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Etude in vivo et in vitro du vieillissement des îlots pancréatiques : Impact de la senescence endothéliale et des Microparticules sur la fonction des îlots

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This thesis is dedicated to my beloved

Father and Mother,

For sacrifices, endless love, encouragement and

Patience make me able to get such success and honor

Brothers

Who never allowed me to give up on the pursuit of my dream

Nour

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Jamal

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Résumé en Français

L'organisation mondiale de la santé (OMS) fait actuellement état de 630 millions de personnes diabétiques dans le monde. L'OMS prévoit qu'en 2030 le diabète sera la 7ème cause de décès dans le monde. En France entre 2,9 et 3 millions de personnes sont diabétiques selon les données de l'assurance maladie avec une augmentation de plus de 6 % du nombre de patients atteints par le diabète entre 2000 et 2009.

On distingue 3 principales formes de diabète : le diabète de type 1, de type 2 et le diabète gestationnel. Le diabète de type 1 (DT1) est un trouble du métabolisme glucidique lié à une carence absolue en insuline du fait de la destruction des cellules β (cellules productrices d'insuline) par un phénomène auto immun. Le diabète de type 1 instable se traduit par des hypoglycémies fréquentes, sévères, malgré un traitement insulinique optimal. Il expose à un risque vital à court terme et à une altération de la qualité de vie en raison d'un risque hypoglycémique permanent.

Le traitement du DT1 consiste en un apport exogène et définitif d'insuline par injections sous cutanées et surveillance de la glycémie capillaire pluri quotidiennes. La greffe d'îlots pancréatiques est une thérapie cellulaire proposée depuis une dizaine d'année à des patients avec un DT1 instable mettant en jeu leur pronostic vital. En effet l'apport de cellules α et β fonctionnelles améliorent de manière spectaculaire l'équilibre glycémique en réduisant notamment les importantes variations glycémiques observées auparavant. La sensibilité aux hypoglycémies est également restaurée, les patients ressentant à nouveau les signes d'appels d'hypoglycémies. Malgré la nécessité d'un traitement immunosuppresseur, le bénéfice pour ces patients dans leur vie quotidienne est important.

Les îlots pancréatiques constituent la part endocrine du pancréas soit environ 1-2% du parenchyme total et sont formés d'un amas de cellules de 5 types principaux : cellules α sécrétant le glucagon, cellules β sécrétant l'insuline, cellules δ sécrétant la somatostatine, cellules ε sécrétant la ghreline et cellules PP sécrétant le polypeptide pancréatique. L'îlot se présente sous une forme arrondie de 200 µm à 500 µm de diamètre en moyenne et est séparé des cellules exocrines par une fine capsule de collagène. La structure des îlots pancréatiques humains est complexe, adoptant une architecture dite "tri-laminaire" avec une couche de cellules β inclue entre deux couches de cellules α.

La greffe d'îlots pancréatiques consiste en l'injection par voie intra portale d'une suspension cellulaire constituée d'environ 50% de cellules endocrines, qui va s'installer dans les micro

vaisseaux hépatiques. Les îlots sont isolés par traitement enzymatique (collagénase) et mécanique du pancréas du donneur. En général l'injection successive de deux à trois préparations issues de donneurs différents est nécessaire pour espérer obtenir l'insulino-indépendance.

On estime que 60% des îlots sont détruits après la greffe du fait d'une réaction inflammatoire nommée IBMIR (Instant Blood-Mediated Inflammatory Reaction). L'IBMIR provoquerait la perte précoce de la fonction du greffon et survient immédiatement après l'injection. Elle est caractérisée par l'activation de la coagulation et du complément conduisant à l'activation et l'agrégation rapide des plaquettes à la surface des îlots pancréatiques suivi du recrutement de leucocytes qui vont infiltrer les îlots. Un des facteurs d'initiation de l'IBMIR est la présence de facteur tissulaire (FT) à la surface des îlots pancréatiques. L'ensemble de ces phénomènes conduit à une perte d'intégrité de l'îlot. De plus, le micro environnement hépatique expose les îlots à des signaux inflammatoires qui vont altérer leur fonction et leur survie ainsi que leur revascularisation.

Malgré l'amélioration des protocoles d'immunosuppression on observe une perte progressive de la fonction des îlots marquant une dysfonction chronique du greffon. Ainsi, à trois ans post-greffe, 50% des patients reprennent un traitement insulinique.

Actuellement les pancréas des donneurs les plus jeunes sont préférentiellement attribués à la greffe pancréatique et ceux des donneurs les plus âgés à la greffe d'îlots. Alors que les techniques d'isolement d'îlots provoquent une ischémie insulaire par la rupture de la vascularisation. Par ailleurs, le vieillissement s'accompagne d'une perte des capacités vasoprotectrices de l'endothélium due à une sénescence endothéliale liée à l'âge, autre cause probable de la dysfonction des îlots.

L'endothélium est la monocouche cellulaire tapissant l'intérieur des vaisseaux sanguins. Il constitue le plus grand compartiment cellulaire en contact direct avec le sang. Il est directement impliqué dans la régulation du tonus vasculaire et le maintien de l'hémostase.

La sénescence endothéliale est définie par l'arrêt définitif et irréversible du cycle cellulaire de la cellule endothéliale s'accompagnant de modifications géniques et chromosomiques et est associée au stress oxydant et à une réponse inflammatoire par recrutement de cellules leucocytaires et libération de cytokines. La sénescence cellulaire s'etablit avec le nombre de réplications cellulaires, en réponse à de forts signaux mitogéniques, la réduction de taille des télomères, les dommages de l'ADN et le stress oxydant notamment. L'entrée en sénescence

implique différents acteurs de la régulation du cycle cellulaire dont p53, p21 et p16 qui appartiennent à des voies suppresseurs de tumeurs. Les capacités anti-oxydantes du pancréas sont faibles, ce qui suggère un organe particulièrement sensible à la dysfonction endothéliale.
La revascularisation des îlots greffés s'effectue en partie à partir des cellules endothéliales intra-insulaires du donneur, des cellules endothéliales du receveur et des progéniteurs endothéliaux sous l'action de facteurs pro-angiogéniques secrétés par les cellules endothéliales du donneur et du receveur. Les microparticules (MPs) sont des vésicules de membrane plasmique de diamètre 50 à 1000 nm libérées par les cellules dans les fluides biologiques et dans l'espace péricellulaire en réponse à une stimulation ou à un stress. Dans le sang, elles ont été décrites par le laboratoire et d'autres équipes comme des biomarqueurs du devenir du greffon. Par ailleurs, les MPs sont des effecteurs cellulaires *in vitro* ou dans les modèles animaux. Récemment, la possibilité d'un effet pro-angiogénique des MPs émises par les progéniteurs endothéliaux a été rapportée dans un modèle d'îlot reconstitué. En greffe d'îlots pancréatiques, les travaux récentes du laboratoire ont montré une augmentation des taux de MPs totales circulantes contemporaine d'une augmentation des besoins en insuline témoignant d'une perte de fonction des îlots pancréatiques, le plus souvent liée à un processus de rejet.

Hypothèse et Objectifs :

Nous faisons l'hypothèse que la sénescence endothéliale des îlots liée au vieillissement physiologique ou induite par les troubles de la vascularisation initiale du greffon, est présente au sein de l'endothélium de l'îlot et participe à sa dysfonction post-greffe. L'IBMIR induit un stress endothélial pouvant entrainer également une sénescence précoce à l'origine d'une libération de MPs aux effets potentiellement délétères sur la fonction de l'îlot pancréatique greffé et sur sa survie.
Le présent travail de thèse vise à étudier l'impact de la sénescence endothéliale sur le devenir de l'îlot pancréatique afin de proposer de nouvelles stratégies susceptibles de préserver la fonction des îlots au cours de la greffe d'îlots pancréatiques en clinique.
L'objectif du projet est de déterminer :

- l'impact du vieillissement du pancréas sur la morphologie, le devenir et la fonction de l'îlot pancréatique par analyse comparative entre pancréas de rats jeunes et d'âge moyen.
- le rôle des MPs endothéliales pro-sénescentes dans la sénescence prématurée des îlots pancréatiques isolés.

Travaux :

Première partie : Etude morphologique et fonctionnelle du pancréas de rats d'âge moyen avec fonction vasculaire normale

Le vieillissement s'accompagne d'une perte des capacités vasoprotectrices de l'endothélium due à une sénescence endothéliale liée à l'âge. Les capacités anti-oxydantes du pancréas sont réduites, ce qui le prédisposerait à la dysfonction endothéliale. Nous avons étudié l'effet de l'âge sur la qualité des îlots et sur le dommage vasculaire et endothélial chez le rat.

Des pancréas de rats jeunes de 12 semaines (n=8) et d'âge moyen de 52 semaines (n=8) ont été prélevés. L'analyse histologique a permis de déterminer la taille des îlots, leur infiltration en collagène et les marqueurs de la fonction insulaire en immunofluorescence (insuline, glucagon). La sénescence du pancréas a été évaluée par l'expression en western blot des protéines p53, p21 et p16, le stress oxydant par l'expression d'eNOS. L'expression des ROS (espèce réactive de l'oxygène) a été étudiée en immunofluorescence par DHE (dihydroéthidium). Des prélèvements sanguins ont été réalisés au sacrifice des rats jeunes et âgés pour dosage de la glycémie, de l'insulinémie, des MPs et du profile lipidique et en même temps le tonus vasculaire a été étudié dans des anneaux d'artère mésentérique secondaire utilisé pour la mesure de la réactivité vasculaire.

Une proportion augmentée d'îlots de petite taille (diamètre = 20-150 µm) est retrouvée chez les rats d'âge moyen vs rats jeunes : 92,7 ± 1,9 % VS 86,8 ± 1,4 % ($p<0,05$), alors que le nombre d'îlots de diamètre supérieur chez les rats jeunes est doublé. Le ratio surface glucagon / surface des îlots des rats d'âge moyen dans l'analyse histologique des coupes pancréatiques augmente de 6,04 ± 0,1 % à 10,40 ± 0,01 % ($p<0,05$) alors que le ratio surface insuline / surface îlot diminue de 93,96 ± 0,01 % à 89,60 ± 0,01 % ($p<0,05$).

Une augmentation significative de l'expression de marqueurs de senescences p53, p21 et p16 (2, 7 et 3 fois respectivement), du facteur tissulaire TF (4 fois) et une diminution de 30 % de l'eNOS sont observées chez les rats d'âge moyen comparativement aux jeunes. L'accumulation d'espèces réactives de l'oxygène (ROS) est doublée chez les rats âgés. Les sources principales de ROS déterminées par des inhibiteurs pharmacologiques sont le NADPH oxydase et l'eNOS découplée. La glycémie est comparable dans les 2 groupes des rats alors que l'insulinémie augmente significativement chez les rats d'âge moyen de 5,69 ± 0,47 à 7,05 ± 0,35 ng/ml ($p<0,05$), ainsi que le taux de cholestérol de 1,59 ± 0,04 à 2,02 ± 0,19 mM ($p<0,05$) et LDL-CHOL 0,15 ± 0,01 à 0,21 ± 0,02 mM ($p<0,05$). Les MPs totales, d'origine leucocytaire,

plaquettaire et endothéliale sont comparables dans les 2 groupes. Lors de l'étude de réactivité vasculaire, l'artère mésentérique secondaire montre une contraction et une relaxation similaire entre les rats jeunes et d'âge moyen.

L'ensemble de ces résultats montrent que :

i. Les altérations morphologiques et fonctionnelles des îlots pancréatiques sont des indicateurs précoces de l'altération du pancréas détectables chez le rat d'âge moyen.
ii. Le vieillissement macro vasculaire est indétectable ni par l'étude de la fonction ni par dosage de MPs chez les rats d'âge moyen.
iii. La sénescence est associée au stress oxydant et au dysfonctionnement du pancréas chez le rat d'âge moyen suggérant un rôle déterminant de l'âge du donneur pour un fonctionnement optimal de la greffe d'îlots pancréatiques.

<u>Deuxième partie :</u> rôle des MPs de cellules endothéliales pro-sénescentes sur le devenir de l'îlot pancréatique

Les cellules endothéliales sénescentes libèrent des MPs capables d'induire la sénescence endothéliale prématurée de cellules jeunes et la revascularisation des îlots post-greffe est un facteur limitant majeur. Cette revascularisation a lieu essentiellement à partir des cellules endothéliales du receveur mais également des cellules endothéliales intra insulaires (du donneur) pour 7% à 40% des nouveaux vaisseaux néoformés d'où l'importance de notre étude sur l'effet des MPs de cellules endothéliales pro-sénescentes sur l'îlot pancréatique greffé.

Des îlots pancréatiques de rats été isolés de pancréas prélevés sur des rats mâles de type WISTAR pesant 250g environ et mis en culture. Une partie des îlots a été traitée pour obtenir une suspension totalement dissociée de cellules insulaires. Pour pallier aux difficultés d'isolement de quantités suffisantes de cellules endothéliales de l'îlot, les MPs endothéliales pro-sénescentes ont été isolées à partir de cellules endothéliales primaires de porc, cultivées selon un modèle de sénescence réplicative disponible au laboratoire (UMR 7213). Les MPs seront récoltées dans le surnageant de culture de cellules endothéliales sénescentes puis isolées par centrifugation différentielle et ont été quantifiées par dosage enzymatique prothrombinase. Après sélection manuelle et mise au repos pendant 24h, les îlots pancréatiques sont stimulés par trois agents : les microparticules P1 (isolées de cellules jeunes), les microparticules P3 (sénescentes récoltées au troisième passage) et le peroxyde d'hydrogène (H_2O_2 de concentration 100 µM) en tant que contrôle positif. Le contrôle négatif correspond à l'ajout d'un volume équivalent de milieu. La concentration des microparticules utilisée a été définie par une courbe

dose réponse. Pour cela des MPs P1 et P3 ont été appliquées sur 200 îlots pancréatiques pendant 24h à la dose de 5, 10 et 20 nM éq PhtdSer. La concentration de MPs retenue pour toutes les expériences ultérieures retenue est de 5 nM éq PhtdSer. Le temps d'application a été fixé à 24h d'après des résultats préliminaires du laboratoire. La réponse aux MPs a été évaluée par l'expression de protéines de la sénescence (p16, p21, p53) par Western-Blot. L'expression de p53, p21 et p16 doublait significativement après 24h de traitement par les MPs P3 par rapport au contrôle négatif.

Concernant la viabilité, le traitement par les MPs P3 ou l'H_2O_2 entraîne une diminution non significative de la viabilité (89±1,7% pour P3 ; 87±2% pour H_2O_2 ; $p>0,05$ respectivement par rapport au contrôle).

La fonctionnalité des îlots pancréatiques a été évaluée par le rapport entre la quantité d'insuline sécrétée après incubation dans un milieu hyperglucidique (25 mM de glucose) et la quantité d'insuline sécrétée après incubation dans un milieu hypoglucidique (2,5 mM de glucose), définissant l'index de stimulation qui est la mesure de la capacité des îlots à secréter d'insuline en réponse au glucose. Une diminution significative de l'index de stimulation est observée après traitement par les MPs pro-sénescentes (1,7±0,2 pour les MPs P3 ; 2,7±0,2 pour le contrôle ; $p<0,05$).

Une quantification de l'apoptose par cytométrie en flux (double marquage IP/Annexine-V) a également été réalisée sur des suspensions cellulaires issues de la dissociation complète des îlots pancréatiques traités. Le traitement par MPs P3 augmente l'apoptose mais de manière non significative (37,5±3,5% pour les MPs P3 ; 24±3,5% pour le contrôle ; $p>0,05$) signant un potentiel pro-apoptotique nettement inférieur à celui du contrôle positif H_2O_2 . Le traitement par H_2O_2 qui augmente l'apoptose cellulaire jusqu'à 42±5% ($p<0,05$ contre le contrôle).

Le marquage des MPs par la sonde lipidique PKH26 permet d'affirmer une interaction entre MPs et îlots pancréatiques. L'observation des îlots après 24h de traitement par 5 nM éq Phtd-Ser de MPs (P1 et P3) marquées met en évidence l'apparition d'une fluorescence des îlots pancréatiques (56% pour P1 et 27% pour P3).

Cette étude a permis de mettre en évidence que le traitement d'îlots pancréatiques de rats par des microparticules endothéliales porcines pro-sénescentes entraînait :

(i) L'intégration de microparticules endothéliales pro-sénescentes, indiquant la possibilité d'un transfert d'information

(ii) L'apparition d'une sénescence cellulaire insulaire induite et accélérée

(iii) La réduction de la capacité de sécrétion d'insuline en réponse aux conditions hyper- et hypo-glucidiques des îlots pancréatiques et cela sans altération importante de la viabilité.

