG-dependent enhancement of IFNα, IL-6 and TNFα production with inactivated virus was suppressed. The potentiation of IFNα production was associated with a high intracellular viral load. Infectious and non-infectious CVB4 can induce the production of inflammatory cytokines but not IFNα by PBMC. High levels of IFNα, in addition to TNFα and IL-6, in culture supernatants were obtained when infectious CVB4 was combined with immune serum or IgG, and they were associated with high amounts of intracellular viral RNA.

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Introduction

Coxsackieviruses are small, non-enveloped, single-stranded positive RNA genome viruses that belong to the *Enterovirus* genus within the *Picornaviridae* family. These ubiquitous pathogens are mainly transmitted via the fecal–oral route [1]. *Coxsackievirus* B (CVB) serotypes B1 to B6 are of particular interest because they have been associated with acute clinical features such as meningitis, encephalitis, pancreatitis or pericarditis [2] and with chronic diseases such as chronic myocarditis, dilated cardiomyopathy and type 1 diabetes (T1D) [1]. Several studies have reported on the role CVB3 plays in the pathogenesis of chronic autoimmune myocarditis, especially during an inflammatory process [3]. Epidemiological, clinical and animal model studies have suggested a close relation between CVB4 and the development of T1D [4–6]. Previous reports have suggested that mechanisms involved in viral pathogenesis of T1D may include the “bystanding” activation of preexisting autoreactive T cells [1,7]. In the proposed model, CVB4 induces significant local inflammation of beta cells, with an increase in stress markers, phagocytosis and presentation of autoantigens by resident macrophages. This leads to the recruitment and activation of autoreactive T cells that may contribute to disease development [8]. Inflammatory cytokines may thus play an important role in the enteroviral pathogenesis of T1D.

Moreover, besides the modulating effect on cells, cytokines may be toxic to beta cells, as shown by *in vitro* and *in vivo* studies, and could be involved in the pathogenesis of T1D [9]. CVB induced production of proinflammatory cytokines when studied in INS-1 [10], Panc-1 [11] and peripheral blood

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mononuclear cells (PBMC) [12,13]. CVB can activate the immune system, especially through sensors of innate immunity including Toll-like receptors (TLR) and RIG-like receptors (RLR), which in turn results in production of various cytokines and other proteins [14]. The intravesicular sensor TLR3 seems to play the most important role in the response to CVB [15]. However, other receptors such as TLR4, TLR7, TLR8 and MDA5 are also involved in the recognition of the virus [11,14].

To enter cells, CVB has to bind to the CAR (*C*oxsackievirus and *A*denovirus *R*eceptor) on the surface of the cell. Internal sensor responses are triggered upon release of viral RNA. The internalization process is similar to that reported for macropinocytosis [16]. However, an alternative mechanism exists for cells that express Fc receptors (FcR) and bind to non-neutralizing antibodies [17]. This phenomenon, termed “antibody-dependant enhancement (ADE)”, has been described for other pathogens [18]. In the most common mechanism of ADE, virus–antibody complexes bind to cells bearing FcγR, such as monocytes or macrophages, which provides a strong bridge for virus entry [17,19].

We have shown that incubation of CVB4 with diluted serum or non-neutralizing anti-CVB4 IgG antibodies before addition to PBMC resulted in production of high levels of interferon alpha (IFNα), while inoculation of the virus led to little or no production of IFNα [20–22]. The production of other cytokines and the role of serum remain uncertain. In the present study, we have determined whether CVB4 and human serum can activate proinflammatory cytokines, especially IL-6 and TNFα, in addition to IFNα in PBMC cultures.

Results

Cytokines, but not IFNα, are detected in culture supernatants of PBMC inoculated with CVB4

PBMC inoculated with CVB4 did not produce IFNα, regardless of the incubation time. This is in contrast to, Sendai virus and herpes simplex virus, which induced production of IFNα by PBMC (Fig. 1a).

PBMC can, however, produce other cytokines when inoculated with CVB4 (see Fig. 1b–f). TNFα was produced at 462 ± 203 pg/mL 24 h after inoculation. This concentration was much lower compared to production in the presence of lipopolysaccharide (LPS) or phytohaemagglutinin (PHA). The minimum concentration of IL-6 produced after 24 h of incubation was 5000 pg/mL. Significant levels of IL-12 (188 ± 5 pg/mL at 24 h) and IFNγ (170 ± 0.5 pg/mL at 48 h) were recorded. However, low levels of IL-10 (80 pg/mL) were recorded. Production of TNFα in the presence of CVB4 was not affected by polymyxin B. However, this anti-endotoxin significantly reduced the LPS-induced production of TNFα (Fig. 1g). This indicated that cytokine production was not due to endotoxins. CB4-induced production of TNFα and IL-6 is virus dose dependent, as shown in Fig. 1h and i, respectively.

Inactivated CVB4 can induce the production of cytokines by PBMC

When culture medium containing infectious CVB4 was heated, exposed to UV light or pleconaril, and then was inoculated to Hep-2 cells, no cytopathic effect was observed (Fig. 2a). Cytokines levels were not significantly lowered in the culture supernatants of PBMC inoculated with inactivated virus (see Fig. 2b and c). Moreover, the incubation of CVB4 with pleconaril did not reduce the production of IL-6 by PBMC (Fig. 2d).

Neutralization of TLR4 did not suppress the production of cytokines induced by CVB4 in PBMC cultures

PBMC were incubated for 30 min in the presence of an anti-TLR4 monoclonal antibody or an irrelevant monoclonal murine IgG2a. The cultures were then inoculated with LPS or CVB4, incubated for 24 h and tested for the presence of TNFα. LPS-induced production of TNFα was inhibited when cells were incubated with the anti-TLR4 monoclonal antibody. CVB4-induced production of TNFα was not inhibited (Fig. 3).

Human serum and polyvalent immunoglobulin G containing anti-CVB4 antibodies enhance the CVB4-induced production of cytokines by PBMC

CVB4 was incubated at 37 °C for 1 h in the presence of diluted serum with high (serum 1, titer 256) or low (serum 2, titer 2) anti-CVB4 neutralizing activity before PBMC were inoculated. The cells were incubated, and after 24 h, the culture supernatants were harvested and the levels of cytokines were determined as before. When CVB4 was added to PBMC, no IFNα was detected in the culture supernatant. However, high levels of IFNα were recorded when the virus was incubated in the presence of 1:10 diluted serum 1 (2040 ± 160 pg/mL) and 1:100 diluted serum 1 (12,008 ± 279 pg/mL). In contrast, no IFNα was detected in the supernatants when CVB4 was suspended in diluted serum 2 (see Fig. 4a). An increase in CVB4-induced production of TNFα and IL-6 by PBMC was recorded when the virus was incubated in the presence of 1:100 diluted serum 1 before inoculation into PBMC: 1686 ± 35 pg/mL *versus* 55 ± 7 pg/mL TNFα and 2831 ± 1353 pg/mL *versus* 551 ± 134 pg/mL IL-6, respectively (Fig. 4b

and c). In contrast, serum 2 did not enhance CVB4-induced production of TNFα and IL-6 by PBMC (data not shown). When CVB4 was added to serum that had been heated for 30 min at 56 °C, IL-6 levels decreased significantly. A reduction in IFNα was also recorded, but this was not significant (see Fig. 4d and e). Culture medium and diluted serum (10^{-1} to 10^{-3}) did not induce production of IFNα and TNFα by PBMC, and low levels of IL-6 (<50 pg/mL) were produced when 1:10 diluted serum was added to PBMC cultures. No IL-6 production was recorded in the presence of 1:100 and 1:1000 diluted serum (Fig. 4f).

Hober *et al.* reported that IgG in serum enhanced CVB4-induced production of IFNα by PBMC [21]. In our study, the anti-CVB4 neutralizing activity titer of polyvalent IgG solution was 128. Three dilutions of this solution (1:10, 1:100 and 1:1000) corresponding to 1.6, 0.16 and 0.016 mg/mL IgG, respectively, were incubated with CVB4 before inoculation into PBMC cultures. An enhanced production of IFNα up

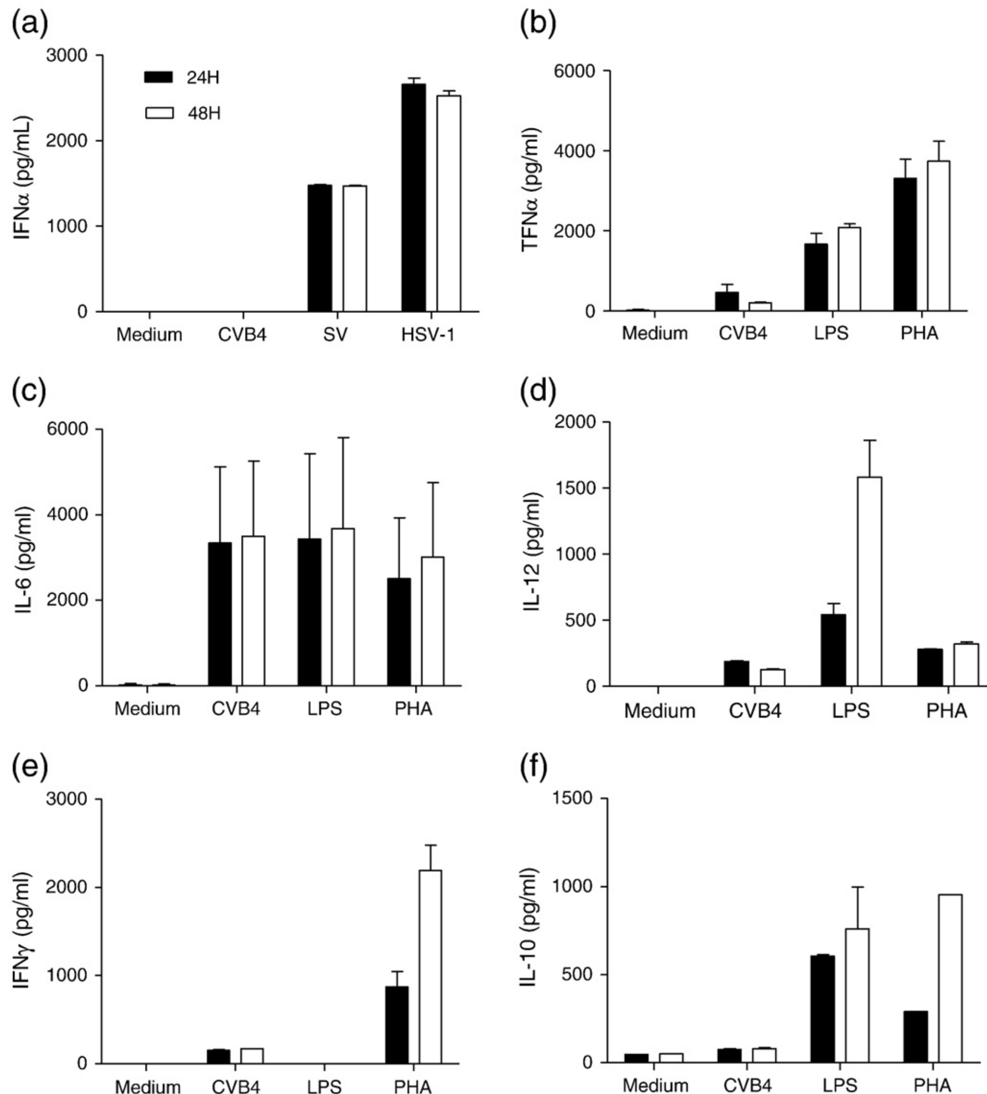

Fig. 1. Cytokine levels in supernatants of PBMC cultures inoculated with CVB4. PBMC were inoculated with CVB4 (MOI 10) or SV (MOI 1) or HSV1 (MOI 1); then, 24 and 48 h later, supernatants were collected for IFNα detection (a). PBMC were inoculated with CVB4 (MOI 10) or LPS (20 µg/mL) or PHA (20 µg/mL); then, 24 and 48 h later, supernatants were collected to determine the levels of TNFα, IL-6, IL-12, IFNγ and IL-10 (b–f). PBMC were incubated with CVB4 (MOI 10) or LPS (10 ng/mL) in the presence of polymyxin B (100 µg/mL). Supernatants were collected at 24 h for TNFα detection (g). PBMC cultures were inoculated with CVB4 at various MOI (0.1–10), and then supernatant fluids were collected at 24 h for TNFα and IL-6 detection (h and i). Cytokine concentrations were measured using ELISA. Results expressed as picograms per milliliter (pg/mL) are the mean ± SD of two independent experiments.

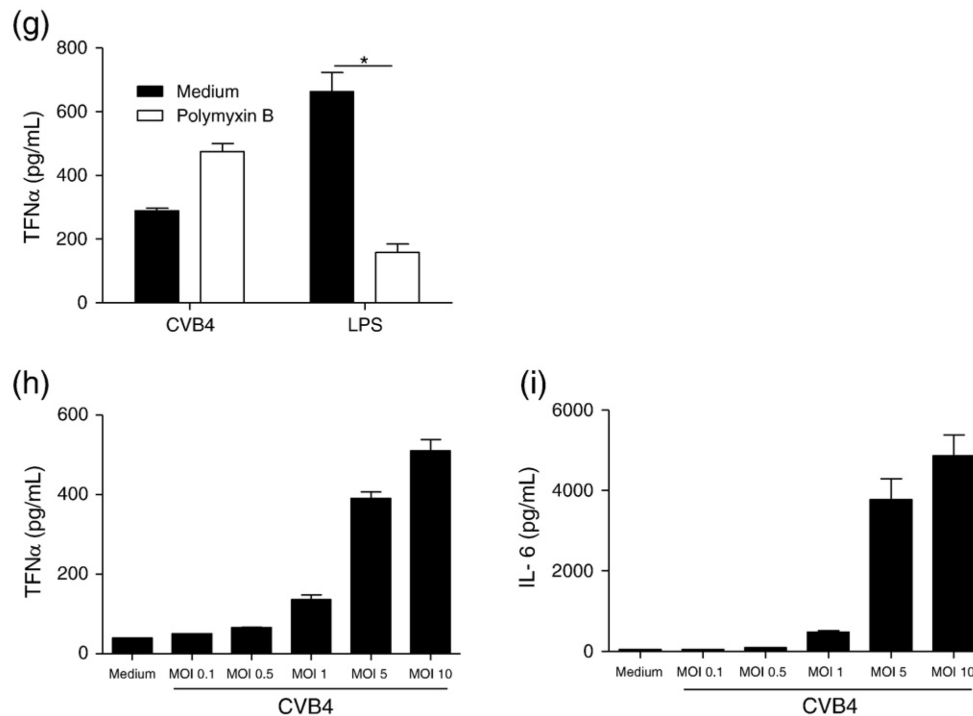

Fig. 1 (*continued*).

to 2219 ± 149 pg/mL was recorded with the 1:10 diluted polyvalent IgG solution (Fig. 5a).

Production of TNFα and IL-6 was also enhanced when the virus was incubated with 1:10 diluted polyvalent IgG solution (Fig. 5b and c).

Culture medium and diluted polyvalent IgG (10^{-1} to 10^{-3}) did not induce the production of IFNα and TNFα by PBMC. Furthermore, the IL-6 levels were very low (<50 pg/mL) with 1:10 diluted polyvalent IgG and zero with 1:100 and 1:1000 diluted polyvalent IgG (data not shown).

The CVB4-induced enhancement of IFNα and IL-6 obtained with polyvalent IgG was inhibited when virus infection was suppressed by exposure to UV light and pleconaril (Fig. 5d–f).

The IgG-dependent enhancement of CVB4-induced production of cytokines by PBMC is associated with an increased level of intracellular viral RNA

CVB4 incubated with culture medium or polyvalent IgG was added to PBMC cultures and incubated for 24 h, supernatants were collected and the cells were extensively washed with phosphate-buffered saline and harvested. The average levels of IFNα in culture supernatants were 0, 6705, 2742 and 0 pg/mL when PBMC were inoculated with CB4, CVB4 incubated in the presence of 1:10, 1:100 and 1:1000 diluted IgG solution, respectively (see Fig. 6a). When PBMC cultures were inoculated with CVB4 or CVB4 incubated with 1:100 and 1:1000 diluted IgG solution, a specific band (435 bp) of low intensity was observed after a one-step enteroviral RNA real-time PCR (RT-PCR). A band of high intensity was obtained when CVB4 was incubated with 1:10 diluted IgG. beta-Actin RNA RT-PCR displayed bands with comparable intensity in every intracellular RNA extract (see Fig. 6b). The levels of intracellular CVB4 RNA in our experiments were further investigated by quantitative RT-PCR. In PBMC inoculated with CVB4, the mean level of viral RNA was 300 copies per nanograms of total RNA, whereas in PBMC inoculated with CVB4 incubated with 1:10 and 1:100 diluted IgG solution, the mean level of viral RNA was approximately 30,000 and 1500 copies per nanograms of total RNA, respectively (Fig. 6c).

Discussion

The absence of IFNα in culture supernatants confirms that CVB4 is a weak inducer of IFNα in PBMC cultures. This is in agreement with our previous findings showing the absence of IFNα mRNA in PBMC inoculated with CVB4 [20].

CVB4 did not induce IFNα in PBMC cultures but stimulated the production of TNFα and to a higher extent IL-6. Furthermore, Th1 pathway cytokines IL-12

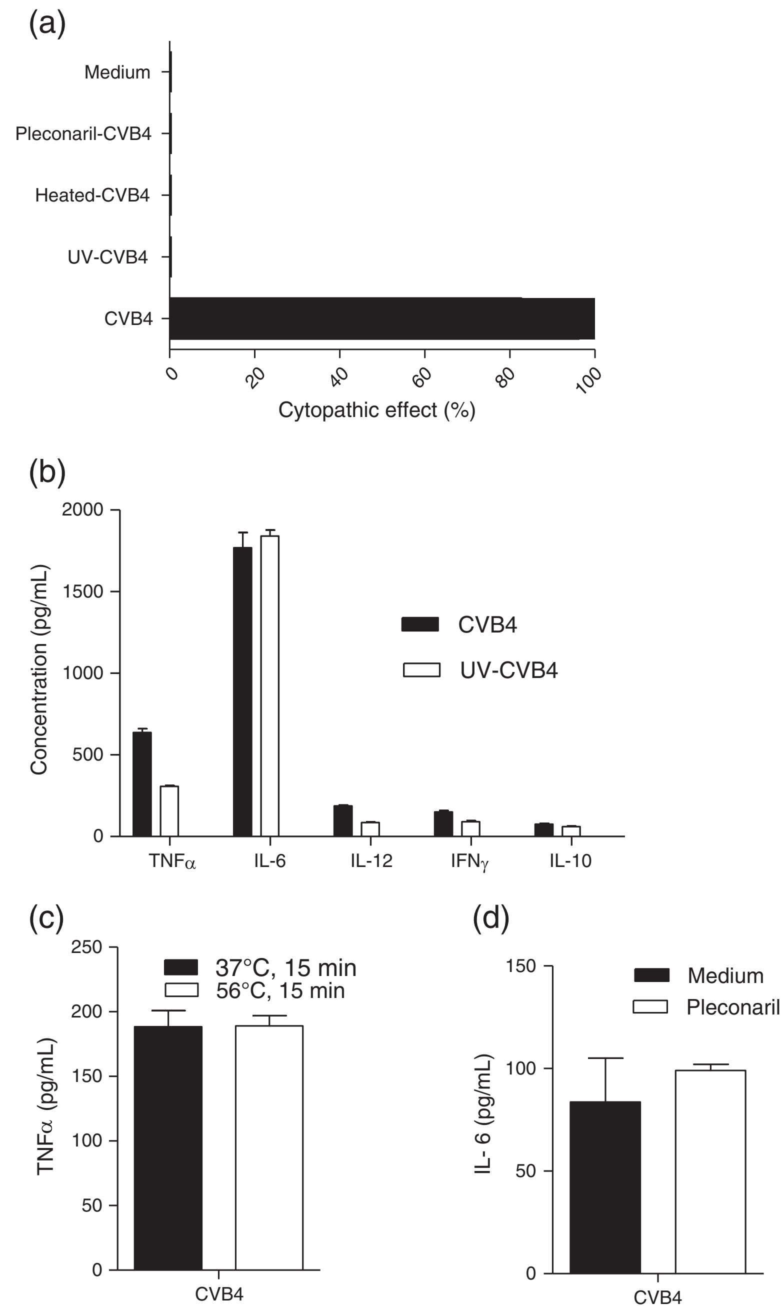

Fig. 2. CVB4 exposed to UV light, heat and pleconaril can induce the production of cytokines by PBMC.Culture medium containing CVB4 (10^5 TCID50) was exposed to UV, heat or pleconaril and then was inoculated to Hep-2 cells. The cells were incubated for 72 h; afterwards, the cell layers were observed under the inversed microscope to evaluate the cytopathic effect. The results are expressed as percentage compared with the cytopathic effect obtained with 10^5 TCID50 CVB4 (100%) (a). CVB4 was inactivated with UV light (b), heat (c) and pleconaril (d) so that the virus was not infectious for Hep-2 cells. PBMC were inoculated with CVB4 exposed to these inactivators; then, 24 h post-inoculation, the supernatants were collected for measuring the concentration of cytokines by ELISA. The results expressed as picograms per milliliter (pg/mL) are the mean ± SD of two independent experiments.

and IFNγ were also produced, but at a low level. The anti-inflammatory response of PBMC, evaluated from the production of IL-10, was not significant. There is a noteworthy technical consideration in our study; the cytokine response to CVB4 in PBMC cultures was not due to the presence of endotoxin since adding

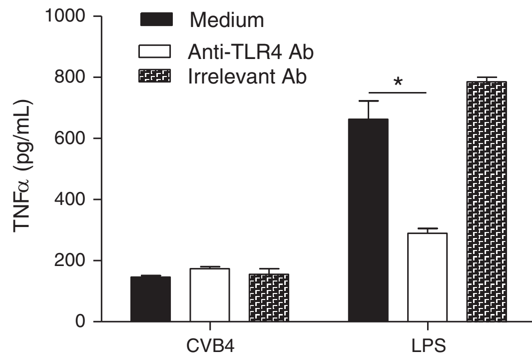

Fig. 3. Neutralization of TLR4 does not inhibit the CVB4-induced production of TNFα by PBMC.PBMC cultures were incubated for 30 min with culture medium or a murine anti-humanTLR4 antibody (20 μg/mL) or an irrelevant murine antibody (20 μg/mL). Then, the cultures were inoculated with CVB4 MOI 5 or LPS (10 ng/mL); afterwards, the supernatants were collected 24 h post-inoculation for measuring the concentration of TNFα by ELISA. The results expressed as picograms per milliliter (pg/mL) are the mean ± SD of two independent experiments. $^{*}p < 0.05$.

polymyxin B did not change the pattern of TNFα, the level of which is a very sensitive assay for the detection of endotoxin [23].

Our results are in agreement with previous findings; that is, CVB4 could induce TNFα, IL-1, IL-2 and IFNγ in PBMC cultures [13]. For the first time, to the best of our knowledge, the present findings show that CVB4 is a strong inducer of IL-6. Moreover, a low inocula (MOI (Multiple of Infection) 0.5) stimulated the production of IL-6 by PBMC. Conflicting results were reported for CVB3-induced production of IL-6 by monocytes [12,24]. Whether the CVB4-induced production of IL-6 can contribute to the pathogenesis in human diseases deserves further investigation. Recently, it has been reported that another enterovirus, *Enterovirus* 71, in a murine model induced high amounts of IL-6 that correlated with the severity of the disease [25].

The discrepancy observed between IFNα and other proinflammatory cytokines in the supernatants of PBMC inoculated with CVB4 can be explained by differences in viral patterns, cell receptors and subsequent signaling pathways involved in the CVB4-induced activation of IFNα and other cytokines. Signaling through endosomal TLR3 is thought to play a major role in IFNα inducing [14], which is strongly supported by a higher mortality in TLR3 KO mice, probably due to their inability to develop a suitable antiviral IFNα response [8]. Whether TLR3 or other intracellular sensors and surface-membrane-bound sensors are involved in the pattern of CVB4-induced production of cytokines by PBMC remains to be investigated.

Virus integrity and decapsidation are not required to induce proinflammatory cytokines in PBMC cultures in contrast with IFNα as demonstrated in our experiments with non-infectious CVB4. Thus, the infection of cells is not mandatory to induce proinflammatory cytokines with CVB4 in PBMC cultures. It is then easily conceivable that defective viral particles that are produced in large amounts during viral replication in cell cultures can induce these cytokines. Whether such non-infectious viral particles are readily produced *in vivo* as well deserves further investigation. It can be hypothesized that cytokine inducing by non-infectious CVB4 depends on activation of cell membrane sensors. It was reported that TLR4 was involved in the CVB4-induced production of proinflammatory cytokines by a pancreatic ductal-like cell line (Panc-1) [11]. The neutralization of TLR4 with an antibody in our experiments did not inhibit the CVB4-induced production of TNFα by PBMC, while the production induced by LPS was strongly decreased.

The incubation of CVB4 with immune serum or IgG induced a production of IFNα, and the production of TNFα/IL-6 was enhanced as well. In both cases, an optimal effect was observed at subneutralizing serum concentrations and was dependent on the level of neutralizing anti-CVB4 antibodies (no enhancing effect with serum 2 containing a low level of anti-CVB4 antibodies), and infectious CVB4 was needed as displayed in our experiments. When serum heated at 56 °C for 30 min was mixed with CVB4, the levels of IL-6 were significantly reduced, and those of IFNα were reduced as well, but at a lower extent. Together, these data suggest that the complement may play a role in the serum-dependant enhancement of these cytokines induced by CVB4, but with a major impact onto the production of IL-6 than the one of IFNα.

It was reported by our team that serum/IgG-dependent enhancement of IFNα involves anti-VP4 viral capsid protein antibodies, Fcγ receptors II and III and specific receptor CAR [20,21,26–28]. Whether the scenario is similar for TNFα/IL-6 production remains to be determined. The level of enteroviral RNA was very low in PBMC inoculated with CVB4, which suggests that viral entry and replication were almost inexistant. These findings are consistent with those of other authors who reported that PBMC were not or little permissive to CVB as suggested by the lack of viral protein detection [12,13]. In contrast, high levels of CVB4 RNA were detected in cells when the virus was previously incubated with IgG, in agreement with other reports using *Enterovirus* 71 *in vitro* (in human monocytes) and *in vivo* (in mice) [29]. Together, the high level of viral RNA in cells associated with the production of IFNα suggests that the amount of viral RNA can be involved in the serum-dependent enhancement of the CVB4-induced production of cytokines by PBMC. The phenomenon was observed with infectious virus only, which is in agreement with the role of the viral replication; nevertheless, further investigation is needed to know whether increased

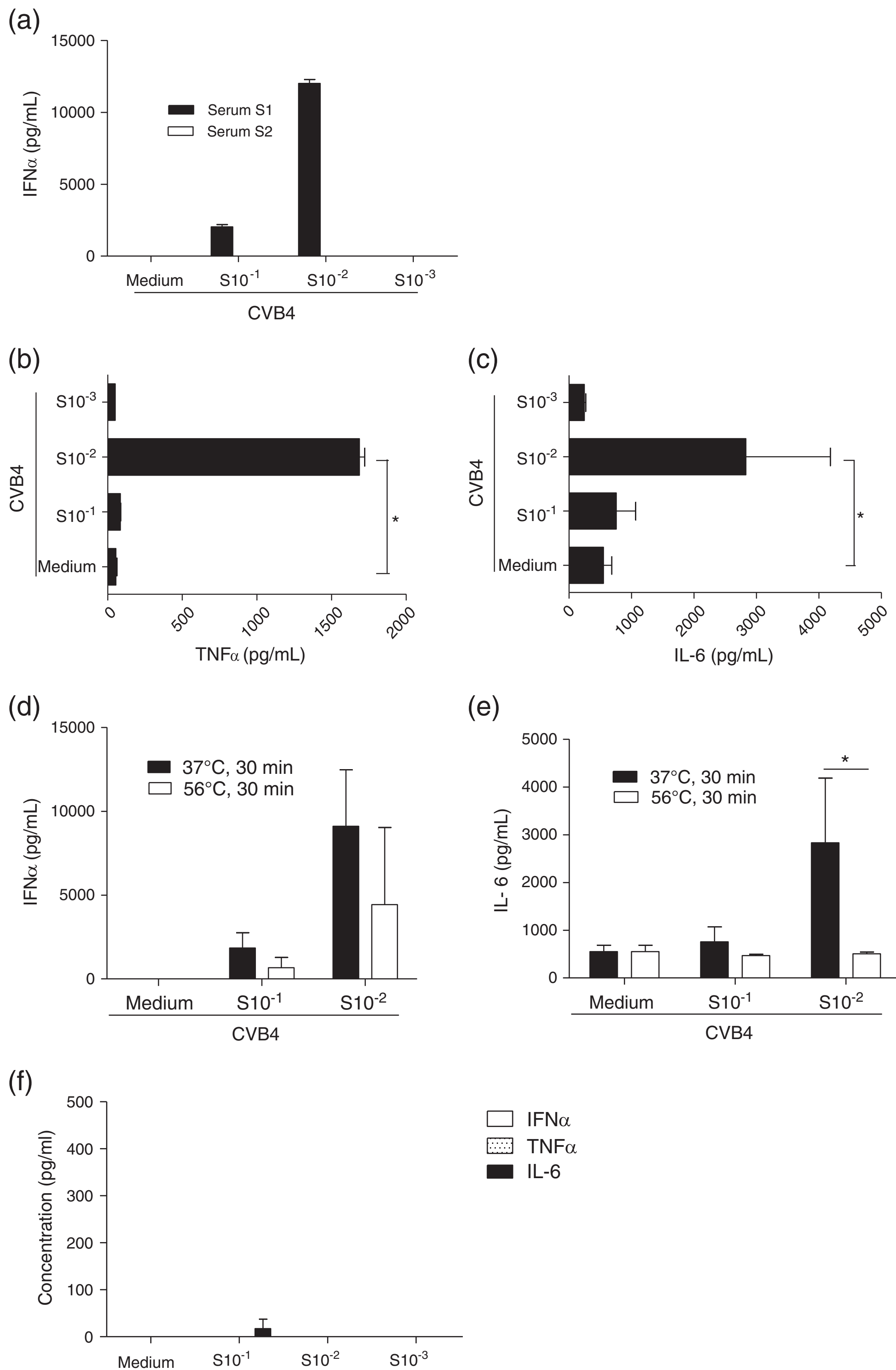

Fig. 4 (*legend on next page*)

virus entry and/or double-stranded viral RNA that appears during the replication of CVB4 are involved as well.

In conclusion, in PBMC cultures, infectious and non-infectious CVB4 stimulate the release of proinflammatory cytokines, whereas IFNα was not detected in supernatants. Nevertheless, infectious CVB4 incubated in the presence of immune serum or IgG was able to stimulate the production of IFNα. In addition, immune serum and IgG enhanced the levels of cytokines induced by CVB4. The enhancing effect of IgG was associated with the infection of cells by CVB4 displayed by the presence of intracellular viral RNA. Together, our data suggest that CVB4 can interact with PBMC and that the role of non-infectious particles (defective or empty viral particles) in the response of these cells to the virus should not be underestimated. It remains to investigate the results of the PBMC response to CVB4 to determine whether the expression of cytokines IL-6, TNFα and IFNα may affect the clearance of or the infection by CVB4 and the development of CVB4-induced diseases such as T1D.

Materials and Methods

Cells

PBMC

Blood was collected by venipuncture in heparinized tubes (Beckton Dickinson), from healthy volunteers, with their consent. One volume of blood was layered on top of one volume of Ficoll-Paque™ PLUS (GE Healthcare). After centrifugation at 500 *g* for 30 min at 20 °C, the PBMC fraction was collected and washed twice with RPMI medium. The cells were then suspended in RPMI supplemented with 10% of heat-inactivated fetal calf serum (FCS), 1% of non-essential amino acids, 1% of penicillin and streptomycin and 1% of L-glutamine, counted and adjusted to 5.10^6 cells/mL.

Hep-2 cells

Hep-2 cells (BioWhittaker) were grown in MEM supplemented with 10% of heat-inactivated FCS, 1% of non-essential amino acids and 1% of penicillin and streptomycin.

Virus

CVB4E2

CVB4E2, the diabetogenic CVB4 strain kindly provided by Ji-Won Yoon (Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada), was propagated in Hep-2 cells. After three freeze–thaw cycles, the suspension was collected and clarified at 2000 *g* for 10 min at 4 °C. Virus titers were determined on Hep-2 cells by limiting dilution assay for 50% tissue culture infection dose by the method of Reed–Muench. Aliquots of virus preparations were stored at −80 °C. For inactivation by UV irradiation, virus preparations were exposed to UV light (Philips® TUV 15 W/G 15 T8) at a distance of 10 cm, during 1 h. The heat inactivation was performed by incubating the virus at 56 °C for 15 min under gentle stirring. The loss of infectivity was ascertained by the lack of viral replication on Hep-2 cells.

Sendai virus

Sendai virus (SV) was kindly provided by D. Garcin (University of Geneva, Switzerland).

Herpes simplex virus 1

Herpes simplex virus 1 (HSV1) is a laboratory strain grown in Vero cells (American Type Culture Collection).

Antigenic stimuli

LPS of *Pseudomonas aeruginosa* and PHA were purchased from Sigma.

Sera and immunoglobulins

Whole blood collected from healthy donors was centrifuged at 2500 rpm for 15 min, and serum was aliquoted and stored at −20 °C. The level of anti-CVB4 antibodies in these serums was determined by seroneutralization assay. Polyvalent human immunoglobulins G (Subcuvia®; Baxter) were also tested for anti-CVB4 activity. A monoclonal neutralizing murine anti-humanTLR4 IgG2a antibody and an irrelevant murine IgG2a antibody were purchased from InvivoGen and Coulter, respectively.

Pleconaril

Pleconaril [3-(3,5-dimethyl-4((3-(3-methyl-5-isoxazolyl) propyl)oxy)phenyl)-5-trifluoromethyl-1,2,4-oxadiazole]

Fig. 4. Human serum can enhance the CVB4-induced production of IFNα, TNFα and IL-6 by PBMC.CVB4 (MOI 5) was incubated for 1 h with 1:10, 1:100 and 1:1000 dilutions of serum with high (serum 1, titer 256) or low (serum 2, titer 2) anti-CVB4 neutralizing activity. The mixtures were then inoculated to PBMC cultures, and supernatants were collected for IFNα detection (a). CVB4 (MOI 5) was incubated for 1 h with dilutions of serum S1 before inoculation to PBMC cultures; afterwards, TNFα and IL-6 were measured in supernatants (b and c). CVB4 (MOI 5) was incubated with dilutions of serum S1 that was heated before inoculation to PBMC cultures. Supernatants were collected for IFNα and IL-6 detection (d and e). Background levels of IFNα, TNFα and IL-6 in culture supernatants of PBMC incubated with culture medium and diluted serum S1 (10^{-1} to 10^{-3}) (f). Cytokine concentrations were measured in supernatants collected 24 h post-inoculation using ELISA. Results expressed as picograms per milliliter (pg/mL) are the mean ± SD of two independent experiments. $^*p < 0.05$.

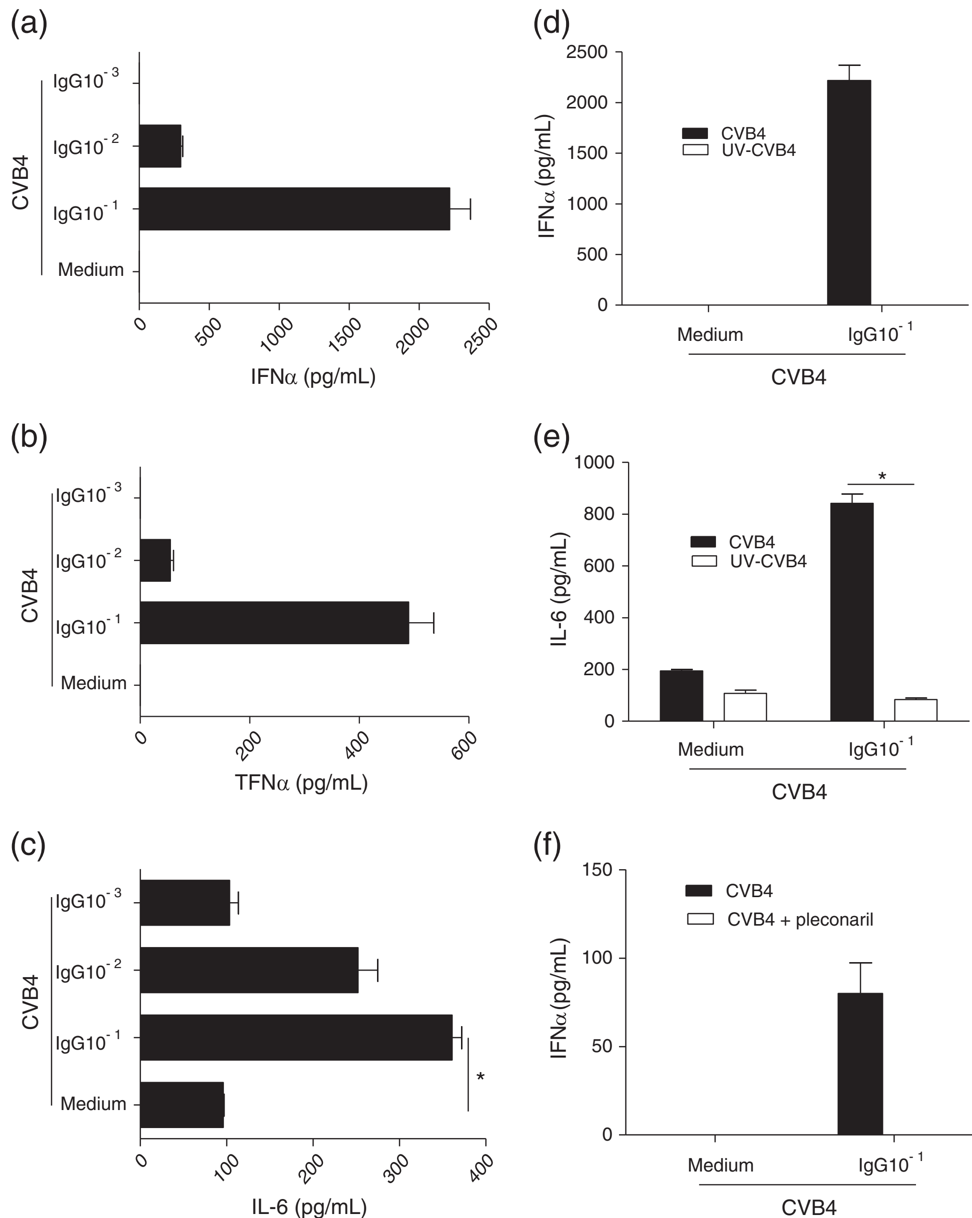

Fig. 5. IgG can enhance the CVB4-induced production of cytokines by PBMC.CVB4 (MOI 1) was incubated for 1 h with 1:10, 1:100 and 1:1000 dilutions of a polyvalent IgG solution corresponding to 1.6, 0.16 and 0.016 mg/mL IgG, respectively. The mixtures were then inoculated to PBMC cultures. Supernatants were collected for IFNα detection (a). CVB4 (MOI 0.5) was incubated for 1 h with dilutions of IgG solution before inoculation to PBMC cultures; afterwards, supernatants were collected for TNFα and IL-6 detection (b and c). UV-inactivated CVB4 (MOI 1) was incubated with 1:10 dilution of IgG solution and then inoculated to PBMC. IFNα and IL-6 were measured in the supernatants (d and e). Pleconaril-inactivated CVB4 (MOI 0.1) was incubated with 1:10 dilution of IgG solution before inoculation to PBMC, and IFNα was measured in the supernatants (f). Cytokine concentrations were measured in supernatants collected 24 h post-inoculation using ELISA. Results expressed as picograms per milliliter (pg/mL) are the mean ± SD of two independent experiments. *$p < 0.05$.

was kindly provided by Dr. Michaela Schmidtke (Virology institute, University Friedrich Schiller, Iéna, Germany). Pleconaril (MM: 381.4 g/mol) has an antiviral activity against picornaviruses (especially enteroviruses and rhinoviruses). It binds into the hydrophobic pocket of VP1 beneath the floor of the canyon of the picornavirus capsid and interferes with the uncoating step necessary to release the viral RNA from the capsid during infection. Pleconaril was dissolved into methanol (VWR) and used at a 10 μM final concentration. This concentration can

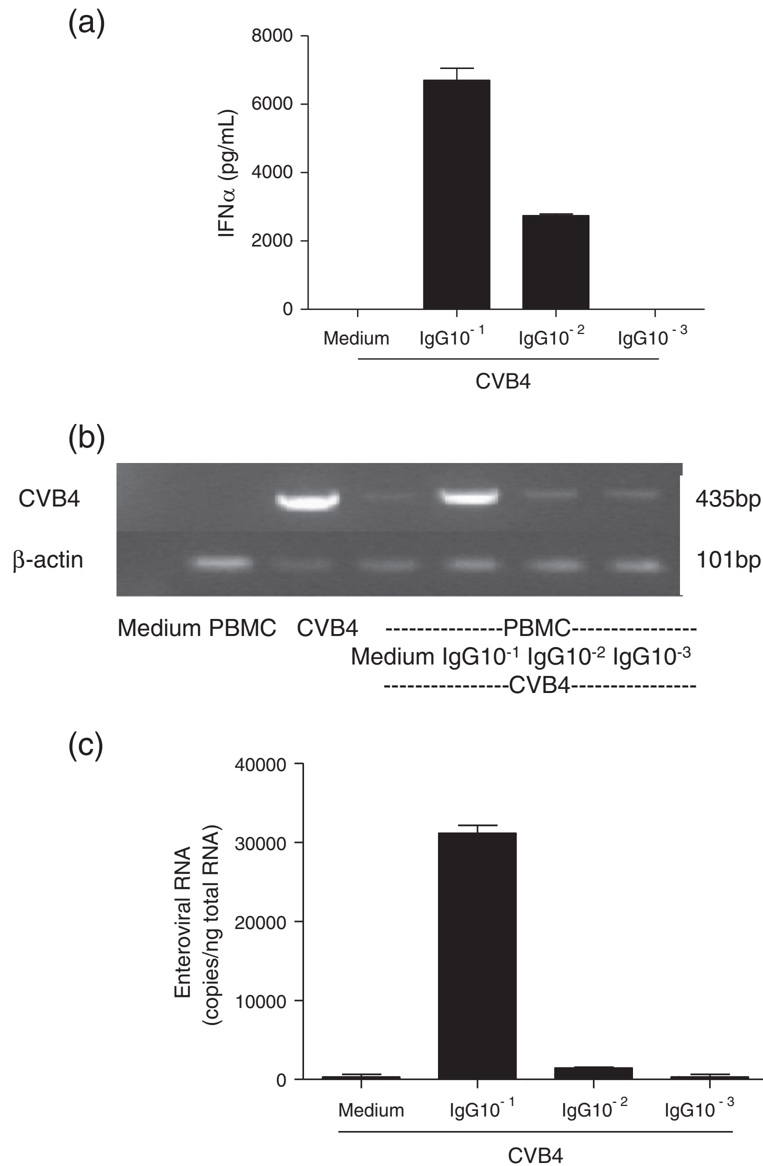

Fig. 6. Relationship between CVB4-induced production of cytokines by PBMC and intracellular viral RNA.CVB4 (MOI 10) was incubated for 1 h with 1:10, 1:100 and 1:1000 dilutions of polyvalent IgG solution, and the mixtures were inoculated to PBMC cultures; then, 24 h post-inoculation, the supernatants were collected for IFNα detection ($n = 2$) (a). Cells were harvested 24 h post-inoculation, and RNA was extracted. Viral RNA and beta-actin mRNA were detected by one-step RT-PCR. PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. One representative out of two independent experiments is shown (b). CVB4 viral loads were determined by quantitative RT-PCR ($n = 2$) (c).

inhibit 10^5 PFU (*p*laque-*f*orming *u*nits)/mL of CVB4 (assessed by the absence of replication on Hep-2 cells) with no cellular toxicity [30].

Plaque neutralization assay

To detect the presence of anti-CVB4 neutralizing antibodies in sera and polyvalent immunoglobulins, we incubated 2-fold serial dilutions in MEM supplemented with 10% of FCS, 1% of non-essential amino acids, 1% of penicillin and streptomycin and 1% of L-glutamine, with 10^3 PFU of CVB4 in 96-well microtiter plates for 2 h at 37 °C. Hep-2 cells in suspension were then added at 2.5×10^4 cells per well, and the plates were incubated for 48 h at 37 °C in a humid atmosphere with 5% CO_2. The results were expressed as the inverse final dilution (titer) of sample that totally inhibited the viral cytopathic effect.

ELISA assays

IFNα was measured using the IFNα pan-specific ELISA kit (Mabtech®) that allowed detection of subtypes 1/13, 2, 4, 5, 6, 7, 8, 10, 14, 16 and 17 of IFNα. TNFα, IL-6, IL-12, IFNγ and IL-10 were quantified with ELISA kits purchased from Peprotech®. The manufacturer's instructions were strictly followed for the different assays. The detection ranges were, respectively, 7–700 pg/mL (IFNα), 23–1500 pg/mL (TNFα), 32–2000 pg/mL (IL-6), 32–2000 pg/mL (IL-12), 46–3000 pg/mL (IFNγ) and 32–2000 pg/mL (IL-10).

RNA extraction

PBMC were previously collected in TriReagent® for cell lysis and stored at −80 °C until RNA extraction. Total RNA was extracted from PBMC using TriReagent® RNA isolation reagent/chloroform procedure (Sigma). Extracted RNA was then dissolved in 50 µL of nuclease free water, quantified with a spectrophotometer (Nanodrop).

DNase treatment

A DNA enzymatic digestion was first performed on RNA extracts. We mixed 10 µL of RNA in a tube with 20 µL of DNase RDD 10× buffer (Qiagen), 0.2 µL of DNase I (Qiagen), 0.25 µL of RNase inhibitor (Invitrogen) and 7.55 µL of nuclease free water. The tube was incubated at 37 °C for 30 min and at 65 °C for 5 min. The tube was then brought to 4 °C and vortexed quickly.

One-step RT-PCR

cDNA synthesis and cDNA amplification were performed in a single tube using the SuperScript® one-step RT-PCR with Platinum® Taq (Invitrogen). beta-Actin gene was co-amplified as control. Primers used for enterovirus were as follows: sens, 5′-CAAGCACTTCTG TTTC CCCGG-3′; reverse, 5′-ATTGT CACCATAAGCAGCCA-3′. Those for beta-actin gene are as follows: sens, 5′-TTGCCGACAGG ATGCAGAA-3′; reverse, 5′-GCCGATCCACACGGAGTACT-3′. The reaction was carried out using a Perkin Elmer 2400 thermocycler. The amplified RT-PCR products (435 bp for CVB4 and 101 bp for beta-actin) were analyzed on 2% agarose gel containing 0.5 µg/mL ethidium bromide (Sigma) and visualized using the Gel Doc 2000 system (Bio-Rad).

Quantitative RT-PCR

The Affinityscript® QPCR cDNA Synthesis Kit (Agilent) was used for the ARN retrotranscription step on a Perkin Elmer 2400 thermocycler. Quantitative RT-PCR for cDNA amplification was performed with the Brilliant® II QPCR Kit (Agilent) on the Mx3000p® (Stratagene). The primers were as follows: sens, 5′-CCC TGA ATG CGG CTA ATC-3′ and reverse, 5′-ATT GTC ACC ATA AGC AGC-3′. The sequence of the probe was FAM-AAC CGA CTA CTT TGG GTG TCC GTG TTT-TAMRA. *Enterovirus* 71 RNA (Vircell) was used as standard for quantification.

Statistical analysis

Data are shown as mean ± SD. Graphs and analysis were performed with GraphPad Prism® V5.0 software. Comparisons were performed with Mann–Whitney *U* test with a significance set at 0.05.

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Abbreviations used:
T1D, type 1 diabetes; RT-PCR, real-time PCR; FCS, fetal calf serum.

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ETUDE N°2 : Les monocytes des patients diabétiques de type 1 hébergent de l'ARN entéroviral.

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Résumé

Objectif

L'ARN des entérovirus a été détecté dans le sang des patients diabétiques de type 1 (DT1), et notamment dans les cellules mononucléées (CMN) du sang périphérique. Le but de ce travail est de rechercher la présence de l'ARN entéroviral (ARN EV) dans les monocytes et d'étudier *in vitro* l'infection de ces cellules avec CVB4.

Méthodes

Un prélèvement de sang a été obtenu chez 42 patients DT1 et 46 sujets contrôles. Les CMN ont été isolées et les monocytes purifiés. L'ARN messager (ARNm) de l'IFNα et l'ARN EV ont été recherchés par RT-PCR. Les taux plasmatiques d'IFNα ont été déterminés par technique d'immuno-analyse. Les cellules ont été inoculées avec CVB4 in vitro et l'infection a été étudiée par technique d'immunofluorescence indirecte, ciblant la protéine de capside virale VP1.

Principaux résultats

L'ARNm de l'IFNα a été détecté dans le sang et dans les monocytes de 12 patients sur 42, mais pas dans les cellules appauvries en monocytes de ces mêmes individus. Des taux significatifs d'IFNα (≥ 5 IU/mL) ont été observés chez 6 patients. L'ARN entéroviral a été retrouvé dans le sang et les monocytes de 7 patients, et le brin négatif de l'ARN détecté chez 6 de ces patients. Lorsque les monocytes des patients avec l'ARNm IFNα et/ou l'ARN EV sont inoculés avec CVB4, la proportion de cellules VP1 positive était de 8,8+/-1% alors qu'aucune cellule positive n'a été détectée dans les monocytes des patients négatifs pour l'ARNm IFNα et l'ARN EV. Par contre, lorsque le virus est préalablement mélangé avec du sérum autologue avant l'inoculation aux cellules, une positivité VP1 dans les monocytes était observée pour tous les patients avec des proportions allant de 12 à 36%.

Conclusion

Les résultats de cette étude montrent que les monocytes des patients DT1 peuvent héberger de l'ARNm d'IFNα et de l'ARN EV ; et ces cellules peuvent être infectées in vitro. Ces données suggèrent un rôle pour les monocytes dans la persistance des entérovirus chez les patients DT1 et requièrent d'autres investigations.

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ORIGINAL ARTICLE

Monocytes of Patients with Type 1 Diabetes Harbour Enterovirus RNA

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ABSTRACT

Background Intracellular enterovirus (EV) RNA was detected in blood of patients with type 1 diabetes (T1D). The presence of EV RNA in subsets of peripheral blood mononuclear cells (PBMCs) of patients, and the *in vitro* infection of these cells with an EV, was investigated.

Materials and methods Blood was collected from 42 patients with T1D, PBMCs were isolated and monocytes were purified. Interferon alpha (IFNα) mRNA and EV RNA were investigated using RT-PCR. Levels of IFNα in plasma were measured using an immunoassay. Cells were inoculated with *Coxsackievirus* B4 (CBV4) *in vitro*, and infection was assessed by indirect immunofluorescence (IFI).

Results Interferon alpha mRNA was detected in blood and in monocytes of 12 of 42 patients with T1D, but not in monocyte-depleted PBMCs of the same individuals. Significant plasma levels of IFNα (≥ 5 IU/mL) were found in six patients. EV RNA was detected in whole blood and in monocytes of seven patients and negative-strand EV RNA was found in monocytes of 6 of them. When monocytes of patients with IFNα and/or EV RNA in their blood were inoculated with CVB4, the proportion of cells stained by an anti-VP1 antibody was 8·8 ± 1%, whereas no VP1 was detected in the monocytes of IFNα, EV RNA negative patients. Nevertheless, when CBV4 was mixed with plasma, VP1 was detected in monocytes of all patients with T1D (staining ranging from 12 to 36%).

Conclusions Our data indicate that monocytes of patients with T1D can harbor EV RNA and IFNα mRNA and can be infected with an EV *in vitro*.

Keywords autologous plasma, Enterovirus, Interferon alpha, monocytes, RNA, type 1 diabetes.

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Introduction

Enteroviruses (EV) are small, nonenveloped, single-stranded positive RNA genome viruses that belong to the *Picornaviridae* family. These ubiquitous pathogens are involved in many acute clinical conditions, but are also thought to play a role in the pathogenesis of chronic disorders such as type 1 diabetes (T1D) [1]. Indeed, data in the literature support a strong relationship between EV and the development of T1D [2–4]. The pancreas is a major target for EV [5,6] and the detection of EV components, especially in islet beta cells of recent-onset or long-term patients with T1D, has been reported [7,8]. In addition, virus has been detected in the gut, which is the primary replication site after faecal-oral route transmission [9], and in the blood which is responsible for the spreading virus to its target organs. A large number of studies have been performed to detect EV in peripheral blood samples from patients with T1D, although most of them have focused on the detection of EV RNA in serum or whole blood [10]. A few groups did specifically investigate the detection of EV in peripheral blood mononuclear cells (PBMCs) of patients with T1D [11–14]; however, the main target cell infected by the virus in the blood is still unknown.

Our team previously reported both increased levels of IFNα in plasma and the presence of EV nucleotide sequences,

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especially Coxsackievirus B4 (CBV4) in peripheral blood of patients with T1D [15]. *In vitro* it has been observed that healthy donors' PBMCs can be infected with CBV4 through an antibody-dependent mechanism [16]. However, the target cells of EV in PBMCs *in vivo* remain an open issue.

The aim of this study was to determine whether IFNα and/or enteroviral RNA were present in monocytes of patients with T1D and to investigate the interaction between CBV4 and these cells.

Materials and methods

Patients and samples

Forty-two patients with T1D (male/female ratio: 1) and 46 healthy control subjects (Male/Female ratio: 1·3) were recruited for this study. The patients' group included 32 children and teenagers (mean age: 12·6 years, range: 2–19 years), and 10 adults (mean age: 43·3 years, range: 21–68 years). Among controls, 24 were children and teenagers (mean age: 12·6 years, range: 7–17 years), and 20 were adults (mean age: 28·5 years, range: 23–45 years). From the date of sample collection, recent onset diabetes patients were those diagnosed within the previous year (n = 29), while long-term patients (n = 13) had been diagnosed for over a year. Subjects were included after informed and written consent (parents' consent was obtained for children and teenagers), and in accordance with local ethics recommendations.

Blood was collected by venipuncture from each individual in one heparin tube and one EDTA tube (Becton Dickinson, Le Pont-de-Claix, France). The EDTA tube was used for direct detection of viral markers, and the heparin one was used for *in vitro* infection experiments.

PBMC and monocytes isolation

PBMCs were isolated from whole blood over a Ficoll–Paque solution (Eurobio). Monocytes were separated from PBMC through positive or negative selections as follows:

Positive selection: Specific immunomagnetic beads (Dynal Dynabeads M-450 CD14) were used for selection of CD14+ cells from PBMCs. The proportion of CD14+ cells in the CD14-negative population was <5% as shown by IFI.

Negative selection: Monocytes were negatively isolated from PBMCs by depletion of T cells, B cells, NK cells, erythrocytes and granulocytes, using Dynabeads (Dynal Monocyte Negative Isolation Kit, Life Technologies, Saint-Aubin, France). The proportion of CD14+ cells varied from 80 to 92% as shown by IFI.

Viruses

Coxsackievirus B4 (JVB strain provided by J. W. Almond, University of Reading, UK) was grown in Hep2 Cells (BioWhittaker) in Eagle's MEM (Gibco BRL) supplemented with 10% FCS (Gibco BRL) and 1% L-glutamine (Eurobio). Virus titration was performed on Hep2 cells using end-point dilution assay, and the titre was estimated by the Reed and Muench statistical method.

Infection of PBMCs

PBMCs were seeded in a 96-well culture plate at $5{\cdot}10^5$ cells/well. CVB4 was pre-incubated either with culture medium or 1:100 diluted autologous plasma for 1 h at 37°, and then, the mixtures were inoculated to PBMCs. CVB4 was used at $2{\cdot}5 \times 10^5$ TCID50 per well, in a total volume of 100 μL. The plate was incubated at 37 °C, 5% CO_2 for the required time.

Immunoassay for IFNα quantification

The plasma concentration of IFNα was determined using a specific and sensitive dissociation-enhanced lanthanide fluoroimmunoassay (DELFIA) as described previously [17]. The detection limit of the assay was 0·5 IU/mL.

Immunofluorescence

Adherent PBMCs were incubated on ice with cold PBS containing 5 mM EDTA for 30 min, and gently detached from wells using a cell scraper. Cells were then washed twice with PBS and cytocentrifuged onto clean glass slides (10^5 cells/slide). The slides were air dried, and cells were fixed in a solution of 4% paraformaldehyde for 20 min at 4 °C and permeabilized with cold acetone/methanol (1 vol/2 vol) for 10 min at −20 °C. Cells were incubated with 5% normal rabbit serum for 1 h at room temperature in a humidified chamber, then with a 1:20 dilution of monoclonal mouse anti-VP1 antibody, clone 5D8/1 (Dako, Les Ulis, France) for 1 h at room temperature. This antibody was reported to be specific to VP1 enteroviral protein [18]. The slides were subsequently stained with a 1:100 dilution of FITC-conjugated anti-mouse IgG antibody (Sigma-Aldrich, Saint-Quentin-Fallavier, France) and counterstained with Evans blue (Sigma-Aldrich). Between each step, slides were washed with PBS. The slides were mounted with permafluor (Coulter Immunotech, Marseille, France), and positive cells were counted using a fluorescence microscope (Leitz Diaplan).

RNA extraction

RNA was extracted from all samples with a commercial kit, involving acidic guanidinium thiocyanate and phenol–chloroform (RNAgents® Total RNA Isolation System, Promega, Charbonnières-les-Bains, France). Extracted RNA was then eluted in 50 μL of diethylpyrocarbonate (DEPC)-treated water (Sigma-Aldrich) and used in the RT-PCR assays.

RT-PCR for IFNα mRNA detection

The cDNA synthesis and amplification were performed in a single tube using the Enhanced Avian RT-PCR Kit (Sigma)

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according to the manufacturer's instructions. GAPDH gene was co-amplified as control. The primers used and reaction conditions are fully described elsewhere [17].

RT-PCR for EV RNA detection

Three EV-specific primers, selected in the highly conserved 5′UTR region were used for EV detection, two external (EV1, 5′-CAAGCACTTCTGTTTCCCCGG-3′; EV2, 5′-TTGTCACCAT AAGCAGCCA-3′) and one internal (EV3, 5′-CTTGCGCGTT ACGAC-3′), as previously described [19]. Briefly EV2 (reverse) or EV1 (sens) was used as template for the cDNA synthesis of positive or negative EV RNA, respectively. EV1 and EV2 were used for PCR giving a 435-bp fragment. A semi-nested PCR was performed when the result was negative with RT-PCR, using EV1 and EV3, which generated a 362-bp PCR product. The reaction conditions are described elsewhere [15]. PCR products were detected by agarose gel electrophoresis. The semi-nested PCR is much more sensitive than RT-PCR only [19,20]. The GAPDH mRNA was amplified by specific primers in a RT-PCR for all specimens.

Statistical analysis

Quantitative data were presented as mean ± standard deviation, and Fischer's exact test or nonparametric Mann–Whitney test were used when appropriate.

Results

IFNα mRNA and EV RNA in whole blood and PBMC subsets of patients with T1D

The results are summarized in Table 1. Whole blood, from T1D patients and controls, was tested for IFNα mRNA, which was detected in 12 of the 42 patients with T1D but not in any of the controls. IFNα was also detected in plasma samples from 9 of these 12 patients (range 1 to 21 IU/mL); significant levels in 6 of the 12 (≥ 5 IU/mL).

In patients with positive detection of IFNα mRNA in whole blood (n = 12), the presence of IFNα mRNA in monocytes and in monocyte-depleted PBMCs was tested. In these patients, IFNα mRNA was detected in monocytes but not in monocyte-depleted PBMCs.

The presence of EV RNA was also assessed. EV RNA was detected in whole blood and monocytes of three patients by RT-PCR and in four other patients by semi-nested RT-PCR; but they were all negative for the detection of EV in monocyte-depleted PBMCs. The same seven patients were positive for the detection of IFNα mRNA in their blood and monocytes. In six of the seven, plasma levels of IFNα ranged from 1 to 21 IU/mL, although in 1 patient, detection was negative. The presence of EV RNA was not detected in whole blood from any of the controls.

To further clarify the pattern of EV infection in monocytes of patients with T1D, the presence of intracellular negative-strand EV RNA, a marker of replicative infection, was investigated by strand-specific semi-nested RT-PCR. Negative-strand EV RNA was detected in monocytes of six of the seven patients found positive for EV RNA.

As shown in Table 1, T1D patients with positive detection of IFNα (intracellular mRNA with/without IFNα in plasma) and/or intracellular EV RNA were classified as group 1 (n = 12), whereas patients with negative detection of these markers in their blood were classified as group 2 (n = 30). Eight of twelve individuals in group 1 and twenty-one of thirty individuals in group 2 were recently diagnosed.

Overall, our results show that, in monocytes of patients with T1D, IFNα is activated, EV RNA is detected and negative-strand EV RNA is expressed. These results can be found in both recently onset and long-term diabetic individuals.

CBV4 Infection of PBMC from patients with T1D

The detection of EV RNA in monocytes of patients with T1D prompted us to study the *in vitro* infection of PBMC subsets with an EV, using CBV4 as the testing agent. Monocytes and monocyte-depleted PBMCs from nine patients (including five patients of group 1 and 4 patients of group 2) were inoculated with either CBV4 or CBV4 that had been previously been incubated with 1:100 diluted autologous plasma for 1 h. The cells were then incubated for 8 h, and stained with anti-VP1 antibody. When monocytes of group 1 patients were inoculated with CBV4, the mean proportion of VP1 positive cells was 8·8 ± 1% (n = 5). In similar conditions, the detection of VP1 in monocytes of 4 group 2 patients was negative (see Table 2).

When monocytes of group 1 and group 2 patients were inoculated with CBV4 and autologous plasma, the mean proportion of VP1 positive monocytes reached 29 ± 7·5% and 17 ± 3·8%, respectively (P = 0·03) (see Table 2). In contrast, in all patients with T1D, the detection of VP1 was negative in monocyte-depleted PBMCs inoculated either with CBV4 or CBV4 pre-incubated with autologous plasma, and in monocytes or monocyte-depleted PBMCs mock-inoculated with medium or autologous plasma.

Discussion

Enteroviral RNA has been found more frequently in PBMCs of patients with T1D compared to healthy subjects [10–14], and frequent detection of IFNα mRNA has also been previously reported in these patients [15]. In the present study of T1D patients, we have determined which of the PBMC subsets harbours EV RNA and IFNα mRNA.

Our data indicate that it is the monocytes of diabetic patients, which harbour EV RNA, especially negative-strand EV RNA,

Table 1 IFNα and EV detection in blood, monocytes and monocyte-depleted PBMCs of T1D patients

Subjects				IFNα (IU/mL)	IFNα mRNA			EV RNA		
Group	N°	Age	Diabetes	Plasma	Blood	Monocytes	Monocyte-depleted PBMCs	Blood	Monocytes	Monocyte-depleted PBMCs
1	P1	16	Long-term	8	Pos	Pos	Neg	Pos	Pos†	Neg
	P2	17	Long-term	5	Pos	Pos	Neg	Pos	Pos†	Neg
	P3	12	Long-term	6	Pos	Pos	Neg	Neg	Neg	Neg
	P4	16	Recent	21	Pos	Pos	Neg	Pos	Pos†	Neg
	P5	13	Long-term	8	Pos	Pos	Neg	Neg	Neg	Neg
	P6	2	Recent	6	Pos	Pos	Neg	Neg	Neg	Neg
	P7	21	Recent	2	Pos	Pos	Neg	Neg	Neg	Neg
	P8	68	Recent	3	Pos	Pos	Neg	Pos*	Pos*†	Neg
	P9	30	Recent	1	Pos	Pos	Neg	Pos*	Pos*†	Neg
	P10	18	Recent	0	Pos	Pos	Neg	Pos*	Pos*†	Neg
	P11	10	Recent	0	Pos	Pos	Neg	Neg	Neg	Neg
	P12	7	Recent	0	Pos	Pos	Neg	Pos*	Pos*	Neg
2	P13-42	-	‡	0	Neg	Neg	Neg	Neg	Neg	Neg
Controls	C1-46	-	NA	ND	Neg	Neg	Neg	Neg	Neg	Neg

Individuals P1-42 are T1D patients and C1-46 are healthy controls.
Pos: positive; Neg: negative; *only detected by semi-nested PCR; †Detection of positive and negative strand of EV RNA; NA: not applicable; ND: not done; ‡Among patients P13-42, nine individuals were long-term diabetics.
Whole blood was collected from T1D patients. Plasma levels of IFNα were determined using a DELFIA assay. PBMCs were isolated and separated in monocyte-enriched and monocyte-depleted cells by positive selection. Detection of IFNα mRNA, EV RNA and Negative strand EV RNA was performed by RT-PCR in monocyte-enriched and monocyte-depleted cells.

as well as IFNα mRNA, as these markers were negative in monocyte-depleted PBMCs.

The viral load was probably low since semi-nested RT-PCR was required to detect EV RNA in four out of seven patients. An explanation for the negative detection of EV RNA in some patients, within the limits of the assay, could be a low EV load, an infection without viremia or no involvement of EVs in these individuals [21].

The presence of EV RNA and IFNα mRNA, obtained by RT-PCR, in CD14+ monocyte-enriched PBMCs but not in CD14+ monocyte-depleted PBMC, excludes the role of non-monocytic cells in harbouring such RNA, as these cells comprised <5% of the cells in the CD14+ samples. Together these data strongly suggest that the major cells harbouring EV RNA and IFNα mRNA in PBMCs of patients with T1D were CD14+ monocytes, as other CD14+ cells which can be infected by EVs are rare in PBMC [22,23]. The present study shows that EV RNA was detected in monocytes of patients with recent-onset diabetes as well as in patients with a long-term diagnosis. These EV PCR results suggest that EVs can be involved at the onset of T1D; however, the positive detection of EV RNA in long-term T1D patients also indicates that EVs can persist throughout the course of the disease.

CBV4 is a member of the *Human Enterovirus* B species that is most frequently associated with T1D [10], hence CBV4 was selected as the testing agent to investigate the *in vitro* infection of T1D patients' monocytes with an EV.

The pattern of interaction between this representative virus and PBMCs of patients contribute to modelling the impact of EVs in these cells.

PBMCs were separated into monocyte-enriched and monocyte-depleted populations, and then were either directly stained with an anti-VP1 antibody or incubated with CBV4 for 8 h prior to staining with the same antibody. There was no positive signal in any PBMCs directly stained whereas VP1 was detected in cells inoculated with CBV4 *in vitro*. A positive signal was observed in monocyte-enriched PBMCs (containing 80–92% CD14+ cells) but not in monocyte-depleted PBMCs, which excludes the role of nonmonocytic contaminating cells

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Table 2 Detection of enteroviral capsid protein VP1 by IFI in cultures of monocytes and monocyte-depleted PBMCs inoculated with CBV4

Patients				VP1 staining (% of positive cells) Monocytes			Monocyte-depleted PBMCs		
Group	N°	Age	EV RNA or/and IFNα in blood	Medium	CBV4	CBV4+ autologous plasma	Medium	CBV4	CBV4+ autologous plasma
1	P1	16	Yes	0	8	32	0	0	0
	P2	17	Yes	0	10	22	0	0	0
	P3	12	Yes	0	10	35	0	0	0
	P4	16	Yes	0	8	36	0	0	0
	P5	13	Yes	0	8	20	0	0	0
2	P17	15	No	0	0	20	0	0	0
	P24	12	No	0	0	12	0	0	0
	P25	6	No	0	0	16	0	0	0
	P42	12	No	0	0	20	0	0	0

PBMC of T1D patients were segregated in monocyte-enriched and monocyte-depleted cells by negative selection. Cells were inoculated with either culture medium, CBV4 or CBV4 mixed with autologous plasma, and incubated for 8 h. Expression of VP1 was then detected by IFI.
The results are the percentage of positive cells for VP1 staining.

(less than 20% of cells) as major host cells harbouring VP1 in the monocyte-enriched fraction of PBMC inoculated with CBV4.

The detection of VP1 in monocyte-enriched PBMCs of T1D patients was negative within the limits of the assay, even when they harboured EV RNA and/or IFNα mRNA. However, *in vitro* the monocyte-enriched PBMCs of these patients harboured VP1 when they were inoculated with CBV4. Taken together our data show that monocytes of patients can be infected with an EV (specifically CBV4) which results in the synthesis of VP1. In contrast the detection of VP1 was negative in CBV4-inoculated monocyte-enriched PBMCs of other patients (those without intracellular EV RNA and/or IFNα mRNA in their blood). Interestingly, VP1 was detected in monocytes of all patients when monocyte-enriched PBMCs were inoculated with CBV4 mixed with autologous plasma. The increased CBV4 infection of monocytes of patients in relation to their plasma could be explained by circulating enhancing antibodies. The role of such antibodies in the infection of monocytes of healthy subjects with CBV4 *in vitro* has been shown previously by our group [16]. In our current experiments we observed that some patients showed an increase in infection of monocytes with CBV4 not mixed with plasma; the mechanism for this increase remains to be elucidated. One hypothesis, involving enhancing antibodies present on the cell surface, was previously reported by our team [24].

Further studies are needed to evaluate the level of replication of viral RNA in these cells and to determine whether the presence of negative-stranded enteroviral RNA is related to viral replication or it simply reflects the presence of double-stranded RNA which can persist in certain cell types [25]. It should also be considered that the presence of EV RNA in circulating monocytes of T1D patients may reflect a persistent EV infection. Furthermore the role of monocytes/macrophages in any viral persistence cannot be ruled out as a persistent CBV infection has previously been observed in hematopoietic cell lines and specifically in monocytic-like cells, when these cells were inoculated with virus mixed with enhancing IgG [26,27]. The hypothesis of the persistence of EV in patients with T1D is supported by a recent report of circulating EV RNA in their PBMCs whereas EV was not detected in throat and stool samples as expected in case of acute of infection [12]. Furthermore, a persistence of EV in human beings has been widely reported [28–35], and has already been observed *in vitro* in human and animal systems and *in vivo* in mice [15,36–40]. Further studies are needed to investigate whether EV persist in patients with T1D.

The presence of EV RNA in monocytes of T1D patients, as demonstrated in this study, indicates that further investigation into the interaction of EV with monocytes and the role of these cells in the persistence of EV and the development of T1D is required. Future studies will be directed along this line in our laboratory.

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ETUDE 3 : Coxsackievirus B4 peut infecter des macrophages dérivés de monocytes du sang périphérique.

Travail publié dans Viruses

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Résumé

Objectif

La pathogenèse virale de maladies chroniques comme le diabète de type 1 repose principalement sur les interactions entre le virus et l'immunité innée, chez des sujets prédisposés. Les macrophages sont des acteurs importants de la réponse de l'immunité innée, au niveau des tissus. Le but du présent travail est d'étudier les interactions entre coxsackievirus B4 (CVB4) et les macrophages issus de monocytes du sang périphérique.

Méthodes

Des cellules mononucléées du sang ont été isolées à partir de concentrés globulaires issus de donneurs sains. Les monocytes ont été purifiés par adhérence en plaque de culture cellulaire, et ensuite différenciés en macrophages dans du milieu exempt de sérum, en présence de M-CSF ou de GM-CSF humain recombinant. Les deux types de macrophages ont été inoculés avec CVB4 préalablement incubé ou non avec du sérum facilitant. La production de cytokines inflammatoires a été évaluée par des technique ELISA. L'infection et la persistance du virus ont été étudiées par RT-PCR et par détermination du titre viral.

Principaux résultats

Les cellules obtenues suite à la différenciation des monocytes au bout de 7 jours en présence de M-CSF ou de GM-CSF, présentaient les caractéristiques morphologiques de macrophages et étaient semblables. Les macrophages obtenus avec du M-CSF, mais pas ceux avec du GM-CSF, étaient infectables par CVB4. Cette infection n'a pas requis la présence de sérum facilitant comme dans le cas des monocytes. L'expression de l'ARN messager du récepteur viral CAR était similaire dans les deux populations de macrophages. CVB4 pouvait induire la production de cytokines inflammatoires dans les deux types de macrophages. CVB4 pouvait établir une infection persistante et productive dans les macrophages différenciés avec du M-CSF, mais l'inflammation était limitée au stade de l'infection aiguë.

Conclusion

Ces résultats démontrent que CVB4 peut infecter et persister dans les macrophages. D'autres investigations sont nécessaires pour préciser le rôle de l'interaction entre le virus et les macrophages résidents, dans la pathogenèse des infections chroniques liées aux CVB.

viruses

MDPI

Article

Coxsackievirus B4 Can Infect Human Peripheral Blood-Derived Macrophages

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Abstract: Beyond acute infections, group B coxsackieviruses (CVB) are also reported to play a role in the development of chronic diseases, like type 1 diabetes. The viral pathogenesis mainly relies on the interplay between the viruses and innate immune response in genetically-susceptible individuals. We investigated the interaction between CVB4 and macrophages considered as major players in immune response. Monocyte-derived macrophages (MDM) generated with either M-CSF or GM-CSF were inoculated with CVB4, and infection, inflammation, viral replication and persistence were assessed. M-CSF-induced MDM, but not GM-CSF-induced MDM, can be infected by CVB4. In addition, enhancing serum was not needed to infect MDM in contrast with parental monocytes. The expression of viral receptor (CAR) mRNA was similar in both M-CSF and GM-CSF MDM. CVB4 induced high levels of pro-inflammatory cytokines (IL-6 and TNFα) in both MDM populations. CVB4 effectively replicated and persisted in M-CSF MDM, but IFNα was produced in the early phase of infection only. Our results demonstrate that CVB4 can replicate and persist in MDM. Further investigations are required to determine whether the interaction between the virus and MDM plays a role in the pathogenesis of CVB-induced chronic diseases.

Keywords: coxsackievirus B; monocyte-derived macrophages; M-CSF; GM-CSF; infection; inflammation; persistence; PACS; J0101

1. Introduction

Enteroviruses (EV) are small (20–30 nm) non-enveloped positive single-strand RNA viruses that belong to the Picornaviridae family. The genus Enterovirus is very important in medicine and includes several major human pathogens. Group B coxsackieviruses (CVB 1–6) are classified within the Human Enterovirus B (HEV-B) species [1,2]. CVB infections are usually mild and asymptomatic, but they can cause severe acute illnesses [2,3]. In addition, accumulating evidence supports a strong association between these viruses and chronic diseases, such as type 1 diabetes (T1D) [4,5]. The virus is thought to trigger autoimmunity in genetically-predisposed individuals through various mechanisms, including activation of inflammation and persistence, and contributes then to the development of the disease [5]. This pathogenesis strongly relies on the response of the immune system and especially the innate immunity to the virus. Therefore, innate immunity cells are believed to play a major role in the orchestration of this process. The interactions between CVB and innate immunity cells are not well understood.

In T1D patients, EV components have been detected more frequently in peripheral blood mononuclear cells (PBMC) [6–9], and monocytes were shown to harbor the virus [10].

In vitro, monocytes and monocytic cell lines are poorly permissive to CVB. However, these cells can be effectively infected when the virus is previously incubated with non-neutralizing serum or IgGs [11,12]. The antibody-dependent enhancement of CVB4 infection results in the production of high amounts of IFNα [11–14] and increased levels of other proinflammatory cytokines [15].

Monocytes are produced from progenitors in the blood marrow and usually circulate via the bloodstream to peripheral tissues. In the steady state or in response to inflammation, monocytes migrate into tissues, attracted by various cytokines or necrotic cells, and mature to replenish resident macrophages and dendritic cells (DC) [16]. Macrophages and DC are well known as potent initiators of immune response. These professional antigen-presenting cells are well equipped with several sensors, namely pattern recognition receptors, and can, on the one hand, initiate and control immune responses to invading pathogens and, on the other, maintain tolerance to self-antigens [17].

Human monocyte-derived DCs can be infected by many viruses [18–21], but were reported to be non-permissive to CVB [22,23].

Macrophages are primarily remarkable phagocytic cells, but they also have a great plasticity that allows them to respond to environmental cues and change their phenotype, depending on the activation state [24]. Macrophages are one of the earliest detectors of danger signals in the host, and their physiology can be highly modified by the mediators of the immune response, especially cytokines. An environmental signal, such as viral infection, can either increase macrophage immune function or give rise to macrophages that are more susceptible to the infection with less antiviral response [24]. Besides being target cells, macrophages could then play a role in the pathogenesis of virus-induced diseases.

In addition to CD8 cytotoxic T lymphocytes, macrophages have been reported to be common in insulitis found in the pancreas of T1D, both at early and later stages of disease progression [25]. Therefore, in the hypothesis of CVB involvement in the pathogenesis of the disease, the role of interactions between macrophages and the virus cannot be ruled out. Such an interaction has not been investigated yet.

In vitro, several viruses can infect monocyte-derived macrophages (MDM) [26–28] with various outcomes, but no study was reported regarding the infection of these cells with CVB.

In this study, we describe the infection of human MDM by CVB4 and further investigate the inflammation induced by the virus, as well as its persistence in these cells.

2. Results

2.1. Monocyte-Derived Macrophages

Monocytes were cultured for seven days in serum-free medium (SFM) containing either M-CSF or GM-CSF. Under these conditions, on Day 7, the cells appeared roughly similar under the microscope, but they were slightly larger in cultures treated with M-CSF (Figure 1a). The staining with MGG displayed that the cells were similar; however, vacuoles or bi-nucleated cells were more common in M-CSF-treated cultures (Figure 1b). M-CSF and GM-CSF-treated cells were macrophages, as they were stained with CD68 antibody (Figure 1c). The macrophages differentiated under these conditions shared the same pattern of staining as regards various markers: CD163+, CD14+, CD80−, CD83−, CD86+, CCR7+ and HLA-DR+ (Figure 1d,e).

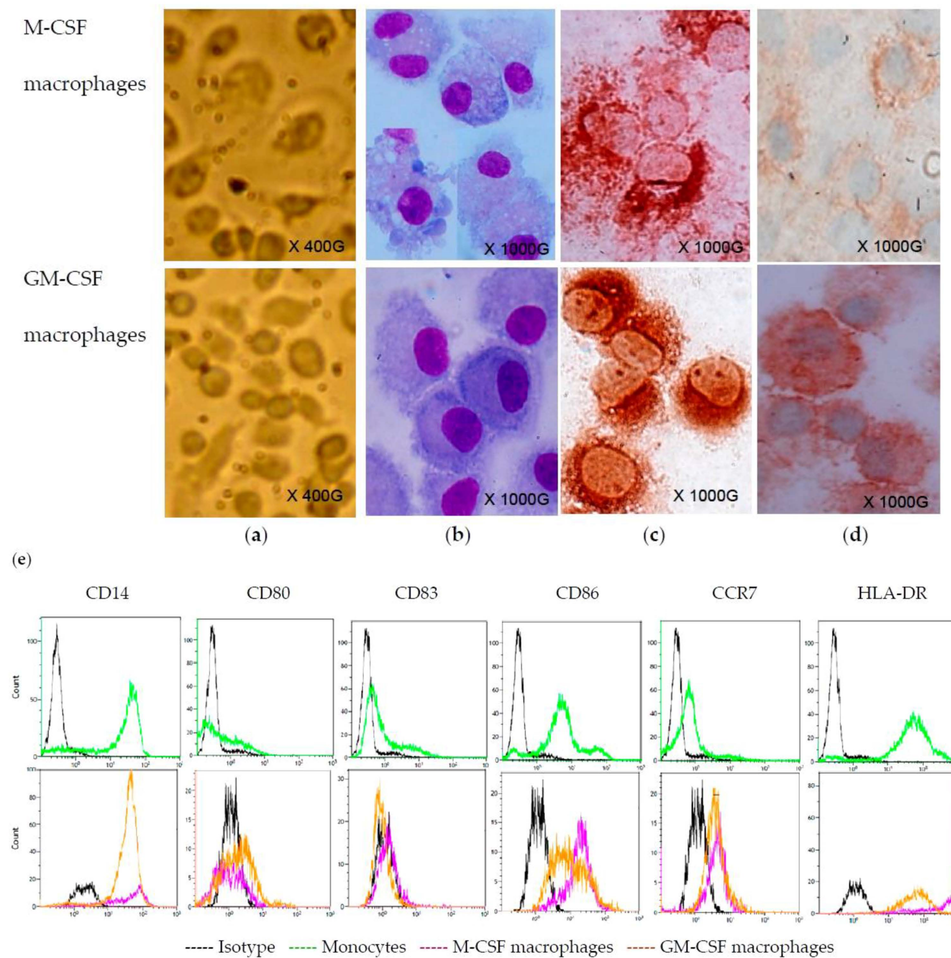

Figure 1. Monocyte-derived macrophages. Monocytes were maintained in serum-free culture medium containing M-CSF or GM-CSF for 7 days. Then cells were directly observed in the culture plate under an inverted microscope (initial magnification X400) (**a**). Cells were cytocentrifuged on slides and stained with MGG (**b**) or labeled with anti-CD68 (**c**) and anti-CD163 (**d**) antibodies by immunocytochemistry (initial magnification X1000). Parental monocytes and derived macrophages were collected and analyzed by flow cytometre (**e**). These observations are representative of three independent experiments.

2.2. CVB4 Can Infect M-CSF-Treated Cells, but not GM-CSF-Treated Cells

Monocytes and monocyte-derived macrophages were inoculated with CVB4 or CVB4 mixed with non-neutralizing dilutions of human serum. In all of the experiments of this study, the multiplicity of infection (MOI) was one. The cultures were incubated; then, 24 and 72 h post-infection (pi), supernatants were collected for IFNα quantification, and the cells were washed five times with PBS and used for viral RNA quantification. As shown in Figure 2, monocytes did not produce IFNα when they were inoculated with CVB4. However, production of IFNα was obtained when the cells were inoculated with CVB4 mixed with diluted human serum (1/100 dilution); the levels of IFNα reached 462 $\pm$ 174 pg/mL and 443 $\pm$ 203 pg/mL at 24 h and 72 h pi, respectively, with an interindividual variability (Figure 2a). When M-CSF-treated cells were inoculated with CVB4, no IFNα was detected in the supernatant collected 24 h pi, whereas a significant amount of IFNα was detected 72 h pi (190 $\pm$ 51 pg/mL). There was no IFNα in the supernatants of cultures inoculated with CVB4 mixed with human serum (1/100 dilution), but the level was 188 $\pm$ 53 pg/mL in supernatants of cultures inoculated with CVB4 mixed with 1000-fold diluted human serum (Figure 2a) and was similar to further dilutions of human serum. Regarding GM-CSF-treated cells, no IFNα was

detected in supernatants, whether CVB4 was previously incubated or not with diluted human serum (Figure 2a). IFNα was not detected in supernatants from control cell cultures.

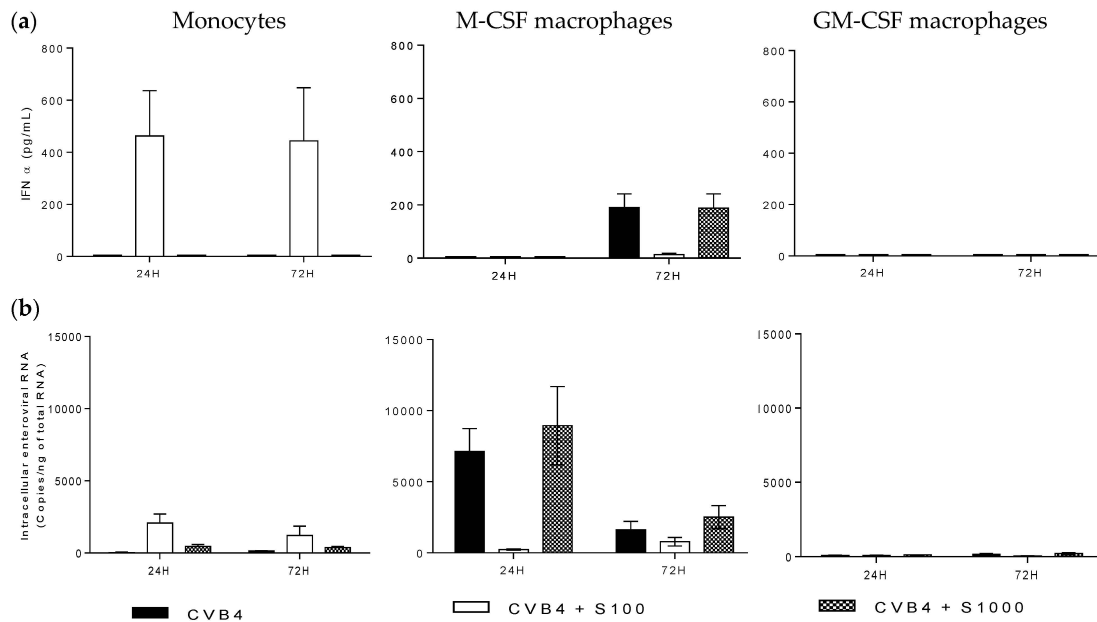

Figure 2. Group B coxsackieviruses 4 (CVB4) can infect M-CSF-induced monocyte-derived macrophages. Monocytes and monocytes treated with M-CSF or GM-CSF for seven days (M-CSF macrophages and GM-CSF macrophages) were inoculated with CVB4 or CVB4 mixed with diluted anti-CVB4 human serum (S100: 1/100 dilution, S1000: 1/1000 dilution). The cell cultures were incubated for 24 h and 72 h, then IFNα levels in the supernatants were measured by using ELISA (**a**), and intracellular viral RNA was quantified by using real-time RT-qPCR (**b**). These results are the mean ± SEM of three independent experiments.