Conclusion :

En conclusion, l'ensemble de nos données suggère que le pancréas est un organe précocement sensible aux dysfonctions vasculaires associées à la sénescence endothéliale et plus particulièrement au cours de la greffe d'îlots pancréatiques, et la présence d'un effet délétère des microparticules endothéliales porcines pro-sénescentes sur la fonctionnalité de l'îlot pancréatique de rat. Cet effet délétère est observé conjointement à une augmentation de la sénescence cellulaire possiblement liée à un effet paracrine des microparticules endothéliales sénescentes. Ces données sont en faveur de l'existence d'un cross-talk délétère entre les cellules endothéliales et les îlots pancréatiques par l'intermédiaire de microparticules endothéliales. Ces résultats suggèrent l'importance de chercher de modulateurs pharmacologiques susceptibles de moduler la réponse des îlots pancréatiques aux microparticules ce qui serait du plus grand intérêt dans le contexte clinique de la transplantation.

List of Abbreviations

ACE	Angiotensin Converting Enzyme
AgII	Angiotensin II
AM	Adrenomedullin
AMPK	AMP Activated Protein Kinase
Ang II	Angiotensin II
APCS	Antigen Presentig Cells
AT1R	Ang II type 1 Receptors
AT2R	Ang II type 2 Receptors
BL	B Lymphocytes
BH4	Tetrahydrobiopterin
BMI	Body Mass Index
BM-MSCs	Bone Marrow – Mesenchymal Stem Cells
CDK	Cyclin dependent kinase
CMRL	Connaught Medical Research Laboratory
CNP	Natriuretic Peptide
COX	Cyclooxygenase
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T Cell
CYP	Cytochrome P450
DDR	DNA Damage Response
Del-1	Developmental Endothelial Locus -1
DHE	Dihydroethidium
DM	Diabetes Mellitus
EC	Endothelial Cell
EDCF	Endothelium derived vasocontracting factors
EDHF	Endothelium derived hyperpolarizing factor
EEI	Exocrine Endocrine Interface
EETs	Epoxyeicosatrienoic acids
EGF-8	Epidermal Growth Factor -8
eNOS	endothelial Nitric oxide synthase
ER	Endoplasmic Reticulum
ERK	Extracellular signal-regulated kinases
ESKD	end stage kidney disease

ET	Endothelin
FAD	Flavin Adenine Dinucleotide
FDA	Fluorescein Diacetate
FGF	Fibroblast Growth Factor
FMN	Flavin Mononucleotide
GAD	Glutamic Acid Decarboxylase
GC	Guanylate Cyclase
GLP-1	Glucagon-like peptide-1
GPI	Glycosylphosphatidylinositol
H_2O_2	Hydrogen Peroxide
H3K9Me	Hypo-acetylated histones , Methylated histones
Hcd20	Anti-Human CD20
hESCs	Human Embryonic Stem Cells
HGF	Hepatocyte Growth Factor
HGP	Hereditary / Genetic Pancreatitis
HLA-DR	Human Leukocyte Antigens DR
HLA-DQ2	Human Leukocyte Antigens DQ class II
HMGB1	High-mobility group box-1
HP1	Heterochromatin Protein 1
HTK	Histidine-Tryptophan-Ketoglutarate
IA-2	Islet Antigen-2
IBMIR	Instant Blood-Mediated Inflammatory Reaction
IE	Islet Equivalent
IEI	Islet-Exocrine Interface
IL	Interleukin
IL-1	Interleukin 1
IL-6	Interleukin 6
IL-8	Interleukin 8
iNOS	Inducible nitric oxide synthase
IPCs	Insulin Producing Cells
LMWDS	Low-Molecular-Weight Dextran Sulfate
LPS	Lipopolysaccharide
MCP-1	Monocyte Chemoattractant Protein-1
MEN	Mitotic Exit Network

MHC	Major Histocompatibility Complex
MMPS	Matrix Metalloproteases
MPs	Microparticles
MRI	Magnetic Resonance Imaging
mTOR	mammalian Target Of Rapamycin
NADPH	Nicotinamide-Adenine-Dinucleotide Phosphate
NF1	Neurofibromin 1
NO	Nitric Oxyde
NOD	Non-Obese Diabetic
NOS	Nitric oxide synthase
$O_2^{\cdot -}$	Superoxide
OIS	Oncogene-Induced Senescence
$ONOO^-$	Peroxynitrite
PAI-1	Plasmingon Activor Inhibitor 1
Pc	Pericytes
PECAM-1	Platelets Endothelial Cell Adhesion Molecule
PET	Positron Emission Tomography
PG12	Prostanoid Prostacyclin
PGI	Prostacyclin
PGIS	Prostacyclin synthase
PhChol	Phosphatidycholine
PhEth	Phosphotidylethanolamine
PhtdSer	Phosphatidylserine
PI	Propidium Iodide
PKA	Protein Kinase A
PLA_2	Phospholipase A_2
POT1	Protection of Telomere 1
pRb	Retinoblastoma Protein
PSGL-1	P-Selectin Glycoprotein Ligand-1
PTEN	Phosphatase and Tensin homolog
Rap1	Repressor activator protein 1
RAS	Renin-Angiotensin System
RB	Retinoblastoma
RBC	Red Blood Cells

ROCK-1	Rho-Kinase-1
ROS	Reactive Oxygen Species
S^{1177}	serine 1177
SAHF	Senescence-Associated Heterochromatic Foci
SASP	Senescence-Associated Secretory Phenotype
SA-βGAL	Senescence Associated-Beta-Galactosaidase
SIPS	Stress Induced Premature Senescence
SKCa	Small conductance Calcium – dependent potassium Channels
IKCa	Intermediate conductance Calcium – dependent potassium Channels
SM	Sphingomyelin
–SNO	S- nitrosothiol
T regs	Regulatory T cells
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
T^{494}	Threonine 494
TAC	Tacrolimus
TL	T Lymphocytes
TF	Tissue Factor
TGFβ	Transforming Growth Factor β
Th1	T helper 1
Th2	T helper 2
Th17	T helper 17
TIN2	TRF1 Interacting Nuclear Protein 2
TM	Thrombomodulin
TP	Thromboxane Prostanoid
TPP1	Tripeptidyl-Peptidase 1
TRF1	Telomere Repeat-binding Factor 1
TRF2	Telomere Repeat-binding Factor 2
TxA_2	Thomboxane A2
VEC	Vascular Endothelial Cells
VE-cadherin	Vascular Endothelial cadherin
VEGF	Vascular Endothelial Growth Factor
VEGF R2	Vascular Endothelial Growth Factors Receptor 2
VEGF-A	Vascular endothelial Growth Factor-A

VHL	Von Hippel Lindau
VSMCs	Vascular Smooth Muscle cells
vWF	von Willebrand Factor
XRP8	Xk-related family Protein 8

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Chapter I

The Physiology of pancreatic islets

I The physiology of pancreatic islets

I.1 Brief description of the pancreatic function and its vascularization

The pancreas, an exocrine and endocrine gland, has a head, body and tail. It is about 15 cm long, tapered organ located in the abdominal cavity behind the stomach [1] (Figure 1). Pancreas serves as two glands in one: a digestive exocrine gland and a hormone-producing endocrine gland.

The endocrine gland constitutes 1-4% of total pancreas volume where islets of Langerhans is the main unit with about 3 million islets [2].

The exocrine pancreas constitutes 96-99% of the total pancreas volume. It is mostly composed of arranged clusters of cells called exocrine acinar cells that secrete the pancreatic juice containing digestive enzymes like trypsin and chymotrypsin. These enzymes are secreted in the inactive form called proenzymes. Once they are secreted in the duodenum by enteropeptidase enzyme, which activate a cascade of enzymatic reaction that leads to proenzymes cleavage. It is initiated by the cleavage activation of proenzyme trypsinogen breakdown to the active form trypsinogen. The activated form of trypsinogen have the ability to activate other reactions including conversion of chymotrysinogen to chymotrypsin. Furthermore, besides their role in helping to digest and absorb nutrients in the small intestine. They are involved in protein, carbohydrates, lipids and nucleic acids digestion [3]. The pancreas vascularization is adequately based on blood flow coming by branches of both the coeliac artery and the superior mesenteric artery. The splenic artery run over the top border of the pancreas and supplied the body and pancreatic tail by its branches, the pancreatic artery. On the other hand, the head of the pancreas is supplied by the superior and inferior pancreatic duodenal arteries [1].

Pancreas dysfunction is defined as the incapability of the pancreas to properly perform its function. It can be caused by many diseases such as pancreatitis, cystic fibrosis, cysts, pancreatic cancer and the most importantly by diabetes.

I.2 The Islets structure and function

The pancreas is derived from two buds (dorsal and ventral endoderm), at 6 weeks of gestation, both bud fused together. This fusion occurs by turning of the ventral bud and then reaching towards the dorsal bud followed by subsequent fusion. In the fetal period, islet cell groups form by differentiating from the pancreatic bud endoderm and the pancreatic islets [4].The first evidence of islets formation in this period can be detected at 9 to 11 weeks of gestation. The

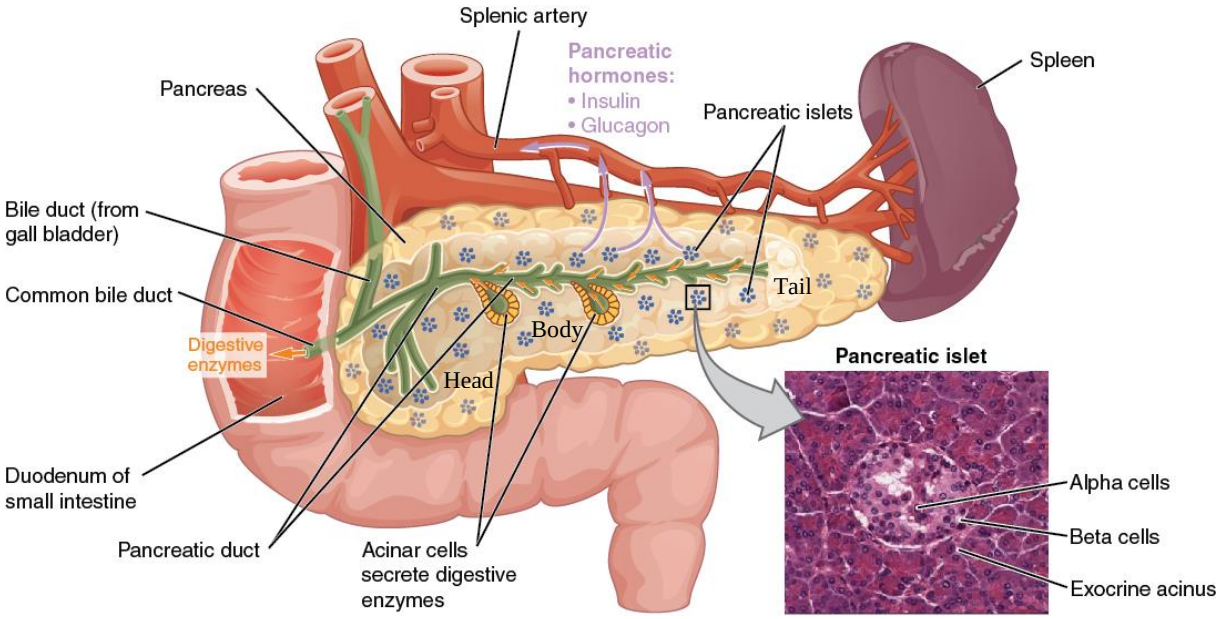


Figure 1 : Pancreas anatomy (OpenStax College, Anatomy and Physiology)

pancreas growth is controlled by a composite cascade of transcription factors [5], where Ngn3 is essential to initiate endocrine differentiation [6]. The main units of the endocrine pancreas are the islets of Langerhans. It is formed as the result of agglomeration of endocrine cells. This crucial unit is scattered between the acini and ductal structures all along the exocrine parenchyma. It is an essential unit for body survival, which controls blood glucose level through insulin secretion. In the human body, islets are made up of distinct cell types [7] including:

- α cells (20–40%), which produce the glucagon hormone that increases blood glucose in response to low blood glucose level.
- β cells (50–70%), which produce the insulin hormone that decreases blood glucose in response to elevated blood glucose level.
- δ cells (<10%), which produce the somatostatin hormone that inhibits the release of both glucagon and insulin.
- ε cells (<1%) which produce the Ghrelin hormone that inhibits glucose-stimulated insulin secretion from beta cells in the pancreatic islets [8].
- PP (<5%) which produces the pancreatic polypeptide hormone that plays a role in appetite and in the regulation of pancreatic exocrine and endocrine secretions [9].

In humans, 70% of the islets are in the size range of 50-250 μm in diameter with an average range of 100-150 μm. It has a regular spherical shape and it is separated from exocrine compartment by a thin collagen capsule [10]. Islet cells are organized into a trilaminar plate where one layer of β-cells is sandwiched between two α-cell-enriched layers. Moreover, this structure of islets has a folded pattern where the vessels circulate along both of its sides [11] (Figure 2).

I.3 Interaction between the islets and the exocrine pancreas

The pancreas has a dual function depends on the intercellular communications between the exocrine and endocrine parts. This islet-exocrine interface (IEI or exocrine-endocrine interface EEI) is a very important anatomical and functional region for the cell-cell communication between the endocrine islets and exocrine acinar cells of the pancreas. In IEI region, a capillary network of islets connect with the surrounding capillaries of acinar cells. Together, they form the insulo-acinar portal system that is defined as the anatomical substrate by which the endocrine pancreas may influence the exocrine pancreas. In addition, the islets have an important structure characterized by its fenestrated capillaries which constitute 8%–10% of

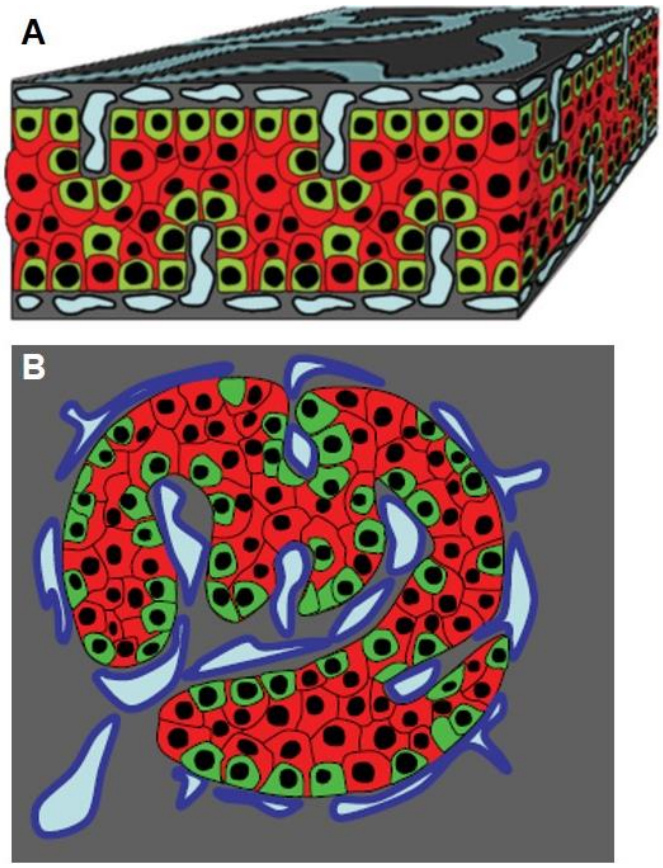


Figure 2 : Human islets organization (Bosco D *et al.* Diabetes; 2010)

A: α-Cells (green) and β-cells (red) are organized into a thick folded plate boarded with vessels on both sides (blue). α-Cells are found on the outer surface of islets in direct contact with vessels. β-Cells, on the other hand, constitute the core part of the islets. Despite being in the core, β-Cells achieve endocrine secretion by developing cytoplasmic extensions that run between α-cells to reach the surface of vessels. B: Islet form by folding of the plate with adjacent vessels.

the islet volume. It is likely that the islets capillaries fenestrations are 10 times wider as compared to exocrine pancreatic capillaries [12].

Most of the studies reveal the inference of oxidative stress in diabetes pathogenesis. Diabetes and oxidative stress can contribute to the loss of cell interface [13] (Figure 3). Moreover, recent studies showed that circulating microparticles (MPs), the small vesicles released from the cell surface upon activation, were significantly elevated in both diabetic patients and diabetic rats. MPs were devised as a distinguishable way to study endocrine-exocrine interactions in many diseases like cystic fibrosis where the mechanisms of these interactions are still unclear [14].