As far as the quantification of intracellular enteroviral RNA was concerned, the levels were very low in monocytes inoculated with CVB4, 85 ± 47 and 146 ± 68 copies/ng of total RNA at 24 and 72 h pi, respectively, and increased significantly when monocytes were inoculated with CVB4 mixed with 100-fold diluted human serum, reaching 2070 ± 626 and 1215 ± 645 copies/ng of total RNA at 24 and 72 h, respectively (see Figure 2b). In M-CSF-treated cells infected with CVB4, the levels of viral RNA (7126 ± 1609 and 1623 ± 601 copies/ng of total RNA at 24 and 72 h) were high. There was no significant increase when the cells were inoculated with CVB4 mixed with 1000-fold diluted human serum (8939 ± 2753 and 2514 ± 801 copies/ng of total RNA) (Figure 2b). In contrast, viral RNA was almost undetectable in GM-CSF-treated cells inoculated with CVB4 or CVB4 mixed with human serum (Figure 2b). No virus was detected in control cell cultures.

Altogether, these results show that CVB4 can infect MDM differentiated in culture medium containing M-CSF and that there is no human serum-dependent enhancement of CVB4 infection of these cells. In contrast, MDM differentiated in culture medium containing GM-CSF were not permissive to CVB4.

We hypothesized that GM-CSF-induced MDM were not permissive to CVB4, compared to M-CSF-induced MDM, because of a reduced expression of CAR, the receptor for CVB4. CAR mRNA was then quantified in both cell populations collected after differentiation on Day 7. As shown in Figure 3, the relative expression of CAR was similar in GM-CSF-induced and M-CSF-induced MDM.

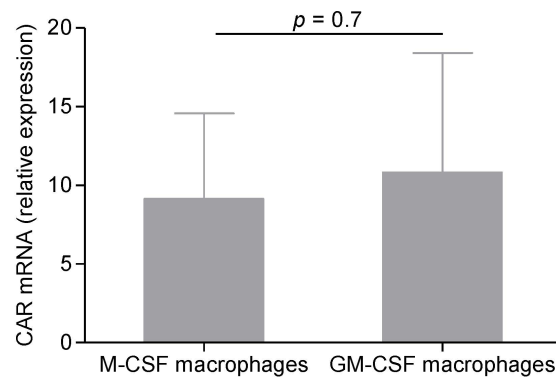

Figure 3. CAR mRNA expression in macrophages. Monocytes and MDM treated with M-CSF or GM-CSF for seven days were collected, and RNA was extracted. CAR mRNA and β-actin were amplified using real-time RT-PCR. CAR relative expression was determined using the $2^{-\Delta\Delta Ct}$ formula, as compared to parental monocytes. These results are the mean ± SEM of three independent experiments.

2.3. CVB4 Can Induce Pro-Inflammatory Cytokines MDM Induced with M-CSF and GM-CSF

We previously observed that PBMCs were not infected when they were inoculated with CVB4; nevertheless, they can produce high levels of pro-inflammatory cytokines [15]. Therefore, the production of these cytokines in MDM cultures was investigated. Cell cultures were inoculated with CVB4, as described above, and supernatants were collected at 24 and 72 h pi. Like monocytes, both M-CSF- and GM-CSF-induced MDM produced high levels of IL-6, up to 3000 pg/mL in both cell cultures (Figure 4a). CVB4 also induced significant amounts of TNFα in both cell cultures (up to 1200 and 1700 pg/mL in M-CSF- and MG-CSF-induced MDM, respectively) (Figure 4b). On the other hand, CVB4 did not induce any significant production of IL-10 in these cells (Figure 4c). None of these cytokines were detected in control wells.

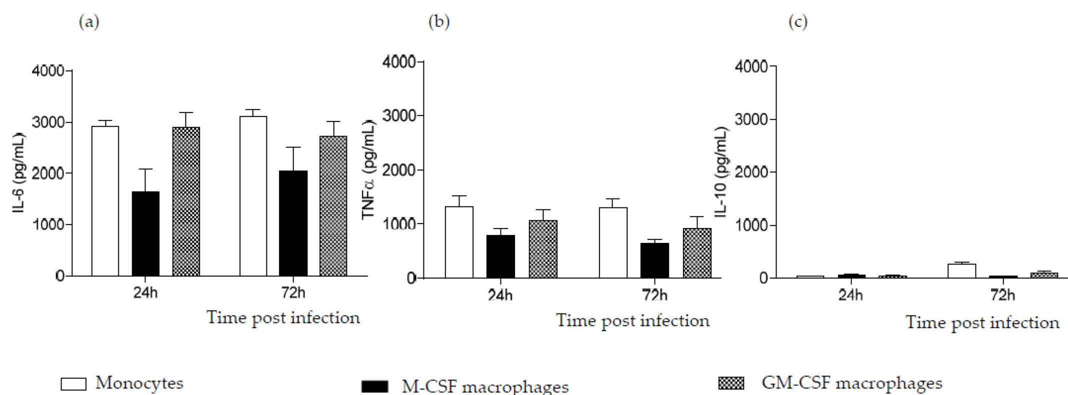

Figure 4. CVB4 can induce pro-inflammatory cytokines in monocyte-derived macrophages (MDM). Monocytes and MDM induced with M-CSF (M-CSF macrophages) or GM-CSF (GM-CSF macrophages) for seven days were inoculated with CVB4. Supernatants were collected at 24 and 72 h post-infection (pi). IL-6 (**a**), TNFα (**b**) and IL-10 (**c**) were measured in supernatants by using ELISA. These results are the mean ± SEM of three independent experiments.

2.4. CVB4 Can Replicate and Persist in M-CSF-Induced MDM

In so far as M-CSF-induced MDM were permissive to CVB4 infection, we investigated further the replication and the persistence of the virus in these cells. MDM were inoculated with CVB4,

and after 2 h, the cells were extensively washed five times with PBS and then cultured in serum-free medium for 12 days. Supernatants and cells were collected every three days. The viability of cells was assessed during the follow-up (Figure 5a).

High levels of IFNα were detected in supernatants on Day 3 pi. Afterwards, the levels of IFNα decreased dramatically and were almost undetectable on Day 6 pi (Figure 5b).

The levels of infectious viral particles in supernatants were assessed by $TCID_{50}$ determination on HEp-2 cells. The viral titers reached a maximum of 8.6 ± 2 log $TCID_{50}$/100 μL on Day 3 and decreased progressively, but were still around 2.5 log TCD_{50}/100 μL on Day 12 (Figure 5c).

The RNA viral load in supernatants was correlated with the viral titers. The maximum value was reached on Day 3 pi (7.4 ± 0.4 log copies/μL) and was 3.6 ± 0.2 log copies/μL on Day 12 (Figure 5d).

Intracellular viral RNA was also detected significantly during the follow-up. The highest levels were around 4 log copies/ng of total RNA and were reached between three and six days pi (Figure 5e).

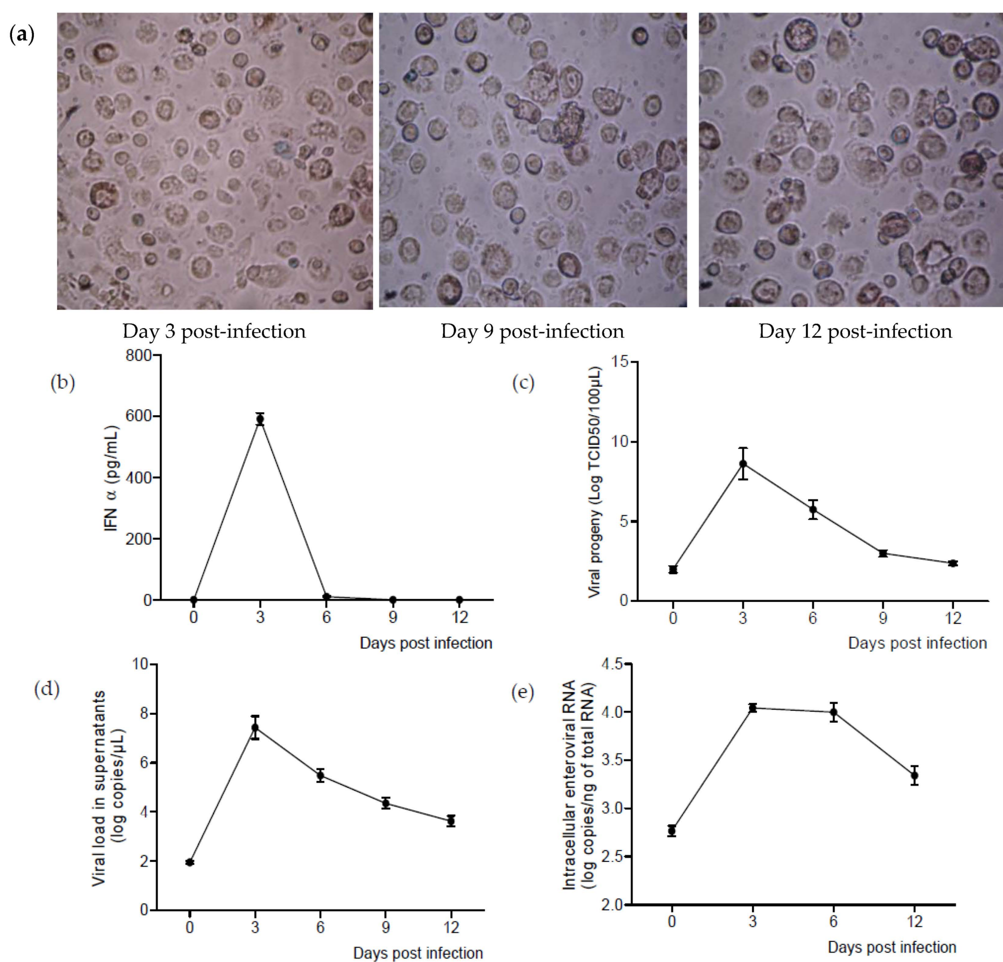

Figure 5. CVB4 can replicate and persist in M-CSF MDM. MDM treated with M-CSF for seven days were inoculated with CVB4 and were cultured for 12 days. The cell viability was assessed using the trypan blue exclusion assay (**a**). In supernatants, the levels of IFNα (**b**), infectious particles (**c**) and enteroviral RNA (**d**) were determined. In cells, enteroviral RNA was quantified by real-time RT-qPCR (**e**). These results are the mean ± SEM of three independent experiments.

3. Discussion

In this study, we described, for the first time, the infection of human primary macrophages by group B coxsackieviruses. We demonstrated that CVB4 can effectively infect M-CSF-induced MDM,

but not GM-CSF-induced MDM. In addition to the production of viral particles, a persistence of viral RNA has been observed in M-CSF-induced MDM cultures.

Previous investigations by our team focused on peripheral blood mononuclear cells (PBMCs) and revealed that among these cells, only monocytes could be infected by CVB *in vitro*, as well as *in vivo* [10]. Monocytes are not spontaneously permissive to the virus; however, the infection can be enhanced by antibodies, as described in previous papers [11,13]. The infection of monocytes with CVB4 can be obtained by pre-incubation of the virus with non-neutralizing dilution of an immune serum before inoculation to cells [11,13]. Enhanced CVB infection of monocytes was shown to rely on both the specific receptor CAR and FCγ receptors, and the target of enhancing antibodies was reported to be the viral protein VP4 [14,29].

However, monocytes are not long-life cells and usually leave the bloodstream after 2–3 days to reach tissues and differentiate into mature cells, such as macrophages [16]. Therefore, the exploration of the interactions between CVB and these cells highly involved in immune response is needed, in the hypothesis of the involvement of the virus in chronic auto-immune diseases, like T1D.

For *in vitro* studies on macrophages, several protocols have been described by researchers to differentiate macrophages from blood monocytes, and usually include the use of: (i) media containing human autologous or heterologous AB serum or fetal bovine serum; or (ii) media containing growth factors, namely M-CSF or GM-CSF [30,31].

In this report, MDM were generated by treating monocytes with a serum-free medium containing either M-CSF or GM-CSF. The phenotypes of cells obtained in both conditions were similar as shown by immunological markers; but surprisingly, only cells treated with M-CSF could be infected by CVB4. It is important to note that the cells were treated for seven days, and then, after washings, they were maintained in culture medium without growth factors. Thus, the opposite patterns of data regarding the infection of MDM in our experiments can be due to some differences between M-CSF-treated cells and GM-CSF-treated cells.

It has been reported that GM-CSF-induced MDM share some transcriptional profiles of classically-activated pro-inflammatory (M1) cells *in vivo*, while those induced with M-CSF tend to replicate alternatively-activated anti-inflammatory (M2) macrophages [30–32], but such an association is overestimated. M-CSF and GM-CSF MDM share many features, but are also different regarding the expression of some gene markers and some functions [31,33,34]. A more realistic *in vitro* approach considers MDM generated with M-CSF and GM-CSF as M0 macrophages, and then, M1 originate from M0 induced with GM-CSF or M-CSF in the presence of IFNγ and/or LPS, while M2 macrophages are triggered from M-CSF-induced cells by the presence of cytokines, such as IL-4 or IL-10 [31,35,36]. In so far as the cells were treated with M-CSF or GM-CSF, but not with additional factors, it can be admitted that MDM were M0 macrophages in our experiments.

The discrepancy observed between M-CSF- and GM-CSF-induced MDM regarding CVB4 infection was not due to a reduced transcriptional expression of the specific viral receptor CAR in GM-CSF-treated cells, as shown by quantitative real-time RT-PCR. It remains to be determined whether the receptor is present at the same level at the surface of both M-CSF and GM-CSF cells. Furthermore, in non-polarized cells, it has been reported that a secondary receptor, such as decay-accelerating factor (DAF), is not mandatory for CVB infection, whereas other entry pathways and molecules seem to be critical for entry [37]. Whether these mechanisms of entry are active in M-CSF-induced MDM, but not in GM-CSF-induced MDM cannot be ruled out.

In this study, it has been observed that CVB4 induced the production of high levels of pro-inflammatory cytokines, such as IL-6 and TNα, in both M-CSF MDM and GM-CSF MDM cultures. Since GM-CSF MDM were not infected with CVB4, the production of cytokines was thought to be triggered mainly by surface sensors on these cells, which is in agreement with previous studies in our laboratory. Indeed, it has been shown that inactivated CVB4 can stimulate the production of IL-6, TNFα and IL-12 by monocytes [15], which suggests that a replicative infection of monocytes/macrophages by the virus is not mandatory for inducing inflammation.

In GM-CSF MDM inoculated with CVB4, a low level of viral RNA was detected by sensitive real-time RT-qPCR, but was not associated with the presence of IFNα in supernatants, suggesting that the infection was at a very low level in these cells or was abortive. This hypothesis is in agreement with the demonstration by another team that HIV replication was suppressed in GM-CSF-induced macrophages, but not in M-CSF-induced macrophages [38]. A similar finding was also reported for Mycobacterium tuberculosis, another intracellular pathogen [34].

M-CSF MDM were infected with CVB4, whereas monocytes were infected when the virus was mixed with human non-neutralizing serum. In addition, no enhancement of CVB4 infection (*i.e.*, no increase in FNα or intracellular viral RNA) was obtained in MDM when the virus was mixed with serum, although both monocytes and MDM were reported to express FcγRI and FcγRII [34]. Thus, this suggests that antibodies do not enhance the infection of M-CSF-induced MDM with CVB4.

The investigation of CVB4 persistence in M-CSF-induced MDM displayed that significant levels of infectious particles in supernatants and viral intracellular RNA can be detected up to 12 days (end of the culture), while the cellular response, especially the production of IFNα, was limited to early steps (Day 3). This finding suggests that CVB4 probably overrides the cellular response to establish persistence in MDM. Further studies are needed to understand the mechanisms of interaction between CVB4 and macrophages.

The micro-environment and the phenotype highly impact the physiology of macrophages. Therefore, the relationship between CVB4 and these cells in the pathogenesis of autoimmune diseases, such as T1D, can depend on a specific micro-environment. Indeed, for example, it has been suggested that GM-CSF plays a role in maintaining macrophage populations in a physiological steady state and in controlling the development of autoimmune diseases by regulating the immune response and the immunological tolerance [39].

Whether the phenotype of macrophages recruited in pancreas plays a role in interactions with CVB and in the development of autoimmunity deserves further investigation.

In conclusion, human primary M-CSF-induced MDM, but not GM-CSF-induced MDM, can be infected by CVB4. A productive infection can be obtained, and the virus can persist in these cells. An inflammatory reaction of the cells is observed during the acute phase of the infection. Macrophages can then be considered not only as target cells, but also as reservoirs for CVB4 in tissues, with implications in the pathogenesis of chronic diseases triggered by these viruses.

4. Materials and Methods

4.1. Virus Stocks

CVB4 E2, the diabetogenic strain of coxsackievirus B4, was used in all experiments. It was kindly provided by Ji-Won Yoon (Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada) and was propagated in Hep-2 cells (BioWhittaker, Walkersville, MD, USA).

4.2. Peripheral Blood Monocyte-Derived Macrophages

Human blood collected from healthy donors was obtained at the Regional Blood Bank (Lille, France). This study was conducted in accordance with the rules of the Declaration of Helsinki of 1975, revised in 2008. Written consent was obtained from donors by the blood bank, and they were informed that the blood would be used for research purposes.

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by density gradient centrifugation using Ficoll-Hypaque™ PLUS (GE Healthcare, Vélizy-Villacoublay, France), as described previously [15]. Cells were resuspended in non-supplemented RPMI 1640 medium, and an average of 10^7 cells per well (~1.5 million/cm^2) was seeded in a Falcon® polystyrene six-well plate (Fischer Scientific, Illkirch-Graffenstaden, France). Monocytes from the PBMCs were allowed to adhere to plastic for 2 h at 37 °C, 5% CO2, and non-adherent cells were subsequently removed by aspiration and extensive washing with PBS. Monocytes were cultivated in serum-free medium

(SFM) containing either macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF) (Peprotech, Neuilly-sur-Seine, France) at 20 ng/mL. The medium was changed on Day 3. On Day 7, the cells were differentiated into macrophages. All media were purchased from Gibco® (Thermo Fischer Scientific, Villebon sur Yvette, France).

4.3. Human Serum

Whole blood was centrifugated at 2500 rpm for 15 min, and serum was aliquoted and stored at −20 °C. The level of anti-CVB4 antibodies was determined by the seroneutralization assay, as previously described [15]. Serum with a titer of 256 was used in all experiments.

4.4. May–Grunwald–Giemsa Staining and Immunocytochemistry

MDM were detached from the plates by incubation at 4 °C with PBS containing 5 mM EDTA followed by gentle scraping. Then, cells were plated on a slide by cytocentrifugation at 500 rpm for 5 min, and slides were air dried. Cells intended for May-Grunwald-Giemsa (MGG) staining were fixed with ethanol for 5 min and stained using the automated SP-10™ slide maker/stainer (Sysmex, Villepinte, France). For immunocytochemistry purposes, cells were fixed with acetone for 10 min. The FLEX monoclonal mouse anti-human CD68 (clone KP1) antibody was purchased from Dako (Dako, Les Ulis, France), and anti-CD163 monoclonal antibody was supplied by DB Biotech (Kosice, Slovak Republic). The staining was performed using the Ultra View Universal DAB Detection Kit, on the VENTANA® Benchmark XT fully-automated IHC/ISH staining instrument (Roche Diagnostics, Meylan, France).

4.5. Flow Cytometry

Monoclonal antibodies directed against human surface markers CD14, CD80, CD83, CD86, CCR7 (Beckman Coulter, Villepinte, France) and HLA-DR (Becton Dickinson Bioscience, Le Pont-de-Claix, France) were used at the supplier's recommended concentrations. The CD14 antibody was fluorescein isothiocyanate (FITC)-conjugated, whereas CD80, CD83, CD86, CCR7 and HLA-DR antibodies were phycoerythrin (PE)-conjugated. Monocytes and monocyte-derived cells were detached and washed once in PBS. Cell suspensions were incubated at 4 °C for 20 min with the appropriate antibodies. The cells were then fixed in 0.5% paraformaldehyde, washed once with PBS and analyzed by flow cytometry on a Navios Flow cytometer (Beckman Coulter, Inc.).

4.6. Quantification of Cytokines

IFNα was measured using the IFNα pan-specific ELISA kit (Mabtech®, Sophia Antipolis, France) that allowed detection of subtypes 1/13, 2, 4, 5, 6, 7, 8, 10, 14, 16 and 17 of IFNα. TNFα, IL-6 and IL-10 were quantified with ELISA kits purchased from Peprotech®. Assays were performed according to the manufacturer's instructions. The detection ranges were 7–700 pg/mL (IFNα), 23–1500 pg/mL (TNFα), 32–2000 pg/mL (IL-6) and 32–2000 pg/mL (IL-10).

4.7. Viral Progeny

The viral titer in supernatants of infected cells was assessed using the end-point dilution assay, and the Spearman–Karber statistical method was used to determine the tissue culture 50% infectious dose ($TCID_{50}$).

4.8. RNA Extraction

Cells were previously collected in TriReagent® for cell lysis and stored at −80 °C until RNA extraction. For the extraction of RNA from supernatants, a volume of 250 μL was used. Total RNA was extracted using the TriReagent® RNA isolation reagent/chloroform procedure (Sigma-Aldrich).

Extracted RNA was then dissolved in 50 μL of nuclease-free water, quantified with a Nanodrop® spectrophotometer (Thermo Fischer Scientific).

4.9. DNase Treatment

A DNA enzymatic digestion was performed on RNA extracts. A volume of 10 μL of RNA was mixed in a tube with 2 μL of DNase RDD10× buffer (Qiagen, Les Ulis, France), 0.2 μL of DNase I (Qiagen), 0.25 μL of RNase inhibitor (Invitrogen/Thermofischer Scientific) and 7.55 μL of nuclease-free water. The tube was incubated at 37 °C for 30 min and at 65 °C for 5 min. The tube was brought to 4 °C and then vortexed.

4.10. Enterovirus Real-Time Quantitative PCR

The Affinityscript® QPCR cDNA Synthesis Kit (Agilent, Les Ulis, France) was used for the RNA retro-transcription step on a Perkin Elmer 2400 thermocycler. Quantitative RT-PCR for cDNA amplification was performed with the Brilliant II® QPCR Kit (Agilent) on the Mx3000p® (Stratagene/ Thermofischer Scientific). Oligonucleotides and RT-PCR conditions were previously described [15]. Enterovirus 71 RNA (Vircell, Granada, Spain) was used as the standard for quantification.

4.11. Quantification of Coxsackie and Adenovirus Receptor mRNA

CAR mRNA was quantified by real-time RT-qPCR using the SuperScript III Platinum One-Step Quantitative RT-PCR System (Life Technologies/Thermofischer Scientific). The oligonucleotides were already published [40]. The quantification was carried out on the Mx3000p® (Stratagene) with the following program: 50 °C for 15 min, 95 °C for 2 min and 40 cycles of amplification consisting of 15 s at 95 °C and 30 s at 60 °C. The expression of the beta-actin gene was used for normalization. CAR mRNA relative expression in macrophages as compared to parental monocytes was determined with the $2^{-\Delta\Delta Ct}$ formula [41].

4.12. Statistical Analysis

Data were presented as the mean ± SEM. Graphs and analyses were performed with GraphPad Prism® V6.0 software. Comparisons were performed with the Mann–Whitney *U* test with the significance set at 0.05.

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Author Contributions: E.K.A. and D.H. conceived of and designed the experiments. E.K.A. performed the experiments and analyzed the data. F.S., J.T. and M.C.C. contributed to performing of the experiments. E.K.A. and D.H. wrote the paper.

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ETUDE 4 : Les cellules pancréatiques chroniquement infectées par coxsackievirus B4 peuvent être guéries par un traitement à la fluoxétine.

Travail publié dans Antiviral Research

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Résumé

Objectif

Les coxsackievirus du groupe B (CVB) peuvent établir des infections persistantes in vitro et in vivo. La fluoxétine est une molécule utilisée dans le traitement de la dépression et autres troubles psychiatriques. Des études ont démontré que cette molécule avait une activité antivirale vis-à-vis de CVB1-3. Dans ce travail, l'effet antiviral de la fluoxétine a été évalué vis-à-vis de CVB4 dans des modèles d'infection aiguë, et dans un modèle d'infection persistante à CVB4 dans une lignée de cellules pancréatiques.

Méthodes

CVB4 a été inoculé à des cellules Hep-2 et Panc-1, en présence de différentes dilutions non toxiques de chlorhydrate de fluoxétine. L'activité antivirale a été évaluée par l'observation de l'effet cytopathogène, la détermination du titre viral dans les surnageants de culture cellulaire et la quantification de l'ARN viral intracellulaire.

Une infection persistante à CVB4 a été établie dans les cellules Panc-1, et la guérison de l'infection a été monitorée par l'évaluation du titre viral et de l'ARN viral intracellulaire.

Principaux résultats

Une inhibition complète de la réplication de CVB4 a été observée sur les cellules Hep-2, en présence de 5,48 µM de fluoxétine. Aucun effet antiviral n'a été observé lorsque la fluoxetine a été mélangée pendant 2 heures au virus, puis éliminée par filtration avant l'inoculation aux cellules. Un résultat identique a été retrouvé lorsque les cellules sont traitées avec de la fluoxétine jusqu'à 96 heures puis lavées avant l'inoculation de CVB4.

La fluoxétine à 5,48 µM réduit de plus de 50% la réplication de CVB4 au cours de l'infection aiguë dans les cellules Panc-1. Dans les cellules Panc-1 chroniquement infectées par CVB4 et traitées par la fluoxétine à raison de 5,48 µM deux fois par semaine, une diminution importante du titre viral a été observée dans les surnageants. Au bout de 3 semaines de traitement, une absence de particules infectieuses a été notée dans les surnageants. L'ARN viral intracellulaire était aussi indétectable.

Conclusion

Ces résultats montrent que la fluoxétine peut inhiber la réplication de CVB4 et guérir l'infection par ce virus dans les cellules chroniquement infectées. Au-delà de l'effet antiviral observé, ce modèle peut permettre de mieux comprendre la biologie de CVB4, et être aussi un outil dans l'investigation des changements cellulaires au cours de l'infection persistante à CVB4.

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Short Communication

Persistent infection of human pancreatic cells with Coxsackievirus B4 is cured by fluoxetine

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ABSTRACT

Group B Coxsackieviruses (CVB) are involved in various acute clinical features and they can play a role in the development of chronic diseases like type 1 diabetes. The persistence of CVB has been described in vitro and in vivo in various models. Fluoxetine was reported to inhibit the replication of CVB1–3, which prompted us to study the in vitro antiviral activity of fluoxetine against CVB4 in models of acute infection. In addition we took advantage of a chronically CVB4-infected Panc-1 cell line to evaluate the antiviral effect of fluoxetine in a model of persistent CVB4 infection.

An inhibition of the CVB4 replication was obtained when fluoxetine was added at 5.48 μM to Hep-2 cell cultures. No inhibitory effect was observed when CVB4 was mixed with fluoxetine for 2 h and filtered to eliminate fluoxetine before inoculation to cells, or when cells were treated up to 96 h and washed before viral inoculation. Fluoxetine (5.48 μM) reduced viral replication by more than 50% in acutely infected Panc-1 cell cultures. A dramatic decrease of infectious particles levels in supernatants of Panc-1 cells chronically infected with CVB4 was obtained a few days after treatment with fluoxetine and no infectious viral particle was found as soon as day 21 of treatment, and intracellular enteroviral RNA was undetectable by RT-PCR after three weeks of treatment.

These data display that fluoxetine can inhibit the replication of CVB4 and can cure Panc-1 cells chronically infected with CVB4.

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Human enteroviruses (HEVs) are small non-enveloped RNA viruses that belong to the *Picornaviridae* family. The genus enterovirus currently includes 7 species involved in human diseases (human enterovirus A–D and human rhinovirus A–C), and over 250 serologically distinct viruses among which several important human pathogens, e.g. poliovirus, coxsackievirus, echovirus, rhinovirus and enterovirus 71 (Knowles et al., 2012; Tapparel et al., 2013). Some of the non-polio enteroviruses can be involved in many severe acute clinical features such as meningitis, myocarditis or fulminate sepsis in newborns (Romero, 2008; Tapparel et al., 2013) while others especially type B Coxsackieviruses (CVB) are also reported to play a role in the development of chronic diseases like type 1 diabetes (Hober and Alidjinou, 2013; Hober and Sauter, 2010). In the pathogenesis of these diseases the persistence of CVB in host tissues is thought to be involved (Jaïdane and Hober, 2008; Jaïdane et al., 2010). The persistence of CVB4 in human pancreatic cell cultures, resulting in an impaired formation and viability of islet-like cell aggregates has been reported (Sane et al., 2013).

The wide range of antivirals currently marketed has not shown any significant activity against enteroviruses. Many compounds have been screened or are under investigation for their antiviral activity against enteroviruses but no antienterovirus drug is currently licensed worldwide (De Palma et al., 2008; Thibaut et al., 2011). Recently, fluoxetine, a selective serotonine reuptake inhibitor (SSRI) has been reported as an inhibitor of enterovirus replication in vitro. This medicine formerly used for the treatment of depression or other mental disorders, has a significant antiviral activity against CVB1–3 in vitro (Ulferts et al., 2013; Zuo et al., 2012).

In the present study the antiviral activity of fluoxetine against CVB4 in cell cultures acutely infected and in a model of in vitro persistent infection has been investigated.

The diabetogenic CVB4 E2 strain, kindly provided by Ji-Won Yoon (Julia McFarlane Diabetes Research Center, Calgary, Alberta, Canada) was propagated in Hep-2 cells. Hep-2 cells (BioWhittaker) were grown in minimum essential medium supplemented with 10% of fetal calf serum (FCS), 1% of L-glutamine, 1% of non-essential amino acids and 1% of penicillin and streptomycin. The human

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ductal cell line Panc-1 (ATCC) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% of FCS, 1% of L-glutamine and 1% of penicillin and streptomycin. A model of persistent infection of Panc-1 cells with CVB4 has been previously reported by our team (Sane et al., 2013).

Fluoxetine chlorhydrate (Lilly France, France) was dissolved in DMSO. A concentration of 21.9 μM or below was shown to have no cytotoxicity on both Hep-2 and Panc-1 cells. To evaluate the antiviral activity of fluoxetine, cells were seeded in a 96 wells cell culture plate at 1.25×10^4 cells per well. Cells were inoculated with the virus at a MOI of 0.01 mixed with various dilutions of fluoxetine or DMSO. The plates were incubated at 37 °C, and the cell cultures were observed every day. The cytopathic effect (CPE) in the wells with fluoxetine was recorded when a CPE of 100% was reached in mock-treated wells inoculated with CVB4.

The viral titers in supernatants of chronically-infected cells were assessed using the end-point dilution assay, and the Spearman-Karber statistical method was used to determine the tissue culture 50% infectious dose (TCID50). Total RNA was extracted from cells using RNeasy kit (Qiagen). The level of intracellular viral RNA in acutely-infected cells was quantified by using a two-step real time PCR as described elsewhere (Alidjinou et al., 2013).

The presence of intracellular enterovirus RNA in persistently-infected cells was investigated by RT-PCR and semi-nested PCR using respectively the SuperScript® one-step RT-PCR and the Platinum® PCR Supermix kits (Invitrogen) as previously described (Sane et al., 2013).

The antiviral activity of fluoxetine has been assessed in Hep-2 and Panc-1 cell cultures inoculated with CVB4. Serial 2-fold dilutions starting from 21.9 μM fluoxetine were tested on both cells. A CPE of 100% was observed on day 2 post-infection (p.i.) in Hep-2 cell cultures. As shown in Fig. 1a, fluoxetine inhibited the CVB4-induced CPE and the production of infectious particles in Hep-2 cells at a concentration of 5.48 μM. The intracellular viral RNA was also significantly reduced (Fig. 1b).

Three situations were tested in Hep-2 cell cultures to study the mode of action of fluoxetine. The molecule used at a final concentration of 5.48 μM was (i) added to cells inoculated with CVB4; (ii) mixed with CVB4 before inoculation to cells (iii) incubated with cells that were subsequently inoculated with CVB4. Fluoxetine inhibited completely the CVB4-induced CPE when added to cell cultures at 0, 2, and 4 h post infection. A residual CPE, 10% and 50%, was observed when fluoxetine was added to cell cultures at 20 and 24 h post infection, respectively (see Fig. 1c). In other experiments, CVB4 was mixed with fluoxetine for 2 h and the mixture was filtered on Illustra MicroSpin Columns® by centrifugation at 735 g for 1 min, and the filtrate was then inoculated to Hep-2 cell cultures. In parallel, Hep-2 cell cultures were also inoculated with filtered CVB4 or with the following mixtures: CVB4 added to filtered fluoxetine or fluoxetine added to filtered CVB4. The results are shown in Fig. 1d. When cells were inoculated with filtered virus, a CPE was observed. In contrast when the filtered virus was mixed with fluoxetine the CPE was inhibited, whereas no inhibition was observed in all conditions that include a step of filtration of fluoxetine. Finally, cell cultures were treated with fluoxetine for various time intervals ranging from 2 to 96 h, then after washings with culture medium, CVB4 was inoculated. No inhibition of the CVB4-induced CPE was observed in these conditions (data not shown).

Fluoxetine was also tested in acutely infected Panc-1 cells. A 100% CPE was observed in control wells on day 5 p.i. A concentration of 5.48 μM reduced the CPE and the viral titer by more than 50% and the level of intracellular enterovirus RNA was also decreased but to a lesser extent (see Fig. 2a and b). When treatment with fluoxetine at 5.48 μM was repeated after 48 h, the viral replication on day 5 p.i. was more extensively reduced up to 100% (data not shown).

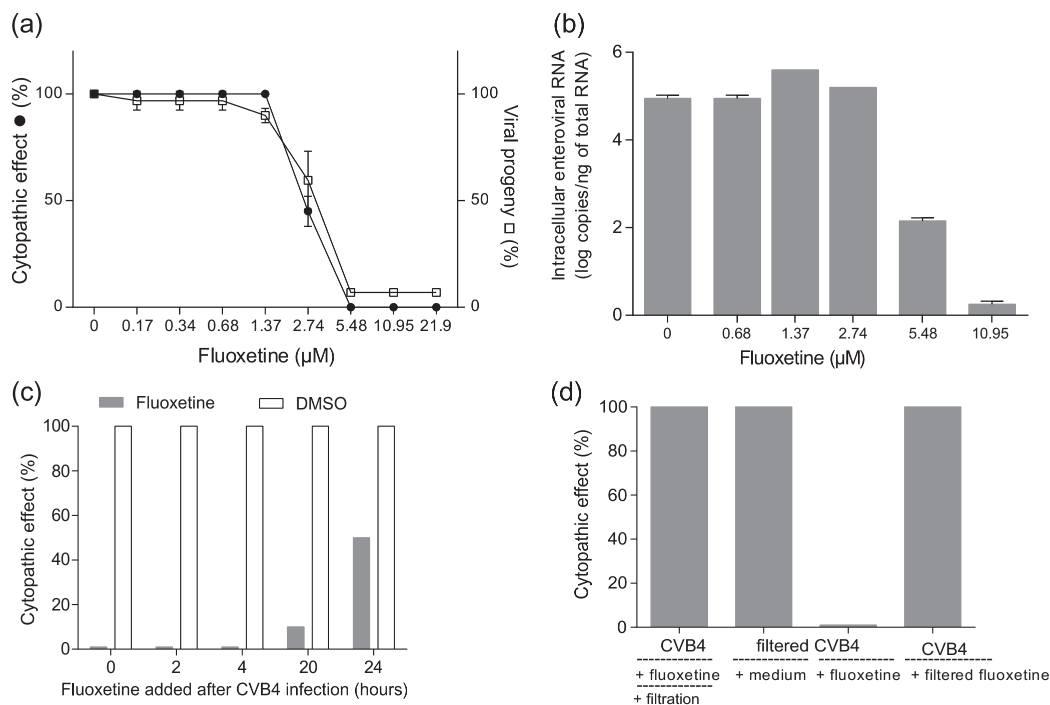

Fig. 1. Fluoxetine inhibits the CVB4 replication in Hep-2 cells. Hep-2 cells were seeded in 96 wells cell culture plates at 1.25×10^4 cells per well. CVB4E2, at MOI 0.01, mixed with various dilutions of fluoxetine was added to cell cultures (a–b). Fluoxetine at 5.48 μM was added to Hep-2 cell cultures at 0, 2, 4, 20 and 24 h post infection with CVB4E2 (c). CVB4E2 was mixed with fluoxetine at 5.48 μM for 2 h and the mixture was filtered and the filtrate was then inoculated to Hep-2 cell cultures. Hep-2 cell cultures were also inoculated with filtered CVB4E2, filtered CVB4E2 mixed with fluoxetine for 2 h, or CVB4E2 mixed for 2 h with filtered fluoxetine (d). The cytopathic effect in the wells with fluoxetine was recorded when it reached 100% in controls. The viral progeny was determined in supernatants using endpoint dilution assay and expressed as percentage of controls, and the level of intracellular enterovirus RNA was quantified by using real-time RT-PCR. The results are representative of two independent experiments.

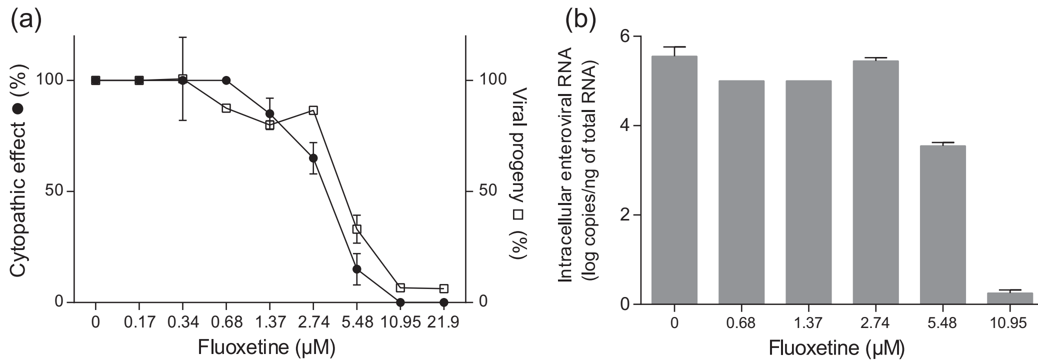

Fig. 2. Fluoxetine inhibits the CVB4 replication in Panc-1 cells. Panc-1 cells were seeded in 96 wells cell culture plates at 1.25×10^4 cells per well. CVB4E2, at MOI 0.01, mixed with various dilutions of fluoxetine was added to cell cultures (a–b). The cytopathic effect in the wells with fluoxetine was recorded when it reached 100% in controls. The viral progeny was determined in supernatants using endpoint dilution assay and expressed as percentage of controls, and the level of intracellular enterovirus RNA was quantified by using real-time RT-PCR. The results are representative of two independent experiments.

Panc-1 cells chronically infected with CVB4 were treated every 3–4 days with fluoxetine at 5.48 μM or with DMSO, and mock-infected cells underwent the same treatment. Once a week, the cell cultures were scraped and subcultured. In so far as there was no CPE in Panc-1 cell cultures chronically infected with CVB4, the impact of fluoxetine onto these cells was evaluated through the level of infectious particles in culture supernatants collected at various time intervals and through the detection of intracellular enteroviral RNA by RT-PCR. The viral titers in supernatants of cell cultures decreased dramatically a few days after treatment with fluoxetine and no infectious viral particle was found as soon as day 21. In contrast, in supernatants of mock-treated cells, the viral titer remained around 4.5 log TCID50/mL all over the follow-up (see Fig. 3). Enteroviral RNA was not detected anymore by both RT-PCR and semi-nested PCR on day 21 post-treatment as shown in Fig. 2.

This study is different in many respects from those of other investigators. Several considerations in the present report are noteworthy. Our results demonstrate that fluoxetine inhibits the CVB4 replication in the Hep-2 cell line and in the human pancreatic cell line, Panc-1. Moreover, we took advantage of a persistent enteroviral infection in this model to display that fluoxetine can cure pancreatic cells chronically infected by CVB4. Indeed, infectious particles and intracellular enteroviral RNA were cleared within three weeks when cultures were treated twice a week. In previous studies, the antiviral effect of fluoxetine against other Coxsackieviruses B (CVB1–3) and other members of the human enterovirus B and D species, and in other cell line models (BGM, Hela) was shown, but not in models of persistent infection (Ulferts et al., 2013; Zuo et al., 2012). Previous authors demonstrated that fluoxetine does not prevent virus entry, and that its antiviral activity relies on an inhibition of viral RNA synthesis. In the present study it has been shown that fluoxetine does not exert a direct inactivation or degradation effect on the viral particle, as far as an extended incubation of CVB4 with fluoxetine does not suppress viral replication, after removal of fluoxetine by filtration. Furthermore, fluoxetine does not induce a stable antiviral state in cells treated up to 96 h, which remain fully susceptible to the virus after removal of fluoxetine by washing. Clearly, the molecule must be present during the infection process to obtain an antiviral activity, and early administration can completely abrogate viral replication.

The total clearance of the virus from chronically-infected cells could be explained by the terms of the persistent infection model. It was reported that in this model, about 50% of cells harbor viral RNA (Sane et al., 2013). We hypothesize that this rate of infection is maintained by a balance between viral replication and cell proliferation. Through the inhibition of viral replication by fluoxetine, this balance is disrupted, and cells harboring viral RNA are gradually eliminated.

The precise mechanism of the inhibition of viral replication by fluoxetine is still unclear. It was suggested that its target was the nonstructural viral protein 2C, but the function of this protein possibly inhibited by fluoxetine remains unknown (Ulferts et al., 2013). Alternatively it cannot be excluded that the impact of fluoxetine on viral replication may involve cellular antiviral pathways through binding to a receptor, in agreement with the effect of fluoxetine on the central nervous system, where it is thought to engage various G protein-coupled neurotransmitter receptors (Beasley et al., 1992). Interestingly, it was reported that fluoxetine can affect the expression of many cellular genes or miRNA, the impact of which onto viral replication should be investigated further (Lauterbach, 2012; Millan, 2011).