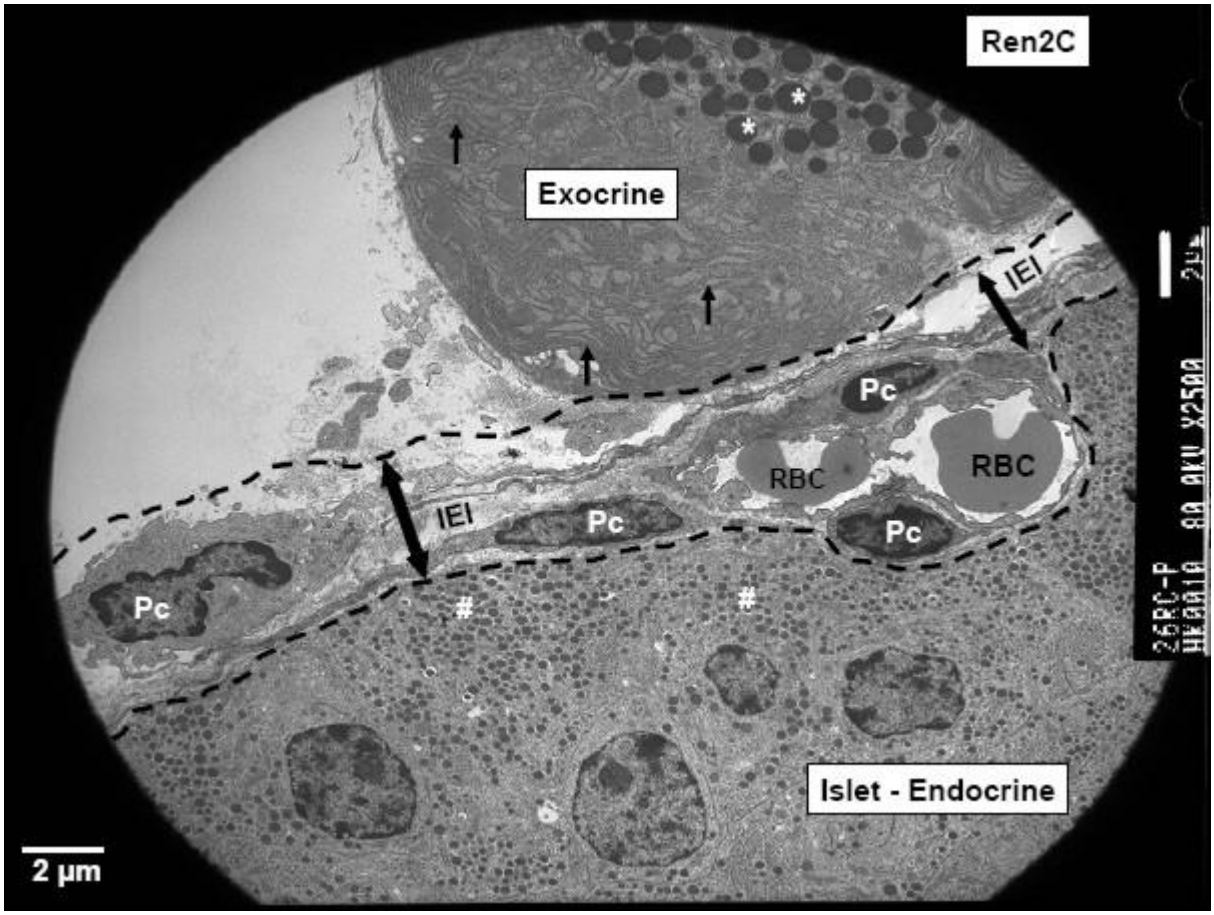


Figure 3 : Islet exocrine interface - IEI in rat model (Hayden MR, JOP 2007)

Electron micrograph of the tail sections of the pancreatic tissue demonstrating the special anatomical area: The islet (endocrine)-exocrine interface (IEI) (double arrows) and is highlighted by bold black dashed lines. This islet-exocrine interface contains an anuclear endothelial capillary containing two red blood cells (RBC) with four adjacent pericytes (Pc) (magnification: 2,500x; bar: 2 µm).

Chapter II

Pancreatic Islet dysfunction: Diabetes

II Pancreatic islet dysfunction: Diabetes

II.1 Diabetes

Diabetes nowadays is a serious public health problem. It is described as a metabolic disorder characterized by chronic hyperglycemia (increase in blood glucose level) either because insulin production is inadequate (insulin deficinecey), or the body's cells don't respond properly to insulin in target tissues especially liver, muscle and adipose tissue. The latter is mainly due to defective insulin receptors in target tissues.

Recently, the number of diabetic patients is constantly increasing. Moreover, diabetes causes and aggravates health complications with time. Over time, diabetes can cause serious complications and leading to damage of many organs such as heart, blood vessels, eyes, kidneys and nerves. The prevelance of diabetes is increasing among all ages. The world health organization (WHO) studies showed that number of diabetic people has abruptly increased from 108 million to 422 million between 1980 and 2014. Noteworthy, there was an increase in diabetes cases from 4.7% to 8.5 % among adults over 18 years old with higher increasing rate in middle and low-income countries. WHO estimated that 1.5 million deaths were directly caused by diabetes in 2012, 2.2 million deaths, were also linked to high blood glucose [15]. Other study by WHO showed that by the year 2030, diabetes will be the 7th cause of death in the world [16]. In France, there was more than 3.3 million according to the statistical data of medical assurance with a 6 % increase in the number of patients with diabetes between 2000 and 2009.

The prevalence of diabetes in adults in France is 4.8 to 7.4 %. Among diabetic patients, two million, seven hundred thousand subjects are diagnosed with Type 2 Diabetes mellitus (T2DM) and one hundred sixty thousand, have type 1 diabetes (T1D).

II.2 Different causes of diabetes and similar outcome

T1D is an autoimmune disease characterized by the destruction of the beta-cells (β-cells) of the pancreas due to aberrant "self" antibodies production that wrongly bind to β-cells , initiating immune-mediated reactions lead to their destruction and lack of insulin.

T1D, is more prevalent in young adults and children, hence the name juvenile diabetes [17]. The T1D autoimmunity progresses for 5 to 10 years or more after onset. Normally, hyperglycemia occurs when patients have less than 10% of functional β-cells. Chronic hyperglycemia plays a major role in the initiation of diabetic vascular damage. On the long

term, it causes severe complications, mainly affecting the vascular endothelium. As a result, diabetes is strongly associated with microvascular and macrovascular complications, including retinopathy, nephropathy and neuropathy [18]. T1D can be diagnosed by systemic detection of auto-antibodies to insulin, islet antigen-2 (IA-2), glutamic acid decarboxylase (GAD 65) and ZnT8A [19, 20].

T2DM, also called insulin-independent diabetes is majorly caused by insulin resistance. It manifests when cells within the muscles, liver and fat tissue are unable to respond correctly to normal levels of insulin [21]. This mainly leads to hyperglycemia induction as the result of the decrease in muscular glucose utilization and increased hepatic glucose production. T2DM is a multi-factorial disease resulting from a combination of several susceptibility genes with differential expression depending on environmental factors such as obesity, sedentary lifestyle, stress and weight of birth [22]. Type 2 diabetes, accounts for ∼90–95% of all diabetes cases [23]. It is a complex disease that combines insufficient insulin production from β-cells that usually precedes 10 to 20 years hyperglycemia and peripheral insulin resistance most often in the context of obesity or overweight.

However, the causes of these two types of diabetes are different, but the consequences are almost the same. Diabetic patients of different types suffer chronically and equally from the unstable blood glucose level which obliges them to be extremely prudent in their life style and stay on treatment to avoid any further complications from progressing.

II.3 Type 1 Diabetes: an auto-immune diseases that destroy islets

II.3.1 Epidemiology of T1D

Types 1 diabetes incidence increase worldwide, it is actually estimated to account for up to 5-10 % of all cases of diabetes [24]. It mainly affects children younger than 15 years. Its incidence has increased from 0.6% in 1989 to 9.3% in 2003. Based on EURODIAB study, the number of new cases of diabetes in Europe will increase from 15,000 in 2005 to 24,400 in 2020. In addition, the prevalence of T1D in children younger than 15 years will increase from 94,000 in 2005 to 160,000 in 2020 [25]. T1D has been found recently to have a higher tendency of earlier onset. It has been speculated that T1D prevalence increases and will be doubled among children less than 5 years of age by 2020 [26]. The prevalence of T1D is growing globally, so cure cannot be done with the current knowledge, but with better understanding of the pathophysiological mechanisms in both the treatment and the management of T1D.

II.3.2 Molecular and cellular causes of T1D

T1D is an autoimmune disease characterized by the destruction of insulin secreting cells (β-Cells) which leads to insulin insufficiency and impaired glucose homeostasis. Multiple causative genes have been identified that are dispersed loci situated in up to 41 distinct genomic locations. The first identified set of genes associated with T1D was found on chromosome 6 and it is called the Human Leukocyte Antigens DR and DQ class II (HLA-DR and HLA-DQ2) [26-28]. In addition to genetic predisposition as aforementioned, T1D can also be inflicted by environmental predispositions that are involved in the pathogenesis of the disease by a T cell-mediated autoimmune attack against β-cells [27].

Many studies suggest that T-lymphocytes (TL), the key players in cellular immunity, play also a pivotal role in the autoimmune attack of β cells [29]. The onset of T1D requires two types of T lymphocytes: $CD4^+$ and $CD8^+$. They were identified after prevention of diabetes by elimination of $CD4^+$ and $CD8^+$ T cells. The activation of TL is debuted by the β-cell antigens recruitment by dendritic cells, members of the family of antigen presenting cells (APCs). Then two possible pancreatic β-cells destruction pathways are possible: i) via direct cell-to-cell contact, by a cytotoxic process, or ii) indirectly the secretion of pro-inflammatory cytokines, perforin, or granzyme B [20].

The $CD4^+$ T cells are primarily involved in the primary immune response by stimulating the activation and proliferation of both $CD8^+$ T cells and B-lymphocytes. Human $CD4^+$ T cells from the pancreatic lymph nodes of subjects with T1D respond to the first 15 amino acids of the insulin A-chain [30]. $CD4^+$ T cells can be divided into two categories: T helper (Th1, Th2 and Th17) and regulatory T cells (Tregs). Both type are differently involved in T1D pathogenesis depending on different cytokines secretion (Figure 4).

The cytotoxic T cell (CTL) are the result of the activation of $CD8^+$ T cells, which recognize pathogen-derived peptides presented by major histocompatibility complex (MHC) class I molecules. Cytotoxicity is expressed by secreting proteins such as Fas and cytokines such as TNF-α and IFN-γ [20]. B cells (B Lymphocytes, BL) are not directly linked to islet-β cells destruction but rather to the progress of the disease by promoting autoimmunity. B-cells promote differentiation of $CD8^+$ T-cells to CTL and provide survival signals for $CD8^+$ T-cells to maintain high levels of aggressive CTLs [31]. Indeed, they stimulate the production of auto-antibodies whose presentation generates self-reaction of TL and the maintenance of $CD4^+$ T cell memory [32]. However, they also play a role in the initial phase of the disease by being

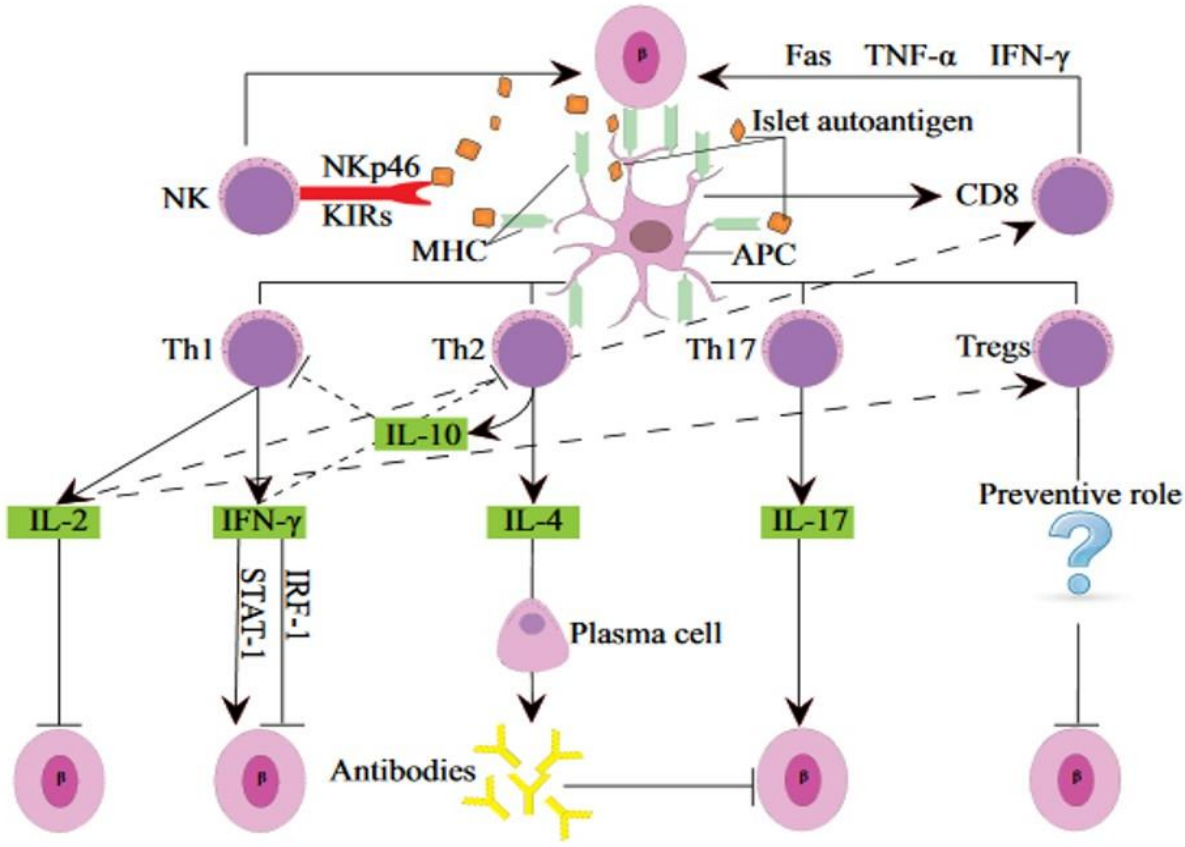


Figure 4 : Immunological factor implicated in the autoimmune destruction of β-cells in T1D. (Min Li *et al.* J Cell Mol Med, 2014)

β-cells are damaged by various factors and the released autoantigens are presented by antigen-presenting cells. Then $CD4^+$ T, $CD8^+$T and NK cells are activated and $CD4^+$ helper T lymphocytes differentiate into Th1, Th2, Th17 and Tregs. Th1 cells can destroy the β-islet cells and accelerate the course of T1DM via production of IL-2 and IFN-γ. IL-2 has been shown to prevent diabetes, while it can activate $CD8^+$ T cells and Tregs. In addition, IFN-γ plays a dual role in β-cells destruction. Via the signal transducer and activator of transcription-1 (STAT-1) pathway and in protection via the IRF-1 pathway. Th2 cells mainly produce IL-4 and IL-10, which are responsible for strong antibody production, have been ascribed with a protective role. Th17 can destroy the β-islet cells by secreting IL-17. Whether Tregs play a preventive role in the pathogenesis of T1DM remains a question. In addition, NK cells are involved in direct killing of β-cells through the interaction of NK cell markers, such as NKp46 and KIRs. Furthermore, $CD8^+$ T cells contribute to the development of T1DM by secreting proteins such as Fas and cytokines such as TNF-α and IFN-γ.

essential antigen presenting cells of the islet but to a less extent compared to dendritic cells [20].

II.4 T1D treatment

T1D is inevitable, so the main target of T1D treatment is to maintain blood glucose levels at normal range, improving functional residual β-cell mass, preventing acute and chronic complications of the disease and to ensure good quality life for the patient. Some researchers postulated that it might be possible to prevent or delay T1D at the latent autoimmune stage and before pancreatic beta cells destruction [33]. To reach these target two types of treatments were developed:

1- Treatments to maintain normal blood glucose level and to prevent the destruction of the β-cells by many techniques like immunotherapy.

2- Treatments to replace the insulin-producing cells with stem cells, pancreas or pancreatic islets transplantation.