In conclusion, fluoxetine inhibits Coxsackievirus B4 replication and can cure a persistent infection of pancreatic cells with this virus. Beyond the antiviral activity of this molecule, an improved knowledge of its impact onto cellular pathways should help for a better understanding of interactions between cell and enterovirus.

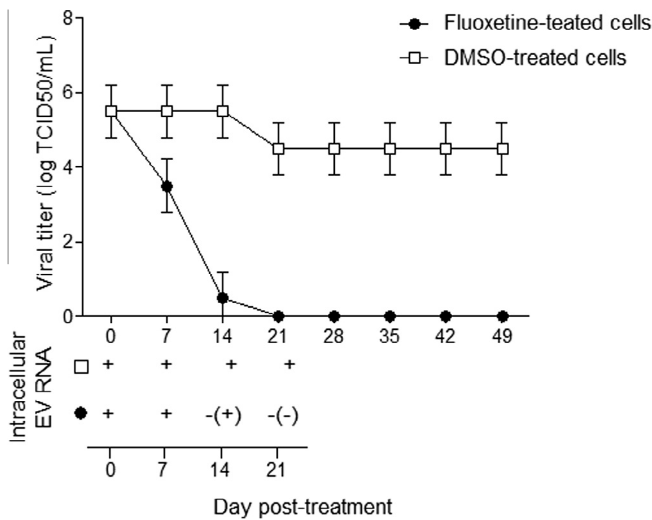

Fig. 3. Fluoxetine can clear CVB4 in chronically-infected Panc-1 cell cultures. Panc-1 cells chronically infected with CVB4 were treated with fluoxetine or with DMSO. The medium was changed every 3–4 days, and the same treatment was performed. Cells were scraped and subcultured once a week. The culture supernatants and cells were collected at various time intervals after treatment initiation. Viral titers in supernatants were determined. Results are mean ± SD of two independent experiments. Intracellular enteroviral RNA (EV RNA) was detected by RT-PCR and by semi-nested PCR when appropriate (results between brackets). The results are representative of two independent experiments.

Acknowledgments

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ETUDE N°5 : La persistance de coxsackievirus B4 dans les cellules canalaires du pancréas s'accompagne de modifications cellulaires et virales.

Travail soumis pour publication

Alidjinou EK, Engelmann I, Bossu J, Villenet C, Figeac M, Sané F, Hober D. Persistence of *Coxsackievirus B4* in pancreatic ductal-like cells results in cellular and viral changes. **Manuscript submitted**

Résumé

Objectif

Les coxackievirus du groupe (CVB) sont généralement considérés comme des virus cytolytiques; cependant ils sont capables d'établir des infections persistantes in vitro et in vivo. La persistence virale a été proposée comme l'un des mécanismes majeurs de la pathogenèse des maladies chroniques liées aux CVB comme le diabète du type 1. Le but de ce travail est d'étudier l'impact de la persistence de CVB4 sur des cellules canalaires pancréatiques.

Méthodes

Une infection persistante à CVB4 a été établie dans une lignée de cellules ductales pancréatiques. L'expression de PDX-1, la résistance à la lyse induite par CVB4 et l'expression de CAR ont été évaluées. Le profil des microARNs (miARNs) cellulaires a été étudié par séquençage. Les changements phénotypiques du virus ont été étudiés et les modifications génomiques ont été évaluées grâce à un séquençage à haut débit du génome viral entier.

Principaux résultats

L'infection persistante à CVB4 dans les cellules canalaires était productive, avec libération continue de particules virales infectieuses. L'expression de PDX-1 était réduite dans les cellules chroniquement infectées par CVB4. De même, ces cellules présentaient une résistance à la lyse cellulaire induite par une surinfection à CVB4, et l'expression de CAR était fortement réduite. Ces changements étaient maintenus, même après l'élimination du virus. Les profils des miARNs cellulaires dans les cellules chroniquement infectées et dans les cellules contrôles étaient complètement différents. Le virus dérivé de l'infection persistante était encore capable d'induire un effet cytopathogène, mais les plages de lyse étaient plus petites que celles induites par le virus initial. Plusieurs mutations sont apparues dans le génome viral au cours de la persistance, mais les substitutions d'acides aminés n'ont pas affecté les sites d'interaction avec CAR.

Conclusion

Des changements cellulaires et viraux surviennent au cours de l'infection persistante à CVB4 dans les cellules canalaires pancréatiques. La persistance des changements cellulaires même après l'élimination du virus est compatible avec l'hypothèse d'un impact durable de l'infection chronique à CVB4 sur les cellules.

1 **Persistence of Coxsackievirus B4 in pancreatic ductal-like cells results in**
2 **cellular and viral changes**

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10 **Running title**: Coxsackievirus B4 persistence in pancreatic cells.

11

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Abstract

Introduction

Although known as cytolytic viruses, group B coxackieviruses (CVB) are able to establish a persistent infection in vitro and in vivo. Viral persistence has been reported as a key mechanism in the pathogenesis of CVB-associated chronic diseases such as type 1 diabetes (T1D). The impact of CVB4 persistence on human pancreas ductal-like cells was investigated.

Methods

A persistent CVB4 infection was established in ductal-like cells. PDX-1 expression, resistance to CVB4-induced lysis and CAR expression were evaluated. The profile of cellular microRNAs (miRNAs) was investigated through miRNA-sequencing. Viral phenotypic changes were examined, and genomic modifications were assessed by sequencing of the viral genome.

Results

The CVB4 persistence in ductal-like cells was productive, with continuous release of infectious particles. Persistently infected cells displayed a resistance to CVB4-induced lysis upon superinfection and expression of PDX-1 and CAR was decreased. These changes were maintained even after virus clearance. The patterns of cellular miRNA expression in mock-infected and in CVB4-persistently infected ductal-like cells were clearly different. The persistent infection-derived virus (PIDV) was still able to induce cytopathic effect but its plaques were smaller than the parental virus. Several mutations appeared in various PIDV genome regions, but amino acid substitutions did not affect the predicted site of interaction with CAR.

Conclusion

Cellular and viral changes occur during persistent infection of human pancreas ductal-like cells with CVB4. The persistence of cellular changes even after virus clearance supports the hypothesis of a deep impact of persistent CVB infection on the cells.

Keywords: Coxsackievirus B4, persistence, pancreatic cells, PDX-1, CAR, miRNA

56 ## Introduction

57 Type B coxsackieviruses (CVB) are small non-envelopped RNA viruses that belong to the
58 *Picornaviridae* family and the *Enterovirus* genus. This highly diverse genus currently includes
59 7 species that are involved in human diseases, and CVB (1-6) are classified in the
60 Enterovirus-B species (Knowles et al., 2012; Tapparel et al., 2013). CVB can cause severe
61 acute clinical features such as meningitis, encephalitis, myocarditis, pancreatitis, hepatitis or
62 fulminant sepsis in newborns (Romero, 2008; Tapparel et al., 2013) but these viruses are
63 also reported to play a role in the development of chronic diseases like dilated
64 cardiomyopathy or type 1 diabetes (T1D) (Hober and Alidjinou, 2013; Hober and Sauter,
65 2010).

66 In T1D patients, the infection of pancreatic cells with enteroviruses, especially CVB, has
67 been reported (Dotta et al., 2007; Richardson et al., 2009; Willcox et al., 2011; Ylipaasto et
68 al., 2004). The pancreas is well known as one of the major targets of CVB. This tropism is
69 supported by the expression of CAR, the specific receptor of CVB in the pancreas (Oikarinen
70 et al., 2008; Spagnuolo et al., 2013). In humans, pancreatic islets and especially beta cells,
71 but not exocrine cells, were found to be susceptible to enteroviral infection (Dotta et al.,
72 2007; Richardson et al., 2009, 2013).

73 CVB can effectively induce lytic infection in pancreatic cells (Anagandula et al., 2014;
74 Elshebani et al., 2007; Hodik et al., 2013). However this scenario of acute massive infection
75 is not compatible with T1D which is an autoimmune disease with a progressive evolution,
76 and the implication of the virus is believed to rely on the immune response (Hober and
77 Sauter, 2010). Therefore, the most likely situation would be a persistent CVB infection
78 probably with low grade viral replication. Indeed, CVB are able to establish persistent
79 infection *in vitro* as well as *in vivo* (Pinkert et al., 2011). Viral persistence has been
80 suggested as a major mechanism in the enteroviral pathogenesis of CVB-related chronic
81 diseases such as T1D (Jaïdane and Hober, 2008; Jaïdane et al., 2010).

82 Previous in vitro studies confirmed that CVB can persist in pancreatic islets. The persistent
83 infection of beta cells resulted in the production of significant levels of IFNα (Chehadeh et

84 al., 2000), and in a disturbance in the function of these cells (Yin et al., 2002). The
85 persistence of CVB4 in human pancreatic ductal cells resulted in an impaired formation and
86 viability of islet-like cell aggregates (Sane et al., 2013).
87 Two main types of persistent viral infections have been described in vitro: steady-state
88 infections and carrier-state infections. In steady-state infections, almost all the cells are
89 infected with no lytic replication cycle, whereas in carrier-state culture systems, only a small
90 proportion of cells are involved with a significant virus replication (Frisk, 2001; Pinkert et al.,
91 2011). Data available on CVB persistence in vitro are compatible with a carrier-state
92 persistent infection (Heim et al., 1992, 1995; Pinkert et al., 2011).
93 Virus persistence is thought to result from a coevolution between the host cell and the virus,
94 and this supposes changes in some of their characteristics (Pinkert et al., 2011)
95 Viral factors during CVB persistence have been studied in several reports. These factors
96 include genomic changes such as mutations with amino acid substitutions (Schmidtke et al.,
97 2000), a deletion in the 5' non coding region (Chapman et al., 2008; Kim et al., 2005, 2008;
98 Tracy et al., 2015), or the persistence as a stable and atypical double-stranded RNA
99 genomic form (Tam and Messner, 1999). Among host factors, the role of a reduced
100 expression of receptors (Fechner et al., 2007; Pinkert et al., 2011; Werk et al., 2005) and the
101 cellular activation status have been reported (Feuer and Whitton, 2008; Feuer et al., 2002,
102 2004). Other cellular factors such as miRNAs could also play a role in viral persistence
103 (Grey, 2015). MicroRNAs are a recently discovered class of small non coding RNAs that act
104 via endogenous RNA interference (Bartel, 2009; Hammond, 2015). An association between
105 miRNAs and non-lytic herpesvirus and hepatitis C virus infection has been reported (El-
106 Diwany et al., 2015; Kim et al., 2016a). Recent reports support the role of miRNAs in
107 enterovirus pathogenesis (Ho et al., 2016).
108 An improved understanding of the impact of viral persistence on the host cell and the virus
109 could provide new insights into the enteroviral pathogenesis of T1D.
110 In this study, we investigated the changes in host cell and virus during persistent CVB4
111 infection in a model of human pancreatic ductal-like cells.

112 ## Material and methods

113 ### *Cells and virus*

114 The human ductal cell line Panc-1 (ATCC) was cultured in Dulbecco's modified Eagle's
115 medium supplemented with 10% of heat inactivated fetal calf serum (FCS), 1% of L-
116 glutamine and 1% of penicillin and streptomycin. Hep-2 cells (BioWhittaker) were grown in
117 minimum essential medium supplemented with 10% of FCS, 1% of L-glutamine, 1% of non-
118 essential amino acids and 1% of penicillin and streptomycin. Hep-2 cells were used for the
119 production and titration of the virus.

120 The diabetogenic CVB4 E2 strain kindly provided by Ji-Won Yoon (Julia McFarlane Diabetes
121 Research Center, Calgary, Alberta, Canada) was propagated in Hep-2 cells. Briefly, after
122 three freeze-thaw cycles, the suspension was collected and clarified at 2000 g for 10 min at
123 4°C. Aliquots of virus preparations were stored at - 80°C.

124 ### *Persistent CVB4 infection in Panc-1 cells*

125 The persistent infection in Panc-1 cells was established as previously described (Sane et al.,
126 2013) with slight modifications. Briefly, a 25 cm^2 Nunc® cell culture flask (Thermofisher
127 Scientific, Villebon, France) containing an average of 10^6 cells was inoculated with CVB4E2
128 at a multiple of infection (MOI) of 0.01. During the acute lytic infection, the virus was regularly
129 washed out and finally a stable equilibrium was found between viral replication and cell
130 proliferation. The cells were scraped and subcultured once a week. Supernatants and cells
131 were periodically collected for analysis. CVB4 and mock-infected cells were processed
132 identically. Persistently infected cells can be frozen and stored in liquid nitrogen, with
133 maintenance of infection after thawing.

134 ### *Viral progeny in supernatants*

135 The viral titer in supernatants of infected cells was assessed using the end-point dilution
136 assay, and the Spearman-Karber statistical method was used to determine the tissue culture
137 50% infectious dose ($TCID_{50}$).

138

139

140 ***Virus plaque assay***

141 Hep-2 cells were cultured in a six-well culture plate as confluent monolayers at a density of
142 10^6 cells/well. After 24 h, medium was removed and cells were washed once with PBS and
143 overlaid with 3 mL of dilutions of the virus suspension, and then incubated at 37°C for 2 h.
144 After removal of the supernatant, cells were overlaid with 3 ml of agar containing Eagle's
145 minimal essential medium (MEM).Three days later, the cells were fixed with 10% PFA for 2
146 h. After removal of PFA and agar, cells were stained with 1% cristal violet for 30 minutes.
147 After washing and drying the plaques were observed and counted.

148 ***Enterovirus RNA quantification***

149 Total RNA was extracted from Panc-1 cells using TriReagent® RNA isolation reagent/
150 Chloroform procedure (Sigma). Extracted RNA was then dissolved in 50 µL of nuclease free
151 water, quantified with a Nanodrop® spectrophotometer (Thermofisher Scientific).

152 The Affinityscript® QPCR cDNA Synthesis kit (Agilent technologies, Les Ulis, France) was
153 used for the ARN retrotranscription step on a Perkin Elmer 2400 thermocycler (Villebon-sur-
154 Yvette, France). Quantitative real-time PCR for cDNA amplification was performed with the
155 Brilliant® II QPCR kit (Agilent technologies) on the Mx3000p® instrument (Agilent
156 technologies). *Enterovirus* 71 RNA (Vircell, Granada, Spain) was used as standard for
157 quantification. The oligonucleotides and the reaction conditions were previously described
158 (Alidjinou et al., 2013).

159 ***Cell viability assay***

160 The cell viability was assessed using the crystal violet assay. Cells seeded in a 96-well plate
161 were washed once with PBS and incubated with 1x crystal violet at 37°C for 20 minutes.
162 Then, crystal violet was removed and cells layers were extensively washed in tap water by
163 immersion in a large beaker. The dissolution of crystal violet remaining inside cells was
164 performed by incubation with 1% SDS at 37°C for 15 minutes. Upon solubilization, the
165 absorbance in each well was read at 595 nm.

166

167

168 ***Fluoxetine treatment***

169 The treatment of persistently infected cells was carried out as previously described (Alidjinou
170 et al., 2015). Briefly Panc-1 cells persistently infected with CVB4 were treated twice a week
171 with fluoxetine at 5.5µM, and virus was completely cleared within 3 weeks.

172 ***PDX-1 and CAR gene expression***

173 RNA extracts were treated with DNAse I (Qiagen, Les Ulis, France). The quantification of
174 PDX-1 mRNA and GAPDH mRNA (house-keeping gene) was carried out by using the
175 Taqman gene expression assays (hs00236830 and hs03929097, respectively) (Thermofisher
176 Scientific). The cDNA synthesis was performed with the High-capacity cDNA reverse
177 transcription kit (Thermofisher Scientific), and the Taqman Universal Master Mix II, no UNG
178 (Thermofisher Scientific) was used for real-time qPCR according to the manufacturer's
179 instructions. CAR mRNA was quantified by real-time RT-qPCR by using the SuperScript III
180 Platinum One-Step Quantitative RT-PCR System (Thermofisher Scientific) and
181 oligonucleotides described by Lam and colleagues (Lam et al., 2015). The quantification was
182 carried out on the Mx3000p® instrument (Agilent technologies) with the following program:
183 50°C for 15 minutes, 95°C for 2 minutes, and 40 cycles of amplification consisting of 15
184 seconds at 95°C and 30 seconds at 60°C. The expression of the beta-actin mRNA was used
185 for normalization.

186 PDX-1 and CAR mRNA relative expression in infected versus noninfected cells was
187 determined with the $2^{-\Delta\Delta Ct}$ formula (Livak and Schmittgen, 2001).

188 ***Flow cytometry***

189 Anti-human CAR mouse IgG1 monoclonal antibody (clone RmcB) and negative control
190 mouse IgG1 monoclonal antibody were purchased from Merck Millipore (Molsheim, France)
191 and used at the supplier's recommended concentrations. Both antibodies were phycoerythrin
192 (PE) – conjugated. Panc-1 cells were detached by trypsinization, washed once in PBS and
193 suspended in FACS Buffer (PBS, 1% BSA). Cell suspensions were incubated at 4°C for 30
194 min with the appropriate antibodies, and washed three times with FACS Buffer. The cells

195 were then fixed in 4% paraformaldehyde, washed once with PBS, and analyzed by flow
196 cytometry on a Navios Flow cytometer (Beckman Coulter, Inc).

197 ***Sequencing of microRNAs***

198 Cells were collected from four replicates of CVB4 and mock persistent infection, and washed
199 with PBS. RNA extraction was performed using the miRNeasy mini kit (Qiagen) with a step of
200 on-column DNA digestion using DNase I (Qiagen). Extracted RNA was quantified with a
201 Nanodrop® spectrophotometer (Thermofisher) and the quality was assessed using the 2100
202 Bioanalyzer (Agilent technologies).

203 Small RNA librairies were prepared from 1µg of total RNA using the Ion Total RNA-Seq Kit
204 v2.0 (Life Technologies, Carlsbad, CA, USA). Barcoded libraries were quantified and
205 assessed for quality using the Agilent 2100 BioAnalyzer (Agilent Technologies). Libraries
206 were pooled in equimolar amounts and sequenced on a Ion PROTON™ Platform using a Ion
207 P1™ Chip Kit v2 and the Ion P1™ Sequencing 200 kit v3 (Life Technologies).

208 Primary analysis transforming signal to DNA sequences was done with the default
209 parameters on a Torrent Server 4.0.2 (Life Technologies). Demultiplexing was done with 0
210 errors allowed in barcodes. Raw reads were analyzed with ncProSeq 1.5.1 (Chen et al.,
211 2012). For each sample, reproducibility of counts within the two barcodes was investigated
212 by Spearman correlation. Each pair of barcodes were then pooled and a new ncProSeq
213 analysis was performed on all samples. The differential Expression of the raw counts
214 obtained from ncProSeq was performed with DESeq2 (Love et al., 2014).

215 ***Quantification of miRNAs by RT-PCR***

216 The expression of miRNAs was quantified using the TaqMan® MicroRNA kits (Thermofisher
217 Scientific). The Taqman MicroRNA Reverse Transcription Kit was used for cDNA synthesis,
218 and the real-time qPCR was performed with the Taqman Small RNA assay (primers and
219 probe) and the Taqman Universal PCR Master Mix II according to the manufacturer
220 recommendations, on the Mx3000p® thermocycler (Agilent technologies). RNU6B was used
221 for normalization and the relative expression was determined using the $2^{-\Delta\Delta Ct}$ formula (Livak
222 and Schmittgen, 2001).

223 ***Transfection of miRNA mimics/inhibitors***

224 Panc-1 cells were seeded at $0.5x10^5$ cells in 0.5 mL complete medium per well in a 24-well
225 plate at 70% confluence. After 24 h, the cells were transfected with specific mirVana™
226 miRNA mimic, miRNA inhibitor or negative controls (Thermofisher Scientific), by using
227 Lipofectamine® RNAiMAX Transfection Reagent (Thermofisher Scientific). After 24h, the
228 transfected or non-transfected cells were then inoculated with CVB4 at MOI 0.01. The virus
229 was removed after 2h by washing three times and fresh medium was added. The plate was
230 incubated at 37°C, 5% CO2.

231 ***CVB4E2 genome sequencing***

232 Full genome sequencing was performed on virus suspensions resulting from acute and
233 persistent CVB4E2 infection. 2 mL of virus suspension was concentrated 10-fold by using a
234 Vivaspin 6 device (Sartorius, Dourdan, France) with a membrane molecular weight cut-off at
235 1,000,000 Dalton. The resulting 200µL were RNAse treated and then used for automated
236 RNA extraction on the Magtration System 12GC with the MagDEA Viral DNA/RNA 200
237 reagents (Precision System Science Co, Ltd, Japan), with a DNAse treatment step. cDNA
238 synthesis was performed with the SuperScript III first-strand synthesis system (Invitrogen)
239 and random hexamer primers.The synthesized cDNA was amplified by Multiple
240 Displacement Amplification technology (MDA) following the protocol of the Quantitect Whole
241 transcriptome kit (Qiagen). The amplified cDNA was used to prepare barcoded librairies by
242 using the Ion Xpress Plus Fragment library kit (Thermofisher). After quality assessement and
243 quantification on the Agilent 2100 BioAnalyzer (Agilent), librairies were pooled in equimolar
244 amounts and sequenced on a Ion PGM™ Platform using a Ion 318™ Chip Kit v2 and the Ion
245 PGM™ HiQ sequencing kit (Life Technologies).

246 The raw sequences were obtained from the intensities on the Torrent-Server software
247 version 5.0.4 (Life Technologies) with no trimming and default calibration. Demultiplexing
248 was performed by allowing one error by index. Alignment on CVB4E2 was made with the
249 tmap tool with default settings for Torrent Server 5.0.4. The detection of variants was carried
250 out with the TSVC tool from Torrent 5.0.4 Server with parameters "Somatic-Low-stringency".

Statistical analysis

Data are presented as the mean ± the standard deviation. Comparisons were performed using Student t or Mann Whitney U tests, as appropriate. $P < 0.05$ was considered statistically significant. All analyses were performed with Graph Pad Prism 6.03 software (GraphPad Software, La Jolla, CA).

Results

Persistent CVB4 infection in ductal-like cells

CVB4 infection in Panc-1 cells usually results in acute lysis with cytopathic effect (CPE) observable within a few days, depending of the infection dose (figure 1a). When the acute infection step was controlled namely by removing excess virus, cell proliferation gradually resumed and was maintained in the presence of the virus. After a few weeks of subculture (an average of 5 weeks), the morphology of infected cells was quite similar to that of uninfected cells (See figure 1a). The persistent infection was productive, and infectious particles were continuously released in the supernatants with a mean viral titer between 3 and 7 logs $TCID_{50}$/mL (figure 1b). The mean amount of intracellular viral RNA was constant between 5 and 6 logs copies/ng of total RNA throughout the follow-up, as shown in figure 1c.

In the model of ductal-like cells persistently infected with CVB4 the impact of the infection on the cells was investigated. The expression of PDX-1 mRNA was reported to decline in CVB4 persistently infected cells (Sane et al., 2013). This observation was confirmed in the current study by using real-time RT-qPCR. The expression of PDX-1 at 20 weeks of chronic infection was around 14%, as compared to mock-infected cells (Figure 1d).

Resistance of CVB4 persistently infected cells to lysis upon superinfection

The impact of superinfection on persistently infected cells was investigated. CVB4 and mock-infected cells were infected with CVB4 (from the same stock that was used to establish persistent infection) at a MOI of 10. On day 2 post infection, cell viability remained unchanged in persistently infected cells, while a dramatic decrease was observed in mock-infected cells (Figure 2a). The level of infectious particles in supernatants collected at 24h

279 and 72h post infection was determined. The virus replication was highly effective in mock
280 persistently-infected cells with a viral titer reaching 10^{10} $TCID_{50}$/mL at 72h post infection. In
281 contrast, viral titers before and after superinfection were similar in CVB4 persistently-infected
282 cells, as shown in figure 2b. At 72h post infection, cells were harvested, washed and the
283 level of intracellular viral RNA was evaluated by real-time RT-qPCR. Upon infection,
284 intracellular viral RNA in mock persistently infected cells was around 6 log copies/ng of total
285 RNA, compatible with an acute infection. The intracellular viral load remained unchanged
286 after superinfection in CVB4 persistently infected cells (figure 2c). These observations
287 suggest that persistent infection protected cells from superinfection. We reasoned that this
288 might be due to reduced expression of CAR, the main receptor used by CVB4. The level of
289 CAR mRNA in persistently-infected cells gradually declined in infected cells and reached less
290 than 3% at 22 weeks post-infection, compared to mock infected cells (Figure 3).

291 ***Persistent reduced expression of PDX-1 and CAR in CVB4 persistently infected ductal-***
292 ***like cells after viral clearance***

293 We hypothesized that changes induced in persistently-infected cells could persist after viral
294 clearance. To test this hypothesis, persistent infection was completely cured by fluoxetine, as
295 previously reported (Alidjinou et al., 2015). One year after the end of treatment we found that
296 PDX-1 expression was still significantly reduced in persistently infected cells that had been
297 cured by fluoxetine (18.2% and 17.8% respectively in untreated and cured cells, compared to
298 mock-infected cells) (figure 4a). In addition, CVB4 superinfection studies were carried out in
299 CVB4 persistently infected cells that were cured by fluoxetine, one year after the end of
300 treatment. There was no cell lysis in cured cells (figure 4b); nevertheless the cells were
301 readily infected, as supported by quantity of viral progeny in supernatants (figure 4c), and of
302 intracellular viral RNA (figure 4d). CAR expression was evaluated in cell cultures cured with
303 fluoxetine. CAR mRNA levels increased significantly in cured cells compared to persistently
304 infected cells (65 % versus 3.8% of the expression in uninfected cells, $p < 0.0001$) (figure
305 4e). However, the membrane expression of CAR on infected and cured cells remained
306 similar (figure 4f).

307 ***Pattern of cellular miRNAs in ductal-like cells persistently infected with CV-B4***

308 The expression or miRNAs in CVB4 and mock persistently infected cells was evaluated
309 through miRNA sequencing. The miRNA profile was clearly different in persistently infected
310 cells, as compared to uninfected cells (Figure 5a). A total of 81 miRNAs displayed a
311 significant differential expression (fold change ≥ 3, $p< 0.05$) including 65 upregulated and 16
312 down-regulated miRNAs (Figure 5b). The list of differentially expressed miRNAs is shown in
313 Table 1. The expression pattern determined by miRNA sequencing was confirmed by
314 taqman RT-PCR for few miRNAs : miR-10a, miR-23a, miR-23b, miR-125, and miR-146 that
315 were described in the literature to be related to enteroviral infection (Ho et al., 2011; Tong et
316 al., 2013; Wen et al., 2013; Zhang et al., 2013) (Figure 5c-d). The levels of miR-146a-5p and
317 miR-23b in ductal-like cells acutely infected with CV-B4 were also determined. These
318 miRNAs were up-regulated and down-regulated respectively in ductal-like cells persistently
319 infected with CVB4 whereas their levels in cells acutely infected harvested on day 2 p.i were
320 unchanged compared to mock-infected cells (see Figure 5e).

321 ***Impact of miRNA mimic transfection on virus replication and CAR mRNA expression***
322 ***in Panc-1 cells***

323 Three miRNAs were selected for further study, including miR-146a-5p, miR-23b, and miR-
324 138-5p that was predicted to target the 3' non coding region of CVB4-E2 using RegRNA 2.0
325 software (with default settings). Mimics of these three miRNAs were transfected in Panc-1
326 cells at 20nM, and 24h later the cultures were inoculated with CVB4 at a MOI of 0.01. The
327 transfection was efficient as suggested by miRNA quantification (Figure 6a-c). The viral titers
328 of supernatants of transfected and non-transfected cell cultures collected at 48h post
329 infection were similar (Figure 6d). There was no difference in the levels of infectious particles
330 collected 6h and 24h post infection (data not shown). Likewise, no difference was observed
331 in intracellular viral RNA levels (Figure 6e). Therefore, overexpression of these miRNAs had
332 no impact on viral replication.

333 MiR-146a-5p was predicted to target CAR gene using miRWalk 2.0 software (with default
334 settings). MiR-146a-5p mimic was transfected at 50nM in Panc-1 cells that were inoculated

335 with CVB at MOI 0.01, 24h after transfection. The transfection of miR-146a mimic increased
336 intracellular miR-146a expression (figure 6f). CAR mRNA expression was similar in cells
337 transfected with miR-146 mimic and mock-transfected cells (figure 6g).

338 ***Characterization of persistent infection-derived CVB4***

339 The persistent infection of ductal-like cells cultures resulted in cellular modifications. We
340 wondered whether changes affecting the virus also occurred during the persistent infection.
341 Similarly to the parental virus, the persistent infection-derived virus (PIDV) can induce
342 cytopathic effect (CPE) in several cell lines such as Hep-2, Panc-1 or RD cells (data not
343 shown). The aspect of plaques induced on Hep-2 cells by the PIDV was compared to the
344 parental virus. The virus emerging from persistent infection induced only tiny plaques on
345 Hep-2 cells, while the parental virus was able to produce larger size plaques (figure 7a). The
346 levels of infectious particles and viral RNA of PIDV and parental virus suspension were
347 determined. The proportion of infectious particles, as evaluated by the ratio between viral
348 titer and the viral RNA load, was lower in PIDV compared with parental virus ($p= 0.01$) (figure
349 7b).

350 Thereafter, whole viral genome deep sequencing was performed on both parental virus and
351 PIDV, using two independent acute and persistent infections. A total of 105 cumulated
352 mutations were observed in various regions of PIDV (as compared to parental virus), with 40
353 amino-acid substitutions.VP1 and 2A proteins were more commonly affected, with 9 and 11
354 amino-acid substitutions respectively. Only one silent mutation (A1854G) was shared by
355 viruses derived from two independent cultures of ductal-like cells persistently infected with
356 CVB4 E2. The number of mutations and amino acid substitutions in each region is presented
357 in table 2.

358 Recently it was reported that amino acid residues highly conserved among type B
359 coxsackieviruses located in the north rim of the canyon (VP1) and in the puff region (VP2),
360 represent the footprints of CVB3 on CAR (Organtini, 2014).

361 To infestigate whether changes had occurred in these regions, mutations in PIDV with a
362 frequency ≥ 20% were integrated in silico in the nucleotide sequence of the CVB4 E2

363 reference strain. Amino acid sequences were obtained by using blastx (NCBI). VP1 and VP2
364 sequences were aligned with sequences corresponding to CVB3/28 and CVB3/Nancy
365 strains, using Uniprot software. Figure 8 shows that the footprints of CVB4 E2 on CAR were
366 conserved in PIDV.
367

368 **Discussion**

369 The persistence of cytolytic viruses such as enteroviruses may be viewed as the result of
370 host immune pressure, or as a pathogenesis mechanism. Group B coxsackieviruses
371 associated chronic diseases such as T1D have been linked at least partially to virus
372 persistence. In T1D, primary target cells are beta cells within the pancreatic islets, but
373 evidence of virus detection in others cell populations such as pancreatic ductal cells, has
374 also been reported (Ylipaasto et al., 2004). The role of pancreatic ductal cells in the
375 homeostasis of pancreas is important since they are thought to contribute to the
376 replenishment of beta cells through transdifferentiation (Lysy et al., 2013).
377 Interestingly, CVB4 persistence in pancreatic ductal cells was previously reported to induce a
378 decrease of PDX1, also known as insulin promoter factor 1, which is a transcription
379 factor necessary for pancreatic development, including β-cell maturation, and duodenal
380 differentiation (Sane et al., 2013). In this study we confirmed this finding using real-time RT-
381 qPCR. Moreover we have observed several changes in ductal-like cells persistently infected
382 with CVB4.
383 The persistently infected cells displayed a resistance to lysis when exposed to a
384 superinfection with CVB4. In addition, viral progeny and intracellular RNA remained
385 unchanged after superinfection, suggesting a lack of entry or a limited entry of the
386 challenging virus in these cells. Reduced virus entry could be explained by a decrease of cell
387 susceptibility, as supported by the significant decrease of the CAR molecule in cells during
388 CVB4 persistent infection. Our results are in agreement with previous reports. Indeed,
389 resistance to lysis associated with a decrease of CAR expression was found during CVB3
390 persistence in a murine cardiac myocyte cell line (Pinkert et al., 2011). CAR is the main

391 receptor for CVB and mediates the attachment of the virus and its internalization in the target
392 cells (Bergelson et al., 1997; Selinka et al., 2004). It is assumed that cells lacking or with low
393 level of CAR expression are protected from CVB infection and cell lysis (Fechner et al.,
394 2000). Indeed, CAR knockdown experiments have shown a decrease of CVB3 replication,
395 and virus-induced cell lysis (Fechner et al., 2007; Werk et al., 2005).
396 The persistently infected cells were completely cured by using fluoxetine, and then
397 subcultured for one year. The expression of PDX-1 was still reduced in cured cells at similar
398 levels than in untreated cells. In addition, fluoxetine cured cells conserved the resistance to
399 CVB4-induced lysis observed in persistently infected cell cultures. A similar observation was
400 reported in CVB3 chronically-infected murine myocytes that were cured using soluble CAR
401 (Pinkert et al., 2011). The sustained cellular changes after virus clearance suggest that the
402 involvement of CVB in chronic diseases may depend on the impact of the virus onto cells,
403 which lingers after viral clearance. Furthermore, the resistance of cured cells was observed
404 in the presence of virus entry and replication in these cells, indicating clearly that this
405 phenotype was not due to the absence of virus entry and replication in the cells.
406 It is noteworthy that the level of CAR mRNA increased significantly in cured cells whereas
407 membrane expression did not change. Indeed CAR was barely detectable at the membrane
408 of cured or untreated CVB4 persistently infected cells. This discrepancy between mRNA and
409 membrane expression may be explained by the fact that flow cytometry evaluated CAR
410 membrane isoforms only. Indeed, the CXADR gene coding for CAR gives rise to five splice
411 variants with two membrane isoforms and three soluble isoforms (Dörner et al., 2004;
412 Thoelen et al., 2001). It cannot be excluded that the expression of CAR soluble isoforms, but
413 not of membrane isoforms, increased in cured cells in our experiments. Such a dissociation
414 between the expression of soluble and membrane isoforms has been already observed in
415 epithelial ovary cancer cells (Reimer et al., 2007).
416 Altogether, observations in cured and untreated persistently -infected cells suggest that the
417 resistant phenotype was associated with a decrease in membrane CAR expression; however

418 cured cells remained significantly susceptible to CVB4 infection. This resistance phenotype
419 probably involves many other cellular changes and deserves further investigation.
420 The persistence of CVBs *in vitro* was reported to be a “carrier state” persistence in which the
421 proportion of infected cells ranges from about 10% to 60% depending on virus and host cell
422 (Heim et al., 1995; Matteucci et al., 1985; Pinkert et al., 2011). Indeed in our model of ductal-
423 like cells persistently infected with CVB4, it was reported that at least 50% of cells harbored
424 enteroviral RNA (Sane et al., 2013). Such an extent of the infection can explain the
425 magnitude of changes observed in this study.
426 To the best of our knowledge, this is the first observation of an impact of enteroviral
427 persistence onto the cellular miRNAs.
428 In this study, the patterns of cellular miRNAs in mock-infected and in CVB4- persistently
429 infected ductal-like cells were clearly different. The technical approach is noteworthy. Indeed
430 miRNA sequencing was carried out instead of analyzing a predefined list of miRNAs. This
431 method yielded a high number of miRNAs with significant differential expression (65 up-
432 regulated and 16 down-regulated). In addition, our results suggest that miRNA expression
433 pattern is different between CVB4 acute and persistent infection. Recently Kim et al.
434 investigated the expression of cellular miRNAs in human pancreatic islets acutely infected
435 with CVB5. The authors reported on day 7 post infection, a modified expression for 33
436 miRNAs (including six up-regulated and 27 down-regulated) out of 754 analyzed miRNAs
437 (Kim et al., 2016b). MiR-663b was the only one of our list that was reported in their study.
438 The specific pattern of miRNA expression in persistentl infection with CVB deserves further
439 studies to evaluate whether miRNAs may be used as markers in patients with diseases
440 possibly related to persistent infections. In the same vein, it has been recently reported that
441 miRNAs expressed during acute infection can predict persistence. Indeed, Kuehl et al. found
442 in patients with CVB3 cardiomyopathy, a distinctive baseline cardiac miRNA profile between
443 patients who spontaneously eliminate the virus, and those with virus persistence and
444 progression to heart failure (Kuehl et al., 2015).

445 We have investigated the impact on CVB4 replication of 3 miRNAs (miR-138, miR-23b and
446 miR-146a-5p) potentially playing a role in enterovirus infection. The transfection of miRNAs
447 mimics in ductal-like cells did not induce any change in viral titers determined in supernatants
448 and in intracellular enteroviral RNA levels. Furthermore, miR-146a was predicted to
449 potentially target CAR, and its impact on CAR expression was evaluated. However, in cells
450 transfected with miR-146a mimic (infected with CVB4 or not) the expression of CAR was not
451 different compared to non-transfected cells.
452 In our experiments, the transfection of miRNAs mimics led to a few hundred-fold increase in
453 miRNA levels; however, it was reported that functional miRNA levels only represent a small
454 proportion of the supraphysiological miRNA levels quantified using RT qPCR after
455 transfection with mimics (Thomson et al., 2013). In addition, it cannot be excluded that the
456 selected miRNAs have other effects than those evaluated in this study. Indeed, during
457 enteroviral infections, cellular miRNAs can directly target enterovirus genome to promote or
458 to suppress viral replication (Tong et al., 2013; Zheng et al., 2013). They can also promote
459 antiviral responses or at the opposite participate in the escape to the immune response (Ho
460 et al., 2016).
461 MiR-23b was reported to reduce EV-71 replication (less than 2 log TCID50/mL) displayed by
462 downregulation of VP1 protein in Vero cells (Wen et al., 2013), but this impact cannot be
463 extended to other systems since the replication of CV-B4 in human ductal-like cells was not
464 modified in our experiments. EV71 infection was also shown to upregulate miR-146a, which
465 on one side targets IRAK1 and TRAF6 involved in TLR signaling and type I interferon
466 production (Ho et al., 2014), and on the other side is involved in EV 71-induced cell
467 apoptosis through targeting SOS1 and GADD45β (Chang et al., 2015). MiR-146a had no
468 effect onto CAR in our studies but other miRNAs may have an impact. Indeed it was reported
469 that miR-466 down-regulated the expression of the molecule CAR in a rat beta cell line
470 infected with CVB4 (Lam et al., 2015).
471 The decrease of CAR expression during chronic infection may result from the combined
472 effect of several miRNAs. Alternatively the impact of a miRNA on CAR may only occur after a

473 long period. We were not able to evaluate a long-term impact of miR-146a in our study,
474 because the cells did not survive after multiple miR-146a mimic transfections (data not
475 shown). Further investigation based on transgenic expression, plasmid transfection or
476 lentiviral transduction is needed to evaluate the impact of miRNAs in long-term cultures (Jin
477 et al., 2015).
478 It is well admitted that persistence results from adaptations of both host cell and virus (Frisk,
479 2001). We therefore analyzed some features of the virus emerging from persistent infection,
480 compared to the parental strain. Both viruses were able to induce a cytopathic effect on
481 several cell lines. However the plaques induced on Hep-2 cells by the persistent infection
482 derived virus (PIDV) were smaller than those induced by the parental strain. This phenotypic
483 change was previously observed during CVB3 persistence in murine cardiac myocyte cell
484 line (Pinkert et al., 2011).
485 In addition, the proportion of infectious particles compared to the levels of viral RNA was
486 lower in PIDV, than in parental strain, which may be explained by genomic changes resulting
487 in an impaired replication of the virus.
488 We report in this study for the first time, a whole genome deep sequencing analysis of an
489 enterovirus emerging from persistent infection. As compared to the parental strain, several
490 mutations appeared throughout the viral genome during the persistence of CVB4 in ductal-
491 like cells. The mutations with amino-acid substitutions affected mainly VP1, VP2, 3D, 2A and
492 2C regions. Whether these mutations play a role in viral adaptation or phenotypic changes
493 requires further investigations.
494 In previous studies various regions of enterovirus genome were analyzed, but no consistent
495 mutation has been reported to be associated with enterovirus persistence (Beaulieux et al.,
496 2005; Duncan and Colbère-Garapin, 1999; Duncan et al., 1998; Pelletier et al., 1998;
497 Schmidtke et al., 2000). However, another team focused on the 5' non coding region and
498 observed deletions with size ranging from 7 to 49 nucleotides. These terminally-deleted
499 persistent variants were not able to induce CPE, and have been observed in vitro as well as
500 in vivo (Chapman et al., 2008; Kim et al., 2005, 2008; Tracy et al., 2015). Genome

501 sequencing did not reveal such variants in our system, in agreement with the fact that PIDV
502 was able to induce a CPE in our experiments.
503 Furthermore, we demonstrated that the amino-acid changes occurring during persistence in
504 VP1 and VP2 viral capsid proteins did not alter CAR footprints on the virus. This finding
505 suggests that CVB4 can replicate in a CAR-restricted environment, but still maintains its
506 interaction properties with CAR. However the efficiency of PIDV binding to CAR requires
507 additional studies. It cannot be excluded that alternative receptors are used by PIDV. In a
508 previous report, Schmidtke et al. sequenced the genome of a CVB3 strain emerging from
509 persistent infection in a low CAR – expressing fibroblast cell line. This strain, exerted higher
510 lytic properties than the parental virus, although it exhibited reduced CAR-binding properties.
511 As a matter of fact the virus emerging from persistent infection acquired the ability to
512 recognize additional receptors, and four VP1 amino-acid substitutions were identified to be
513 associated with this phenotype (Schmidtke et al., 2000).
514 In conclusion, cellular and viral changes occurring during CVB4 persistence in pancreatic
515 ductal-like cells have been investigated. Persistently-infected cells gained a sustained
516 resistance to CVB4 induced cell lysis. CAR expression was reduced and the miRNA profile
517 was modified. Phenotypic and genotypic changes of the virus were also observed reflecting
518 virus adaptation during the persistence. The persistence of CVB in pancreatic cells induced
519 changes that were maintained even after virus clearance. Further investigations are needed
520 to elucidate the mechanisms of the persistence of CVB4 in pancreatic cells and of the
521 sustained impact of the virus on these cells.
522

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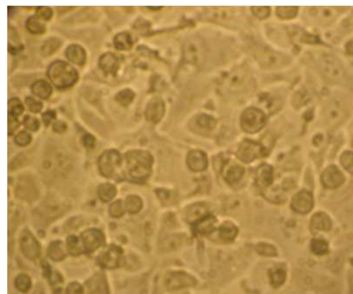
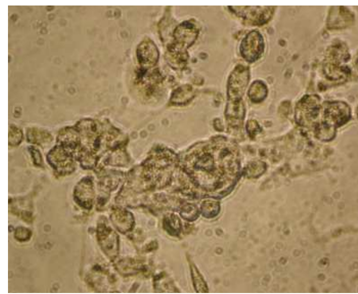
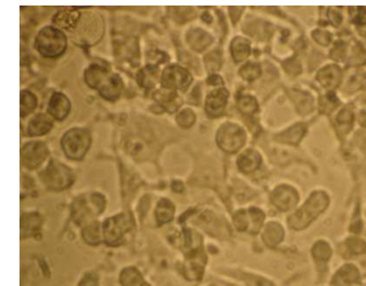


Uninfected cells

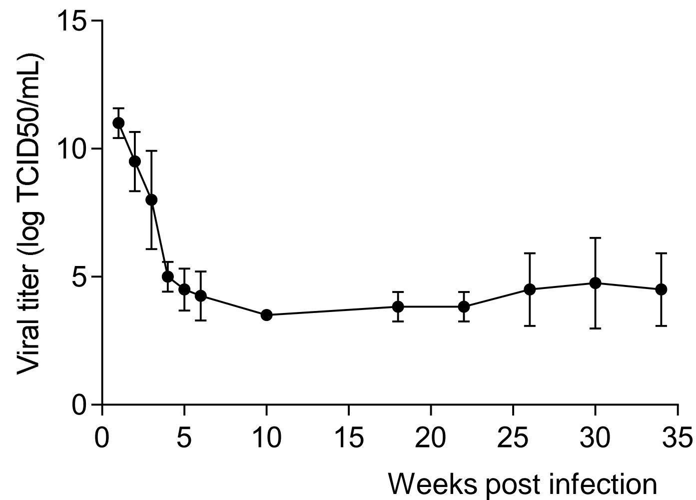


Acute infection

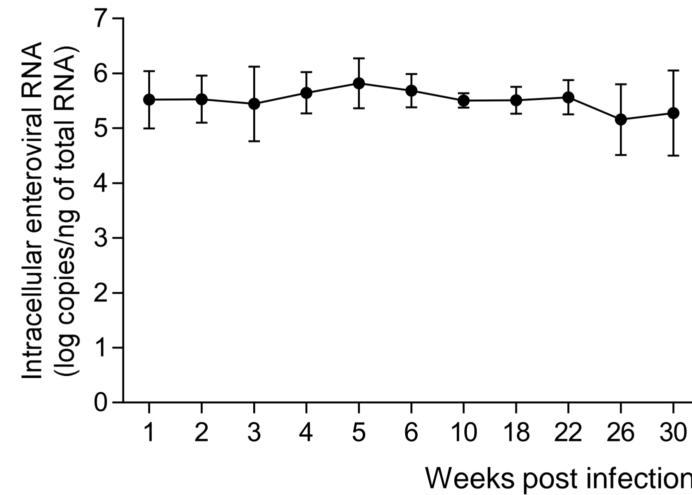


Persistent infection, 30 weeks

(a) Macroscopic aspect of Panc-1 cells (magnification X 200G)



(b) Kinetics of viral titer during persistent infection



(c) Kinetics of intracellular viral load during persistent infection

(d)

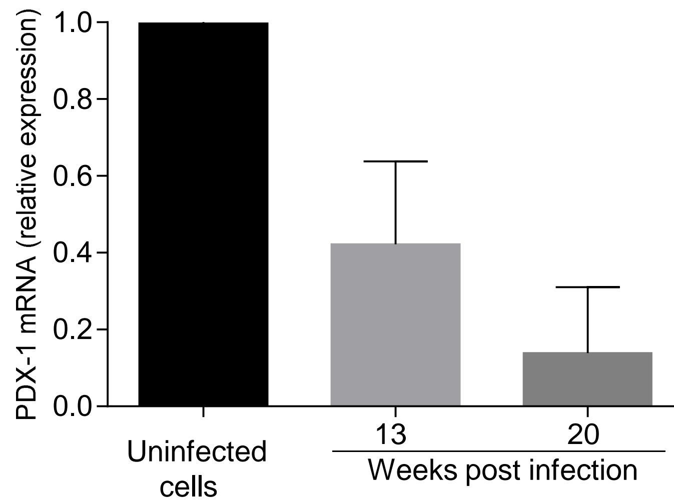
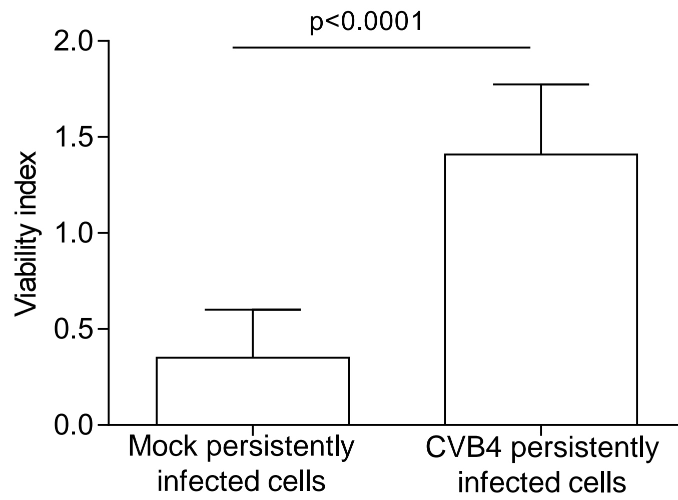


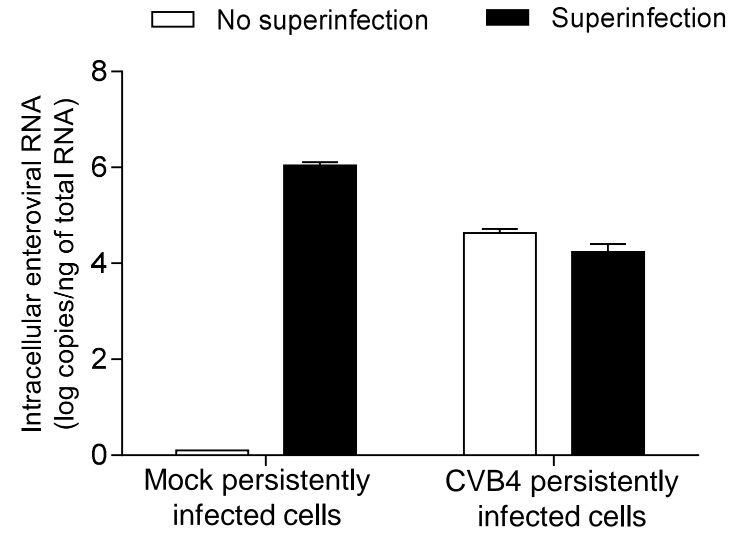
Figure 1. Characterization of persistent CVB4 infection in Panc-1 cells

A persistent CVB4 infection was established in Panc-1 cells. Cells were observed under an inverted microscope (initial magnification X 200) (a). Viral titer in supernatants was determined using endpoint dilution assay (b). Intracellular viral RNA was quantified using a real-time RT-qPCR (c). PDX-1 mRNA was assessed during persistent infection by using real-time RT-qPCR and expressed as fold-change as compared to uninfected cells (d). Results presented (b-e) are mean +/- SD of three independent persistent infections.

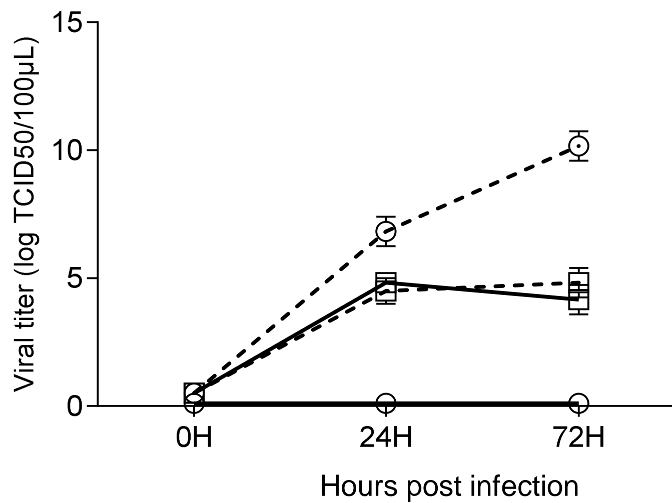
(a)



(c)



(b)



○ Mock persistently infected cells

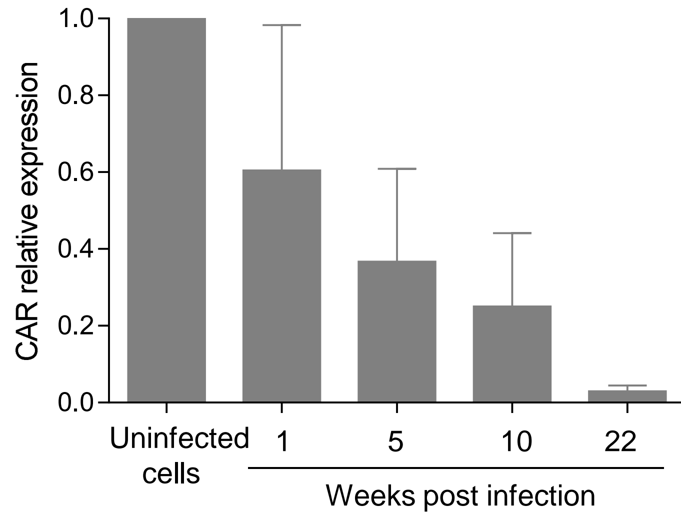
□ CVB4 persistently infected cells

—— No superinfection

- - - - Superinfection

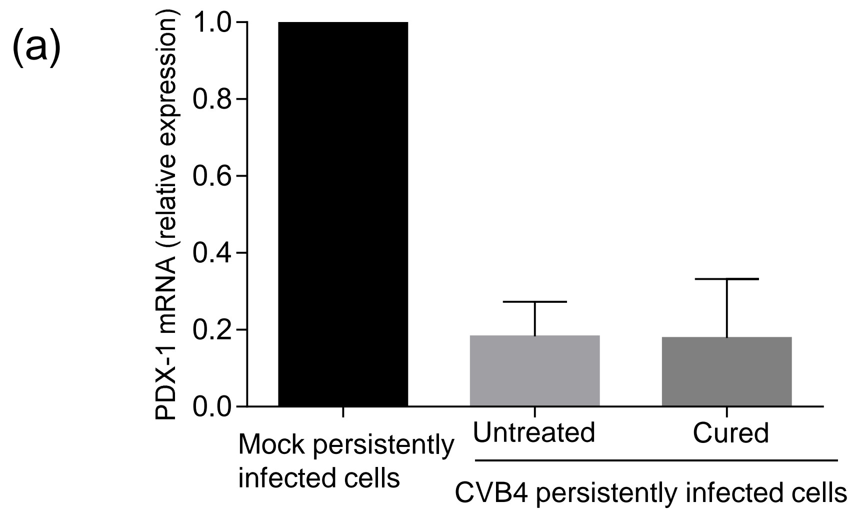
Figure 2. Acute CVB4 superinfection of persistently infected cells did not induce cell lysis

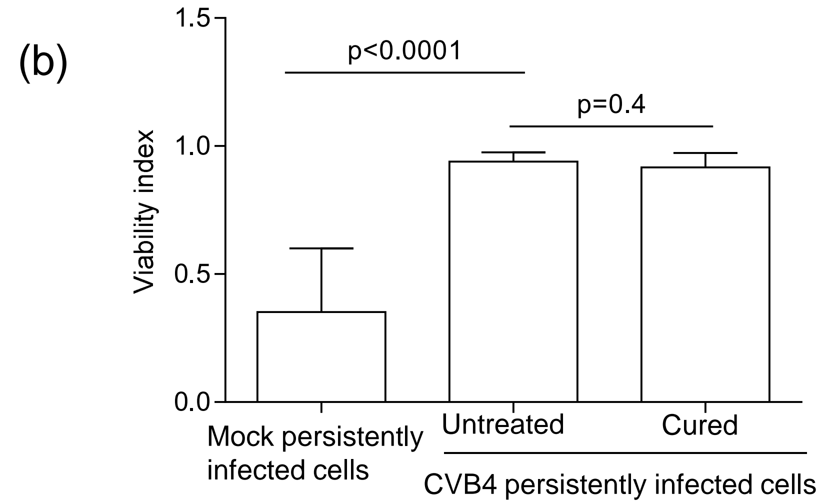
CVB4 and mock-persistently infected cells were infected with CVB4 at a MOI of 10. Cell viability was assessed at 48h post infection by using the cristal violet assay (a). Supernatants were collected and viral progeny was determined (b), Cells were harvested, washed, and intracellular viral RNA was quantified by using RT-qPCR (c). Results are mean+/-SD of 3 independent experiments.

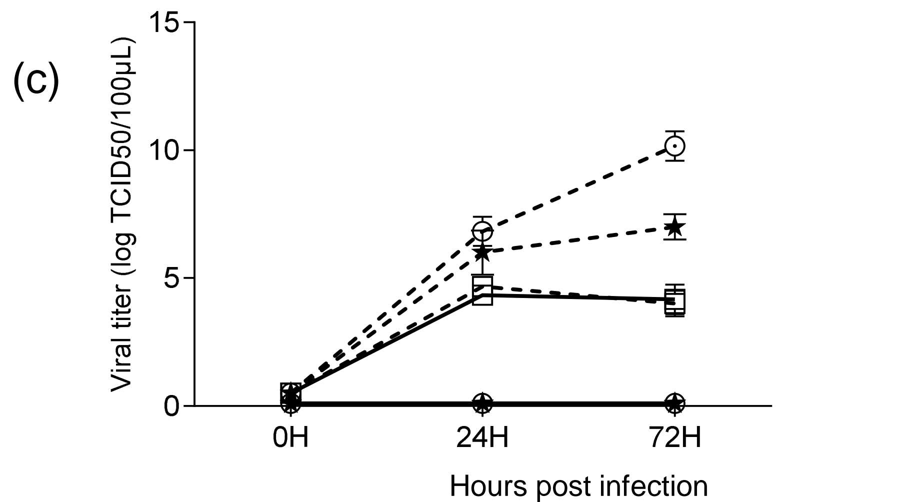


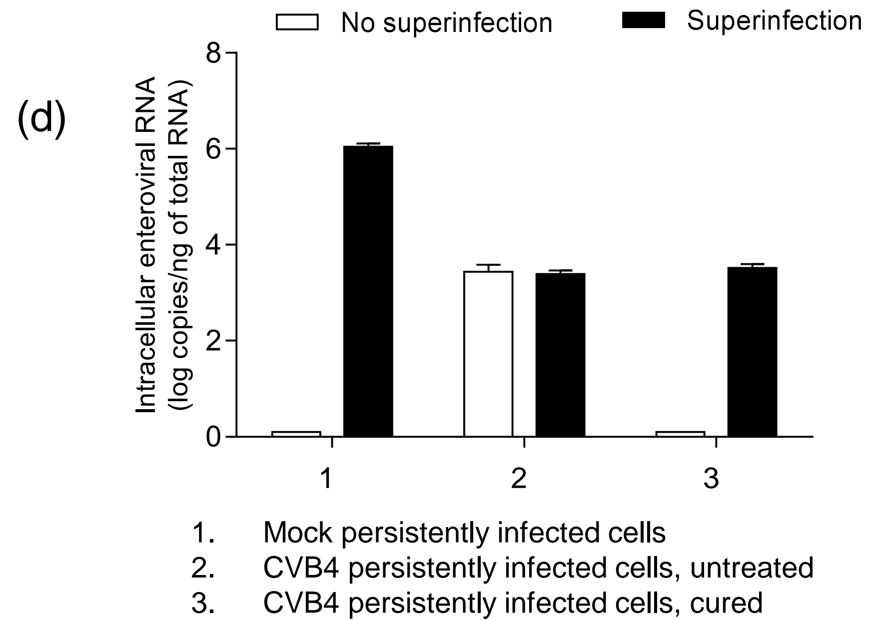
<u>Figure 3</u>. CAR expression is significantly decreased during persistent infection

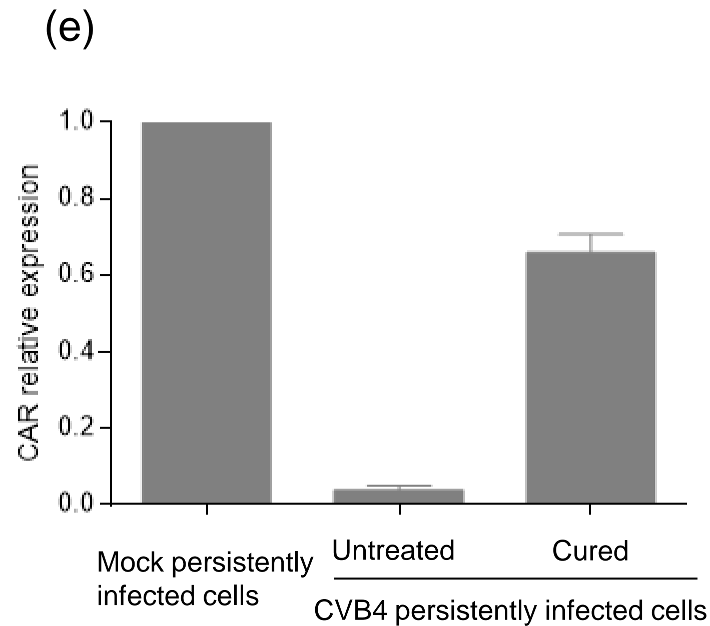
CAR mRNA was quantified by real-time RT-qPCR during persistent infection. Results are mean+/-SD of three independent experiments.

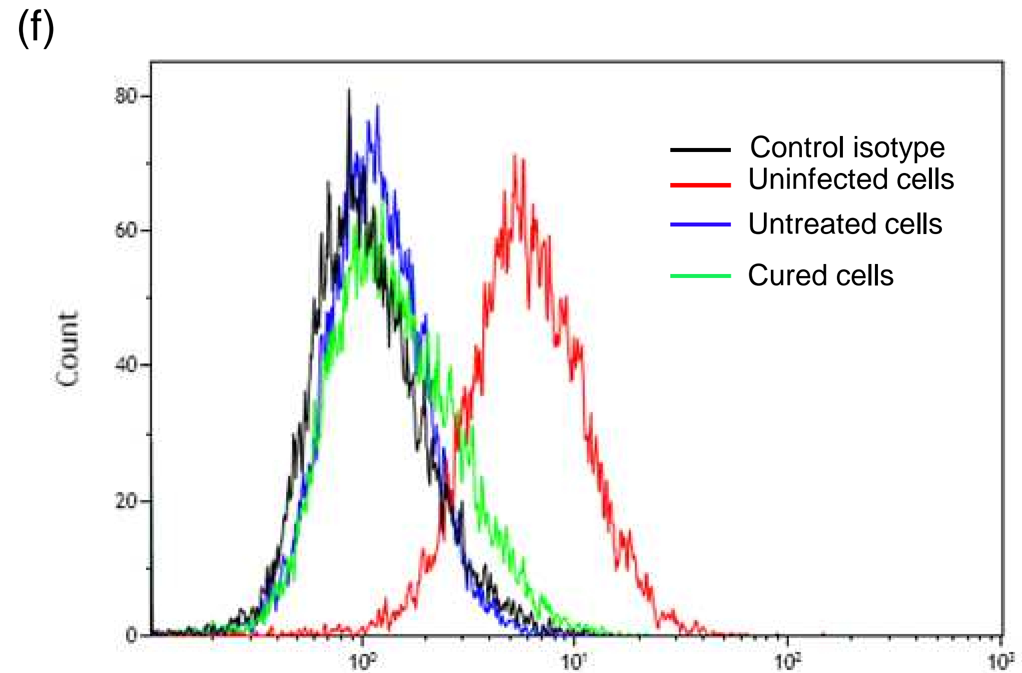

(a)
PDX-1 mRNA (relative expression)
1.0
0.8
0.6
0.4
0.2
0.0
Mock persistently infected cells
Untreated
Cured
CVB4 persistently infected cells


(b)
Viability index
1.5
1.0
0.5
0.0
p<0.0001
p=0.4
Mock persistently infected cells
Untreated
Cured
CVB4 persistently infected cells


(c)
Viral titer (log TCID50/100µL)
15
10
5
0
0H
24H
72H
Hours post infection
Mock persistently infected cells
CVB4 persistently infected cells, untreated
CVB4 persistently infected cells, cured
Superinfection
No superinfection


(d)
No superinfection
Superinfection
Intracellular enteroviral RNA (log copies/ng of total RNA)
8
6
4
2
0
1
2
3
1. Mock persistently infected cells
2. CVB4 persistently infected cells, untreated
3. CVB4 persistently infected cells, cured

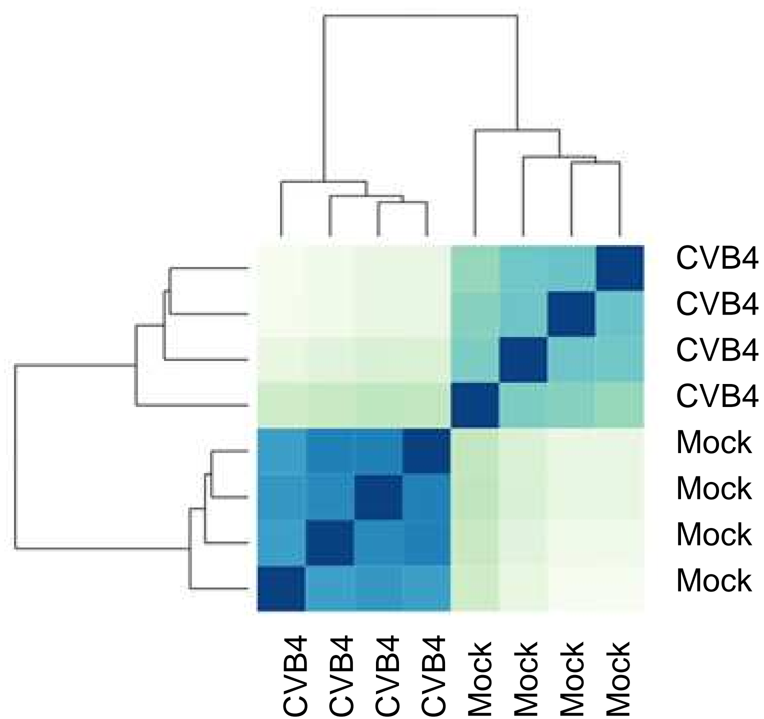


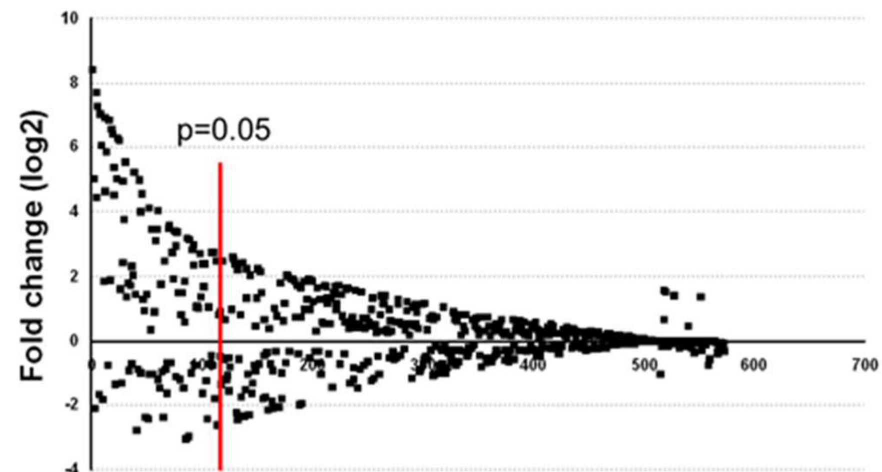


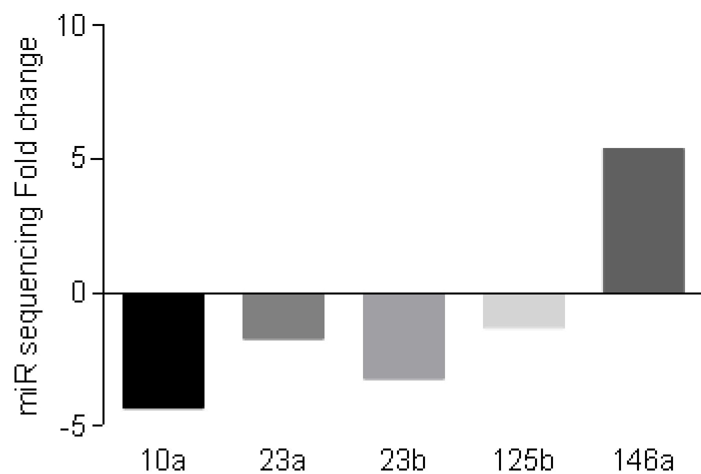
<u>Figure 4</u>. Persistence of changes induced in CVB4 persistently infected cells after virus clearance

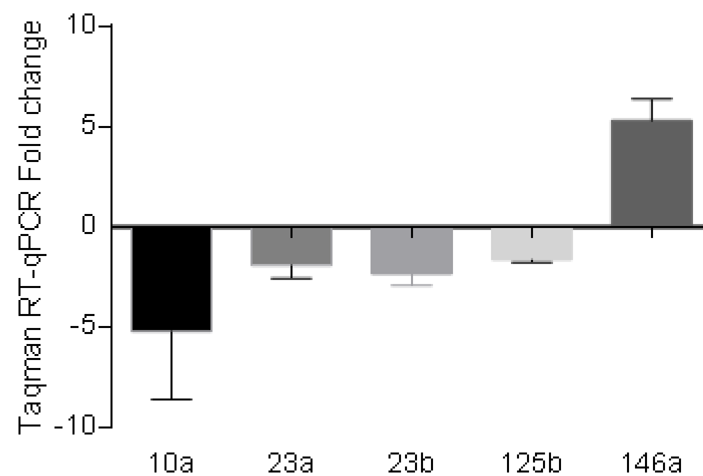
CVB4 persistent infection was cured using fluoxetine, and then PDX-1 mRNA expression was evaluated in CVB4 peristently infected cells that were cleared (a).

Cured cells and untreated cells were infected with CVB4 at a MOI of 10. Cell viability (b), viral progeny (c), and intracellular viral RNA (d) were investigated. CAR mRNA was quantified by real-time RT-qPCR (e). The membrane expression of CAR was evaluated by flow cytometry (f). Results are mean+/-SD of 3 independent experiments, and one representative experiment is shown for flow cytometry.


(a)
CVB4
CVB4
CVB4
CVB4
Mock
Mock
Mock
Mock
CVB4 CVB4 CVB4 CVB4 Mock Mock Mock Mock


(b)
Fold change (log2)
p=0.05
10
8
6
4
2
0
-2
-4
0
500
600
700


(c)
miR sequencing Fold change
10
5
0
-5
10a
23a
23b
125b
146a


(d)
Taqman RT-qPCR Fold change
10
5
0
-5
-10
10a
23a
23b
125b
146a

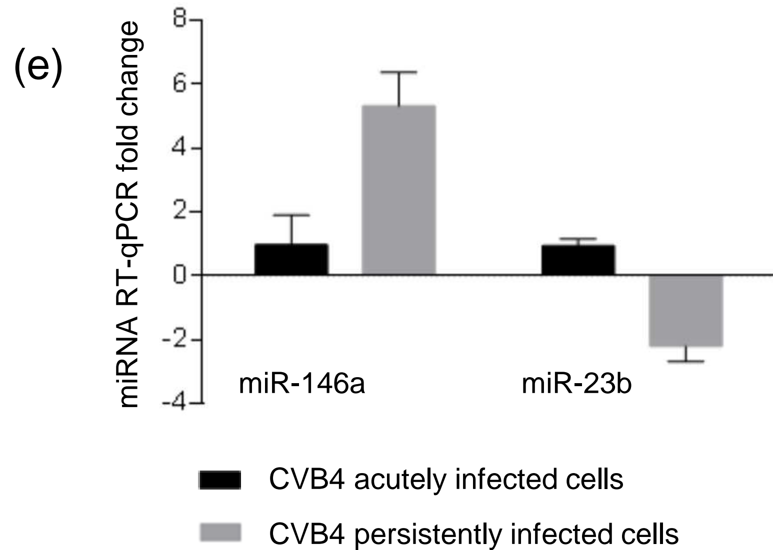
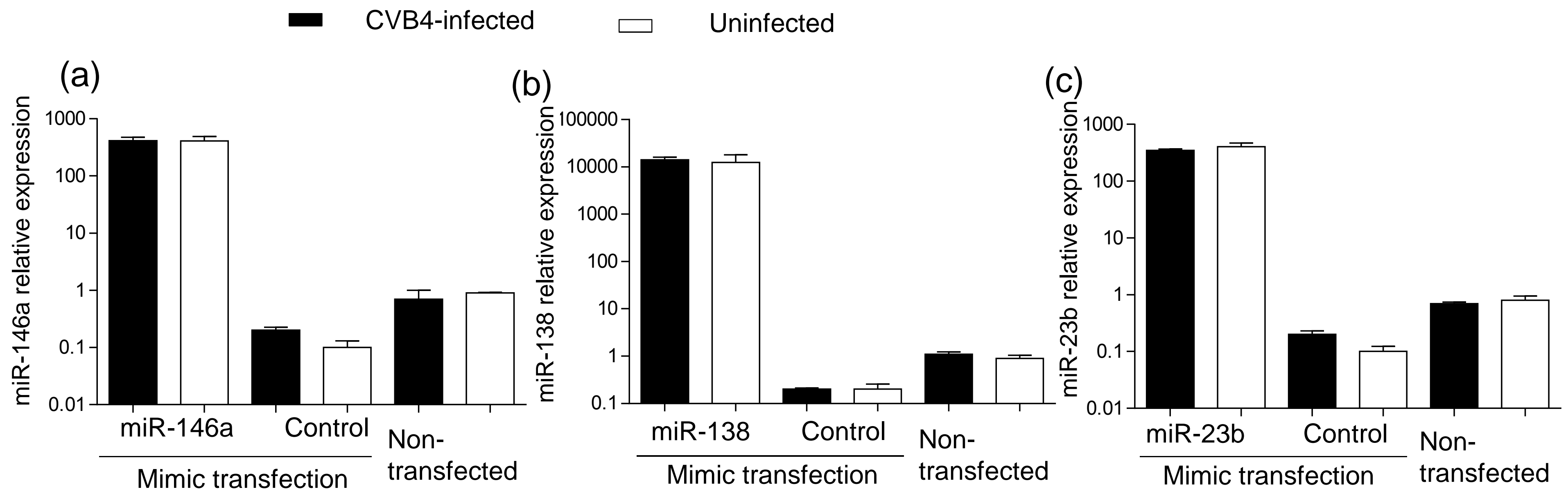
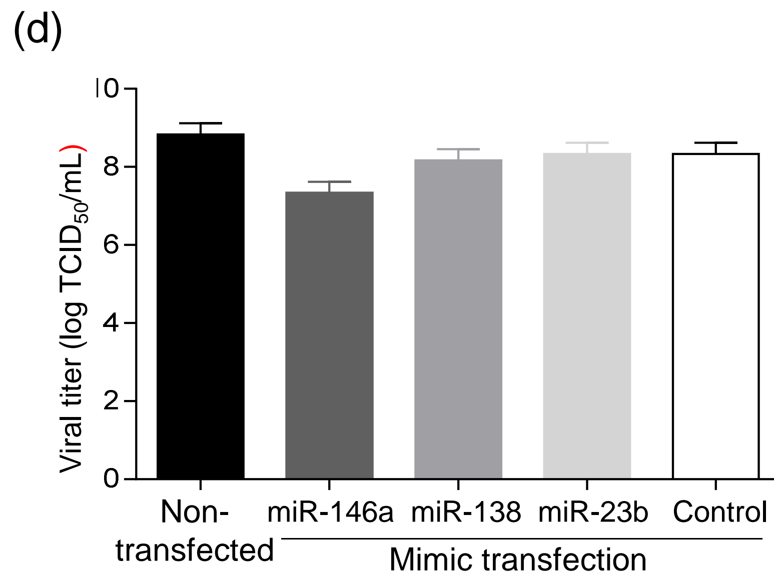


Figure 5. Cellular microRNA profile during CVB4 persistent infection

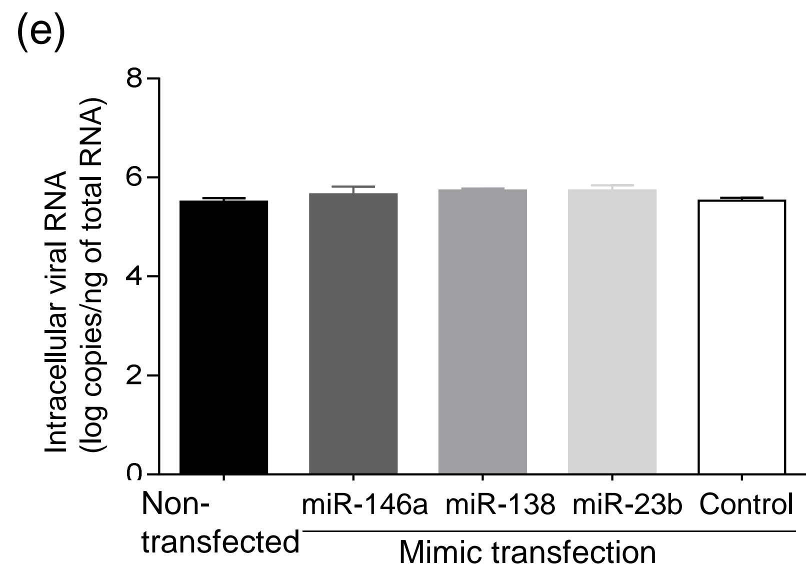
MiRNA sequencing was performed on CVB4 and mock persistently infected Panc-1 cells. The profile is compared between CVB4 and mock persistently infected cells (a). MiRNAs with a fold change ≥ 3 and $p<0.05$ were considered as differentially expressed (b). The fold change determined by miR-sequencing was confirmed using taqman RT-qPCR assays for few miRNAs (c, d). Taqman RT-qPCR was used to to quantify miR-146a and miR-23b in CVB4 acutely and persistently infected cells (e). RT-qPCR results are mean +/- SD of 3 independent experiments.



CVB4-infected
Uninfected
(a)
miR-146a relative expression
1000
100
10
1
0.1
0.01
miR-146a
Control
Mimic transfection
Non-
transfected
(b)
miR-138 relative expression
100000
10000
1000
100
10
1
0.1
miR-138
Control
Mimic transfection
Non-
transfected
(c)
miR-23b relative expression
1000
100
10
1
0.1
0.01
miR-23b
Control
Mimic transfection
Non-
transfected



(d)
Viral titer (log TCID50/mL)
10
8
6
4
2
0
Non-
transfected
miR-146a
miR-138
miR-23b
Control
Mimic transfection



(e)
Intracellular viral RNA
(log copies/ng of total RNA)
8
6
4
2
0
Non-
transfected
miR-146a
miR-138
miR-23b
Control
Mimic transfection

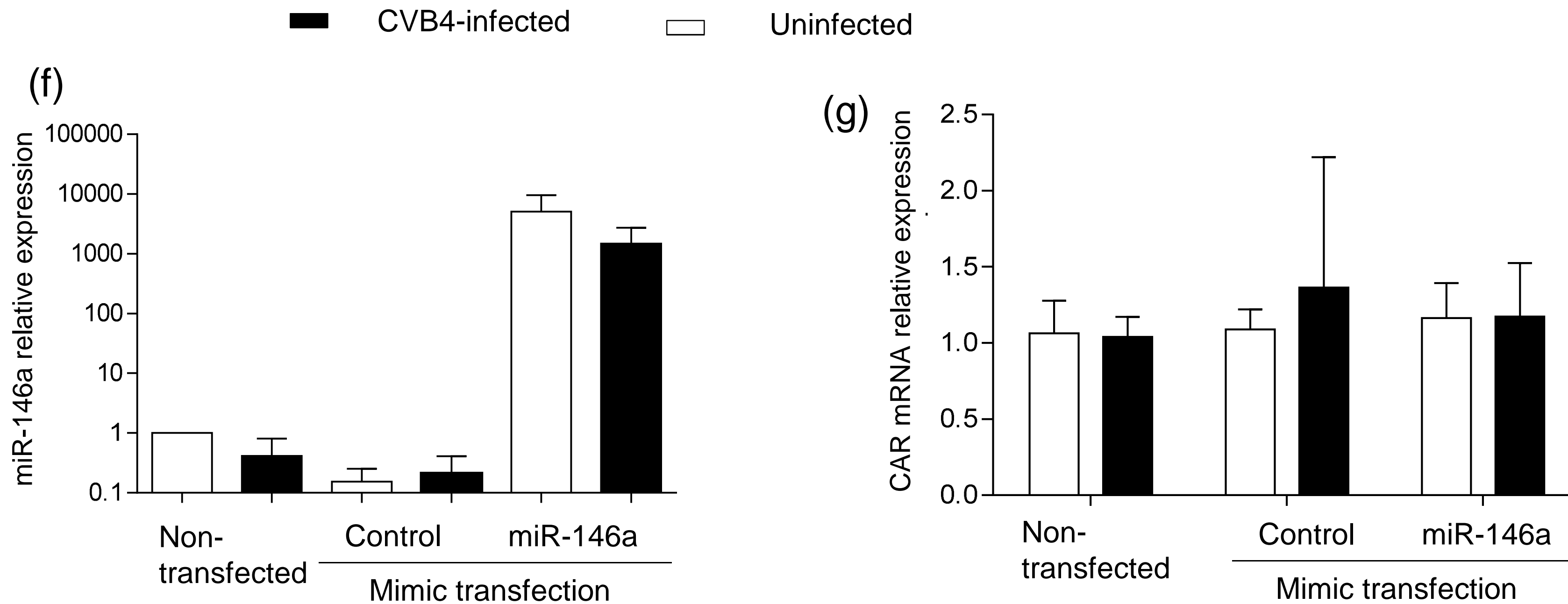
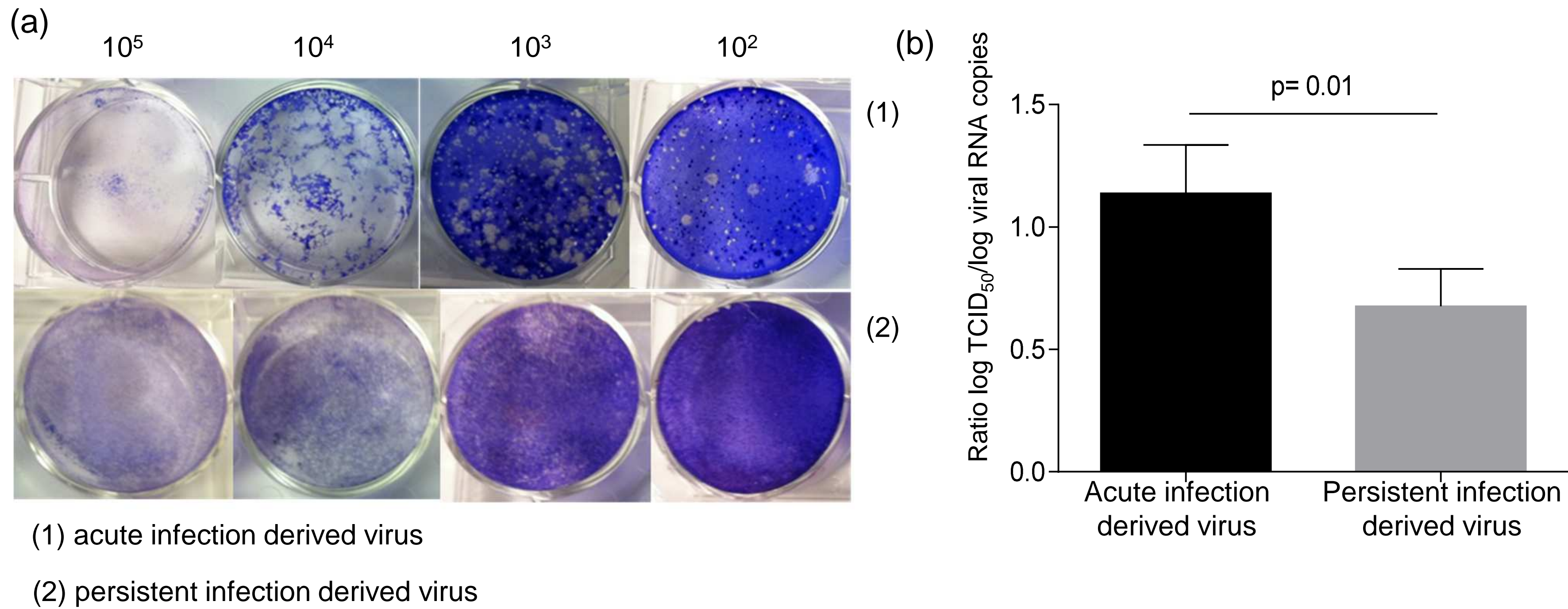


Figure 6. Impact of miRNA mimic transfection on virus replication and CAR mRNA expression in Panc-1 cells

Panc-1 cells were transfected with miR-146a, miR-138 and miR-23b mimics at 20nM, and then infected with with CVB4 at MOI of 1, 24h after transfection. Levels of MiRNAs were quantified in cells (a-c). Viral titers in supernatants (d) and intracellular viral RNA levels (e) were determined at 48h post infection.

MiR-146a mimic was transfected in Panc-1 cells at 50 nM, which were subsequently inoculated with CVB4 at MOI of 0.01. The expression of miR-146a (f) and CAR mRNA (g) was assessed in cells. Results are mean+/-SD of 3 independent experiments.



<u>Figure 7</u>. Characterisation of persistent infection derived virus

The aspect of the plaque induced by the persistent infection derived virus and the parental virus were compared (a). The ratio between the viral titer and the viral RNA load was evaluated for both viruses (b).