II.4.1 Stabilization and prevention of T1D

T1D disease stabilization can be maintained by several day-to-day practices. Daily-based insulin injections, several times per day, are the common and effective way to maintain normoglycaemia. Glucose level is then constantly monitored by a blood glucose monitor that helps adjusting insulin doses. Insulin treatment should also be accompanied by adopting a healthier life style with a low-carbohydrate diet [34], physical exercise and quitting both smoking and alcohol intake.

Immuno-suppressors have been also used to treat T1D such as Cyclosporine, the first successful immunosuppressive agent used. Unfortunately, despite retaining β-cell function, Cyclosporine showed high kidney toxicity along with other side effects of long-term treatment. Altogether, forced the termination of the cyclosporine therapy [33].

In autoimmune diabetes, studies in NOD mice using anti-CD20 antibody have shown contrasting effects of B-cell depletion on reversal or prevention of T1D. Recently, researchers have also shown that the disease progression was totally stopped by the removal of the antibodies at a preinsulitis stage in NOD mice [35]. B cells depletion in NOD mice using anti-CD20 mAb showed effective in reversing hyperglycemia at onset [36]. The combined treatment of anti-human CD20 (hCD20) and oral anti-CD3 have also been shown to reverse diabetes in more than 60% of NOD mice diagnosed with diabetes. Additional studies proved

that the suppressive function of regulatory T cells was synergistically improved by the addition of oral anti-CD3 to the B-cell depletion therapy [37]. Most significantly, T1D can be reversed by using a small number of antigen-specific T_{regs}, which propose a novel approach to cellular immunotherapy for autoimmunity [38].

Several therapeutic approaches were studied to prevent the onset of T1D symptoms by improving immune tolerance for β-cells and maintaining its function. However, despite the promising results, few treatments stood out. Moreover, most treatments have been found either toxic on the long term or failed to maintain sustainable pancreatic β-cell function [39].

II.4.2 Replacement of defective β-cells by Stem cell: an alternative therapy

Scientist have tried to replace the defective β-cells with a bio-mechanical pump. The method is based on the development of an artificial pancreas combining continuous analysis of blood glucose system coupled with an automatic insulin delivery system. Nevertheless, the injection of exogenous insulin didn't succeed to stop the development of T1D and may have caused serious complication such as kidney failure, vascular diseases and retinopathies [40].

As part of a predominantly later management of T1D, the replacement of defective β-cells is as an essential therapeutic strategy. Therefore, the next step in research was the replacement of defective β-cells by Stem-cell therapy or pancreatic islet transplantation.

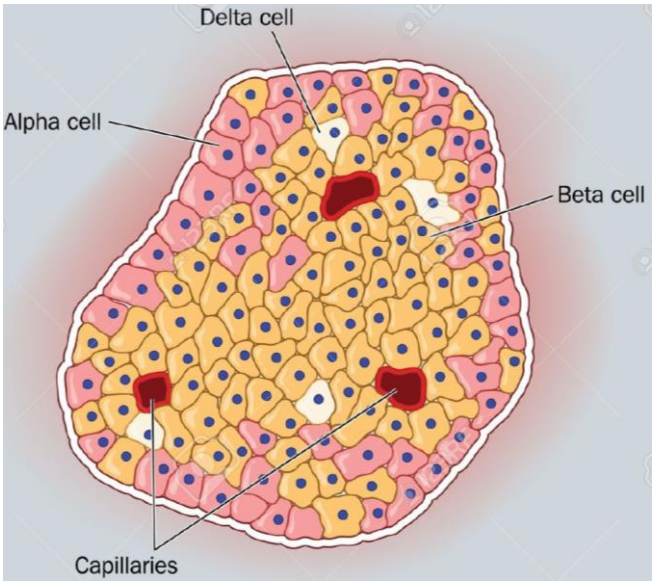
β-cells replacement approaches aim to generate stem cell derived insulin-producing cells (IPCs), which could make diabetes cellular therapy available for millions diabetic patients. Human embryonic stem cells (hESCs), a cell line with unlimited proliferative capacity and a high differentiating potential, was the main source to generate IPCs [41]. The principal characteristic of hESCs was the potential to differentiate into specialized cells of all three primary germ-layers, including pancreatic IPCs. When transplanted into the epididymal fat pads of SCID/NOD mice, the hESC-derived pancreatic IPCs were able to reverse hyperglycemia for ≥8 weeks [42]. The principal challenge to scientists now is to have safe and efficient transplanted cells without the risk of immune rejection or tumorigenesis, not to mention, the big concern regarding the ethical issue [40].

Despite the huge advancement in studies to obtain an unlimited source of functional and immunocompetent β-cells, their application to humans is limited by the need to graft a sufficient amount of cells with complete cell functionality. For this purpose, the grafting of the functional units of insulin secretion directly has been used to quickly help re-establish the normal blood glucose level.

Chapter III

Pancreatic islet transplantation:

A cellular treatment of type 1 diabetes



III Pancreatic islet transplantation: A cellular treatment of T1D

III.1 Islet graft

The development of islet cell transplantation aims at decreasing surgical complications and to limit or eliminate immunosuppression by protection of grafted islets. Islet transplantation started in 1972 by Ballinger *et al.* in rodents, which proved that diabetes mellitus could be reversed by pancreatic islets transplantation [43]. Then in 1990, a 1 month insulin independence was reported in T1D patient by Scharp *et al.* [44]. In the year 2000, Shapiro *et al.* were successful in maintaining insulin independence for 1 year in seven T1D patients using a protocol called Edmonton. It had an overwhelming success that lead to enormous development in clinical islet transplantation [45]. This technique was normally used in patients with unstable T1D and aged > 50 years. Islet auto-transplantation after total pancreatectomy represents a big hope for patients with chronic pancreatitis due to hereditary/genetic pancreatitis (HGP). It offers long-term pain relief (90%) and β-cell function preservation. Islet auto-transplantation done prior to pancreatic inflammation results in a higher degree of pancreatic fibrosis risk, pancreatic cancer and islet cell function loss [46]. Cystic fibrosis patients develop hyperglycemia after lung transplantation. This aggravates if patients are diabetic causing complication that might lead to mortality [47]. Combination of lung and islet transplantation from the same donor can minimize and even ablate complications. This combination has been shown to lead to optimal blood glucose level with no sign of hypoglycemic and 50 % reduction of used insulin [48]. A 5-year follow-up, in a large cohort showed that islet transplantation was safe and efficient in patients with T1D [49].

III.2 Pancreatic islet transplantation protocol

III.2.1 Islet transplantation steps

Islet transplantation proceeded in two steps. The first step started by the isolation of the islets. A pancreas of a brain-dead patient is harvested then processed to isolate only islets. The pancreas is digested by collagenase treatment. Then by using density gradient, islet cells are separated from exocrine cells, purified and then used for culture. The second step occurred in the hospital by the surgical intervention performed in diabetic patients, usually under local anesthesia. The procedure is summarized by the infusion of islets into the patient's through the portal venous circulation under ultrasound guidance (Figure 5). To overcome the severe shortage of available pancreas islet transplantation and experimental status of other sources of

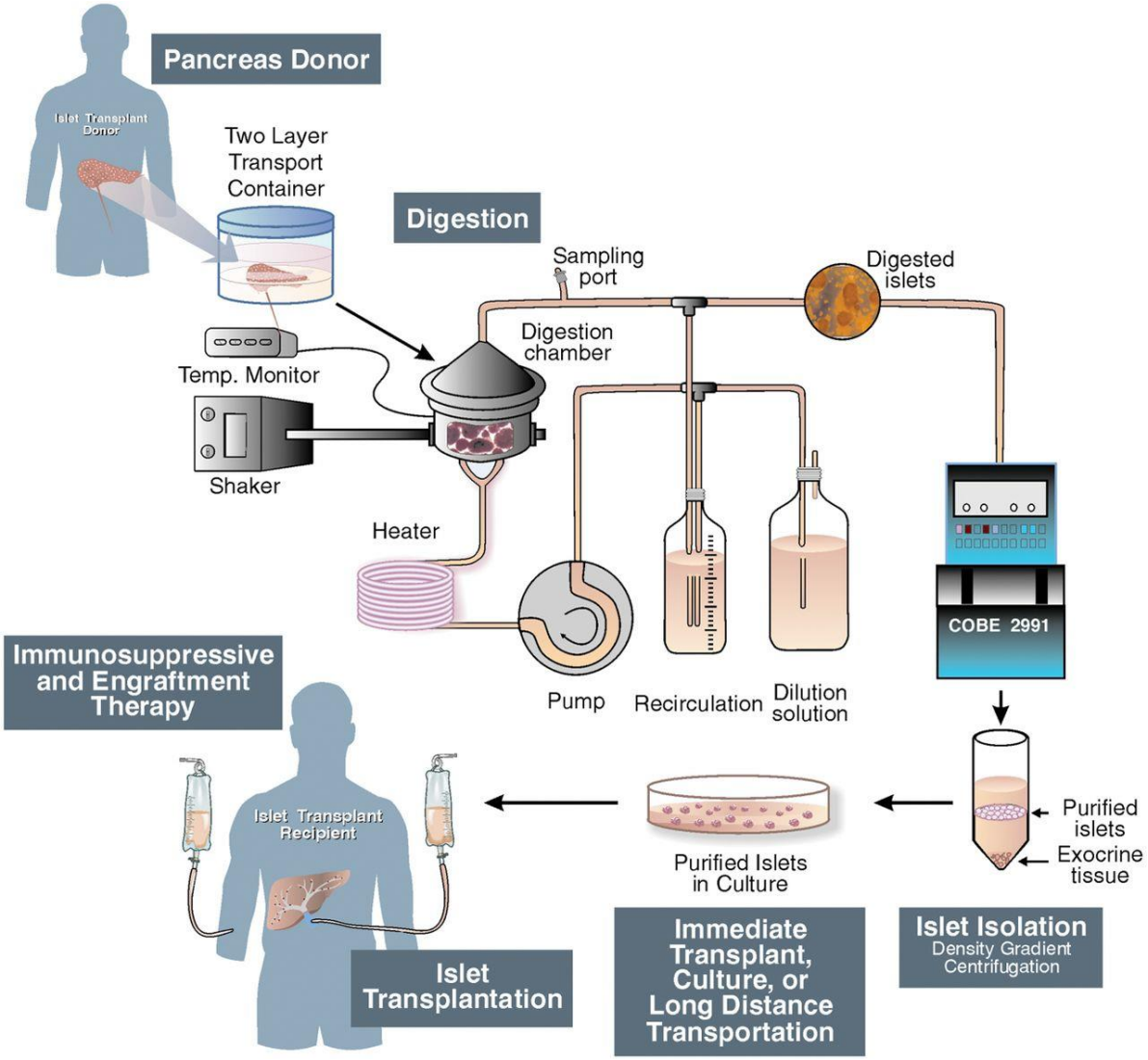


Figure 5 : Islet isolation and transplantation (Merani S, Shapiro A.M, Clin Sci 2006)

β-cells, optimization rate, viability, integrity and functionality of the islets at each stage of the procedure appears to be crucial in the long-term success of the islets.

III.2.2 Donor selection

The main source of donors is from brain-dead patients but actual studies have shown a comparable results from non-heart beating donor [50]. The establishment of criteria, which determine the best donor selection, is very important to improve the success of islet transplantation due to the high cost procedure and the complications that may occur. Previous studies review several selection criteria that should be taken into careful consideration, for which they have been proven effectively in multiple transplantation surgeries. These include, the donor's age, high body mass index (BMI), cold ischemia time and blood glucose level [4]. The donor's age is one of the most essential factor, where a pancreas isolated from donors older than 50 years old with BMI greater than 30 is undesirable for a whole organ transplant, but is favored for islet transplantation [51]. This is because the higher the BMI the higher the quantity of isolated islets [52]. Putative donors with HbA_{1c} levels higher than 7% are not selected as islet transplantation donors. HbA_{1c} is correlated with Diabetes onset. Evidently, T2DM patients are considered unsuitable to be islets donors [53].

III.2.3 Pancreas dissociation and islet isolation

After pancreas isolation, it is preserved prior to islet isolation in a UW or histidine–tryptophan–ketoglutarate (HTK) solution. Recent studies have shown a similar preservation characteristics of these 2 solutions especially in terms of islets yield and functionality [54]. The principal protein structure in the exocrine-islet interface is collagen [55]. As a result, after preservation, collagenases dissociate the strong collagen interface structure [56].This is an essential part that will affect whole procedure and downstream steps. Especially, it affects both the yield and the viability of islets.

In 1990s, Roche introduced the liberase, which is the first blend of enzymes made up of collagenase type (I & II) and thermolysin (a derivative of *Bacillus thermoproteolyticus* as a non-collagenase impurity that is needed to enhance pancreas dissociation). This new product helped reducing the variability of enzyme effectiveness [57]. Another collagenase cocktail called collagenase NB1 that is supplemented with neutral protease NB, showed more improvements in islet morphology, viability and *in vitro* function that makes it a promising product for human-islets isolation to avoid the inter-batch variability [58]. Camillo Ricordi has

developed a semi-automated pancreas dissociation by enzymatic digestion. This method match the following requirements: minimal disturbing action, continuous digestion, minimal technical or surgical intervention in the digestion process, which leads to high yield and purity of the isolated islets [59]. After this step, islets need to be purified from exocrine tissue. The most simple and effective method was developed by ficoll density gradient separation where the difference between islet density (~1.059 g/ml) and exocrine tissue density (between 1.059-1.074 g/ml) [59], would allow their separation. It is noteworthy that the bigger the difference in density the better and more refined the islet separation is. Pure islets are then washed and prepared to culture before they are injected in recipient patient.

III.2.4 Assessment of isolated islets

The size distribution of islets in pancreas is very heterogeneous. It is likely, such heterogeneity means that the exact determination of islet yields should take in to consideration the islet number and size. Therefore, islets were categorized according to diameter size range: 50–100, 101–150, 151–200, 201–250, 251–300, 301–350 and over 350 μm. An islet equivalent (IE) is defined as an islet with a diameter of 150 μm. Then IE number from each size range is multiplied by a conversion factor [60].

The pancreas size is a crucial factor that determines the number of IE per pancreas that can varies from 500,000 and 1000,000 IEs [61]. The yield of islets that are isolated is 300,000 to 400,000 pancreatic islets. This accounts for 50% of total number of IEs present in the pancreas. In addition to the number of isolated islets, the viability of these islets forms an important factor that may affect the success of transplantation. The islet preparation viability prior to transplantation is assessed by a quick and easy technic consisting of the proportion of two fluorescents stains. The fluorescein diacetate (FDA) taken up by the viable cells, which convert the non-fluorescent FDA into the green fluorescent metabolite fluorescein. In contrast, the nuclei staining dye the propidium iodide (PI) cannot pass through a viable cell membrane. It reaches the nucleus by passing through disordered areas of dead cell membranes [4].

III.2.5 Islet culture

The rapid advancement in the islet isolation techniques, especially after Edmonton protocol, has made islet transplantation one of the most promising treatment for T1D. At the infancy of this technique, Edmonton protocol consisted of the injection of isolated islets in recipients within 4 hours after isolation [62]. Other studies have shown to have a higher success when

isolated islets are put in culture prior to injection. The advantage lies in the capability to do additional tests to verify islet purity, viability and functionality. Moreover, it permited the time to prepare the recipient by starting the immunosuppressive treatments, performing microbiological tests and also allows patients to receive their transplants from faraway centers without the trouble of traveling [63]. However, it is well known that islet culturing leads to 30-35% decrease in islet yield by apoptosis and other factors. A study have shown that fresh human islet injection in nude diabetic mice was more effective for islet transplantation than cultured islets [64]. To optimize the culturing parameters, scientists tried changing parameters such a culture medium, supplements addition and incubation temperature. Connaught Medical Research Laboratory 1066 (CMRL) is the most used media for islet culture, as well as M199. To recover the highest number of islets, the addition of various supplements has been proven crucial for optimum islet recovery. The use of serum has a very important role in neutralizing the endogenous pancreatic enzymes [65] or exogenous enzymes left over from the isolation process [66]. Not to mention the beneficial effect that serum addition has on islet viability [67]. The incubation of islet with Liraglutide has also shown to improve graft survival mainly due to its anti-inflammatory and anti-oxidative properties [68]. Co-culture of extracellular matrix ameliorates the pancreatic islet viability [69].

The incubation temperature is another crucial factor. Studies done on different temperatures: 4, 22-24, 37°C to monitor their effect on isolated islets. Noguchi H *et al.* showed that storage at 4°C in the UW solution was better than either the 22°C or 37°C culture [70]. Conversely, many investigators recommend to set the culture system at 22–24°C prior to transplantation [71]. Lakey JR *et al.* demonstrated that the mean recovery rate of human islets after a 24 hours of culturing varies at different temperatures, 73% at 22°C compared to 55% at 37°C [72]. All these data prove that it is very important to standardize culture protocols in all centers of islet isolation to avoid discrepancies.