(a)

```
CVB3/28/VP1         1   FFQGPVEDAITAAIGRVADTVGTGPTNSEAIPALTAAETGHTSQVVPGDTMQTRHVKNYH   60
CVB3/Nancy/VP1      1   FFQGPVEDAITAAIGRVADTVGTGPTNSEAIPALTAAETGHTSQVVPGDTMQTRHVKNYH   60
CVB4/E2/ref/VP1     1   FYQGPTEESVERAMGRVADTIARGPSNSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYH   60
CVB4/E2/AI1/VP1     1   FYQGPTEESVERAMGRVADTIARGPSNSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYH   60
CVB4/E2/AI2/VP1     1   FYQGPTEESVERAMGRVADTIARGPSNSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYH   60
CVB4/E2/CI1/VP1     1   FYQGPTEESVERAMGRVADTIARGPSNSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYH   60
CVB4/E2/CI2/VP1     1   FYQGPTEESVERAIGRVADTIARGPSNSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYH   60
                        *:***.*:::  *:******:. **:*** ******.******** *.********:***

CVB3/28/VP1        61   SRSESTIENFLCRSACVYFTEYEN---SGAKRYAEWVLTPRQAAQLRRKLEFFTYVRFDL  117
CVB3/Nancy/VP1     61   SRSESTIENFLCRSACVYFTEYKN---SGAKRYAEWVLTPRQAAQLRRKLEFFTYVRFDL  117
CVB4/E2/ref/VP1    61   SRSESSIENFLCRSACVIYIKYSSAESNNLKRYAEWVINTRQVAQLRRKMEMFTYIRCDM  120
CVB4/E2/AI1/VP1    61   SRSESSIENFLCRSACVIYIKYSSAESNNLKRYAEWVINTRQVAQLRRKMEMFTYIRCDM  120
CVB4/E2/AI2/VP1    61   SRSESSIENFLCRSACVIYIKYSSAESNNLKRYAEWVINTRQVAQLRRKMEMFTYIRCDM  120
CVB4/E2/CI1/VP1    61   SRSESSIENFLCRSACVIYIKYSSAESNNLKRYAEWVINTRQAAQLRRKMEMFTYIRCDM  120
CVB4/E2/CI2/VP1    61   SRSESSIENFLCRSACVIYIKYSSAESNNLKRYAEWVINTRQVAQLRRKMEMFTYIRCDM  120
                        *****:*********** : :*..    . *******:. **.******:*:***:* *:

CVB3/28/VP1       118   ELTFVITSTQQPSTTQNQDAQILTHQIMYVPPGGPVPDKVDSYVWQTSTNPSVFWTEGNA  177
CVB3/Nancy/VP1    118   ELTFVITSTQQPSTTQNQDAQILTHQIMYVPPGGPVPDKVDSYVWQTSTNPSVFWTEGNA  177
CVB4/E2/ref/VP1   121   EQTFVITSHQEMSTATNSVVPVQTHQIMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNA  180
CVB4/E2/AI1/VP1   121   ELTFVITSHQEMSTATNSDVPVQTHQIMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNA  180
CVB4/E2/AI2/VP1   121   ELTFVITSHQEMSTATNSDVPVQTHQIMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNA  180
CVB4/E2/CI1/VP1   121   ELTFVITSHQEMSTATNSDIPVQTHQIMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNA  180
CVB4/E2/CI2/VP1   121   ELTFVITSHQEMSTATNSDVPVQTHQIMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNA  180
                        * ****** *: **: *.   : ************** .*:.**********:*******

CVB3/28/VP1       178   PPRMSIPFLSIGNAYSNFYDGWSEFSRNGVYGINTLNNMGTLYARHVNAGSTGPIKSTIR  237
CVB3/Nancy/VP1    178   PPRMSIPFLSIGNAYSNFYDGWSEFSRNGVYGINTLNNMGTLYARHVNAGSTGPIKSTIR  237
CVB4/E2/ref/VP1   181   PPRMSIPFMSIGNAYTMFYDGWSNFSRDGIYGYNSLNNMGTIYARHVNDSSPGGLTSTIR  240
CVB4/E2/AI1/VP1   181   PPRMSIPFMSIGNAYTMFYDGWSNFSRDGIYGYNSLNNMGTIYARHVNDSSPGGLTSTIR  240
CVB4/E2/AI2/VP1   181   PPRMSIPFMSIGNAYTMFYDGWSNFSRDGIYGYNSLNNMGTIYARHVNDSSPGGLTSTIR  240
CVB4/E2/CI1/VP1   181   PPRMSIPFMSIGNAYTMFYDGWSNFSRDGIYGYNSLNNMGTIYARHVNDSSPGGLTSTIR  240
CVB4/E2/CI2/VP1   181   PPRMSIPFMSIGNAYTMFYDGWSNFARDGIYGYNTLNNMGTIYARHVNDSSPGGLTSTIR  240
                        ********:******: ******:*:*:*:** *:******:****** .* *  :.****

CVB3/28/VP1       238   IYFKPKHVKAWIPRPPRLCQYEKAKNVNFQPSGVTTTRQSITTMTNT  284
CVB3/Nancy/VP1    238   IYFKPKHVKAWIPRPPRLCQYEKAKNVNFQPSGVTTTRQSITTMTNT  284
CVB4/E2/ref/VP1   241   IYFKPKHVKAYVRPPRRLCQYKKAKNVNFDVEAVTTERASLVTT---  284
CVB4/E2/AI1/VP1   241   IYFKPKHVKAYVPRPPRLC-YKKAKNVNFDVEAVTTKRASLVTT---  283
CVB4/E2/AI2/VP1   241   IYFKPKHVKAYVPRPPRLC-YKKAKNVNFDVEAVTTKRASLVTT---  283
CVB4/E2/CI1/VP1   241   IYFKPKHIKAYVPRPPRLC-YKKAKSVNFDVEAVTTKRASLVTT---  283
CVB4/E2/CI2/VP1   241   IYFKPKHVKAYVPRPPRLC-YKKAMNVNFDVEAVTTKRASLVTT---  283
                        *******.**..  * *** *.**  ***.   *** * *. *
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(b)

```
CVB3/28/VP2          1  SPTVEECGYSDRARSITLGNSTITTQECANVVVGYGVWPDYLKDSEATAEDQPTQPDVAT  60
CVB3/Nancy/VP2       1  SPTVEECGYSDRARSITLGNSTITTQECANVVVGYGVWPDYLKDSEATAEDQPTQPDVAT  60
CVB4/E2/ref/VP2      1  SPTVEECGYSDRVRSITLGNSTITTQECANVVVGYGVWPDYLSDEEATAEDQPTQPDVAT  60
CVB4/E2/AI1/VP2      1  SPTVEECGYSDRVRSITLGNSTITTQECANVVVGYGVWPDYLSDEEATAEDQPTQPDVAT  60
CVB4/E2/AI2/VP2      1  SPTVEECGYSDRVRSITLGNSTITTQECANVVVGYGVWPDYLSDEEATAEDQPTQPDVAT  60
CVB4/E2/CI1/VP2      1  SPTVEECGYSDRVRSITMGNSTITTQECANVVVGYGVWPDYLSDEEATAEDQPTQPDVAT  60
CVB4/E2/CI2/VP2      1  SPTVEECGYSDRVRSITLGNSTITTQECANVVVGYGVWPDYLSDEEATAEDQPTQPDVAT  60
                        ************.****:************************.*.***************

CVB3/28/VP2         61  CRFYTLDSVQWQKTSPGWWWKLPDALSNLGLFGQNMQYHYLGRTGYTVHVQCNASKFHQG  120
CVB3/Nancy/VP2      61  CRFYTLDSVQWQKTSPGWWWKLPDALSNLGLFGQNMQYHYLGRTGYTVHVQCNASKFHQG  120
CVB4/E2/ref/VP2     61  CRFYTLKSVKWEMQSAGWWWKFPDALSEMGLFGQNMQYHYLGRSGYTIHVQCNASKFHQG  120
CVB4/E2/AI1/VP2     61  CRFYTLNSVKWEMQSAGWWWKFPDALSEMGLFGQNMQYHYLGRSGYTIHVQCNASKFHQG  120
CVB4/E2/AI2/VP2     61  CRFYTLNSVKWEMQSAGWWWKFPDALSEMGLFGQNMQYHYLGRSGYTIHVQCNASKFHQG  120
CVB4/E2/CI1/VP2     61  CRFYTLNSVKWEMQSAGWWWKFPDALSEMGLFGQNMQYHYLGRSGYTIHVQCNASKFHQG  120
CVB4/E2/CI2/VP2     61  CRFYTLNSVKWEMQSAGWWWKFPDALSEMGLFGQNMQYHYLGRSGYTIHVQCNASKFHQG  120
                        ******.**:*:  * *****:*****::**************:***:************

CVB3/28/VP2        121  CLLVVCVPEAEMGCATLDNTPSSAELLGGDSAKEFADKPVASGSNKLVQRVVYNAGMGVG  180
CVB3/Nancy/VP2     121  CLLVVCVPEAEMGCATLDNTPSSAELLGGDTAKEFADKPVASGSNKLVQRVVYNAGMGVG  180
CVB4/E2/ref/VP2    121  CLLVVCVPEAEMGCTNAENAPTYGDLCGGETAKQFEQNAVT--GETAVQTAVCNAGMGVG  178
CVB4/E2/AI1/VP2    121  CLLVVCVPEAEMGCTNAENAPTYGDLCGGETAKQFEQNAVT--GKTAVQTAVCNAGMGVG  178
CVB4/E2/AI2/VP2    121  CLLVVCVPEAEMGCTNAENAPTYGDLCGGETAKQFEQNAVT--GKTAVQTAVCNAGMGVG  178
CVB4/E2/CI1/VP2    121  CLLVVCVPEAEMGCTNVENAPTYGDLCGGETAKQFEQNAAT--GKTAVQTAVCNAGMGVG  178
CVB4/E2/CI2/VP2    121  CLLVVCVPEAEMGCANAENAPTYGDLCGGETAKQFEQNAVT--GKTAVQTAVCNAGMGVG  178
                        **************:. .*:*: .:* **::**:* :: .:  .:. ** .* *******

CVB3/28/VP2        181  VGNLTIFPHQWINLRTNNSATIVMPYTNSVPMDNMFRHNNVTLMVIPFVPLDYCPGSTTY  240
CVB3/Nancy/VP2     181  VGNLTIFPHQWINLRTNNSATIVMPYTNSVPMDNMFRHNNVTLMVIPFVPLDYCPGSTTY  240
CVB4/E2/ref/VP2    179  VGNLTIYPHQWINLRTNNSATIVMPYINSVPMDNMFRHNNFTLMIIPFAPLDYVTGASSY  238
CVB4/E2/AI1/VP2    179  VGNLTIYPHQWINLRTNNSATIVMPYINSVPMDNMFRHNNFTLMIIPFAPLDYVAGASSY  238
CVB4/E2/AI2/VP2    179  VGNLTIYPHQWINLRTNNSATIVMPYINSVPMDNMFRHNNFTLMIIPFAPLDYVAGASSY  238
CVB4/E2/CI1/VP2    179  VGNLTIYPHQWINLRTNNSATIVMPYINSVPMDNMFRHNNFTLMIIPFAPLDYVAGASSY  238
CVB4/E2/CI2/VP2    179  VGNLTIYPHQWINLRTNNSATIVMPYINSVPMDNMFRHNNFTLMIIPFAPLDYVAGASSY  238
                        ******.******************* *************.***:***.****  *:::*

CVB3/28/VP2        241  VPITVTIAPMCAEYNGLRLAGHQ  263
CVB3/Nancy/VP2     241  VPITVTIAPMCAEYNGLRLAGHQ  263
CVB4/E2/ref/VP2    239  IPITVTVAPMSAEYNGLRLAGHQ  261
CVB4/E2/AI1/VP2    239  IPITVTVAPMSAEYNGLRLAGHQ  261
CVB4/E2/AI2/VP2    239  IPITVTVAPMSAEYNGLRLAGHQ  261
CVB4/E2/CI1/VP2    239  IPITVTVAPMSAEYNGLRLAGHQ  261
CVB4/E2/CI2/VP2    239  IPITVTVAPMSAEYNGLRLAGHQ  261
                        :*****:***.************
```

Figure 8. CVB4 footprints on CAR during persistent infection in Panc-1 cells

Mutations with a frequency ≥ 20% obtained by deep sequencing of 2 CVB4 independent acute infections and 2 independent persistent infections in Panc-1 cells, were integrated in the sequence of the CVB4E2 reference strain (Accession number: AF311939.1). Translated amino acid sequences were obtained using blastx (NCBI). VP1(a) and VP2(b) sequences were aligned along with sequences corresponding to CVB3/28 and CVB3/Nancy strains, using Uniprot software. Footprints of the virus on CAR are shown.

Table 1. List of differentially expressed miRNAs

MicroRNA	Fold change	MicroRNA	Fold change	MicroRNA	Fold change
hsa-miR-6087	341	hsa-miR-6821-5p	12	hsa-miR-138-5p	5
hsa-miR-663b	209	hsa-miR-6808-3p	11	hsa-miR-7704	4
hsa-miR-4516	156	hsa-miR-4758-5p	11	hsa-miR-668-3p	4
hsa-miR-4532	133	hsa-miR-664a-5p	11	hsa-miR-376b-3p	4
hsa-miR-1913	120	hsa-miR-922	11	hsa-miR-222-3p	4
hsa-miR-3621	116	hsa-miR-6834-3p	11	hsa-miR-3117-3p	4
hsa-miR-6730-3p	96	hsa-miR-4710	9	hsa-miR-665	3
hsa-miR-4492	85	hsa-miR-6892-5p	9	hsa-miR-1248	3
hsa-miR-6800-3p	78	hsa-miR-204-5p	9	hsa-miR-376a-3p	3
hsa-miR-1292-3p	73	hsa-miR-582-5p	8	hsa-miR-675-3p	3
hsa-miR-3687	66	hsa-miR-1268b	8	hsa-miR-1307-5p	3
hsa-miR-6858-3p	58	hsa-miR-551a	7	hsa-let-7f-1-3p	-8
hsa-miR-4449	46	hsa-miR-4417	7	hsa-miR-23b-5p	-8
hsa-miR-663a	42	hsa-miR-668-5p	7	hsa-let-7b-3p	-7
hsa-miR-885-3p	38	hsa-miR-483-3p	7	hsa-miR-335-3p	-6
hsa-miR-7641	33	hsa-miR-8072	7	hsa-miR-194-5p	-6
hsa-miR-4488	33	hsa-miR-137	6	hsa-miR-3178	-5
hsa-miR-3656	32	hsa-miR-30d-3p	6	hsa-miR-34c-5p	-5
hsa-miR-1470	31	hsa-miR-15a-3p	6	hsa-miR-187-3p	-5
hsa-miR-4497	25	hsa-miR-483-5p	6	hsa-let-7d-3p	-5
hsa-miR-6858-5p	24	hsa-miR-5701	6	hsa-miR-132-5p	-5
hsa-miR-4508	23	hsa-miR-146a-5p	5	hsa-miR-10a-5p	-4
hsa-miR-3182	22	hsa-miR-1908-3p	5	hsa-miR-10b-5p	-4
hsa-miR-3654	18	hsa-miR-412-5p	5	hsa-miR-98-3p	-3
hsa-miR-4477a	16	hsa-miR-1227-3p	5	hsa-miR-23b-3p	-3
hsa-miR-664b-5p	16	hsa-miR-380-5p	5	hsa-miR-335-5p	-3
hsa-miR-1914-3p	14	hsa-miR-147b	5	hsa-miR-1260b	-3

Table 2. Mutations observed in chronic infection derived virus*

Genome regions	All mutations (number)		Mutations with frequency ≥ 20%	
	Nucleotide changes	Amino-acid substitutions	Nucleotide changes	Amino-acid substitutions
5’NCR	12	0	7	0
1A (VP4)	1	0	0	0
1B (VP2)	10	6	5	4
1C (VP3)	9	1	7	1
1D (VP1)	17	9	11	7
2A	18	12	7	3
2B	1	0	1	0
2C	10	5	7	3
3A	5	1	3	1
3C	4	1	4	1
3D	18	5	13	4

*Cumulative data from two independent chronic infections

CONCLUSION GENERALE ET PERSPECTIVES

Conclusion générale

Les coxsackievirus B (CVB) sont des virus ubiquitaires dont l'intérêt en médecine humaine ne se limite pas aux infections aiguës, car leur rôle est également évoqué dans des pathologies d'évolution chronique. La pathogenèse virale dans ce type d'affections implique certes des déterminants de virulence du virus, mais ce sont les modalités de l'interaction entre le virus et l'hôte qui sont le plus en cause. Les données épidémio-cliniques sont en faveur d'une association statistique entre les infections à entérovirus et notamment celles à CVB et le développement du diabète de type 1 (DT1). En effet, si les anciennes études basées sur la sérologie étaient moins convaincantes (Green et al., 2004), les plus récentes utilisant notamment les techniques de biologie moléculaire, retrouvent de façon consensuelle cette association (Yeung et al., 2011). De plus, au-delà des études observationnelle cas-témoins de détection de composants viraux, de véritables études prospectives ont retrouvé le rôle des CVB et des entérovirus en général dans l'induction et dans l'accélération de l'auto-immunité anti-îlot. Il s'agit notamment des études DiMe (Hyöty et al., 1995; Lönnrot et al., 2000a), DIPP (Lönnrot et al., 2000b; Salminen et al., 2003) et TIGR (Sadeharju et al., 2003) en Finlande, BABYDIAB (Füchtenbusch et al., 2001) et Babydiet (Simonen-Tikka et al., 2011) en Allemagne, DAISY aux Etats-Unis (Graves et al., 2003; Stene et al., 2010) et MIDIA en Norvège (Tapia et al., 2011).

Cependant, le DT1 est une maladie chronique multifactorielle et l'affirmation d'un lien de causalité incontestable avec l'infection virale constitue un véritable défi. Une association statistique est définie par la différence de maladie entre un groupe non exposé et un groupe exposé à l'agent causal proposé. Il est parfois difficile d'affirmer si la différence observée est due au hasard, à un biais ou à un véritable lien de causalité (Stene and Rewers, 2013).

Les postulats de Koch-Henle sont difficiles à satisfaire dans le cadre de maladies multifactorielles comme le DT1. Ces critères avaient été établis initialement pour des infections bactériennes, et ne peuvent pas s'appliquer à des maladies chroniques dues à des virus, survenant souvent à distance de l'infection initiale. De plus, il existe de nombreux exemples de relations de causalité bien établies qui ne satisfont pas ces critères. Les causes d'une maladie multifactorielle peuvent être catégorisées en nécessaire, suffisante, ou les deux, ou ni l'un ni l'autre (Stene and Rewers, 2013). Dans l'état actuel des connaissances, l'infection entérovirale ne semble être ni indispensable ni suffisante pour l'induction de l'auto-immunité anti-îlot et le développement du DT1. Cependant les EV font probablement partie d'un faisceau d'arguments qui concourent à la survenue de la maladie. Le modèle causal du DT1 auto-immun n'est pas encore entièrement élucidé, mais il inclut certainement des composants tels que la prédisposition génétique, les facteurs exogènes dont notamment les

entérovirus et la réponse de l'immunité innée (Hober and Alidjinou, 2013; Hober and Sauter, 2010).

Au-delà donc de l'association statistique retrouvée à la faveur les études épidémio-cliniques, des données expérimentales sont nécessaires pour mieux définir la place des infections entérovirales dans ce modèle causal du DT1, et plusieurs équipes dans le monde ont initié des études dans ce sens.

Les différents travaux réalisés dans le cadre de cette thèse représentent une contribution à la compréhension de certains facteurs de la pathogenèse des CVB qui sont incriminés dans le DT1.

Ces travaux suggèrent que CVB4 (et probablement l'ensemble des CVB) est un virus inflammatoire. Il peut induire la production de quantités importantes d'interféron alpha (IFNα). Le rôle des interférons de type I (α/β) est suspecté dans l'initiation et dans la progression de l'auto-immunité dans plusieurs maladies auto-immunes dont DT1 (Crow, 2010). Dans nos travaux, l'IFNα a été détecté dans le plasma de sujets diabétiques, et fréquemment associé à la présence d'ARN entéroviral. Des travaux récents ont montré dans les cellules mononucléées (CMN) du sang périphérique, la présence d'une signature transcriptionnelle de type IFN I précédant l'installation de l'auto-immunité chez les enfants génétiquement prédisposés au DT1 (Ferreira et al., 2014; Kallionpää et al., 2014). La réponse interféron au niveau systémique peut donc refléter le processus auto-immun chez certains patients. Les modalités de cette réponse sont liées à des facteurs génétiques (voir annexe 4, Alidjinou and Hober, 2015). MDA5 est un senseur important dans la réponse aux CVB. Certains polymorphismes du gène IFIH1 codant pour cette protéine sont impliqués dans une forte réponse inflammatoire aux CVB et constituent un facteur de risque pour le DT1 (Lincez et al., 2015; Nejentsev et al., 2009).

Dans le sang périphérique, nous avons identifié les monocytes comme les principales cellules productrices d'IFNα. Néanmoins ces cellules sont peu infectables par CVB, sauf en cas de facilitation de l'infection par des anticorps non neutralisants. In vitro, cette facilitation de l'infection entraîne la production de grandes quantités d'IFNα. Cette observation suggère que le profil d'anticorps anti-CVB pourrait jouer un rôle important dans l'induction de l'IFNα par les CVB. Ces faits expérimentaux peuvent être corrélés à des données épidémiologiques qui retrouvent une prévalence plus élevée du DT1 dans les pays du Nord, en comparaison à ceux du Sud. Les patients prédisposés dans les pays occidentaux et soumis à une « pression entérovirale » réduite, pourraient ainsi avoir de faibles quantités d'anticorps neutralisants, et donc des conditions favorables pour une production accrue d'IFNα en cas d'infection par les CVB. Dans cette hypothèse, l'âge des premières infections à entérovirus serait critique, et une exposition précoce à ces virus entraînerait une protection grâce à l'induction de taux importants d'anticorps neutralisants qui seront efficaces lors d'une

infection qui peut jouer un rôle dans l'initiation d'un processus auto-immun. La facilitation de l'infection à CVB a fait l'objet de nombreux travaux dans notre laboratoire. Une partie de la protéine de capside VP4 a été identifiée comme la cible potentielle des anticorps facilitants (Chehadeh et al., 2005; Sauter et al., 2007, 2008), et une technique ELISA est actuellement en cours de développement pour quantifier ces anticorps. Un tel outil permettra d'évaluer le profil de ces anticorps et leur impact réel dans la réponse aux CVB.

En dehors de l'IFNα, nous avons montré que CVB4 peut induire la synthèse de plusieurs autres cytokines pro-inflammatoires notamment l'IL-6 et le TNFα. De façon intéressante, l'infection des cellules n'est pas indispensable, et des particules non infectieuses ou même des composants structuraux du virus pourraient donc y contribuer de façon significative. Cette production de cytokines pro-inflammatoires par les CMN peut également être amplifiée par la facilitation de l'infection en présence de particules infectieuses de CVB. L'impact de cette réponse inflammatoire systémique sur l'issue de l'infection n'est pas bien élucidé. De façon théorique, une réponse faible pourrait être bénéfique pour le virus, permettant une réplication importante et la dissémination aux organes cibles. A l'opposé, une réponse forte pourrait empêcher une infection massive et une atteinte des organes par le virus. Cependant, au-delà de l'ampleur de la réponse, c'est son efficacité qui doit être prise en compte. Ainsi, avec une réponse inefficace même importante, il pourrait y avoir une persistance du virus dans les cellules sanguines qui peuvent alors servir de véhicule vers les cibles comme le pancréas (Lind et al., 2012).

Localement au niveau des îlots pancréatiques, l'inflammation induite par les CVB est une composante essentielle de la pathogenèse entérovirale du DT1, par le biais d'une « activation en passant » du processus auto-immun chez les individus prédisposés. Les cellules effectrices de l'immunité innée comme les macrophages et les cellules dendritiques jouent un rôle important dans cette réponse.

Nous avons montré que les macrophages peuvent produire en présence de CVB4 des quantités significatives d'IFNα et d'autres cytokines pro-inflammatoires et sont ainsi susceptibles de contribuer à la constitution d'un environnement inflammatoire au niveau des îlots pancréatiques. Cet environnement inflammatoire peut d'une part fragiliser les cellules bêta, et d'autre part faciliter l'hyper-expression des molécules CMH de classe I et une présentation de peptides de cellules bêta. Cet impact peut favoriser le recrutement de lymphocytes auto-réactifs préexistants.

Nous avons également étudié la persistance de l'infection à CVB4. En effet, le rôle des CVB dans les pathologies chroniques comme le DT1 ne repose pas sur une infection aiguë lytique, mais plutôt sur la persistance du virus dans les tissus. Il est donc important de comprendre les mécanismes et les conséquences de cette persistance. Nos résultats montrent que le virus peut infecter les macrophages dérivés de monocytes sanguins et y

persister. Cependant des caractéristiques particulières sont probablement nécessaires pour cette infection. En effet, nous avons montré que CVB4 pouvait infecter les macrophages obtenus par culture des cellules en présence de M-CSF mais pas de GM-CSF. La persistance de CVB4 dans les macrophages est cohérente avec l'hypothèse d'une persistance virale au niveau des cellules de l'immunité innée résidant au niveau du pancréas pour maintenir une inflammation chronique. Cependant dans notre modèle d'infection de macrophages in vitro, l'inflammation était seulement limitée à la phase aiguë de l'infection.

La persistance de CVB4 a été également étudiée dans un modèle de cellules canalaires du pancréas. Le virus peut établir une infection chronique productive de type « état porteur » dans une lignée de cellules canalaires. Des modifications cellulaires ont été observées au niveau des cellules chroniquement infectées notamment une résistance à la lyse au cours d'une réinfection, associée à une diminution très importante de l'expression du récepteur CAR. Ces cellules présentent également un profil de microARNs très différent de celui des cellules non infectées. Les miARNs peuvent être des acteurs intermédiaires entre l'infection virale et les modifications cellulaires. Récemment, une analyse fonctionnelle a montré que plusieurs miARNs induits par CVB5 dans des îlots pancréatiques, avaient pour cibles des gènes associés au DT1. Une évolution du virus a été également observée avec des changements phénotypiques et génotypiques. De façon intéressante, nous avons obtenu une guérison complète des cellules infectées grâce à un traitement par de la fluoxétine. Cette molécule utilisée dans le traitement de troubles psychiatriques, présente in vitro une activité antivirale vis-à-vis de certains entérovirus, et permet d'éliminer complètement en quelques semaines le virus des cellules chroniquement infectées par CVB4. Cet outil nous a permis de démontrer que certaines caractéristiques cellulaires acquises au cours de l'infection chronique pouvaient persister après l'élimination du virus. Ces observations confortent l'hypothèse de changements durables au cours et même au décours de la persistance des CVB, susceptibles de jouer un rôle dans le développement de pathologies chroniques comme le diabète de type 1.

Perspectives

Plusieurs investigations sont envisagées et s'inscrivent dans la continuité des travaux décrits dans cette thèse afin de :

- Comprendre le lien entre le profil du macrophage et l'infection à CVB4.

Nous allons identifier les facteurs qui conditionnent l'infection par CVB4 des macrophages différenciés avec du M-CSF (MM), alors que ceux obtenus avec du GM-CSF (MG) ne sont pas infectables. Il peut s'agir d'expression de récepteurs ou d'autres mécanismes de l'hôte qui empêchent l'entrée et/ou la réplication du virus. L'identification d'un marqueur

critique permettra de vérifier dans un modèle murin, son existence au niveau des macrophages résidant des îlots pancréatiques, et la corrélation avec l'infection par CVB4. De plus le marqueur identifié sera recherché au niveau des macrophages isolés d'ilots humains provenant de sujets diabétiques et de sujets non diabétiques.

- Compléter et approfondir l'étude des conséquences de l'infection persistante à CVB4 sur les cellules canalaires pancréatiques.

Nous envisageons de réaliser une analyse transcriptomique comparant les cellules non infectées, les cellules avec une infection aigue et les cellules chroniquement infectées. Cela permettra d'identifier le profil particulier d'expression génique, associé à la persistance de CVB4 dans les cellules canalaires pancréatiques. Des études de méthylation de l'ADN seront également réalisées pour apprécier le rôle de l'épigénétique dans les changements cellulaires survenant au cours de la persistance.

- Mesurer l'impact des modifications du virus survenues au cours de la persistance, sur la réponse et la physiologie des cellules bêta. Il s'agira de comparer les effets sur la cellule bêta, du virus initial et celui émergeant après la persistance. Les changements acquis par le nouveau virus sont associés à son adaptation à la cellule, mais notre hypothèse est qu'ils confèrent également au virus des propriétés nouvelles dans ses interactions avec l'immunité innée, dans ses effets sur la physiologie ou les fonctions de la cellule bêta. Les effets seront étudiés dans des lignées et des cellules bêta de première explantation (inflammation, apoptose, production d'insuline) et dans un modèle murin de souris NOD.

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ANNEXES

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Enterovirus Persistence as a Mechanism in the Pathogenesis of Type 1 Diabetes

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Abstract: Beyond acute clinical conditions, the role of enteroviruses (EVs) in chronic human diseases has been described. Although they are considered as highly cytolytic viruses, EVs can persist in various tissues. The persistence is believed to play a major role in the pathogenesis of EV related chronic diseases such as type 1 diabetes (T1D). T1D is characterized by an autoimmune destruction of pancreatic beta cells, and results from interplay between a genetic predisposition, the immune system, and environmental factors. EVs and especially group B coxsackieviruses (CVB) have been the most incriminated as exogenous agents involved in the development of T1D. Enteroviral persistence is the result of a virus-host coevolution combining a cell resistance to lysis through mutations or down-regulation of viral receptor, and a decrease of the viral replication by genomic modifications or the production of a stable double-stranded RNA form. CVB can persist in pancreatic cells and therefore could trigger, in genetically predisposed individuals, the autoimmune destruction of beta cells mainly through an activation of inflammation. The persistence of the virus in other tissues such as intestine, blood cells, and thymus has been described, and could also contribute to some extent to the enteroviral pathogenesis of T1D. The molecular and cellular mechanisms of CVB persistence and the link with the development of T1D should be investigated further. [*Discovery Medicine* 18(100):273-282, November 2014]

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Introduction

Human enteroviruses (HEVs) include many major human pathogens such as poliovirus, rhinovirus, enterovirus 71, coxsackievirus, and echovirus. These small non-enveloped RNA viruses belong to the Picornaviridae family, and the genus Enterovirus currently encompasses 7 species involved in human diseases (Human enterovirus A-D and Human rhinovirus A-C) (Knowles *et al*., 2012; Tapparel *et al*., 2013). Non-polio enteroviruses are ubiquitous pathogens and can infect a wide range of tissues (Harvala *et al*., 2002). They can be involved in many severe acute clinical features such as meningitis, encephalitis, myocarditis, pancreatitis, hepatitis, or fulminant sepsis in newborns (Romero, 2008; Tapparel *et al*., 2013).

Enteroviruses (EVs), especially the group B Coxackieviruses (CVB1-6), have also been associated with the development of chronic diseases like type 1 diabetes (T1D). T1D is characterized by a defect of insulin production as a result of an autoimmune destruction/dysfunction of pancreatic β cells in genetically predisposed individuals with an impaired immune regulation (Roep and Tree, 2014); but the role of exogenous factors in the initiation and progression of this disorder seems obvious, since only a small proportion of genetically susceptible individuals progress to clinical disease (Knip and Simell, 2012). Enteroviruses and especially CVB have been the most incriminated as environmental factors, and a relationship between these viruses and the development of T1D has been reported (Hober and Alidjinou, 2013; Hober and Sauter, 2010; Morgan and Richardson, 2014).

Although EVs are cytolytic viruses, they can establish persistent infections *in vitro* as well as *in vivo* (Pinkert *et al*., 2011), and viral persistence has been suggested as a major mechanism in the enteroviral pathogenesis of T1D (Jaïdane and Hober, 2008; Jaïdane *et al*., 2010).

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Epidemiological studies have found, in T1D patients, a more frequent detection of enteroviral (EV) components in blood, in the intestine, and in pancreas (Yeung *et al*., 2011), most often beyond the stage of acute infection.

The persistence of EVs has already been associated in humans to other syndromes, including post-polio syndrome (Julien *et al*., 1999; Leparc-Goffart *et al*., 1996) and chronic fatigue syndrome (Chia *et al*., 2010). Furthermore, CVB persistence was shown to contribute significantly to the occurrence of chronic myocarditis and dilated cardiomyopathy through direct effects of viral replication as well as induction of inflammation in the heart (Chapman and Kim, 2008).

EVs are transmitted mainly by fecal-oral route and their primary replication occurs in the intestine mucosa. From the gut, a systemic infection can lead to dissemination of the virus to other target organs such as pancreas. Although the presence or the persistence of the virus in the pancreas is believed to be a major component of the enteroviral pathogenesis of T1D, the virus can also persist in other sites such as intestine or blood cells that could act as reservoir and contribute to the circulation of the virus and the maintenance of pancreatic cells infection.

In addition, CVB can infect the thymus, whose most important role is the induction of central tolerance, i.e., the ability of T cells to discriminate 'self' from 'non-self.' A persistent CVB infection of thymic cells could lead to the disturbance of immune tolerance and contribute to the autoimmune process in T1D, by loss of central self-tolerance to insulin-secreting pancreatic β cells (Jaïdane *et al*., 2012a).

After a brief presentation of the currently known molecular mechanisms of EV persistence, the cumulative evidence *in vitro* and *in vivo* regarding EV persistence (with a focus on CVB) will be described in pancreatic cells and also in the other potential sites, and the link between the persistence and the pathological process leading to the development of T1D will be analyzed.

Factors Involved in the Persistence of EVs in Tissues

EVs are considered as cytolytic viruses; however, they can establish persistent infections *in vitro* as well as *in vivo* (Frisk, 2001; Pinkert *et al*., 2011). This suggests the role of a regulatory mechanism of viral replication under certain circumstances. Two major groups of persistent viral infections have been described: steady-state infections and carrier-state infections. The first group is characterized by infection of all cells (without lytic replication cycle), whereas in carrier-state culture systems, only a small proportion of cells are involved (with productive virus replication) (Frisk, 2001; Pinkert *et al*., 2011). EVs and especially CVB were shown to establish carrier-state persistent infections *in vitro* (Heim *et al*., 1992; 1995; Pinkert *et al*., 2011).

Most of the knowledge on viral persistence comes from *in vitro* systems, with some from *in vivo* models. Actually persistent infection by cytolytic viruses such as EVs is thought to result from a virus-host coevolution which combines a resistance developed by the cell, and an adaptation of the virulence of the viral strain (Pinkert *et al*., 2011). In this section, viral and cellular factors involved in the persistence of EVs are reviewed.

Viral factors are undoubtedly the most studied parameters during EV persistence. Since RNA-dependent RNA polymerases lack proofreading, the main mechanism reported is the selection of virus mutants that are less cytopathic for cells or that result in low-level viral replication. Some mutations were reported to affect the binding properties of the virus. A combination of mutations in the VP1 and VP2 capsid genes of poliovirus (PV) was shown to affect the cell binding and the receptor-mediated conformational changes necessary for viral penetration and uncoating. This modification has been suggested as the mechanism by which PV is able to establish persistent infections in HEp-2 cell cultures (Duncan and Colbère-Garapin, 1999; Duncan *et al*., 1998; Pelletier *et al*., 1998). Some amino-acid substitutions described in CVB3 strain emerging during viral persistence were associated with a weak interaction with the coxsackie and adenovirus receptor (CAR) but strong binding to the decay accelerating factor (DAF), as compared to the parental virus (Schmidtke *et al*., 2000).

Other genomic alterations have been reported in the EV highly conserved 5′NTR region. This region was shown to harbor the genomic determinants of EV replication (Bedard and Semler, 2004). Chapman and colleagues have demonstrated that *in vivo* CVB3 persistent infection of mouse or human heart, as well as *in vitro* infection of cardiomyocytes, was associated with a deletion in the 5′ end of the RNA. These 'terminally deleted' viruses have a lower replication rate and can persist in host cells over a prolonged period (Chapman *et al*., 2008; Kim *et al*., 2005; 2008). Recently, this deletion was also reported in a murine model during CVB persistence in the pancreas (Tracy *et al*., 2014).

The genomic modifications during EV persistence could explain at least partially the low detection rate of EV RNA by RT-PCR in samples from patients with EV

associated chronic diseases. However, an alternative viral persistence mechanism is possible especially *in vivo*. Indeed, it has been described that EV persistence in muscle and probably in other nondividing cells was not associated with the selection of mutant virus, but with the presence of a stable and atypical double-stranded RNA genomic form. Myofibers can harbor this RNA form for extended times without a production of detectable levels of infectious virus (Cunningham *et al*., 1990; Klingel *et al*., 1992; Tam and Messner, 1999).

Few authors have focused on the cellular factors involved in EV persistence. Feuer *et al*. (2002; 2004) reported that the cell cycle status affects CVB3 replication and suggested that the persistence of CVB3 *in vivo* may rely on infection of quiescent cells in which viral replication is lowered or suppressed. Cellular activation may also play a role in the outcome of CVB infection (Feuer and Whitton, 2008). The role of receptor mutations or reduction of receptor expression has been reported for EV persistence. Specific mutations in the domain 1 of poliovirus receptor (PVR) were associated with an increase of cell resistance to lysis (Pavio *et al*., 2000), and a decrease of PV-induced apoptosis (Gosselin *et al*., 2003). A down-regulation of CAR has been reported during CVB3 persistence (Pinkert *et al*., 2011), and a decrease of CAR expression was known to be associated with a decrease of CVB infection and cell lysis (Fechner *et al*., 2007; Werk *et al*., 2005). A heart-specific deletion of CAR in mice resulted in a resistance to CVB infection (Shi *et al*., 2009).

In summary, EV persistence depends strictly on the interactions within the virus-cell system (Figure 1). It probably combines many of the mechanisms described above, and others unknown. A better understanding of this phenomenon will provide a molecular basis to the pathogenesis of enterovirus-related chronic diseases like T1D.

EV Persistence in Pancreatic Cells and Relationship with T1D

The understanding of the pathogenesis of T1D requires undoubtedly focusing on pancreas. The pancreatic tropism of EVs both in animals and humans is well known. In humans, the evidence of enteroviral infection within pancreatic cells at the onset or during the progression of the disease has been difficult to obtain since this requires a biopsy that is invasive and often risky. Therefore, most of data available come from necropsies (Dotta *et al*., 2007; Richardson *et al*., 2009; Willcox *et al*., 2011; Ylipaasto *et al*., 2004). Pancreatic islets and especially β-cells, but not exocrine cells, were found to be susceptible to enteroviral infection (Dotta *et al*., 2007; Richardson *et al*., 2009; 2013). Interestingly, the specific receptor of cosackieviruses, the CAR molecule, is expressed in the pancreas mainly by these β-cells (Oikarinen *et al*., 2008a; Spagnuolo *et al*., 2013).

CVB can effectively replicate in pancreatic cells and cause massive cell lysis (Anagandula *et al*., 2014; Elshebani *et al*., 2007; Hodik *et al*., 2013). *In vivo*, this extensive cell destruction upon CVB infection could lead to what is known as "fulminant diabetes" (Kobayashi *et al*., 2011; Tanaka *et al*., 2013), a particular and rare clinical feature especially described in Japanese patients (out of the scope of this review).

Things are different in CVB associated autoimmune T1D since a clinical disease occurs often many years after the appearance of islet specific autoantibodies which have been reported to be a result of enteroviral infection (Laitinen *et al*., 2014; Oikarinen *et al*., 2011). Such important damage is not observed in pancreatic cells of patients in which the virus components have been detected. The most likely scenario would be a persistent infection with probably a low grade viral replication.

In fact the outcome of CVB infection within pancreatic cells seems to depend on the serotype and even the strain of the virus (Elshebani *et al*., 2007; Frisk and Diderholm, 2000; Frisk *et al*., 2001; Hindersson *et al*., 2004; Roivainen *et al*., 2002; Tracy *et al*., 2000). In addition, the route of transmission was reported to impact the effect of CVB on pancreatic cells. Indeed, a study has compared intraperitoneal injection and oral administration in mice, and concluded that though both routes lead to systemic and pancreas infection, the oral administration that is the natural transmission route in humans, protects pancreas from damage (Bopegamage *et al*., 2005). This finding suggests that the viral titer reaching the pancreas after oral administration is lower, resulting in a non-highly cytopathic phenomenon.

It is well accepted that the selective destruction of beta cells in T1D patients is an autoimmune process (Pugliese, 2014; Roep and Tree, 2014). The main hypothesis addressing the relationship between CVB persistence and T1D is that non-cytopathic CVB infection triggers autoimmunity against beta cells through activation of inflammation.

Actually, pathological studies on pancreases from died T1D patients (Richardson *et al*., 2014) show a quasi-absence of beta cells and the presence of an inflammatory cell infiltrate (insulitis) composed mainly of CD8 cytotoxic T cells and at lesser extent CD4 T cells and macrophages, and sometimes NK cells were reported

(Dotta *et al*., 2007; Willcox *et al*., 2009).

Persistent CVB infection is thought to induce an inflammatory response (and especially IFNα production) in pancreatic endocrine cells. Yet, this response might depend on a genetic background since some polymorphisms of IFIH1 gene have been epidemiologically associated with an increased risk of T1D (Nejentsev *et al*., 2009; Smyth *et al*., 2006). This gene encodes for MDA5 protein which is a cytoplasmic innate immune sensor for CVB (Kato *et al*., 2006). The local inflammation could lead to a beta cell antigen presentation that is enhanced by the hyperexpression of class I major histocompatibility complex (MHC) by endocrine cells (Richardson *et al*., 2014). The result of this antigen presentation is a destruction of beta cells by CD8 cytotoxic T cells that interestingly were found to be antigen-specific (Coppieters and von Herrath, 2009). These T cells probably preexist in predisposed individuals and are recruited to islets, guided by antigen presentation and driven by chemokines (Roep *et al*., 2010; Sarkar *et al*., 2012).

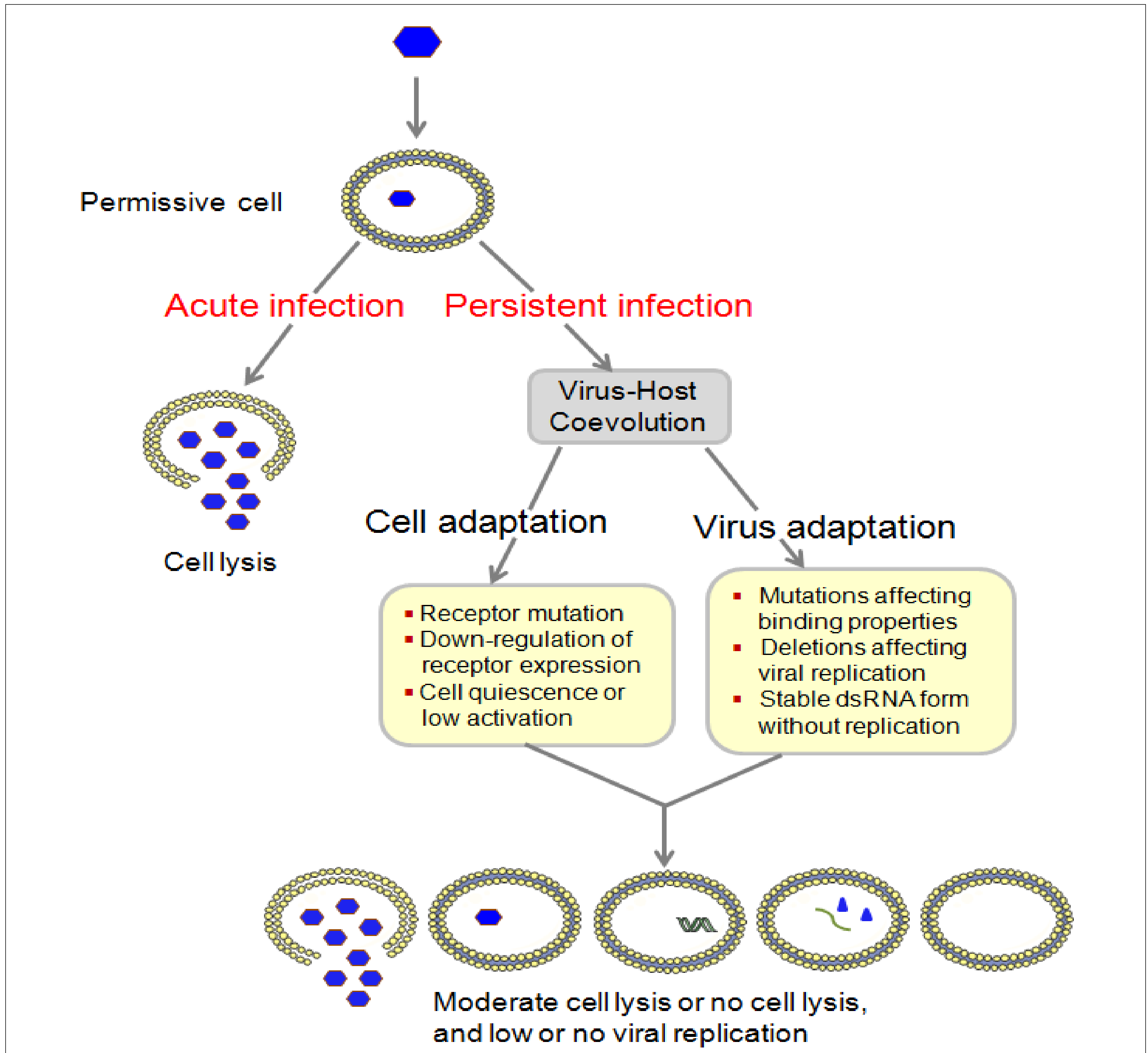

Figure 1. Possible mechanisms involved in the persistence of coxsackievirus B. A cytolytic virus such as coxackievirus B (CVB) can establish under certain circumstances a persistent infection in susceptible cells. Changes in cell and virus characteristics leading to a decreased or suppressed viral replication can be observed when the infection is persistent.

In contrast to the non-obese diabetic (NOD) mouse model, the insulitis seems to be moderate in humans, and only a limited number of infiltrating cells are observed (Carrero *et al*., 2013; Willcox *et al*., 2009). *In vitro* studies confirmed that pancreatic islets can support persistent CVB infection which results in a production of IFNα (Chehadeh *et al*., 2000a), and a disturbance in the function of beta cells (Yin *et al*., 2002a).

Other mechanisms involving persistent CVB infection in T1D could include molecular mimicry and an inhibition of the trans-differentiation of pancreatic ductal cells. The hypothesis of molecular mimicry is supported by the homology between a conserved sequence of the enteroviral 2C protein and glutamate decarboxylase (GAD), an autoantigen frequently detected in T1D patients (Hou *et al*., 1994; Kaufman *et al*., 1992). This possibility has not been investigated further, since CVB infections have been associated with T1D only in some patients, and this autoantigen was also reported to share some homologies with other viral peptides (Hiemstra *et al*., 2001; Honeyman *et al*., 2010).

The trans-differentiation of pancreatic ductal cells is thought to be a renewal process of beta cells following a loss of these cells in a context of T1D, for example. An inhibition of this phenomenon could contribute to a rapid development of T1D (Lysy *et al*., 2013; Sane *et al*., 2013). Interestingly, our team has established a persistent CVB infection in a pancreatic ductal cell line (Panc-1 cells), and found that the persistent infection reduced the expression of Pdx-1, a transcriptional factor required for the differentiation of ductal cells (Sane *et al*., 2013).

EV Persistence in Other Tissues and Relationship with T1D

EV persistence in the intestine

After transmission most of time by oral route, CVB can replicate effectively in the gastrointestinal tract and especially in the intestine, and thereafter can spread from this site to the pancreas or other target organs. However, intestine is not just a crossing for the virus and there is some evidence that EV can establish a persistent infection in the intestine. Oikarinen *et al*. (2008b; 2012) have detected the presence of EV in the mucosa of small intestine of T1D patients but not in controls. Interestingly, patients remained EV positive 12 months after, and evidence of intense viral replication was not observed, suggesting a persistence of the virus in the gut of these patients (Oikarinen *et al*., 2012). In addition, enteroviral infection was associated to a chronic inflammation in the intestine (Oikarinen *et al*., 2012). This finding is compatible with previous reports which found an enhanced immune activation in the small intestine of T1D patients (Westerholm-Ormio *et al*., 2003). This environment could constitute a reservoir from which the virus spreads to the pancreas and triggers autoimmunity, since intestine is highly vascularized. Nevertheless, the hypothesis of the role of gut in the persistence of enteroviruses in patients with T1D should be investigated further, since data reported by Oikarinen *et al*. were not confirmed by those of another team (Mercalli *et al*., 2012). *In vitro*, the persistence of CVB in human intestinal cell line (Caco-2 cells) has been demonstrated (Harrath *et al*., 2004; Riabi *et al*., 2012). However, cells involved in the replication and the persistence of CVB *in vivo* in the intestine have not been precisely identified. In infected mice, the virus was reported to predominate in the lymphoid cells of the gut mucosa (Harrath *et al*., 2004).

EV persistence in blood cells

The blood is the main vehicle that spreads the virus in the whole body. The majority of epidemiological studies that investigated the relationship between EVs and T1D have focused on blood because it can be easily sampled by venipuncture. Thus, a large number of reports have found a more frequent detection of enteroviral RNA in the blood of T1D patients as compared to healthy individuals. EV RNA has been detected in the blood long before onset of clinical T1D and up to 6 months before the appearance of diabetes-associated autoantibodies (Oikarinen *et al*., 2011), and moreover the virus has been found both in recent and long-term diabetic patients (Yeung *et al*., 2011).

Most of these investigations were performed using whole blood or serum, and few authors focused on the blood cells that could harbor the virus (Chehadeh *et al*., 2000b; Salvatoni *et al*., 2013; Schulte *et al*., 2010; Toniolo *et al*., 2010; Yin *et al*., 2002b). EV RNA has been detected in peripheral blood mononuclear cells (PBMCs) of T1D at a relatively higher rate than in plasma or serum (Schulte *et al*., 2010; Yin *et al*., 2002b). In addition, EV RNA was still detected in blood beyond the stage of acute infection, after the detection of EV RNA in throat and stool samples was negative (Schulte *et al*., 2010). These data suggest that EVs can be detected in PBMCs during and after the "viremic" stage.

Experiments performed in our laboratory have shown that among PBMCs of T1D patients, EV RNA was harbored mainly by monocytes, which also displayed an increase in susceptibility to enteroviral infection *in vitro* (Alidjinou *et al*., submitted). Although monocytes are poorly permissive to enteroviral infection *in vitro*,

they can be efficiently infected under some circumstances, especially the presence of enhancing antibodies (Chehadeh *et al*., 2005; Hober *et al*., 2001). Moreover, our team has shown that CVB mixed with enhancing IgG can establish a persistent infection in a monocytic cell line (Goffard *et al*., 2013).

It can therefore be hypothesized that PBMCs and especially monocytes could constitute a reservoir contributing to the enteroviral infection or reinfection of target organs such as pancreas. Whether macrophages can play a role in the persistence of CVB deserves further investigations.

EV persistence in the thymus

The thymus, a primary lymphoid organ, is a major component of immune system and the site of initiation of self-tolerance. The self-antigens are expressed within the thymus, and self-tolerance is established during T-cell ontogeny by elimination of autoreactive T lymphocytes (negative selection). In addition, self-antigen-specific natural regulatory T cells (nTregs) are generated to inactivate periphery self-reactive T cells that have escaped negative selection (Klein *et al*., 2009). A disturbance of thymus function can initiate an autoimmune process, and since T1D is an autoimmune disease, it makes sense to explore the involvement of the thymus in its pathogenesis.

The thymus is a target for EV infection as supported by reports in humans (Cavalcante *et al*., 2010) and in animal models (Jaïdane *et al*., 2006). After inoculation by oral route in mice, CVB can infect the thymus and viral RNA is still detected until 70 days post-inoculation

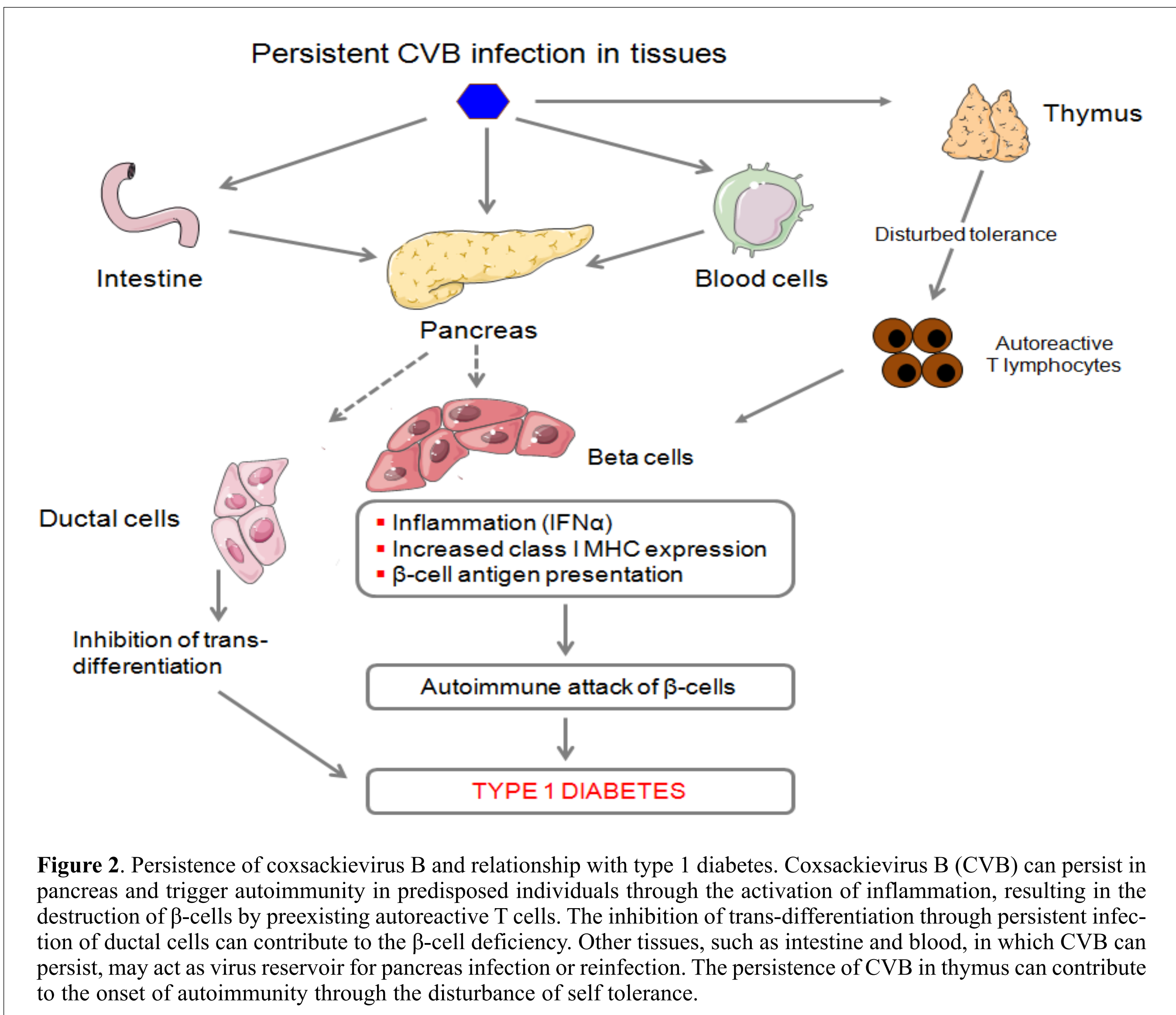

Figure 2. Persistence of coxsackievirus B and relationship with type 1 diabetes. Coxsackievirus B (CVB) can persist in pancreas and trigger autoimmunity in predisposed individuals through the activation of inflammation, resulting in the destruction of β-cells by preexisting autoreactive T cells. The inhibition of trans-differentiation through persistent infection of ductal cells can contribute to the β-cell deficiency. Other tissues, such as intestine and blood, in which CVB can persist, may act as virus reservoir for pancreas infection or reinfection. The persistence of CVB in thymus can contribute to the onset of autoimmunity through the disturbance of self tolerance.

(Jaïdane *et al*., 2006).

In vitro, human epithelial thymic cells can be infected by various strains of CVB4. The virus can replicate and persist in these cells, and induces the production of interleukin (IL)-6, leucocyte migration inhibition factor (LIF), and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Brilot *et al*., 2002). CVB4 can infect immature thymocytes in human fetal thymus, which results in an increased expression of class I MHC molecules and a severe depletion of thymocytes (Brilot *et al*., 2004).

The CVB infection of murine thymic cells *in vitro* was reported to disturb the T-cell maturation and differentiation processes (Brilot *et al*., 2008; Jaïdane *et al*., 2012a).

Recently our team established a persistent CVB4 infection in murine thymic epithelial cell line. The infection led to a decrease in the production of type 2 insulin-like growth factor (Igf2), the dominant polypeptide of the insulin family, which has a tolerogenic effect towards insulin (Jaïdane *et al*., 2012b). A defect of Igf2 expression in the thymus was suggested to play a role in the development of autoimmune diabetes in a BBDP rat model (Kecha-Kamoun *et al*., 2001).

These data suggest that it cannot be excluded that a persistent CVB infection of the thymus could disturb self-tolerance at the central level, and could then play a role in the pathogenesis of T1D.

The link between EV persistence and T1D is summarized in Figure 2.

Conclusion

Enteroviruses can be involved in acute and lytic infections, but they can also persist in tissues through an adaptation of characteristics of both virus and host cell. This persistence is thought to be the main mechanism in the pathogenesis of chronic enterovirus-related diseases. EVs and especially CVB can persist in pancreas, leading to, in predisposed individuals, a progressive and moderate inflammatory response that can activate the beta-cell autoimmune destruction process by preexisting cytotoxic T cells. In addition, a persistence of CVB can also occur in other sites such as intestine or blood cells that could serve as a reservoir for infection or reinfection of pancreas, and in thymus resulting in a defect of central self-tolerance that could lead to autoimmune diseases such as T1D.

Further *in vivo* and *in vitro* studies are still needed for a better understanding of the molecular mechanisms of enteroviral persistence in these tissues, and its contribution to the pathogenesis of T1D.

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Disclosure

The authors report no conflicts of interest.

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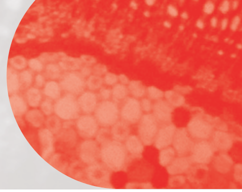

L'infection virale joue-t-elle un rôle dans la genèse du diabète de type 1 ?

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RÉSUMÉ

Le rôle des virus dans le développement du diabète de type 1 (DT1) est suspecté depuis de nombreuses années. Certains d'entre eux peuvent provoquer une altération des cellules β des îlots pancréatiques qui produisent l'insuline et induire l'apparition d'auto-anticorps et d'un DT1 auto-immun : *Herpesviridae, Parvoviridae, Retroviridae, Togaviridae, Paramyxoviridae* et *Picornaviridae.* Certains sont des virus animaux qui déclenchent chez la souris ou le rat un DT1, comme le virus Kilham du rat, le rotavirus simien, le virus de l'encéphalomyocardite et le virus Ljungan, ces deux derniers appartenant à la famille des *Picornaviridae.* Ces modèles sont riches d'enseignement concernant la pathogenèse virale de la maladie. Chez l'Homme, les entérovirus, qui font partie aussi de la famille des *Picornaviridae,* font l'objet d'une attention particulière car leur implication dans le DT1 est fortement suspectée. Des études épidémiologiques ont mis en évidence une association entre ces virus, plus particulièrement les coxsackievirus B, et la maladie. De l'ARN et des protéines des entérovirus ainsi que des marqueurs d'infection virale ont été détectés dans les cellules β pancréatiques des patients. Des travaux expérimentaux ont permis d'établir les mécanismes permettant à ces agents infectieux de contribuer à la pathogenèse de la maladie.

MOTS-CLÉS : diabète de type 1, virus, entérovirus, coxsackievirus B, inflammation, persistance, auto-immunité, pancréas.

I. - INTRODUCTION

Le diabète de type 1 (DT1) est une maladie chronique due à un défaut de production d'insuline consécutif à une destruction ou à une altération de fonction sélective des cellules β du pancréas. Il survient habituellement après une longue phase préclinique asymptomatique. Le DT1A, que nous nommons DT1 dans cet article, est une maladie auto-immune qui implique le rôle d'effecteurs de la réponse immunitaire et une altération de la tolérance vis-à-vis d'antigènes des cellules β. La prédisposition au DT1 est influencée par des facteurs génétiques, les plus importants étant situés sur les loci HLA-DR et DQ (1). Néanmoins, la maladie ne survient que chez une faible proportion d'individus avec ce patrimoine génétique, ce qui suggère le rôle de facteurs exogènes. De nombreux arguments plaident en faveur de l'implication de facteurs environnementaux dans le développement du DT1. Ainsi, il a été observé des différences dans la pénétrance et dans l'âge de survenue de la maladie chez les jumeaux homozygotes. L'étude des migrants montre également que ces derniers acquièrent un risque de développement de la maladie similaire à celui du nouveau pays d'accueil. De même, l'augmentation rapide durant ces dernières décennies de l'incidence du DT1, notamment dans les pays du Nord, ainsi que les différences d'incidence de la maladie d'un pays européen à l'autre ne peuvent pas être attribuables qu'à des changements environnementaux ou à la réponse des individus à ces variations (2). Plusieurs facteurs exogènes ont été incriminés (des protéines du lait de vache, la carence en vitamine D, le gluten, les nitrites) (3) et, en raison de l'épidémiologie de la maladie, notamment de la distribution

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des cas dans le temps et dans l'espace, l'implication de virus dans le DT1 a été évoquée : cet article les recense et développe plus particulièrement les entérovirus dont le rôle dans la maladie est fortement suspecté.

II. - DES VIRUS APPARTENANT À DIFFÉRENTES FAMILLES SONT ASSOCIÉS AU DT1

Nous proposons d'examiner les principales familles de virus incriminées en commençant par les virus à ADN, *Herpesviridae* (virus enveloppés, à ADN double brin) et *Parvoviridae* (virus dépourvus d'enveloppe, à ADN simple brin). Nous poursuivrons avec les virus à ARN, *Retroviridae, Togaviridae* et *Paramyxoviridae* (virus enveloppés, à ARN simple brin), puis avec ceux qui sont davantage suspectés, *Reoviridae* (virus non enveloppés, à ARN double brin segmenté) et surtout *Picornaviridae* (virus non enveloppés, à ARN simple brin).

A) *Herpesviridae*

Des travaux dans des modèles animaux suggèrent que le cytomégalovirus (CMV) de rat peut induire un DT1 chez ce rongeur (4-6), et l'hypothèse qu'une réaction croisée entre des lymphocytes T anti-CMV et anti-GAD65 (auto-antigène des cellules β) pourrait jouer un rôle dans le développement de la maladie a été émise. Des épitopes d'antigènes du virus pourraient être présentés par les cellules dendritiques et reconnus par des cellules T anti-GAD65 (7). Cependant, les infections périnatales à CMV ne sont ni associées à des marqueurs sérologiques d'auto-immunité, ni à l'évolution en DT1 chez les enfants ayant un génotype HLA à risque (8).

B) *Parvoviridae*

Le virus Kilham du rat (KRV) est un virus de la famille des *Parvoviridae* qui induit un DT1 auto-immun chez le rat *BioBreeding Diabetes-Resistant* (BBDR) ne développant pas la maladie par ailleurs (9, 10). Il a été montré que le virus perturbe la tolérance vis-à-vis des cellules β, avec diminution du nombre de lymphocytes Treg ($CD4^+CD25^+$) spléniques, mais que le mimétisme moléculaire ne joue pas de rôle dans la pathogenèse de la maladie (11, 12). Le développement du DT1 chez le rat BBDR provoqué par KRV repose sur des perturbations du système immunitaire induites par la stimulation du récepteur TLR9 (13, 14). Depuis de nombreuses années, il était admis que la destruction des îlots induite par KRV ne reposait pas sur l'infection des cellules β (15). Or, il a été récemment constaté qu'au cinquième jour suivant l'inoculation de KRV au rat Lewis LEW1.WR1, de l'ADN et des protéines du virus sont détectés dans les cellules β des îlots et que l'immunité innée impliquant TLR9 et la voie NF-κB est activée (12, 16). Ainsi, l'infection des cellules β joue un rôle dans les mécanismes physiopathologiques du diabète induit par ce virus. Chez l'Homme, des *Parvoviridae* pourraient intervenir dans le DT1 car la maladie a été observée chez des sujets infectés par le parvovirus B19 (17-19) : elle serait la conséquence de l'effet lymphoprolifératif (cellules T) de ce virus et /ou d'une modulation de la réponse auto-immune chez des sujets prédisposés (20, 21).

C) *Retroviridae*

Dix pour cent du génome humain est constitué de séquences de rétrovirus endogènes. L'impact des *Human Endogenous Retroviruses* (HERVs) dans l'auto-immunité peut se concevoir de plusieurs manières. La transcription et la traduction de gènes d'HERVs peuvent aboutir à l'expression de protéines qui stimulent l'apparition d'auto-anticorps reconnaissant des antigènes du soi. L'insertion de séquences d'HERVs, à proximité de gènes impliqués dans la régulation du système immunitaire, peut contribuer au déclenchement de processus pathologiques. En outre, il a été rapporté que des HERVs induisent la production de cytokines inflammatoires, notamment par des monocytes (22). L'ARNm d'HERV-K18 a été trouvé en quantité plus élevée chez les patients avec un diabète récent que chez les sujets contrôles, mais le lien avec la maladie reste à élucider (23). HERV-K18 n'est pas transcrit en principe dans des conditions normales, mais il peut l'être par un effet direct des *Herpesviridae* EBV et HHV-6 ou indirect par le biais de l'IFNα induit par ces virus (24, 25).

D) *Togaviridae*

Une récente étude clinique a mis en évidence une association entre l'incidence du DT1 et la rubéole chez des enfants (26). Elle corrobore les données expérimentales obtenues chez le hamster nouveau-né infecté par le virus rubéoleux qui développe une réaction auto-immune et un diabète (27). Il convient toutefois de noter que des marqueurs d'auto-immunité (anticorps anti-îlots, anti-insuline, anti-GAD65) n'ont pas été détectés chez 37 sujets avec un syndrome de rubéole congénitale ou exposés au virus durant la vie fœtale (28).

E) *Paramyxoviridae*

Des anticorps anti-îlots ont été décelés dans le sérum d'enfants infectés par le virus ourlien, et l'un d'entre eux a évolué vers un DT1 trois semaines après la survenue des oreillons (29). Une séquence de 7 acides aminés de l'une des protéines virales est partagée avec la chaîne invariante constitutive du complexe majeur d'histocompatibilité de classe II, et elle pourrait être à l'origine d'une réaction immunologique croisée vis-à-vis de ce constituant essentiel du système immunitaire (30).

F) *Reoviridae*

Chez des enfants devenus diabétiques ou à haut risque de développer la maladie (avec des marqueurs sériques d'auto-immunité), une étude prospective a montré qu'une augmentation du titre des auto-anticorps était associée à la détection (ou à l'augmentation du titre) d'IgG ou d'IgA anti-rotavirus sériques (31). De plus, les profils de prolifération des cellules T répondeuses à des peptides de la pro-

téine capsidale VP7 du rotavirus et à des épitopes des protéines IA-2 et GAD65 sont semblables (32). Ces observations suggèrent que l'infection par ce virus pourrait déclencher ou exacerber l'auto-immunité anti-îlots par mimétisme moléculaire.

Chez des souris NOD, un rotavirus simien administré par voie orale accélère le développement du DT1. Chez ces animaux, l'infection est asymptomatique et sans virémie, et le virus n'est pas détecté dans le pancréas (33). Dans ce modèle, il se propage aux ganglions lymphatiques mésentériques et pancréatiques, active les cellules présentatrices d'antigènes et stimule une réponse de type Th1 induisant une production d'anticorps et de cytokines (34). De plus, le virus stimule des lymphocytes T auto-réactifs, anti-îlots de pancréas (35). Il reste à démontrer la pertinence de ces résultats pour rendre compte du rôle éventuel des rotavirus humains dans le DT1 de l'Homme. Il est intéressant à ce titre de noter qu'à l'instar de l'ARN de rotavirus simien présent dans les ganglions lymphatiques de la souris NOD, l'ARN de rotavirus humain est détecté dans les ganglions lymphatiques mésentériques d'enfants (34, 36).

G) *Picornaviridae*

- Le genre *Cardiovirus*

Le virus de l'encéphalomyocardite (EMCV) est capable de provoquer un diabète d'installation très rapide qui s'apparente au DT1 fulminant, un diabète non auto-immun (37). Deux variants d'EMCV ont été décrits : EMCV-B, non diabétogène et EMCV-D, diabétogène. Ce dernier a un tropisme préférentiel pour les cellules β du pancréas et induit un diabète chez la souris, DBA/2 notamment (38). Le caractère diabétogène du virus est conféré par un changement d'un seul acide aminé en position 152 de la protéine de capside VP1 (39), la thréonine remplacée par l'alanine (40). Cette substitution facilite la liaison de la protéine capsidale au récepteur du virus présent à la surface des cellules β et de ce fait, l'infection de celles-ci (41).

Le rôle d'acteurs de l'immunité innée dans le diabète induit par l'EMCV a été étudié : *Melanoma Differentiation-Associated gene-5* (MDA5), produit du gène *IFIH-1,* détecteur cytoplasmique d'ARN viral spécialisé dans la perception des *Picornaviridae* et qui stimule la synthèse d'IFNα ; *Toll-Like Receptor 3* (TLR3), capteur endosomal d'ARN double brin ; et IFNα. Dans le modèle murin de diabète induit par EMCV-D, au cours duquel le virus altère directement les cellules β, le rôle préventif de MDA5 et TLR3 a été rapporté (38). Les auteurs de cette étude émettent l'hypothèse que dans le cas d'infection à virus avec un tropisme pour les cellules β, un défaut de réponse de ces détecteurs, notamment de TLR3, conduirait à une faible stimulation d'IFNα ayant pour conséquence une réplication virale accrue et un diabète. À l'opposé, dans le diabète auto-immun, les détecteurs d'ARN double brin seraient sollicités par des acides nucléiques viraux au cours d'infections ne ciblant d'ailleurs pas forcément les cellules β. La stimulation excessive de l'IFNα qui en découle, serait capable d'activer des cellules T auto-réactives latentes (38).

- Le genre *Parechovirus*

Le genre *Parechovirus* comporte notamment 16 virus humains dont les anciens échovirus 22 et 23 (devenus paréchovirus humain 1 et paréchovirus humain 2), et un virus animal, le virus Ljungan. Ce virus de rongeurs (*Myodes glareolus* ou campagnol roussâtre), découvert dans les années 1990, provoque un DT1 auto-immun (auto-anticorps anti-GAD, IA-2, et insuline) et des protéines virales sont décelées dans les îlots pancréatiques (42). Le rat *BioBreeding Diabetes-Prone* (BBDP), issu de la lignée Wistar, développe un diabète de type 1 avec acidocétose (43), qui est la conséquence de l'infection virale. En effet, la présence d'antigènes du virus Ljungan dans les cellules β du rat BBDP a été rapportée, et le diabète peut être prévenu quand le rongeur est traité par le pléconaril, un inhibiteur de décapsidation actif notamment sur certains entérovirus, ou la ribavirine dont le spectre d'action est large (44, 45). L'intérêt pour ce virus animal a été amplifié lorsque d'une part, des anticorps spécifiques ont été découverts chez des patients avec un diabète récemment diagnostiqué (42) et d'autre part, une association entre la présence de ces anticorps et d'anticorps anti-insuline a été mise au jour chez des sujets HLA-DQ8 qui avaient débuté un diabète avant l'âge de 10 ans (46).