III.3 Monitoring of grafted islets

Up to now, along with all the development of monitoring tools, it is still difficult to detect the transplanted islet dysfunction. The most used technique in the context of organ graft monitoring is the biopsy. However, with islet transplantation, biopsies are difficult, non-informative and it is random due to the dispersion of islets in the hepatic vascular tree. In addition, changes in markers related to graft, as the need for insulin or C-peptide, are observable only when 95% of the graft is destroyed.

Imaging techniques such as MRI (magnetic resonance imaging) and PET (positron emission tomography) help the follow up of grafted islets. Nevertheless, it is also challenging due to the difficulty in attaching the imaging agent (dye-loaded nanoparticles) to the islets and its loss after contact with blood which reduces the monitoring time [73, 74]. Due to the limitation of the aforementioned techniques, researchers have been trying to find prognostic markers to follow the function and the viability of β-cells. One of this promising marker is miRNAs [75].A team research in Geneva demonstrated that dosage of insulin mRNA in the peripheral blood is a promising method for the prediction of islet graft damage [76]. In our lab, our team has shown that the microparticles (MPs) level, which are microvesicles made by a cell under stress conditions and circulating among the grafted islets in patients, varies according to the state of the graft. Indeed, a peak of total MPs before a fall in C-peptide was reflected the loss of function of the graft. Thus, circulating MPs in the transplanted patients could be an early marker of graft loss [77].

III.4 Favorable sites for islet transplantation

An islet is a viable unit that needs a continuous nutrient and oxygen supply. As a result, after isolation, it should be grafted in a site that is able to respond to its need. Lacy *et al.* suggested that the liver is the best site for islet transplantation after a study on rat model [78]. The insulin independence after islet infusion by the portal vein in a T1D patient confirmed Lacy *et al's*. choice of liver [44]. Under X-ray, infusion of islets is performed in the portal vein by catheterization while monitoring the portal venous pressure. Portal venography is performed using iso-osmolar contrast to confirm position. The islet preparation is resuspended in a medium that contains 20% human albumin and heparin (35 U/kg recipient body weight if pellet volume is < 5 ml; 70 U/kg recipient body weight if pellet is 5 to 10 ml). Islets are infused via a catheter by gravity pressure directly into the main portal vein. The catheter is then removed and a biological glue (Avitene) is inserted to limit the bleeding at the injection site [79]. The huge advantage of using the liver as transplantation site is the low risk of this operation and the rapid physiological secretion of insulin in the liver [80]. On the contrary, the down sides include risk factor of thrombosis, lower oxygen tension in comparison with the pancreas, induction of IBMIR (instant blood-mediated inflammatory reaction), noxious side effects of the high level of immunosuppression treatment, delayed revascularization of infused islets and glucolipotoxicity with loss of 50-75% of infused islets [4, 81]. Due to the number of undesirable complications, researchers have showen the need to find another site that will be more receptive

to the islet graft and with less complication. Spleen, pancreas, kidney capsule, bone marrow, subcutaneous space, peritoneum and omental pouch could be the promising locations for islets transplantation [82]. However, the application of these sites from experimental to clinical is big challenge. For example, the use of kidney capsule as an implantation site requires less islets to reverse hyperglycemia in mice but in humans, a larger quantity of islets was needed in comparison to the intraportal injections. Furthermore, the surgery is more invasive [83]. The subcutaneous space as an alternative site currently presents a very interesting domain for research. The advantage of this site is the easy access and receptiveness to allow biopsies, which is very difficult to do in the case of intraportal injections. These advantages stimulated researchers like Barkai *et al.* to develop a different subcutaneous or encapsulated system that allows islet to have sufficient nutritional and gaseous environment with no immune or inflammatory responses [84]. For meantime, the intra-portal infusion in the liver remain the preferred site for islet transplantation, until other sites of transplantation have been proven effectively with no complications.

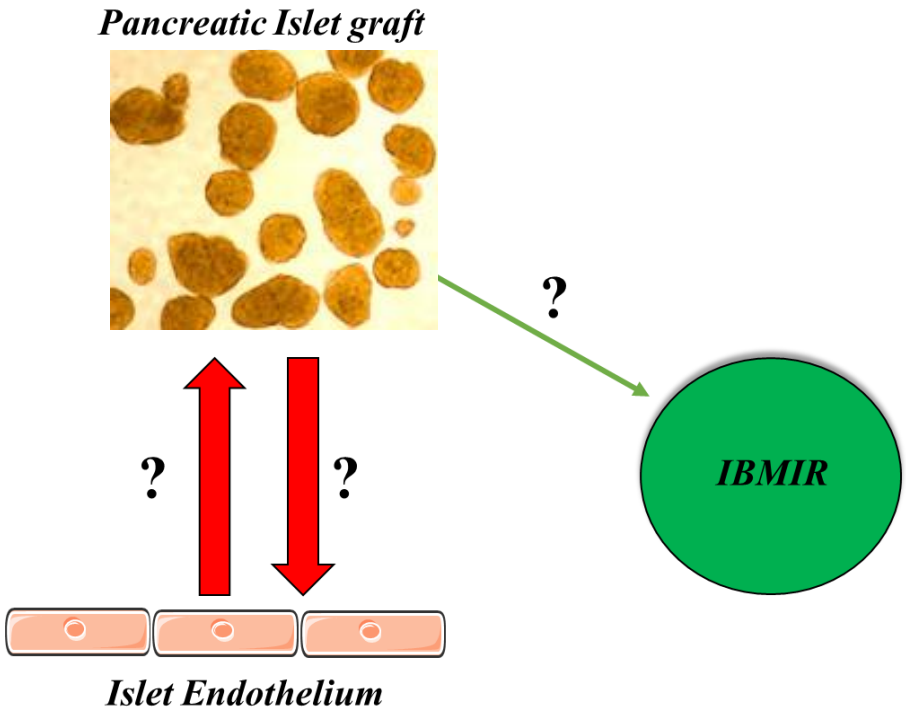
III.5 Advantages of islets transplantation

The debate regarding the efficiency of islet grafts compared to whole pancreas grafts and the optimized insulin therapy by external or implanted insulin pump with continued glucose measure is yet ongoing. A prospective study comparing islet transplantation to intensive insulin therapy in T1D patients showed that the islet transplantation is associated with less progression of microvascular complications than intensive insulin therapy [85]. Other studies comparing intraperitoneal insulin infusion to islet transplantation, suggested that metabolic results improve using both methods, but were significantly better with islet transplantation versus intraperitoneal insulin infusion [85]. When comparing islets to pancreas transplantation, Gerber PA *et al.* showed that islet transplantation results in glucose control were comparable to those achieved with pancreas transplantation. A key advantage of islet versus pancreas transplantation is that 40% of patients with pancreas transplantation need a second surgical intervention versus 0% in islet's case [86]. Over last decades, the field of islet transplantation has evolved significantly and with the success in all above mention trials showing a big improvement in results and long-term data, this transplantation domain is now ready for the next step by evaluating its long term safety, efficacy and cost [87].

Despite all these advantages, there are still many limitations in islet transplantation, which appear in all different steps of the process such as pre-transplantation, peri-transplant and post-transplant. The most limited factor is the scarcity of isolated islets justifying the need to use more than one pancreas for islet transplantation. As such, other complications rise to the surface where organ variability from one donor to the other necessitates further verification steps. The quality assessment of the grafted cells constitutes the pre-transplantation limits. The instant blood mediated inflammatory reaction (IBMIR), difficulty in islet revascularization, recurrence of autoimmunity, allo-reactivity, immunosuppression treatment, limitless expandable source of stem cell or xenograft-derived insulin-secreting cells and the lack of early markers of graft loss are the major limitations post-transplant. Hence, understanding better these difficulties may improve the success of islet transplantation.

Chapter IV

Difficulties and prospects for better islet graft outcome



IV Difficulties and prospects for better islet graft outcome

IV.1 Non vascular difficulties

IV.1.1 Immunosuppressive treatment: a double-edged weapon

Immunosuppressive treatments are administered to graft recipients before and even after islet transplantation to avoid rejection of grafted islet by decreasing the body's immune response. From 1990 to date, a vast development was done in the amelioration of the immunosuppressive treatment. However, these treatments remain destructive to β-cells. This is why a compromise between efficacy and toxicity has to be met for an optimum transplantation results.

As it is well known, the T-lymphocytes (TL) are the main actors in the immune response against islet transplantation next to the B-lymphocytes (BL) which, upon activation, they produce allo-antibodies against donor HLA antigens. Like all immune responses, TL need three types of signals to be activated. It starts by the presence of allo-antigens combined with a co-stimulatory molecules presented by APC, that initiate the TL response by activating three signal transduction pathways including the calcium–calcineurin pathway. The signal transduction pathways induction constitutes the second signal to activate TL. Finally, the third and final signal debuts when the mammalian target of rapamycin (mTOR) pathway is activated by the release of interleukin-2. This third signal leads to a large number of T effector cells after initiation of TL proliferation [88]. In the year 2000, Edmonton group made great contribution by the establishment of a steroids-free immunosuppressive protocol. It consisted of a combination of Sirolimus (Rapamune, mTOR inhibitor) in high doses, low doses of Tacrolimus (Prograf, calcineurin inhibitor) and a monoclonal antibody against the interleukin-2 receptor (daclizumab). Despite that Sirolimus induce TL apoptosis and the generation of regulatory T-cells (Treg), it has been reported that it has a destructive effects on β-cell regeneration [89]. Tacrolimus, also has proven very useful in islet transplantation but at the same time, also has been found toxic to β-cells. Therefore, Sirolimus and Tacrolimus combination as immunosuppressive treatment is not the best solution to avoid graft rejection. This is because they have nephrotoxic effects, can lead to the development of hypertension and hyperlipidemia which, in the end, may contribute to increase micro- and macrovascular complications risk [88]. After the year 2000, the most favorable immunosuppressants in this domain remain the anti-TNF-α (etanercept) and recombinant TNF-α receptor protein [90]. When etanercept was used in combination with prednisone (daclizumab) and rabbit anti-thymocyte globulin, resulted in high success rate having 5 from 8 patients still insulin independent after 1 year [91]. Studies

have also shown a reduction in the number of islets needed to achieve insulin independence by the combination of etanercept with an exenatide like glucagon-like peptide-1 (GLP-1) and Edmonton immunosuppressive protocol [92].

Some studies showed that the inhibition of the mTOR signaling could help avoiding cellular senescence (see chapter 5) [93, 94]. Not to forget that senescence may leads to the reduction in the number of functional islets that can take place during the islet graft, or pre-existent before isolation from the donor depending on his/her age.

A promising, novel approach, which is under development, is the islet encapsulation. Islet encapsulation aims to mimic the physical properties of natural biological barriers. This method will allow the selective permeability, which allows the passage of nutrients and oxygen to the graft sustaining its function. The advantage of protecting transplanted islet from immune system attack is that it eliminates the need for immunosuppressive treatment or at least reducing it.

This means that immunosuppressive treatment is inevitable for the success of islets graft. This is quite challenging, because this treatment should be taken over all life span. Therefore, the fact that it has deleterious effect should be taken with consideration and trying to use as little as possible to avoid graft rejection and little side effects.

IV.1.2 IBMIR: an early non-immune alteration of grafted islet

IV.1.2.a IBMIR description

IBMIR (Instant Blood Mediated Inflammatory Reaction) is another major difficulty for grafts, especially in islet transplantation. It is considered as one of the earlier cause for graft failure, which occurs immediately after islets injection. It is launched directly after the first contact of injected islets with the portal blood of the recipient. IBMIR is a nonspecific response mediated by the innate immune system characterized by coagulation and complement activation, which leads to rapid activation and aggregation of platelets to the surface of the pancreatic isles. It then followed by the recruitment of leukocytes that infiltrate into the islets [95]. In addition, it is characterized by the expression of tissue factor (TF), which is one of the initiation factors of IBMIR. The onset of these different cellular mechanisms leads to premature deterioration of the integrity and functionality of the islets (Figure 6). Under normal conditions, TF is not expressed on the surface of the endothelial or other cells that are in direct contact with blood, but certain pathological stimulus (lipopolysaccharide (LPS), cytokines, etc.) can induce its expression. It has been shown that TF is expressed by α and β cells of pancreatic islet while is not present on exocrine cells [96]. In an experimental model by contacting human pancreatic

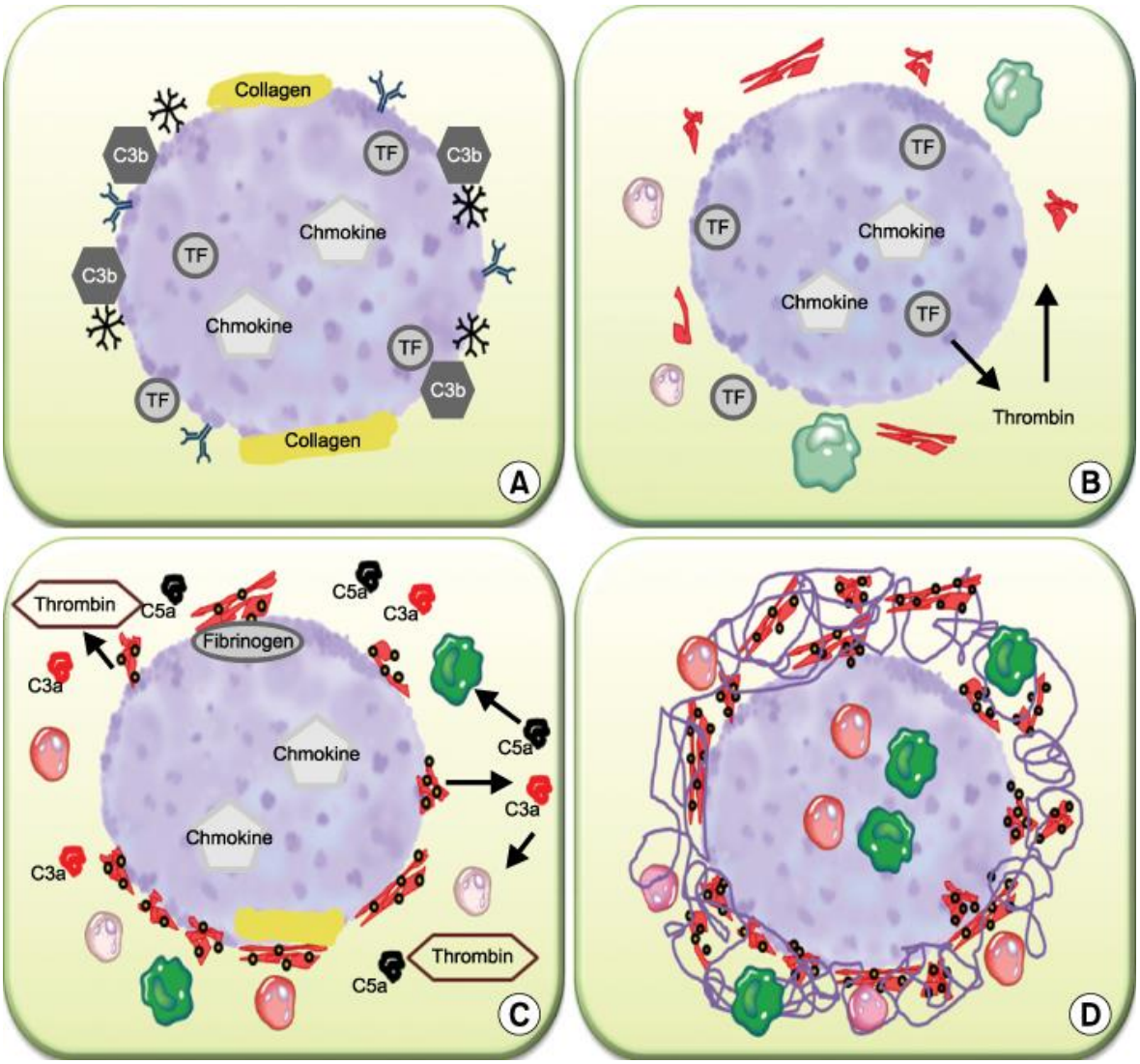


Figure 6 : IBMIR (Instant Blood Mediated Inflammatory Reaction) (Nilsson B, Xenotransplantation, 2008)

(A) In contact with blood, IgG and IgM antibodies bind to the islet surface and activate complements. This then leads to the deposition of C3b/iC3b to the surface. (B) Tissue factor (TF) activates the coagulation system via the extrinsic pathway. As a result of extrinsic pathway activation, prothrombin is cleaved into thrombin. Thrombin subsequently generates fibrin and activates platelets. (C) Platelet activation increases the affinity of the integrins GPIIb-IIIa and a2b1 for fibrin and collagen, respectively. Activated platelets bind to fibrin and collagen on the islet surface. (D) Amplified by platelets, thrombin generates more fibrin creating a capsule containing platelets, PMNs and monocytes surrounding the islets. Chemotactic factors (e.g., C5a and IL-8) that were released as a consequence of IBMIR or released directly from the islets (e.g., MCP-1, IL-8 etc.), exert their action on PMNs and monocytes that infiltrate the islets in large numbers after 30 min.

islets and compatible ABO human blood, the initiation of coagulation, platelets consumption and infiltration of the islet by many leukocytes (principally neutrophilic granulocytes) has been observed directly after 1 hour. Interestingly, all of these reactions were inhibited by the addition of a TF blocking antibody [97-99].