Concernant les paréchovirus humains, une étude prospective regroupant des enfants ayant développé une auto-immunité vis-à-vis des cellules β (anticorps anti-GAD65 ; anit-IA2 et anti-insuline) n'a pas dépisté (par RT-PCR) l'ARN viral plus fréquemment dans leurs selles par rapport aux sujets contrôles, sauf dans les échantillons collectés 3 mois avant la séroconversion (OR = 3,17, p = 0,022) (47).

H) Conclusion

Des virus appartenant à des familles distinctes sont capables d'induire une production d'auto-anticorps anti-cellules β et même, pour certains, de provoquer un DT1 chez l'Homme. D'autres virus infectent des animaux qui développent alors un DT1 (Tableau). Ces modèles expérimentaux ont permis d'étudier les mécanismes par lesquels des virus peuvent initier ou aggraver le développement de la maladie. Ainsi, l'infection des cellules β du pancréas déclenchant la libération d'auto-antigènes, le mimétisme moléculaire entre des composants viraux et des protéines des cellules β, ou encore l'activation passive (*bystander activation*) de lymphocytes T auto-réactifs, font partie des processus mis en œuvre. Nous avons mentionné le pouvoir diabétogène de deux *Picornaviridae,* l'EMVC et le virus Ljungan, et la suite de cette revue sera consacrée à d'autres virus de cette même famille mais faisant partie du genre *Enterovirus,* dont le rôle dans le DT1 est fortement suspecté.

III. - *ENTÉROVIRUS* (EV) ET DT1

L'établissement du lien entre EV et DT1 remonte aux années 1960, lorsque Gamble *et al.* (48) ont constaté une fréquence plus élevée d'anticorps neutralisants anti-coxsackievirus B4 (CVB4) chez les sujets DT1 que chez des

Tableau - Principaux virus associés au diabète de type 1.
Les virus sont regroupés en fonction de leurs caractères structuraux, acide nucléique et enveloppe et en fonction de la famille à laquelle ils appartiennent. Dans le cas des virus de la famille des *Picornaviridae,* le genre (*) est indiqué.

	Virus humains	Virus animaux
Virus à ADN		
Enveloppé, ADN double brin		
Herpesviridae	*Cytomegalovirus*	
Non enveloppé, ADN simple brin		
Parvoviridae	*Parvovirus* B19	Virus Kilham du Rat (KRV)
Virus à ARN		
Enveloppé, ARN simple brin		
Togaviridae	Virus de la rubéole	
Paramyxoviridae	Virus des oreillons	
Retroviridae	HERVK[1]	
Non enveloppé, ARN double brin segmenté		
Reoviridae	*Rotavirus*	*Rotavirus*
Non enveloppé, ARN simple brin		
Picornaviridae		
*Cardiovirus**		Virus de l'encéphalomyocardite (EMCV)
*Parechovirus**	*Parechovirus*	Virus Ljungan
*Enterovirus**	*Enterovirus*	

[1]HERVK : *Human Endogenous Retrovirus K,* séquence d'acide nucléique rétroviral endogène présente sous forme d'ADN intégré dans le génome cellulaire.

témoins. Mais la principale observation historique, ayant donné un coup d'accélérateur aux travaux sur le rôle des EV dans le DT1, date de 1979 quand CVB4 a été isolé du pancréas d'un enfant décédé d'une acidocétose diabétique et que l'inoculation à la souris de ce virus a déclenché un diabète auto-immun (49). Un an plus tard, un cas similaire impliquant coxsackievirus B5 fut rapporté (50). Depuis, des données épidémiologiques, clinico-biologiques et expérimentales se sont accumulées et l'association entre les EV et le DT1 est la plus convaincante et la plus documentée parmi toutes celles précédemment rapportées.

A) Brève présentation du genre

Le genre *Enterovirus* regroupe actuellement plus de 250 petits organismes (environ 30 nm) comprenant, dans une capside icosaédrique constituée de 4 protéines (VP1-4) et non recouverte d'une enveloppe, un ARN dit « positif » (Figure 1). Certains d'entre eux sont des pathogènes humains majeurs, le plus connu étant le virus de la poliomyélite. Sur la base d'analyses phylogénétiques, la classification actuelle du genre individualise 7 espèces pouvant entraîner des maladies chez l'Homme : HEV (*Human Enterovirus*) A, B, C et D et *Rhinovirus* A-C. Les coxsackievirus B (1-6) appartiennent à l'espèce HEV-B (51, 52). La taxonomie des entérovirus est en constante évolution (www.picornaviridae.com). Le terme EV utilisé dans cet article fait essentiellement référence aux EV « non-poliomyélitiques » des espèces HEV A-D.

Les EV sont transmis principalement par voie oro-fécale et l'incubation de la maladie qu'ils induisent peut varier de 2 à 30 jours. Après une réplication primaire dans la muqueuse intestinale, les virus peuvent se propager vers divers organes cibles (53) qui déterminent les types de symptômes. La plupart des infections entérovirales sont asymptomatiques ou responsables de manifestations cliniques bénignes : hyperthermie isolée, pharyngite, céphalées, myalgies, troubles gastro-intestinaux ou éruption cutanée. Néanmoins, ces virus peuvent être impliqués dans des pathologies aiguës sévères (méningite, encéphalite, myocardite, pancréatite, hépatite) (52, 54), ainsi que dans la pathogenèse d'affections chroniques notamment la myocardite chronique pouvant évoluer vers une cardiomyopathie dilatée, et le DT1. L'ARN entéroviral peut être dépisté dans des tissus par RT-PCR, en recourant à des amorces oligonucléotidiques reconnaissant des séquences communes à l'ensemble des EV et qui sont situées dans la partie 5' non codante du génome. Lorsque la quantité d'ARN viral dans les échantillons biologiques est suffisante, le séquençage du produit obtenu par PCR permet de préciser le virus en cause.

B) Des marqueurs d'infection entérovirale sont fréquemment décelés chez les patients avec un DT1

L'un des arguments majeurs qui supporte fortement un lien entre les EV et le DT1 est la prévalence plus élevée des marqueurs d'infection entérovirale chez les malades que chez les sujets sains. Les fréquences rapportées varient largement dans la littérature et dépendent de la technique utilisée ainsi que de la nature du produit biologique analysé ; néanmoins, la majorité des données disponibles convergent vers leur détection plus fréquente chez les patients DT1. Une méta-analyse d'environ 4 500 patients a confirmé l'association statistiquement significative entre l'infection à EV, principalement à coxsackievirus B, et l'auto-immunité associée au DT1 (Odds ratio: 3,7) et le développement de la maladie (Odds ratio: 9,8) (55).

- Dans le sang

La majorité des études de prévalence disponibles ont été réalisées à partir du sang périphérique de patients DT1 et plusieurs auteurs y ont décelé de l'ARN entéroviral plus fréquemment que chez les sujets contrôles (55), associé de façon significative à l'auto-immunité anti-îlot pancréatique (56). Des études prospectives ont démontré la présence d'ARN entéroviral dans le sang des patients bien en amont de l'apparition de l'auto-immunité anti-îlot et du développement du DT1 (57, 58). La détection d'ARN a été rapportée aussi bien chez des patients débutant la maladie que chez des diabétiques anciennement diagnostiqués (55). Dans la plupart des études, l'ARN entéroviral a été recherché dans le sang total ou dans le sérum/plasma ; toutefois, dans quelques unes, l'acide nucléique a été pisté dans les cellules sanguines (59-63) et a été décelé plus volontiers dans les cellules mononucléées que dans le sérum/plasma (61, 62). Les quantités d'ARN étaient basses dans le sang et indétectables dans les prélèvements pharyngés et dans les selles. Ces observations suggèrent que la liaison entre EV et DT1 dépasse le cadre de l'infection aiguë, et sont en faveur de l'hypothèse d'une persistance tissulaire du virus dont le taux de réplication cellulaire est bas (64). Nous avons récemment rapporté que les cellules contenant l'ARN entéroviral dans le sang périphérique des patients sont les monocytes et qu'ils sont davantage susceptibles, comme les macrophages qui en dérivent, d'être infectés par CVB4 *in vitro* que ceux de sujets contrôles (63, 65) et que l'infection des monocytes est facilitée par des anticorps dirigés contre la protéine de capside VP4 (66, 67).

- Dans le pancréas

Le DT1, avec le défaut de production d'insuline par les cellules β qui le caractérise, se présente comme une pathologie du pancréas endocrine. L'étude de cet organe est donc pertinente pour mieux comprendre le lien entre EV et DT1. Leur tropisme pancréatique est bien connu mais, chez l'Homme, les preuves directes d'une infection entérovirale du pancréas endocrine, au moment du diagnostic ou au cours de l'évolution de la maladie, sont difficiles à obtenir compte tenu du caractère invasif du geste nécessaire pour biopsier cet organe. De ce fait, les données disponibles proviennent davantage de nécropsies que de biopsies réalisées chez des patients vivants. Ainsi, Ylipaasto *et al.* (68) ont mis en évidence la présence d'ARN d'EV dans le pancréas endocrine de 4 parmi 65 patients. Par immunohistochimie, Dotta *et al.* (69) ont rapporté la présence de la protéine de capside virale VP1 dans 3 échantillons pancréatiques de patients nouvellement diagnostiqués ou ayant subi une greffe de pancréas. En recourant à cette même approche, Richardson *et al.* (70) ont décelé, lors de l'analyse de 72 échantillons pancréatiques issus de patients atteints de DT1, cette même protéine dans 44 d'entre eux (soit 61 %) et seulement dans 3 parmi 39 (soit 7,7 %) échantillons témoins ; ce marquage était limité aux seules cellules β des îlots de Langerhans (70, 71). Tout récemment, l'étude de biopsies pancréatiques

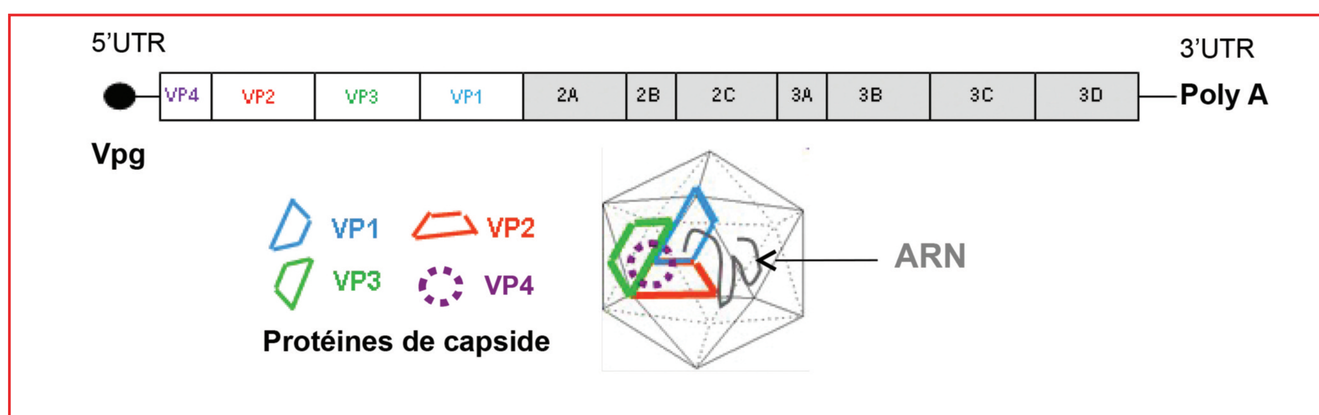

Fig. 1 - Représentation schématique du génome et de la structure des entérovirus.
Le génome à ARN simple brin positif, constitué de 7 400 bases environ, comporte un seul cadre de lecture flanqué aux extrémités de deux régions non codantes qui sont dénommées UTR (pour *UnTranslated Region*) : l'extrémité 5' comprend une région interagissant avec la petite sous-unité du ribosome (IRES) et l'extrémité 3' présente une queue poly A. Le génome est traduit en une polyprotéine qui, après clivage, génère 7 protéines non structurales (en gris) et 4 protéines structurales (VP1, VP2, VP3 et VP4) formant un capsomère répété 60 fois pour constituer la capside de symétrie icosaédrique. La protéine VP4 située à l'intérieur de la capside est représentée en pointillés. À l'extrémité de la zone 5' non codante est liée la protéine Vpg issue du gène 3B.

réalisées chez des sujets vivants a révélé la présence d'ARN entéroviral dans les îlots de 4 parmi 6 patients DT1 et chez aucun sujet contrôle (72).

Au-delà de la détection de composants viraux, des signes indirects d'infection virale ont été constatés dans le pancréas au début et au cours de l'évolution du DT1 : expression d'interféron α et des protéines antivirales induites comme la protéine kinase R, surexpression des molécules du complexe majeur d'histocompatibilité de classe I ou de capteurs de l'immunité innée (73).

Des initiatives internationales comme nPOD (*Network for Pancreatic Organ Donors with Diabetes,* http://www.jdrfnpod.org/) and PEVNET (*persistent virus infection as a cause of pathogenic inflammation in T1D – an innovative research program of biobanks and expertise,* http://www.uta.fi/med/pevnet/index.html, 7e PCRDT CE) ont pour objectif de rassembler des prélèvements de patients et des données concernant l'infection à EV chez ces individus.

- Dans les selles et dans les intestins

Après transmission oro-fécale des EV, leur réplication primaire se déroule dans l'intestin et ils sont fréquemment détectés dans les selles au cours d'une infection aiguë, symptomatique ou non. Il n'a pas été démontré d'association entre la détection d'ARN entéroviral dans les selles et la progression vers le DT1 (56). Cependant, Oikarinen *et al.* ont rapporté la présence d'ARN entéroviral dans la muqueuse de l'intestin grêle de patients DT1 (mais pas chez des sujets contrôles) et pendant plus de 12 mois, sans aucun signe de réplication intense, suggérant une persistance du virus (74, 75). Une telle observation n'a pas été confirmée par d'autres chercheurs et des études supplémentaires sont donc nécessaires pour explorer l'hypothèse d'une infection intestinale persistante à EV (76).

C) Un mécanisme complexe qui met en jeu une prédisposition génétique, le système immunitaire de l'hôte et le virus

Il est admis que l'atteinte sélective des cellules β dans le DT1 est la conséquence d'un processus auto-immun (77). La contribution des EV à la genèse du DT1 ne doit donc pas être envisagée comme résultant d'une infection virale lysant massivement le pancréas ; une lyse pancréatique peut, par contre, être impliquée dans le diabète fulminant qui n'est pas auto-immun et qui survient chez des patients asiatiques, notamment japonais (78).

Plusieurs mécanismes ont été proposés pour expliquer l'implication des EV dans le DT1, notamment : (a) le mimétisme moléculaire dû à l'homologie entre la protéine entérovirale conservée 2C et la glutamate décarboxylase GAD65, un auto-antigène des cellules β ; (b) l'activation passive (*bystander activation*) de lymphocytes T auto-réactifs préexistants, grâce à des phénomènes inflammatoires et (c) la persistance virale (79). Le scénario retenu actuellement pour comprendre la pathogenèse entérovirale du DT1 combine les deux dernières hypothèses et repose sur l'induction de l'auto-immunité via l'activation virale de processus inflammatoires. Une infection persistante, sans lyse cellulaire, est capable d'induire une inflammation des îlots (production d'interféron α et d'autres cytokines pro-inflammatoires) et une surexpression des molécules du complexe majeur d'histocompatabilité de classe I. Cette inflammation pourrait s'accompagner d'une présentation accrue d'auto-antigènes par les cellules β et de leur destruction par des lymphocytes T $CD8^+$ cytotoxiques auto-réactifs (1). De tels lymphocytes T, spécifiques des cellules β préexistent chez des sujets génétiquement prédisposés (80). Les modalités de l'interaction entre le virus et le système immunitaire, notamment inné, jouent un rôle majeur dans la pathogenèse. Ainsi, une réponse initiale très forte pourrait prévenir une infection productive efficace chez l'hôte et une dissémination du virus vers le pancréas. En revanche, une réponse modérée ou faible permettrait au virus d'atteindre cet organe cible, voire d'y persister et d'initier une réponse inflammatoire notamment via l'activation des TLR (81). Par ailleurs, l'interaction entre le virus et le système immunitaire semble également dépendre de facteurs génétiques. Certains polymorphismes du gène *IFIH1* (*Interferon-Induced Helicase 1*) ont été associés de façon significative à un risque élevé de DT1. Ce gène code pour la protéine MDA5, un récepteur intra-cytoplasmique responsable d'une activation de l'inflammation en réponse aux EV et notamment aux coxsackievirus B qui sont les plus incriminés dans le DT1 (82). Récemment, le rôle de l'activation de l'immunité innée dans l'initiation de l'auto-immunité anti-îlot a été conforté par la mise en évidence d'une signature transcriptionnelle interféron de type 1 dans les cellules du sang périphérique, avant l'apparition des auto-anticorps chez les sujets prédisposés (83, 84) (Figure 2).

Des résultats expérimentaux, obtenus *in vitro* et *in vivo,* permettent d'étayer, au moins en partie, l'hypothèse d'une implication des EV dans le DT1. Les cellules β des îlots pancréatiques humains peuvent être infectées *in vitro* par les coxsackievirus B qui peuvent y persister et induire la production d'interféron α (64, 85). En outre, une infection chronique des cellules canalaires du pancréas peut être réalisée par le coxsackievirus B4, altérant alors leur capacité à se différencier en cellules endocrines et à former des amas cellulaires ressemblant à des îlots (86). L'infection cellulaire *in vitro* peut être potentialisée par des anticorps facilitateurs, susceptibles de participer à la dissémination du virus dans l'organisme (87). Par ailleurs, le rôle de l'infection à coxsackievirus B dans l'apparition de lymphocytes T auto-réactifs a été étudié *in vivo* et *in vitro* par nous-mêmes, en collaboration avec le groupe de recherche liégeois du Pr Vincent Geenen. Nous avons notamment rapporté que le virus B4 peut infecter le thymus et y persister, aboutissant à une perte de la tolérance vis-à-vis du soi et à l'initiation du processus auto-immun ; par ailleurs, des anomalies des cellules thymiques *in vitro* et des troubles de maturation des lymphocytes T dans des cultures de thymus fœtal ont été notées, avec en particulier une inhibition de l'expression d'IgF2, une protéine tolérogène de la famille de l'insuline (88-93).

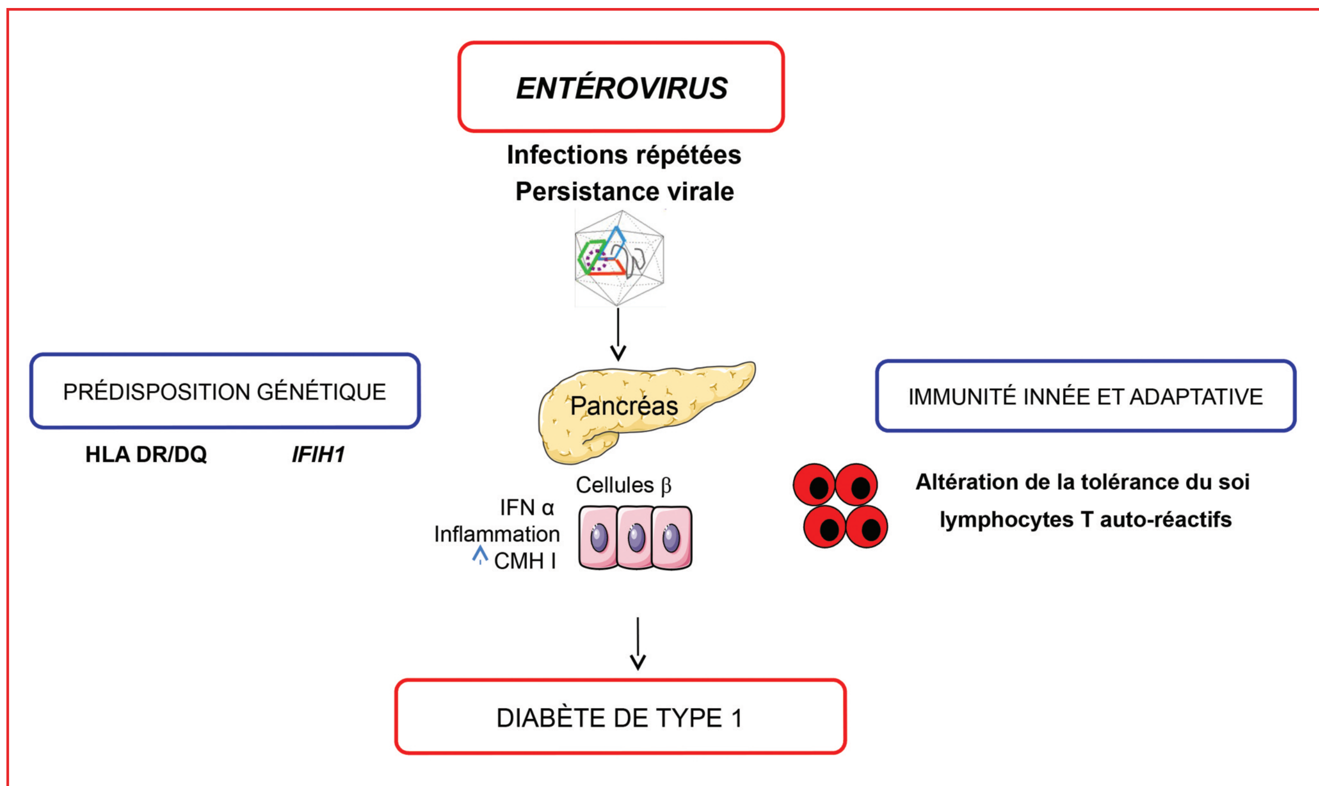

Fig. 2 - Entérovirus et pathogenèse du diabète de type.
L'impact de la prédisposition génétique, du système immunitaire inné et acquis et des entérovirus est présenté. Des infection répétées et /ou persistantes des cellules β provoquent une production d'interféron alpha (IFNα), une réaction inflammatoire, une surexpression d'antigènes du complexe majeur d'histocompatibilité de classe I (CMH I). L'infection des cellules β a pour conséquence une réaction auto-immune dirigée contre ces cellules.

D) Des questions en attente de réponses !

De nombreux arguments en faveur d'un lien entre les EV et le DT1 existent mais des zones d'ombre subsistent.

- Sont-ils des agents initiateurs ou des facteurs aggravants dans le développement du DT1 ?

Les EV peuvent initier le processus auto-immun à la faveur d'infections successives ou persistantes. Par ailleurs, dans la mesure où il a été observé une progression de l'auto-immunité anti-îlot vers le DT1 au décours d'infections entérovirales, il ne peut pas être exclu que les virus jouent un rôle aggravant dans le développement de l'auto-immunité. Ainsi, les virus interviennent probablement à la fois en tant qu'agent initiateur et aggravant dans la pathogenèse de la maladie.

- Quelles espèces ou sérotypes sont impliqués ?

Parmi les entérovirus, les coxsackievirus B sont les plus incriminés, mais les échovirus ont été également isolés chez des patients. Parmi les 6 sérotypes de coxsackievirus B, le sérotype 4 est fortement suspecté mais les autres sérotypes ne peuvent être exclus. Une étude européenne de séroprévalence plaide en faveur du rôle du sérotype B1 (1, 94). Les quantités de virus isolés de patients sont habituellement faibles et le typage difficile : la mise au point de marqueurs spécifiques et de méthodes de détection sensibles permettrait d'apporter de nouveaux éléments de réponse.

- Existe-t-il une corrélation entre l'épidémiologie des infections à EV et l'incidence du DT1 ?

La répartition géographique des infections à EV n'est pas superposable à l'apparition des cas de DT1. Néanmoins, à la faveur du suivi d'individus infectés par des échovirus, l'apparition d'auto-anticorps a été mise en évidence, avec cependant une rare survenue d'un DT1 (95). Par ailleurs, une équipe italienne a montré que les membres de familles de patients avec un DT1 partageaient le même sérotype d'entérovirus : 4 parmi 20 avaient développé la maladie dans les 3 à 25 mois suivant la détection sanguine d'ARN entéroviral (60).

Il a été constaté dans les pays occidentaux une corrélation inverse entre l'incidence du DT1, qui est en augmentation, et la fréquence des infections à EV. Cette observation, *a priori* paradoxale par rapport à l'hypothèse du rôle de ces virus dans le DT1, pourrait en réalité être compatible avec la théorie de l'hygiène, évoquée initialement à propos de la prévalence accrue des maladies allergiques dans les pays développés. Selon cette théorie, l'amélioration des conditions sanitaires pourrait entraîner

une diminution de la circulation des EV et donc de l'immunité à leur égard dans la population. Le maintien d'une immunité anti-EV optimale dans la population pourrait avoir un effet bénéfique sur l'incidence du DT1, et constitue un argument pour la mise au point de stratégies vaccinales contre les EV. La relation entre les EV et le DT1 s'inscrit dans le cadre de la notion de virome humain. Dans ce modèle, les individus sont exposés fréquemment à des virus banals comme les EV, provoquant des infections des muqueuses, intestinale en l'occurrence, qui sont asymptomatiques ou modérées dans la plupart des cas. Le virome est un composant de l'environnement interagissant avec les gènes de l'hôte qui peut être impliqué dans la pathogenèse de maladies tel que le DT1 (96).

IV. - CONCLUSION

Des virus peuvent jouer un rôle dans le développement du DT1 qui est une maladie multifactorielle. Un impact majeur des virus du genre *Enterovirus* et notamment des coxsackievirus B est fortement suspecté. Une association entre ces virus et la maladie est documentée par des études épidémiologiques, et les résultats de travaux expérimentaux chez l'animal plaident en faveur de leur rôle dans la pathogenèse de la maladie. Il reste à déterminer s'ils interviennent en tant qu'agent déclenchant et /ou aggravant du DT1. Des études sont nécessaires pour préciser encore davantage tous les contours de l'association entre les EV et le DT1, pour en déduire des stratégies de prise en charge et de prévention optimales de la maladie.

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REVIEW

Enteroviral pathogenesis of type 1 diabetes: queries and answers

Didier Hober and Enagnon K. Alidjinou**

Purpose of review

Type 1 diabetes (T1D) results from interplay between genetic predisposition, immune system, and environmental factors. Epidemiological and experimental data strongly suggest a role for enteroviruses in the development of T1D, but a lot of controversies and unanswered questions remained. This review focuses on issues that are fueling debate.

Recent findings

Beyond HLA genes, which provide genetic susceptibility for T1D, other loci have been identified to be associated with the disease. There is a link between T1D and single-nucleotide polymorphisms (SNPs) in the *interferon-induced helicase 1* (*IFIH1*) gene that encodes melanoma differentiation-associated protein 5 (MDA5). This protein is a cytoplasmic sensor for viruses especially coxsackieviruses B, the most incriminated enteroviruses in T1D pathogenesis. Upon viral infection, MDA5 stimulates the production of mediators of the innate antiviral immune response, which is believed to play a role in a 'bystander activation' scenario. Rare variants of *IFIH1* through a lost or reduced expression of the protein are protective against T1D, whereas common *IFIH1* SNPs are associated with the disease. However, a clear association has not been yet established between T1D-associated *IFIH1* polymorphisms and enterovirus detection.

Summary

Literature have accumulated a lot of evidence supporting that enteroviruses can contribute, at least in some patients, to the pathogenesis of T1D through various mechanisms. But it is still a challenge to date to prove a causal relationship between enteroviruses and T1D. Future studies may lead to a better understanding of this relationship and ultimately can help toward disease prevention.

Keywords

enterovirus, genetic factors, immune system, type 1 diabetes

INTRODUCTION

An increasing incidence rate of type 1 diabetes (T1D) has been observed for the last few decades especially in young individuals (less than five years old) [1]. T1D is a multifactorial autoimmune disease resulting from the loss of functional insulin-producing pancreatic islet β cells in genetically susceptible individuals, but it is not entirely clear how β cells are damaged or destroyed. It is believed to result from a complex interplay between genetic predisposition, the immune system, and environmental factors [2]. It is well known that the major histocompatibility complex (MHC) genes on chromosome 6 provide a strong genetic susceptibility. However, evolution patterns and other evidence suggest that nongenetic factors are involved, supporting the implication of one or perhaps multiple environmental events in the disease development [3,4]. Viruses, especially human enteroviruses (HEV) have been long suspected as environmental agents in the development of T1D [5], but the difficulty in biopsying pancreas has made it impossible to assay for virus detection in the pancreas at the time of T1D onset, which could satisfy Koch's postulates for a causal relation [4]. The relation between enterovirus infection and diabetes is not consistent across all human studies, and the subject remains controversial [6]. Moreover, in most of cases, diagnosis of

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KEY POINTS

- The role of enterovirus is highly suggested in the development of T1D through interplay with host genes and immune system.
- Enterovirus infections could initiate islet autoimmunity or precipitate progression from islet autoimmunity to T1D, or both.
- Rare variants of *IFIH1* through a lost or reduced expression of MDA5 are protective against T1D, probably through a weaker immune response to enterovirus.
- Enterovirus infections may play a role in both T1D initiation and disease protection.

T1D follows a long period of preclinical islet autoimmunity, and enterovirus infections may contribute either to initiation of autoimmunity, or to progression from islet autoimmunity to clinical T1D or both [7]. Whether enteroviruses are involved in T1D in every patient or in some of them only remains an open question; nevertheless, experimental studies displayed different mechanisms that could be involved in the enteroviral pathogenesis of T1D.

In this review, we will bring answers dealing with questions regarding the role of enteroviruses in the development of T1D.

WHAT IS THE RELATIONSHIP BETWEEN THE EPIDEMIOLOGY OF ENTEROVIRUSES AND THE INCIDENCE OF TYPE 1 DIABETES?

Although infections in modern countries are drastically decreasing, T1D incidence is rapidly increasing. There is an inverse correlation between T1D incidence and the prevalence of enteroviral infections. The increased incidence of T1D could be correlated with a change in the epidemiology of enterovirus infections [2]. The hygiene hypothesis could explain that the incidence of T1D is higher in industrialized countries where the sanitary conditions are better and where enteroviral infections are less well spread. Observational data of T1D incidence generally support the concept that more rural or less developed populations have a lower T1D risk [8]. Indeed, T1D, which was rare in the past but common nowadays, may be linked to the decrease of HEV infections. A low prevalence of enteroviral infections in a population could increase the risk of complications like β-cell destruction. A primary infection would occur in children when they are no longer protected by maternal antibodies. Owing to the restricted exposure to enteroviruses, mothers had been exposed to a limited panel of enteroviruses; therefore, they transmit in *utero* a limited protection. Thus, a low prevalence of enteroviral infections in a population would increase the risk of complications (like β-cell destruction) because primary infections would occur at an advanced age, when individuals are no longer protected by maternal antibodies. Furthermore, the risk of developing T1D during childhood and later in life has been associated with maternal enterovirus infections during pregnancy [9–11]. This hygiene hypothesis is also supported by experimental data showing that autoimmune T1D is suppressed in non-obese diabetic (NOD) mice following inoculation with HEV [12] and that such exposure can promote expansion of a protective regulatory T cell (T reg) population [13].

ARE ENTEROVIRUSES COMMONLY DETECTED IN TYPE 1 DIABETES PATIENTS?

Enteroviruses are ubiquitous with fecal–oral route transmission and primary replication in the gut. Systemic infection may lead to dissemination to other target organs. The reported occurrence of enterovirus infection depends critically on methodology. Throughout the literature, both serological and molecular assays have been widely used to find a relationship between enteroviruses and T1D. Green *et al.* [14] reviewed studies of coxsackievirus B (CVB) serology and T1D and claimed that no convincing evidence existed for an association between CVB virus serology and T1D from 26 included studies and found a great heterogeneity in assays and study design. The problem of using antibodies as a marker is the possible involvement of several viral serotypes and a lack of standardized methods.

On the other hand, a lot of studies have linked enterovirus RNA or protein detection in blood, stool, or pancreatic tissue to T1D onset. A meta-analysis by Yeung *et al.* [6] of studies using molecular methods of enterovirus detection found a significant association between enterovirus infection and T1D-related autoimmunity, and clinical T1D. The majority of published studies have used reverse transcriptase-PCR for enterovirus RNA detection in blood samples. Few authors have studied detection in the small intestine, the primary replication site of enteroviruses, which in addition was reported to show a stronger local immune activation in T1D patients [15]. Oikarinen *et al.* [16] first reported more frequent enterovirus detection in the mucosa of small intestine of T1D patients. A recent study by

the same team confirmed more frequent enterovirus RNA detection in the intestine of T1D patients, but not VP1 protein, and suggested prolonged or persistent enterovirus in gut mucosa of these patients [17▪], but these results are not confirmed by Mercalli *et al.* [18▪]. This group found no evidence of enteroviruses in the small intestine biopsy samples from 25 patients at different stages of T1D [18▪]. Only a few studies have also reported detection of enteroviruses in human pancreas biopsy samples and especially in islet β cells [19,20]. Taken together, these studies suggest that enteroviruses are more frequently detected in patients with recent or longer-term T1D. However, enteroviruses detected in patients at or after diagnosis may well have infected the host after disease onset. The available longitudinal studies investigating the potential link between enterovirus infections and islet autoimmunity or T1D were conducted in Finland (DIPP, DiMe, and TRIGR studies), in the United States (DAISY study), in Norway (MIDIA study), in Germany (BABYDIAB and Babydiet studies), and in Australia (VIGR study), and were reviewed by Yeung *et al.* [6] and Stene *et al.* [3]. In these studies, results are inconsistent regarding temporal association. Some factors such as seasonal variation in enterovirus infection or data analysis are still challenging [3]. Furthermore, a lack of enterovirus diagnosis at the onset of the disease does not exclude a role of virus in the etiology, as 'hit-and-run'-type mechanisms may have been involved [3].

Ultimately, important advances have been made in recent years, but further investigations and methodological improvements are needed to provide a conclusive proof in this area. New initiatives are projects aimed to study the presence of enteroviruses in pancreas tissue of T1D patients: nPOD (Network for Pancreatic Organ Donors with Diabetes, http://www.jdrfnpod.org/) and PEVNET (persistent virus infection as a cause of pathogenic inflammation in T1D – an innovative research program of biobanks and expertise, http://www.uta.fi/med/pevnet/index.html).

ARE ENTEROVIRUSES INDUCERS OR AGGRAVATING FACTORS OF TYPE 1 DIABETES DEVELOPMENT?

Enteroviruses could act in the early phases of T1D (in the initiation of autoimmunity). They target β cells and damage these cells and therefore may be involved in the development of T1D through the activation of inflammation and innate immunity. One single infection may initiate pathogenic processes, as displayed in experimental models. Alternatively, multiple infections of pancreas β cells may provoke cumulative injuries that may trigger or aggravate the process [21–23]. Viruses targeting β cells could be repeatedly released from sites of chronic infection like intestine [24].

Enteroviral infections could stimulate the proliferation of specific β-cell self-antigen autoreactive T lymphocytes and successive infections would enrich the pool of such T lymphocytes. In the case of infection with a diabetes 'inductor' profile virus, the pancreatic β-cell attack would develop, because such viruses infect β cells, and activate the presentation of β-cell antigens by antigen-presenting cells, which would activate specific T lymphocytes produced during the preceding phase.

It is also believed that enterovirus infections can probably also precipitate diagnosis nonspecifically in those with advanced preclinical disease. Stene *et al.* [25] reported that progression from islet autoimmunity to T1D may increase after an enterovirus infection characterized by the presence of viral RNA in blood. This hypothesis is consistent with experimental findings obtained with NOD mice. Indeed, it was reported that the inoculation of CVB to NOD mice did not trigger T1D when insulitis was absent. The virus is thought to exploit a changed host environment (insulitis) to productively replicate in islets and trigger T1D [12]. In fact, the hypotheses that enterovirus infections can initiate islet autoimmunity or enhance progression from islet autoimmunity to T1D are not mutually exclusive.

IS THERE AN INTERPLAY BETWEEN ENTEROVIRUSES AND HOST GENES?

Although it is admitted that HLA genes are involved in T1D susceptibility, recent genome-wide association studies have identified additional T1D risk loci containing candidate genes. More than 20 loci are associated with T1D, like the gene for insulin, a major autoantigen in the disease [26]. Interestingly, some of the loci are associated with antiviral activities. An association has been found between T1D and single-nucleotide polymorphisms (SNPs) in the *interferon-induced helicase 1* (*IFIH1*) gene that encodes melanoma differentiation-associated protein 5 (MDA5), a cytoplasmic sensor of viruses, especially CVB [27–29]. Upon viral infection, MDA5 stimulates the production of type 1 interferons (IFNs), which are mediators of the innate antiviral immune response, and able to play a role in the activation of the adaptive immune response. This viral sensor is expressed at low levels in numerous cell types, including human pancreatic islets. Rare variants of *IFIH1* through a lost or reduced expression of the protein are protective against T1D, whereas common variants are associated with the disease. *IFIH1* is

expressed at higher levels in peripheral blood mononuclear cells (PBMCs) from individuals with common alleles than in those with protective alleles [30–33]. Elevated IFIH1 levels in response to enteroviruses may have an increased stimulating capacity of dendritic cells and an elevated production of proinflammatory cytokines, which may promote the development of T1D. Other findings confirm the notion that protective genotypes downregulate responses to environmental insults after initiation of autoimmunity [34]. A present hypothesis is that a weaker immune response to enterovirus (associated with the minor alleles) should be mirrored by increased replication of enterovirus. However, to date, no clear association has been found between *IFIH1* polymorphisms and enterovirus detection in the gut or in the blood [35▪▪,36▪▪].

Further research is needed to know how the different polymorphisms in *IFIH1* affect the host immune response to enterovirus infections and thereafter mechanisms by which these viruses contribute to T1D development.

IS THERE AN INTERPLAY BETWEEN ENTEROVIRUSES, INNATE AND ADAPTIVE IMMUNITY?

Enteroviruses need to access the pancreas and the β cell in order to promote T1D. It is then believed that a primary strong innate immune response in the intestine, the port of entry for most enteroviruses, is likely to prevent systemic spread of the virus and will be protective. A weak response would be counterproductive, as it may allow the virus to replicate and spread systemically [3].

The spreading of CVB in the host could be facilitated by antibodies that target the VP4 capsid protein. CVB/antibodies immune complexes can bind to coxsackievirus and adenovirus receptor, FcγRII, and FcγRIII and enhance the infection of monocytes, which are able to disseminate the virus to the pancreas. Moreover, the antibody-dependent enhancement of infection of these cells results in the production of IFN-α, which can stimulate autoimmune processes [37].

Enteroviruses can infect β cells, which results in production of type I IFN (IFN-α and IFN-β), and proinflammatory cytokines, triggering of apoptosis, and overexpression of MHC class I antigens. These effects of enteroviruses on β cells can play a role in the pathogenesis of β-cell impairment [38]. The involvement of enterovirus-induced cytokines in T1D has been studied. Schulte *et al.* [39] provided an overview of inflammatory mediators that are secreted by human islets of Langerhans upon CVB infection, whereas findings by Ylipaasto *et al.* [40] suggested a distinct, virus strain-specific, gene expression pattern leading to pancreatic islet destruction and proinflammatory effects after enterovirus infection. However, it was reported in children with islet autoimmunity that the higher levels of multiple cytokines, consistent with an active inflammatory process in the prediabetic state, were not related to coincident enterovirus infection [41▪▪].

The enterovirus-induced activation of the innate immune system results in the presentation of virus antigens to cells of the adaptive immune system, which leads to the production of antiviral effectors: specific antibodies, of which some can enhance the infection of PBMCs and antiviral cytotoxic T lymphocytes (CTLs) aimed to clear the infected cells. Successive enterovirus infections, even localized to the gut, enrich the pool of antiviral CTL able to attack infected β cells, which results in release of β-cell antigens [2].

Thus, because of the infection, β-cell self-antigens can be presented and together with the inflammation stimulate an adaptive immune response with proliferation and activation of T cells directed toward these antigens. Using a transgenic model of virus-induced T1D, a recent report showed that among the CD8 CTLs infiltrating the β cells, only 1–2% are antigen-specific recognizing the immunodominant virus CD8 T-cell epitope expressed on β cells [42▪▪].

T1D is an autoimmune disease that is the fruit of a disturbed tolerance. At the peripheral level (through T reg dysfunction), and maybe at a central level (thymus), the tolerance is disturbed, possibly because of enterovirus infections [43–45].

The established role of inflammation in the different stages of the insulitic process [46] and increasing evidence in support of the contribution of viral infections to a proinflammatory islet scenario are strongly suggestive that enteroviruses may indeed contribute to T1D development. However, an alternative hypothesis should not be ruled out. If the proinflammatory markers found in the pancreas of T1D patients are expressed typically in response to viral infection, they may also be a consequence of generalized or local inflammation. This raises the question of whether these findings are a result of the viral infection or just a consequence of a nonspecific ongoing inflammation in a scenario where viral infection will be a simple epiphenomenon [4].

The suggested enteroviral pathogenesis of T1D is summarized in Fig. 1, which displays the role of viruses, host genes, and innate and adaptive immunity.

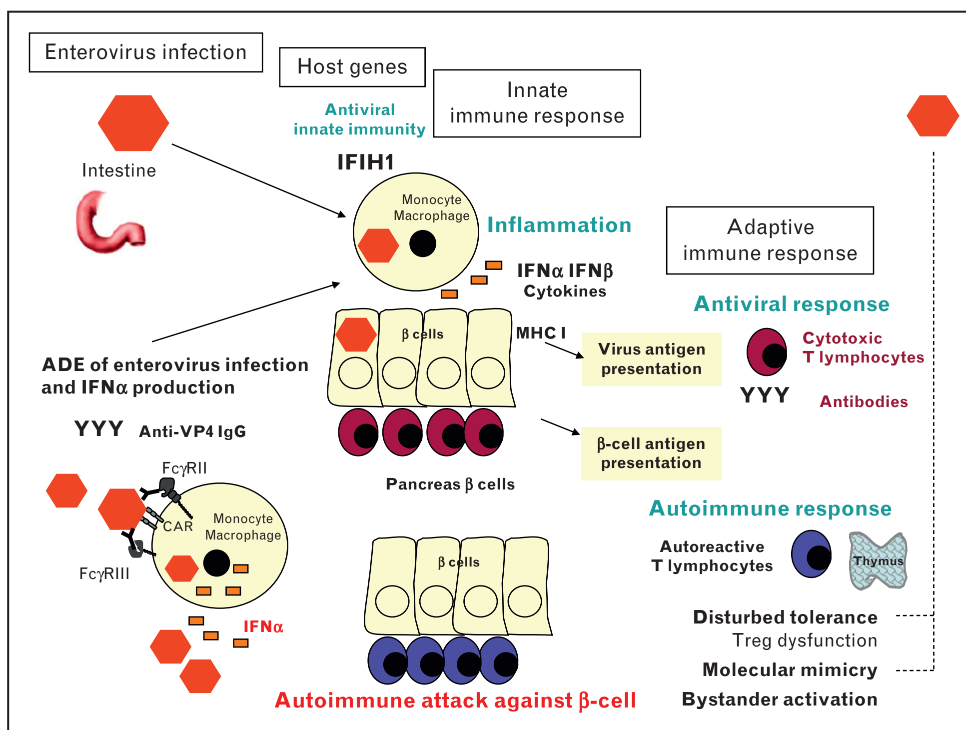

FIGURE 1. Enteroviral pathogenesis of type 1 diabetes. Enteroviruses spread to β cells in pancreas islets. The infection of monocytes with coxsackievirus B can be enhanced by antibodies [antibody-dependent enhancement (ADE)], which results in production of interferon (IFN)-α by these cells. Persistent and/or successive infections of β cells (from sites of chronic infection like intestine) activate the innate immune system. The innate immune response to viruses, influenced by the genetic background, results in the production of type 1 IFN (IFN-α and IFN-β) and cytokines, which are mediators of inflammation and increased expression of major histocompatibility complex class I antigens (MHC I) by β cells. The activated innate immune response through the presentation of viral antigens stimulates the adaptive immune response that produces antibodies (YYY) and cytolytic antiviral T lymphocytes, which are able to damage infected β cells, resulting in release of self-antigens. These β-cell antigens in an inflammatory context because of the infection can stimulate the production and the activation of anti-β-cell autoreactive T lymphocytes through bystander activation, molecular mimicry, and disturbed tolerance [regulatory T lymphocytes (Treg)]. Enterovirus infections outside the pancreas can participate in the development of autoimmune response to β cells, through a molecular mimicry mechanism, and the infection of thymus can disturb the function of that organ involved in the tolerance to self-antigens. Thus, enteroviruses, the genetic background, and the innate and adaptive immune systems interact to participate in the development of the autoimmune attack to β cells. CAR, coxsackievirus and adenovirus receptor; IFIH1, interferon-induced helicase 1.

DO ENTEROVIRUSES PLAY A DUAL ROLE IN TYPE 1 DIABETES?

There is a possible relationship between perinatal infections and risk of T1D in childhood. It is hypothesized that declining proportions of pregnant women providing their infants with antienterovirus antibodies may explain some of the increasing incidence of T1D over time [3]. Gestational enterovirus infection was reported to be associated with an increased risk for T1D in the offspring [10] or at least to play a role in some susceptible subjects [11]. In animal models, the nature of the virus, its tropism for β cells, and the timing of the infection have an important role in T1D occurrence. A virus with a protective effect induces a different inflammatory profile from that provoked by a virus with a diabetogenic effect [47]. The results of experimental studies in NOD mice suggest that CVB infections before weaning would reduce diabetes incidence, whereas infections after this critical period would have an opposite effect [48]. In individuals genetically predisposed to T1D, the virus-induced inflammation would not necessarily have a pathological effect. Together, these data suggest that the consequences of viral infections can be different according to conditions under which they occur such as the age of individuals. Enterovirus may then be a major player in both disease initiation and disease protection.

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CONCLUSION

Enteroviruses, especially CVB, and/or their components (RNA, protein), have been detected in patients with T1D and in some 'at-risk' individuals with antiself antibodies progressing to overt disease. The negative detection of enterovirus in some patients is questioning. Further investigation with highly sensitive methods is needed to determine whether low levels of enteroviral components can be found in tissue of patients and at-risk individuals.

Enteroviral infections and type 1 IFN could play a role in the development of T1D as suggested by recent findings [49]. The pathogenic processes of T1D would be based on interactions between enteroviruses and maybe other environmental factors, the genetic background, and the innate and adaptive immune system. Further studies will improve the understanding of the relationship between enteroviruses and T1D pathogenesis, which will open the possibility to develop strategies aimed to prevent or to treat the disease [50].

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

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▪ of special interest
▪▪ of outstanding interest
Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 292).

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Commentary

Enteroviruses and Type 1 Diabetes: Candidate Genes Linked With Innate Immune Response

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In addition to HLA associated genetic predisposition, the role of exogenous factors in the development of type 1 diabetes (T1D) is well admitted. Since many decades, accumulating evidence strongly has supported an association between enteroviruses (EV) and T1D (Hober and Sauter, 2010). Moreover, the potential mechanisms underlying this enteroviral pathogenesis are getting better understood, and may also be linked to background susceptibility.

Beta cell destruction/dysfunction in T1D would result from an autoimmune process (Roep and Tree, 2014) and the role of EV in the scenario should not be thought as a massive lytic replication in islets. The implication of the virus relies on the immune response, and especially the production of type 1 interferons (IFNs) and other pro-inflammatory cytokines. Indeed, the terms of the interaction between the virus and the innate immune system determine the susceptibility to this EV-mediated autoimmune diabetes, and could justify why such infection do not trigger T1D in every patient. The scenario leading to the disease is thought to include the production of significant amounts of IFNs, through activation of pathogen recognition receptors (PRRs). This inflammatory environment contributes to the initiation of autoimmune destruction of beta cells.

Candidate genes for T1D have been identified by genome-wide association studies. These genes may be involved in (i) innate immune response to infectious agents such as EV, (ii) modulation of antigen presentation and expansion of self-reactive cells or (iii) regulation of beta cell apoptosis (Concannon et al., 2009; Santin and Eizirik, 2013).

Innate immune system gene polymorphisms that are specifically related to response to viruses are very attractive to understand the early events in the enteroviral pathogenesis of T1D. This may cover molecules and pathways involved in virus recognition and IFN production as well as IFN-dependent downstream signaling and antiviral responses.

At PRR signaling level, some rare polymorphisms of IFIH1 (or MDA5) were shown to be associated to a reduced risk of T1D (Nejentsev et al., 2009). MDA5 is a well-known intracellular sensor for EV. Protective variants displaying reduced MDA5 expression are associated with a lesser response to viral infection and reduced inflammation in islets (Lincez et al., 2015).

In addition, PTPN2, a candidate gene for T1D is a negative regulator of signal transducer and activator of transcription (STAT) signaling pathway in beta cells and modulate IFN-induced beta cell apoptosis. Polymorphisms leading to a reduced expression of PTPN2 (T1D risk variant) could predispose β-cells to increased apoptosis following type 1 interferon induced by a viral infection (Colli et al., 2010).

Type 1 IFN signaling pathway also includes TYK2, another candidate gene associated with T1D (Wallace et al., 2010). Such association was already described with other autoimmune diseases like systemic lupus erythematosus or inflammatory bowel diseases. It was earlier shown that mice with natural mutation within this gene or KO models were more susceptible to infection and displayed an alteration of the response to IFNs and others pro-inflammatory cytokines. More recently this gene was clearly linked to the susceptibility of mice to a rapidly-progressing hyperglycemia induced by encephalomyocarditis virus (EMCV) (Izumi et al., 2015).

In their study, Nagafuchi et al. (2015) investigated in humans the link between TYK2 polymorphisms and the risk for diabetes. The authors identified a TYK2 promoter haplotype in patients with suspected viral infection at T1D onset. Interestingly, this variant was associated with increased risk of both type 1 and type 2 diabetes. Through its impact in the signaling of many cytokines such as types 1 & 2 IFNs, IL-6, IL-10, IL-12, IL-23, and probably others, TYK2 could be associated to inflammatory and autoimmune diseases that involve a disturbed production of these cytokines. However, due to the broad potential effect of TYK2, its specific role in the pathogenesis of virus-, and especially enterovirus-induced T1D remains an open issue.

Nagafuchi et al. (2015) claimed that the TYK2 promoter variant was associated with a more significant increased T1D risk in individuals with flu-like syndrome and in anti-GAD antibody-negative patients, but not in those with positive anti-GAD antibody. This pattern of data suggests that a TYK2 variant may be more likely associated with susceptibility to virus-induced diabetes appearing as fulminant diabetes in human beings, observed in Japan more frequently, rather than associated with autoimmune T1D.

The relationship between TYK2 and EV-related autoimmune T1D deserves further investigations. It cannot be excluded that TYK2 contributes to the pathogenesis of this disease, insofar as a strong association was reported (Wallace et al., 2010). Considering that the T1D risk allele leads to a reduced expression of TYK2 and a subsequent decreased expression of type 1 IFN-induced genes, the overall result would be a decreased antiviral response possibly involved in the mechanisms of virus persistence which can maintain an inflammatory status.

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In conclusion, T1D is undoubtedly a multifactorial and polygenic disease. The interaction between environmental insults such as EV and innate immunity probably relies on a cross-talk between many genes, which confers a susceptible background.

Disclosure

The authors declared no conflicts of interest.

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Résumé

Les coxsackievirus du groupe B (CVB) sont des petits virus à ARN appartenant à au genre *Enterovirus* et à la famille des *Picornaviridae*. Chez, l'homme, les CVB sont responsables de nombreuses infections aiguës bénignes ou sévères. Ils sont également incriminés dans le développement de maladies chroniques telles que le diabète de type 1 (DT1). En effet, plusieurs données épidémio-cliniques sont en faveur d'un lien entre les entérovirus et notamment les CVB et le DT1. Deux mécanismes majeurs ont été proposés pour expliquer la pathogenèse entérovirale du DT1. Il s'agit de l'activation « en passant » d'un environnement inflammatoire et la persistance virale qui concourent à l'initiation du processus auto-immun. Nos travaux visent à mieux connaitre les caractéristiques et conséquences de l'infection à CVB. Les résultats obtenus suggèrent que CVB4 (utilisé comme modèle des CVB) a un potentiel inflammatoire. In vitro, il induit la production de grandes quantités d'IFNα par les cellules mononucléées du sang (CMN). Néanmoins cette induction d'IFNα n'est possible qu'en cas de facilitation de l'infection par des anticorps non neutralisants, qui se traduit par une entrée importante du virus dans les cellules. Dans nos travaux, l'IFNα a été détecté dans le plasma de sujets diabétiques, et fréquemment associé à la présence d'ARN entéroviral. De même, parmi les CMN, les monocytes ont été identifiés comme les principales cellules cibles du virus. En dehors de l'IFNα, nous avons montré que CVB4 peut induire la synthèse de plusieurs autres cytokines pro-inflammatoires notamment l'IL-6 et le TNFα. De façon intéressante, l'infection des cellules n'est pas indispensable car cette induction est possible par des particules non infectieuses. Cette production de cytokines pro-inflammatoires par les CMN peut également être amplifiée par la facilitation de l'infection en présence de particules infectieuses de CVB4. Nous avons montré que les macrophages, cellules effectrices importantes de l'immunité innée au niveau tissulaire, peuvent produire en présence de CVB4 de l'IFNα et d'autres cytokines pro-inflammatoires. Les macrophages dérivés de CMN en présence de M-CSF (mais pas de GM-CSF) sont infectables par CVB4 et le virus peut persister dans ces cellules. CVB4 peut également établir une infection chronique productive de type « état porteur » dans une lignée de cellules canalaires pancréatiques. Les cellules chroniquement infectées peuvent être guéries grâce à un traitement par de la fluoxétine. Cette molécule présente in vitro une activité antivirale vis-à-vis de CVB4 et permet d'éliminer complètement en quelques semaines le virus des cellules chroniquement infectées. Des modifications cellulaires ont été observées au niveau des cellules chroniquement infectées notamment une diminution de l'expression de PDX-1, une résistance à la lyse au cours d'une réinfection par CVB4, ainsi qu'une diminution très importante de l'expression du récepteur CAR. Ces modifications cellulaires acquises au cours de l'infection chronique persistent après l'élimination du virus. Les cellules chroniquement infectées présentent également un profil de microARNs très différent de celui des cellules non infectées. Une évolution du virus a été également observée avec des changements phénotypiques et génotypiques. L'ensemble de nos observations montre que les caractéristiques de l'infection à CVB4 peuvent contribuer aux mécanismes évoqués dans la pathogenèse entérovirale du DT1 et renforcent l'hypothèse de l'implication des CVB dans cette maladie.

Summary

Group B coxsackieviruses (CVB) are small RNA viruses belonging to *Enterovirus* genus and to the *Picornaviridae* family. In humans, CVB can cause numerous mild and severe acute infections. They are also thought to be involved in the development of chronic diseases such as type 1 diabetes (T1D). Several epidemiological and clinical data support a link between enteroviruses, especially CVB and T1D. Two main mechanisms have been described to explain this enteroviral pathogenesis of T1D including a “bystander activation” of an inflammatory environment and viral persistence. These mechanisms contribute to initiation of the autoimmune process. Our studies aimed to understand the features and outcomes of CVB infection that could explain their involvement in these mechanisms. The results suggest that CVB4 (used as CVB model) is an inflammatory virus. CVB4 induces in vitro the production by peripheral blood mononuclear cells (PBMCs) of high amounts of IFNα. However this induction is only possible when CVB4 infection is enhanced by non-neutralizing antibodies, resulting in increased viral entry in cells. We also reported detection of IFNα in plasma of T1D patients, commonly associated with enteroviral RNA. In addition, monocytes have been identified as major targets of enteroviruses among PBMCs. Besides IFNα, CVB4 can induce the synthesis of other proinflammatory cytokines, mainly IL-6 and TNFα. Interestingly, infection is not needed, since inactivated viral particles can induce these proinflammatory cytokines. In addition, the enhancing of CVB4 infection in PBMCs results in increased production of these cytokines. We have shown that macrophages that are known as major innate immunity effectors can produce IFNα and other proinflammatory cytokines upon infection with CVB4. Macrophages derived from PBMCs in presence of M-CSF (but not GM-CSF) can be infected by CVB4, and the virus can persist in these cells. CVB4 can also establish a productive, carrier-sate persistent infection in pancreatic ductal-like cells. The virus can be completely cleared from chronically-infected cells using fluoxetine. This molecule has displayed antiviral activity against CVB4, and can completely clear CVB4 from chronically-infected cells within few weeks. Cellular changes have been observed during chronic infection including a reduced expression of PDX-1, a resistant profile to lysis upon superinfection with CVB4, and an important decrease of CAR expression. These changes can linger even after the clearance of CVB4. In addition the miRNA profile in chronically-infected ductal-like cells was clearly different from that of mock-infected cells. Some phenotypic and genotypic changes were also observed in the virus derived from chronic infection. Altogether, these findings show the features of CVB4 infection can contribute to mechanisms reported in the enteroviral pathogenesis of T1D, and support the hypothesis of involvement of CVB in this disease.