The pre-transplant pro-inflammatory processes prepare the base and the elements by which IBMIR will start in the peri-transplant and amplifies in the post-transplantation phase. The islet isolation procedure maintains and amplifies the pro-inflammatory state, where in fact, the enzymatic digestion and mechanical stress induce the secretion of inflammatory mediators. In this context, studies have shown that islets express TF and over-express the genes associated with inflammation and apoptosis via activation of the NF-κB pathway, directly after isolation and culturing. Moreover, the secretion of pro-inflammatory molecules such as IL-8, IL-6 and cyclo-oxigenase-2 is mainly high 1 day after culturing [100].

After transplantation, during IBMIR the activation of inflammation is clear by the high amount of circulating inflammatory mediators such as IL-6, IL-8 and interferon-inducible protein-10 [101]. Infiltrated macrophages secrete IL-1β which induces the secretion of other inflammatory mediators by the islets such as IL-6, IL-8, TNF-α and MCP-1 [102]. This inflammatory context induces significant production of ROS where β cells are particularly sensitive because of their low expression of the antioxidant enzymes such as glutathione peroxidase, catalase and superoxide dismutase. On the other hand, the presence of APCs in islets would be responsible for amplification of the inflammatory response by lymphocytes. Indeed, a recent study showed that 67% of immune cells in the islets are APCs and 50% of these APCs are BL [103]. During the first 3 hours after islets injection, the damage inflicted by IBMIR on transplanted islets is revealed by a significant increase in coagulation markers such as thrombin-antithrombin and proinflammatory cytokines such as interleukin IL-6, IL-8 and IP-10 in conjunction with C-peptide level increase [102].

Coagulation is stimulated directly after injection when the islets come in direct contact with the portal blood. The launch of coagulation is done through TF, a membrane protein whose expression is inducible in monocytes-macrophages and vascular endothelial cells. TF expressed on the surface of isolated islets interacts with factor VIIa and initiates the coagulation cascade by the formation of thrombin and generation of fibrin clots [99, 104, 105]. The presence of thrombin at high concentration has a proinflammatory effect that causes apoptosis. In turns, it aggravate the destruction of pancreatic islets [102]. For that, a study showed that low-

molecular-weight dextran sulfate (LMWDS), a potent inhibitor of coagulation and complement activation, could efficiently suppress IBMIR [106].

Complement activation is major IBMIR element. The complement system is composed of nine plasma proteins (C1 to C9), where they are activated in a cascade following the fixation of an Ag-Ab complex on the C1 fraction. This assembly is very important because it allows the lysis of bacterial agents or recruitment of neutrophils and monocytes. Activation of complement proteins C3a and C5a leads to the recruitment and activation of leukocytes, platelet adhesion, production of ROS and cytokines [107]. Additionally, the complement protein C5a is responsible for the activation of coagulation and inflammation by modulating the expression of TF in neutrophils [108]. Complement activation is demonstrated in the serum of transplanted islets recipients by increased complement proteins [109].

IV.1.2.b Possible treatment

In order to limit the earlier loss of grafted islets and maintaining a sufficient quantity, preventing the destructive effect of IBMIR on islets appears as a crucial factor. This has been validated by approaches targeting the control of IBMIR that confirmed the importance of control of the initial pro-inflammatory, pro-apoptotic mechanisms of graft. In addition, these studies support the use of a combination of anticoagulants and anti-inflammatory as well as the association of the pre-treatment of islets and the post-treatment of the patient.

As anti-inflammatory treatment, actually TNF-α inhibitor (Etanercept) constitute a very important part in islet transplantation. These maneuvers began with a small portion (11.8%) in 1999-2002 and increased to 33.8% between 2007 and 2010 [90]. Important improvement in the insulin independence rate was proven by a meta-analysis study involving TNF-α inhibitor treatment coupled with a TL depletion regimen [110]. The results using this combination is now likely comparable to that of whole-organ pancreas transplantation alone, offering approximately 50% success at 5 years post-transplantation [90, 110, 111]. In addition, McCall M *et al.* study showed that the use of Anakinra (IL-1β receptor antagonist) in combination with etanercept led to notable improvement in islet engraftment [112].

In a mouse model of syngeneic transplantation, it was shown that the damaged islets contain a high level of High-mobility group box-1 (HMGB1), which is released immediately after transplantation. This release causes the activation of Kupffer cells, which secrete in turn the HMGB-1, IL-12 and IL-6. IL-12 promoting the activation of NK cells and production of INF-γ that is deleterious to the islets. The HMGB1 release was also observed following cytokine

stimulation and anti-HMGB1 treatment was able to restore glycemic control in diabetic mice [113].

The strategy to avoid or limit coagulation is based on the use of heparin, dextran and inhibitors of factor VIIa or thrombin. Although these molecules have contributed to the improvement of the survival of transplanted islets, they expose patients to an increased risk of bleeding during percutaneous puncture of the portal vein [114]. Moreover, the heparinization of the islets surface before their injection, has also been proposed and has reduced the IBMIR, without increasing bleeding risk [115].

In addition of anti-inflammatory and anti-coagulation treatments, different agents directed against the complement system have been developed one of them is C5a inhibitory peptide. A recent study show that the combined effect of anticoagulant gabexate mesilate and complement protein C5a inhibitory peptide improves the success of transplantation by reducing the cross-talk between coagulation and the complement system and that this combination is free of side effects [116].

TF is the main element of the IBMIR at the interface between inflammation and coagulation. Its ability to amplify the deleterious mechanisms is based, in part, on its high expression in islets. What amplifies its effect is due to its ability to spread in its active form, the MPs. It is interesting to note that the study of islet culture supernatants also showed a procoagulant activity that may be linked to the presence of MPs revealed by electron microscopy.

IV.1.3 Ischemia reperfusion

During islet preparation prior to transplantation and many days after transplantation before complete revascularization, islet are exceptionally sensitive to what is known as ischemia reperfusion injury. Before transplantation, islets suffer from hypoxic until complete revascularization process. During this period, hypoxia can lead to apoptosis, activation of the NF-κB pathway as well as the MCP-1 secretion (Monocyte Chimoattractant Protein 1) for macrophage infiltration in the islets. For that, it appears that the ischemia duration is very important in obtaining high quality islets. A duration superior to 8 hours of cold ischemia causes significant damage, loss of functional human islets and decreased isolation performance [117].

During the integration of the graft, reperfusion follows ischemia. It starts in tissues by the restoration of blood flow by islet revascularization. Reperfusion is well characterized by the release of cytokines, stimulation of adhesion molecules on vascular endothelial cells and the adhesion and extravasation of leukocytes into post ischemic tissue. In addition to secreted

factors, reactive oxygen species (ROS), which is a major effector in oxidant stress and that may lead to the cellular senescence in the islets, are also produced [118]. The release of all these factors contribute in disturbing the integrity of the vascular endothelium, in the secretion of the chemokine and in the platelet aggregation, which participate in the loss of the grafted islets [119]. Furthermore, we have found that tissue damage started during the ischemic period and progressed during the reperfusion period. Many studies have shown that the effect of the ischemia reperfusion injury can be inhibited or decreased by using adiponectin. Adiponectin works through the COX-2-TNFα-NF-κB-dependent signal transduction pathways [120]. Injures were also found to be reduced by the immunosuppressive role of the Adenosine A_{2A} receptor. The use of adenosine as an agonists of the A_{2A} receptor resulted in more than 2-fold improvement in the efficiency of islet transplantation [121].

IV.1.4 Efficiency of the islet isolation

Islet isolation yield ranges between 200000 and 400000 islets per pancreas that constitutes one-half of the human pancreas. However, for transplantation we need almost 9000 islets/kg, meaning that the islet number is not sufficient and more than one pancreas for each operation is needed. In addition, after culturing or preconditioning and due to many factors like ischemia, we found that almost 30% of islets are available for transplantation. Therefore, researchers have been trying to optimize islets isolation in order to minimize loss and avoid using another pancreas that is frequently not available or poses complications in regards to histocompatibility that might accelerate the grafted islet loss [122].

IV.2 Vascular difficulties: islet revascularization

In addition to all the non-vascular difficulties, islet revascularization is a vascular complication facing a successful graft. Although pancreatic islets constitute 1-2% of the pancreas mass, they require 5-10% of blood flow. Pancreatic islets have a very special feature that allows them to have more oxygen pressure compared to that of exocrine tissue by the higher density of blood vessels that is also lined with fenestrated endothelial cells that allow the exchange of oxygen and nutrients [123]. Directly after transplantation, islets need quick access to oxygen, glucose and other metabolites. This is because the isolation is very harmful for islets' structure where all connections with blood vessels will be destroyed. This might lead to ischemia and stress [124]. In contrast to whole pancreas transplantation, where blood perfusion is re-established directly after operation by the reconnection of blood vessels, the islets revascularization need

between 7 to 14 days after transplantation [125]. The islets revascularization constitute a major limiting factor and is dependent on endothelial cells [126]. Endothelial cells originate mainly from three different sources: 1) from the transplant recipient, 2) from the intra-islet of the donor where it constitutes 7-40% of the endothelial cells [127]. However, large proportions of endothelial cells from the donor decrease when islets are cultured for several days before transplantation [128]. Molnar C *et al.* found that transplantation of fresh islets without the culture phase may improve islet revascularization results. Direct grafting results mild hypoxia and can lead to the decrease in glucose induced insulin release [129]. 3) The last source being the minor source is from bone marrow [130] (Figure 7). All data have shown that 1-3 days post transplantation the cell mass, islet insulin content and islet viability decrease. This is why we require a big number of transplanted islet pooled from more than one donor [124]. The liver that is a highly perfused organ has been thought of for long time, as a very promising site for transplantation. However, it is still facing a big problem that inner islet core does not receive sufficient oxygen and nutrients supply. This leads to the decrease in insulin secretion due to hypoxia and cell death [131]. After transplantation, islets produce angiogenic factors like vascular endothelial growth factor-A (VEGF-A) and angiopoietin-1, which help islets to improve and accelerate the revascularization by the recruitment of endothelial cells from the transplant recipient to the site of transplantation and utilization of intraislet endothelial cells. [132]. The overexpression of VEGF-A in the islets of mouse before transplantation allowed better islets revascularization and faster insulin independence [133]. In fact, there are many strategies used to increase revascularization: The first of which is by increasing the action of proangiogenic agents or by inhibiting antiangiogenic factors. This leads to the stimulation of proliferation, migration and maturation of endothelial cells. To optimize this approach it seems that the accurate control of timing, dose and duration of angiogenic factor action in the post-transplant period is crucial for ideal formation of mature and completely functional islet vasculature [123]. A second approach can be by direct targeting of endothelial cells or by improving their capability to form mature and functional vessels. This approach can also happen by the addition of preactivated endothelial cells or some type of endothelial progenitor cell populations [123]. Recently Cao XK *et al.* proposed a new approach using tissue engineering where they found that the co-combination of islets with bone marrow mesenchymal stem cells (BM-MSCs) promotes angiogenesis by the migration of BM-MSCs to transplanted islet, where it will differentiate into vascular smooth muscle cells (VSMC), vascular endothelial cells (VEC) and secrete vascular endothelial growth factor (VEGF). In addition, they found that it improved

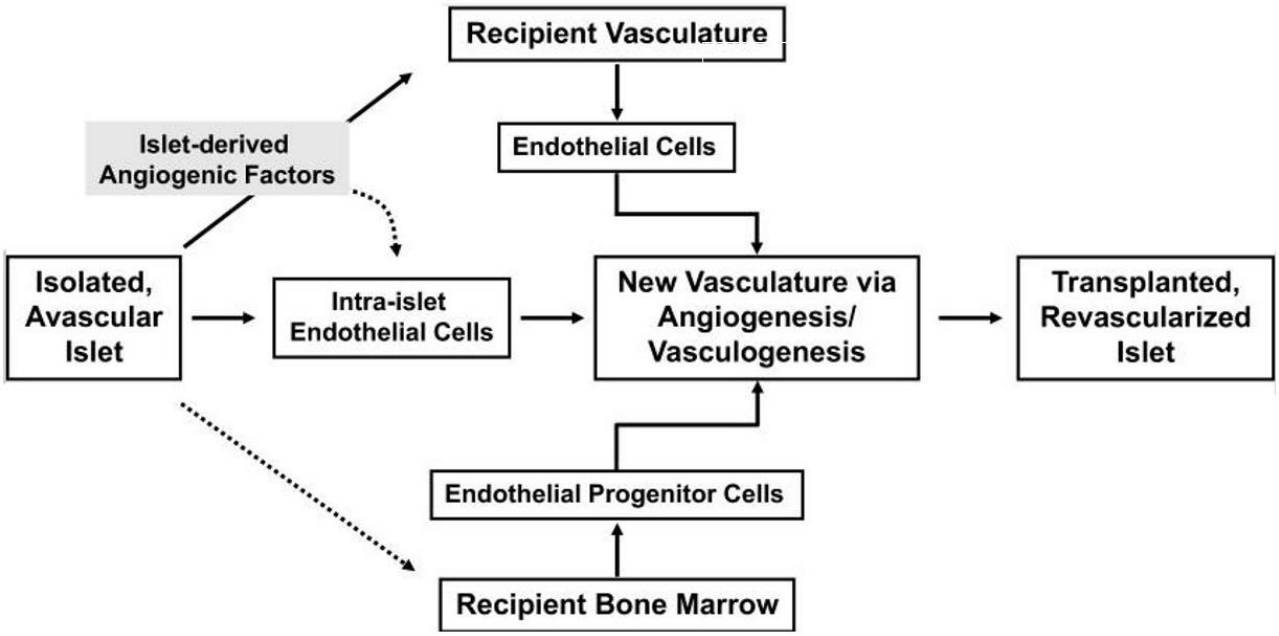


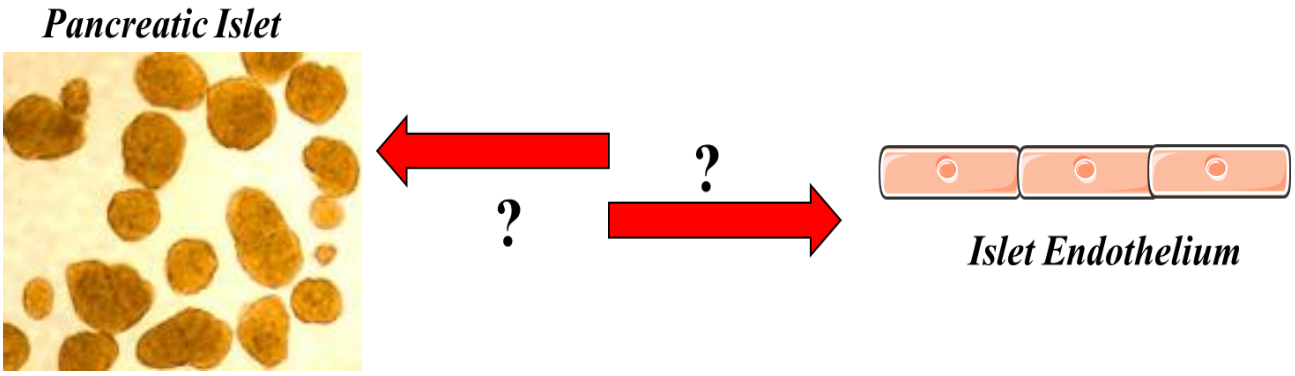
Figure 7 : Sources of endothelial cells in islets revascularization (Brissova M, Powers AC, Diabetes 2008)

allograft immune tolerance of islet through the increasing of donor lymphocytic chimerism [134]. The use of immunosuppressive drugs has long been an inevitable treatment after islets transplantation. It can also be a detrimental cause of poor outcome after islet allotransplantation. The effects of both current standard immunosuppressant rapamycin and tacrolimus have been finally elucidated by recent studies that have shown to have an inhibitory effect on the revascularization of isolated pancreatic islets. These results clarify that to enhance the success rate of islet allotransplantation, we need a real improvement in the immunosuppressive regimen, particularly regarding the revascularization of islet grafts [135, 136].

Based on the above mentioned results, we can say that in order to have a proper function of the graft many requirements should be met. Sufficient number of intact islets with minimal stress or damage should be obtained. To have that, proper revascularization of the islets should occur. In order to do so, functioning islet endothelium should be obtained not only to ensure oxygen and nutrient but also it contribute in the insulin secretion. It is also involved in the growth of the endocrine part, the regulation of the β-cell mass and release of other important factor like NO and cytokine. All combined sheds light on the importance of the islet endothelium as a therapeutic target especially in the islet transplantation. Thus, we need to better understand the islet endothelium and its signaling and its role in the islet physiology and pathology to optimize the islet graft success.

Chapter V

Islet Endothelium: An important structure for islet survival and function



V Islet endothelium: An important structure for islet survival and function

V.1 General structure and definition of the endothelium

In the human body, studies have found that blood vessels play an essential role in vascular homeostasis [137], through a complex network that constitutes the circulatory system, which is mainly made up of: 1) the heart, vessels and blood, all together form the cardiovascular system, 2) the capillaries and lymph vessels which constitute the lymphatic system. The wall of the blood vessels histologically can be divided into three distinguished layers that contain smooth muscle cells and elastin, which are the intima, the media and the adventitia [138].

1- The intima (tunica interna/intima) is the thinnest layer, which consists of a monocellular layer of epithelial cells known as the endothelium, at the interface between the blood flow and the vessel wall, maintained by connective tissues and the interior elastic lamina.

2- The media (tunica media) is the muscular middle layer of the arteries and veins essentially buildup of smooth muscle cells, elastic connective tissue and elastic lamina that separated it from adventitia. It constitutes the thickest layer in arteries. The main role of the media is the regulation of vascular tone by controlling the vasoconstriction and the vasodilatation.

3-The adventitia (tunica externa) is the thickest layer in veins and the outermost layer of the blood vessels. It has collagen, elastin, fibroblasts, macrophages, nerve endings and *vasa vasorum*, which are small blood vessels consisting of arterioles [138, 139] (Figure 8).

In 1865, the embryologist Wilhelm Hirsch used the word endothelium to label the cell layer covering the lumen of the vascular system and the internal cavities of the body. After that it was well defined as the lining of the blood and lymph vessels [140], where it is a spatially dispersed system that spans the whole human body. The endothelium forms a semipermeable wall consisting of an endothelial cell (EC) layer [141] that are in direct contact with the blood and form a selective barrier between the vessel lumen and the surrounding tissues. ECs contain multiple transport systems for amino acids and facilitative glucose transporters. That latter of which is very important in diabetes [142]. These EC cells are structurally heterogeneous, electron microscopy analysis have identified differences in intercellular junctions. This has led to the classification of three types: i) continuous endothelium: found in most arteries, veins and capillaries of the brain, skin, lung, heart and muscle. In this type of endothelium, ECs are attached by tight junctions to a continuous basal membrane. ii) Fenestrated endothelium: similarly, to continuous endothelium, it is connected to a continuous basal membrane but is

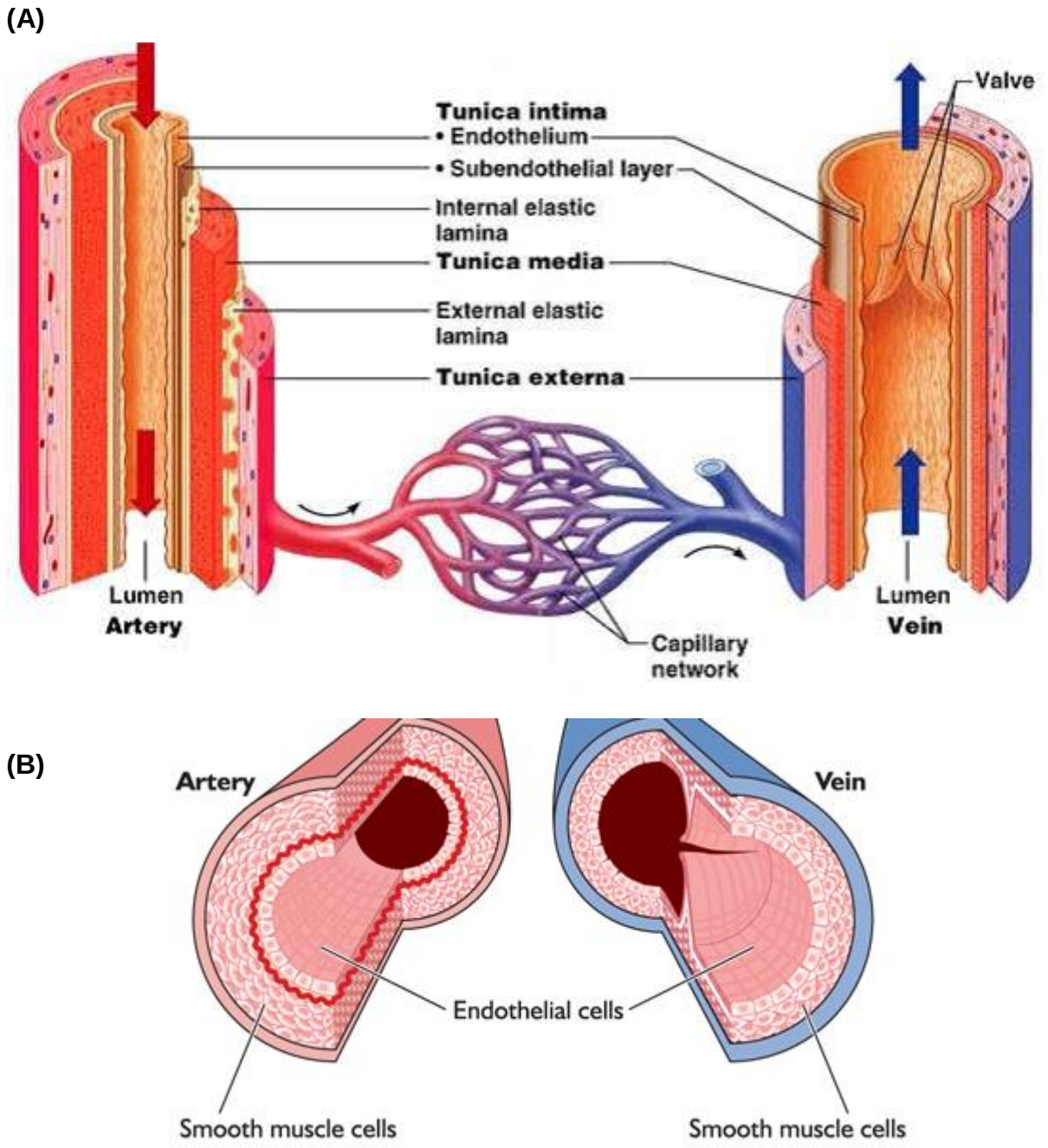


Figure 8 : Structure of Blood Vessel

(A) (Adopted from Wayne W. La Morte, MD, PhD, MPH, Boston University School of Public Health), **(B)** (Topol Rric, J Science Translational Medicine, March 2012).

characterized by the existence of transcellular 50–60 nm wide pores. This structure is very important in tissues with an elevated trans-endothelial transport, like endocrine and exocrine glands. iii) Discontinuous endothelium: is associated with a poorly structured basal membrane where large fenestrations of 100-200 nm wide without diaphragm. This type of endothelium is largely found in the liver, spleen and bone marrow [143, 144].

Additionally, ECs have a group of components that ensure both metabolic and synthetic functions, which are involved in many aspects of vascular biology [145] (Figure 9).

V.2 Islet endothelium structure and function

In all organs, the main role of blood vessels is to ensure the supply of nutrients, oxygen and growth factors that are the indispensable elements for any cell survival. Moreover, the islet endothelial cells in the pancreas have an essential role especially in the early stages of development by its positive paracrine signals that induce insulin expression in the endoderm [146]. The islet endothelium also affects β-cell proliferation that influences adult β-cell functions and produce a number of growth factors along with angiogenic substances [147]. The islet vasculature appears to be crucially implicates in glucose homeostasis. This occurs by quickly responding to any fluctuation in plasma glucose concentration by secreting islet hormones into the circulation. Furthermore, the islet endothelial cells supply the pancreatic β-cells with a high oxygen quantity, this is essential for its function [4].

V.2.1 Morphology and characteristics of islets vasculature

Pancreatic islet of Langerhans are highly vascularized. In fact, it is one of the most vascularized organs despite it only compromising 1% of the total pancreas volume. It also needs at least 10% of the blood perfusion [147]. As a result, the pancreatic islets receive about 10-times more blood than the exocrine part [12]. The islet endothelium has a 95 nm fenestration closed by a diaphragm [4]. One of the characteristic of the islet vasculature is that it contains approximately 10 times more fenestrae compared to the exocrine pancreatic capillaries [148]. This special characteristic is promoted by high local production of vascular endothelial growth factor-A (VEGF-A) from the islet β-cells [149]. The VEGF family represents an essential stimulator of neovascularisation by inducing proliferation and migration of endothelial cells. β-cells both proliferate and form blood vessels after the migration of endothelial cells towards its location as being a main source for VEGF-A that is responsible for the dense islet vascularization. This why islets have five times higher capillary density than the exocrine tissue [147, 150].

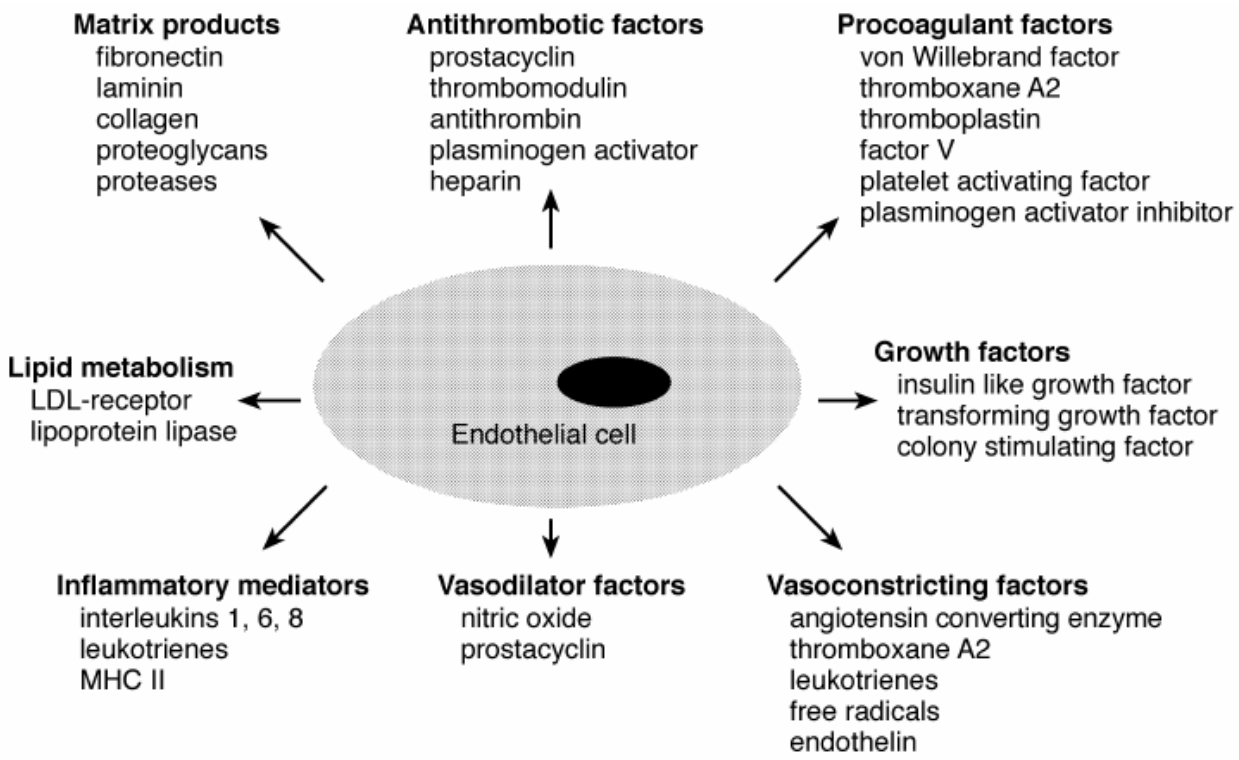


Figure 9: Different mediators that show metabolic and synthetic functions of the endothelial cells (Galley H. F., Webster N. R., British Journal of Anaesthesia, 2004)

V.2.2 The structures of islet microvessels

Pancreatic islets of Langerhans contain three types of microvessels:

(i) Mostly larger islets have three afferent arterioles. These arterioles contain a layer of vascular smooth muscle cells (VMSC) that are responsive to hormones and to locally secreted endothelium-derived vasoactive substances.

(ii) The islet capillaries are five times denser than that of the exocrine tissues of the pancreas. It is described as glomerulus-like. Capillaries structure are made up of a tube of single ECs surrounded by a basement membrane. This explains the fact that capillaries do not possess any blood flow regulatory properties.

(iii) The veins drain the islets. However, their anatomy is different between species depending on the islet size. The venous drainage forms 1 to 4 larger veins that are connected to lobular veins and lastly with the portal vein. The morphology of the islet veins is the same as all other veins in the human body [151].

In humans, a structure called the insulo-acinar portal system is formed when the islet capillaries lead blood to capillaries surrounding acini due to their predominant location within the lobules. Conversely in mice, another structure called the insulo-venous system is formed where the venous blood influenced by the interlobular location of islets is collected by the interlobular veins [12]. Generally, islet β-cells are delimited by at least one capillary and show polarity in their cytoplasm with the secretory granules oriented at the apical pole in the direction of the blood vessel [152]. Human islet capillaries are surrounded by two basement membrane layers consisting of specifically structured and closely associated parenchymal and endothelial basement membranes. In contrast, rodent islet capillaries are only surrounded by the vascular basement membrane [153].

An example of the islet blood flow perfusion threw microvessels is in mice where three different patterns of this flow exist: 1- periphery to center, 2- from center to periphery or 3- from one pole of the islet to the other [12]. Taking into consideration the human islet organization described by Bosco D *et al.* [11] it is possible that the arterial blood comes first in contact with α-cells then follows to β-cells [12].

V.2.3 Islet endothelial cell specialized functions and crosstalk with islet endocrine cells

Islet endothelium has many functions. In capillaries and arterioles it secretes molecules such as NO, that contributes to the regulation of β-cell mass in islets [154], ET-1 and other mediators that affect the VSMC [151]. In addition to its general role of assuring oxygen and nutrients to the endocrine cells, studies have shown that the islet endothelium mainly participates in the

trans-endothelial rapid passage of secreted insulin into the circulation. This contributes to the control the blood glucose level [146, 150]. This function is proven by studies done on mice with pancreatic VEGF-A deletion. It clearly showed a loss of endothelial fenestrations and alteration in blood glucose level [150]. Furthermore, islet endothelial cells also stimulate β-cells proliferation by the secretion of hepatocyte growth factor (HGF). The results of this secretion could be due to the stimulation by the insulin and VEGF-A that are secreted by the islet endocrine [155]. Another study by Kaido T *et al.* suggested that the islet endothelium could secrete collagen IV, which potentiates insulin secretion *via* the interaction with its receptor integrin α1β1 on β-cell [156] (Figure 10).

V.3 Endothelium function in larger vessels:

The endothelium is crucial for body homeostasis. Uncontrolled endothelial cell responses has been shown to be involved in many diseases. This can be the results of endothelial injury, dysfunction and activation [142]. It plays a main role in the modulation of vascular wall tone. The wall tone regulation is controlled by the secretion of biomechanical and chemical stimuli in order to maintain the balance of the contractile status of the vascular smooth muscle cells (VSMCs). It selectively secretes many vasodilator and vasoconstrictor mediators [157] (Figure 11). In addition, it plays a role in the control of the coagulation cascade, the formation of blood clots, permeability, leukocyte trafficking, angiogenesis and immunity [144].

V.3.1 Endothelium-derived vasorelaxing factors

V.3.1.a Nitric Oxide (NO)

NO is an essential signaling cellular molecule involved in several physiological and pathological processes [158]. It is the main physiological vasodilator secreted by the endothelium with a short half-life of a few seconds in the blood, especially in the arteries [159]. NO is produced by the endothelial NO synthase (eNOS) from the cationic amino acid L-arginine. The enzyme eNOS is activated through intracellular calcium in response to modifications in mechanical distension (shear stress) caused by the blood flow on the vascular wall or via receptor-mediated processes [160]. Directly after release, nitric oxide activates soluble guanylate cyclase (GC) in smooth muscle cells, converting GTP to cGMP. This process activates a protein kinase, which leads to the inhibition of calcium influx into the smooth muscle cell and decreased calcium-calmodulin stimulation of myosin light chain kinase. This in turn, decreases the phosphorylation of myosin light chains, decreasing smooth muscle tension

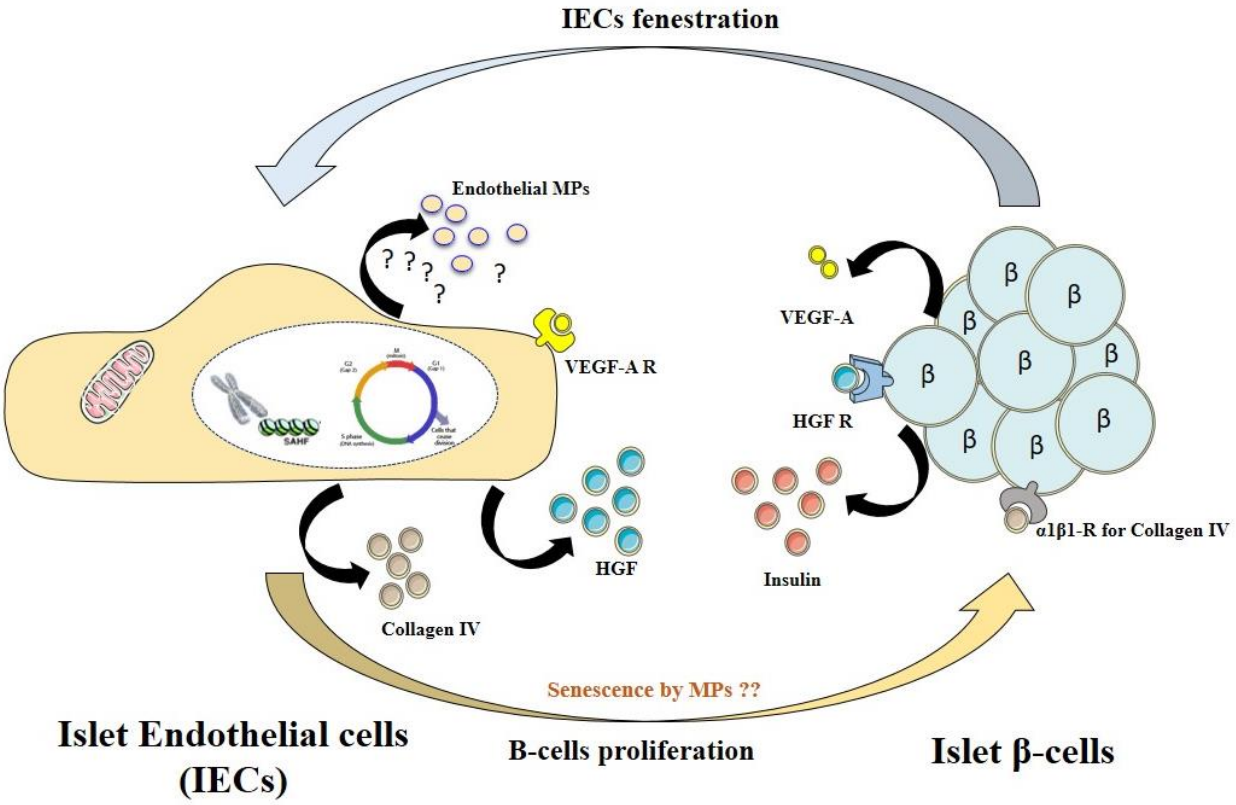


Figure 10 : Cross-talk relationship between islet endothelial cells (IECs) and β-cells

β-cells secrete VEGF-A that is responsible of the ECs fenestration and the high vascularization of the endocrine pancreas, it also secretes the insulin. Secreted VEGF-A and insulin can stimulate the release of the HGF by the ECs that in turns stimulate β-cells proliferation. In addition, islet endothelium could secrete collagen IV, which then potentiates insulin secretion *via* the interaction with its receptor integrin α1β1 on β-cell.

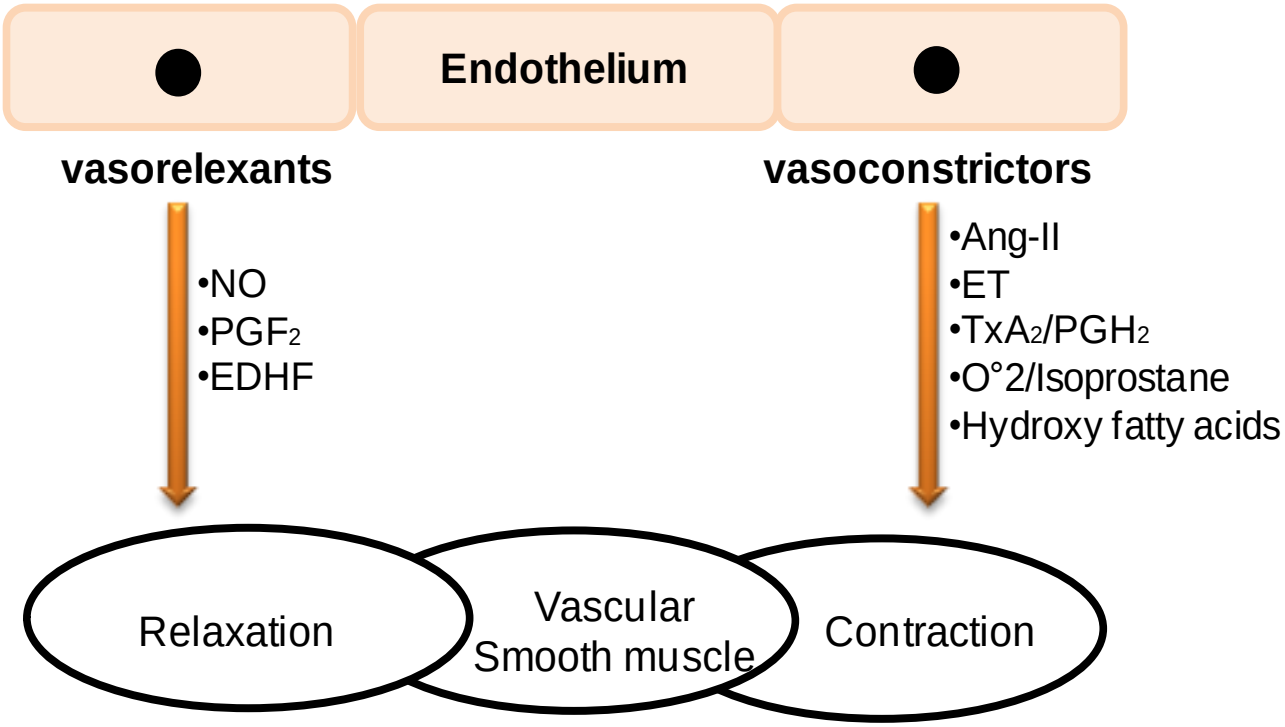


Figure 11: Biochemical mediators of vasocontracting and vasorelaxing released by the endothelium (Abeywardena M. Y., Head R. J., J of Cardiovascular Research, 2001)

NO, nitric oxide; PGI_2, prostacyclin; EDHF, endothelium-derived hyperpolarizing factor; AII, angiotensin II; ET, endothelin; TxA_2/PGH2, thromboxaneA2/prostaglandin H2; O_2^- superoxide anion.

development and yield vasodilatation [142] (Figure 12).

In addition to its role in regulation of vascular tone, NO inhibits platelet aggregation, leukocytes adhesion and has anti-apoptotic and antithrombotic effects, all these prove that NO is a main factor of endothelium viability, longevity and cardiovascular health.

There are three distinctive isoforms of NO synthase (NOS). Each isoform is associated with many characteristics and expression forms, which have been used to establish the isoform's nomenclature:

i) Neuronal NOS (NOS1 or nNOS) that is expressed in neurons, in the heart and in the brain. ii) Inducible NOS (NOS2 or iNOS) that is expressed by induction in response to cellular activation. iii) Endothelial NOS (NOS3 or eNOS) that is associated with the endothelium [161].

The most probable mechanism that might lead to endothelial dysfunction is the reduction of bioavailability of NO. NO reacts rapidly with amino acid residues, ions and oxygen-derived species, specifically superoxide anion (O_2^-) [162]. The latter reaction is very important since it aids in the reduction of cellular O_2^-. NO acts an antioxidant molecule. As a result, the more it interacts with O_2^- the more its bioavailability is reduced. This reaction of NO with O_2^- leads to the formation of peroxynitrite. The generated latter is a powerful oxidant species that has been implicated in established clinical conditions such as hypercholesterolemia, diabetes and coronary artery disease [163].

The role of NOS is compromised in endothelial aging. eNOS activity is decreased due to the increase in the interaction with caveolin that is over-expressed, reduced phosphorylation of Serine 1177 ($S^{1177)}$, increased phosphorylation of Threonine 494 (T^{494}) and also by the reduction in the expression and association with hsp90. However, the most important cause of depletion is the decreased availability of L-arginine and tetrahydrobiopterin (BH4). This can lead to eNOS uncoupling and catalytic activity change that stimulates superoxide anion generation. In turn, this would lead to vascular dysfunction, a phenomenon called NOS uncoupling.

Aged endothelial cells up-regulate the inducible NOS (iNOS) expression that might be caused by the NF-κB-induced vascular inflammation. As a result, high levels of NO will be produced causing the build up of NO levels in the cells. This forces overproduced NO to react with cysteine residues of proteins (like arginase), forming S-nitrosothiol (-SNO)–arginase, which increases arginase activity and L-arginine consumption. As a result, NO directly reacts with O_2^- produced by uncoupled eNOS and production of dangerous reactive nitrogen species, peroxynitrite ($ONOO^-$), which contribute to vascular dysfunction [164].

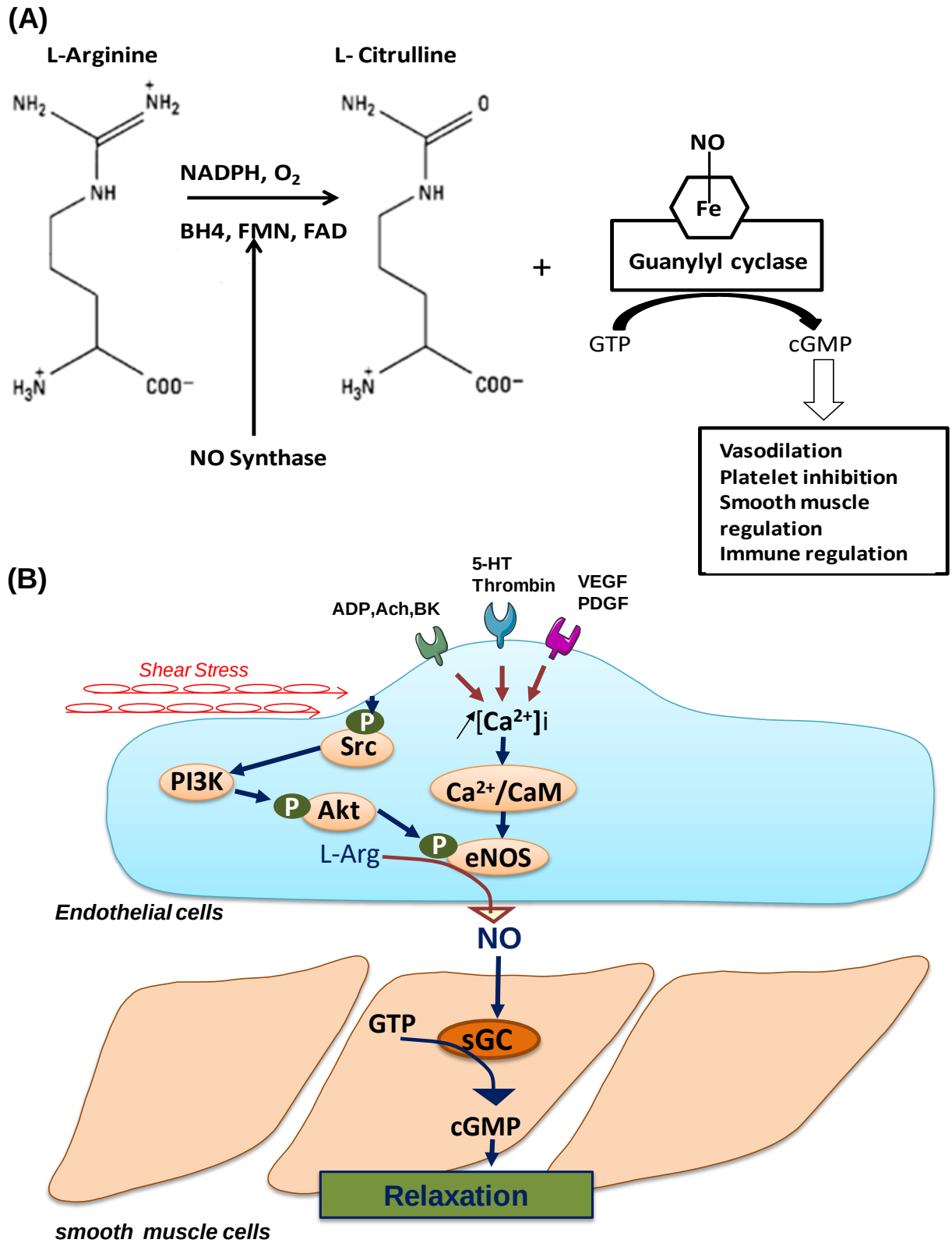


Figure 12: NO generation from L-Arginine and its functional properties in the endothelial cell and its actions in the vascular smooth muscle cell. (Ghalayini I. F., International Journal of Impotence Research, 2004)

V.3.1.b Prostacyclin

In 1976, Needleman P *et al.* discovered the first endothelium-derived vasorelaxant, which was the prostanoid prostacyclin (PGI_2) [165] (Figure 13). The prostacyclin synthesis starts with the liberation of arachidonic acid from membrane bound lipids via the enzymatic actions of phospholipase A_2 (PLA_2) [166]. In endothelial cells, PLA_2 activation is a calcium-dependent step. PGI_2 constitute the final product derived from a series of metabolic reaction starting from arachidonic acid by the cyclooxygenase-2 (COX-2) and prostacyclin synthase (PGIS). Cyclo-oxygenase is present in two isoforms: COX-1 and COX-2 encoded by two separate genes. Cyclo-oxygenase-1 (COX-1) is constitutively expressed, largely associated with physiological functions and is present in many tissues, including healthy endothelial cells being the predominate isoform. COX-2 is induced at sites of inflammation. It is largely associated with pathological functions. COX-2 is not constitutively expressed. However, it can be induced rapidly and transiently in many cells, including vascular endothelial cells and smooth muscle cells, under the effect of specific stimuli such as cytokines, growth factors, bacterial endotoxins, tumor promoters and hormones by macrophages, neutrophils and activated mesenchymal cells [167, 168].

V.3.1.c Endothelium-derived hyperpolarizing factor (EDHF)

Endothelium-derived hyperpolarization factor (EDHF), currently called EDH, is another important mechanism of endothelium-dependent relaxation. It is mostly observed in small arteries and arterioles such as second and third-branch mesenteric artery, induced by diverse stimuli including locally released factors, blood flow and low pH [169, 170]. After suppressing the synthesis of both NO and PGI_2 by pharmacologic or genetic manipulations in vessels, an endothelium-generated signaling mechanism still remains and causes hyperpolarization and relaxation of vascular smooth muscle cells (VSMCs) (Figure 14). In addition of NO and prostacyclin, EDHF constitutes the third vasodilator that is triggered by the vascular endothelium [159].

V.3.2 Endothelium-derived vasocontracting factors (EDCF)

V.3.2.a Thromboxane A_2 & Prostacyclin I_2 (TxA_2 & PGI_2)

Endothelial dysfunction, as in the case of hypertension, can be yielded by endothelium-derived vasocontracting factors (EDCF) that result from cyclooxygenase (COX)-mediated metabolism of arachidonic acid [171]. The two isoforms of COX, COX1 and COX-2, convert the

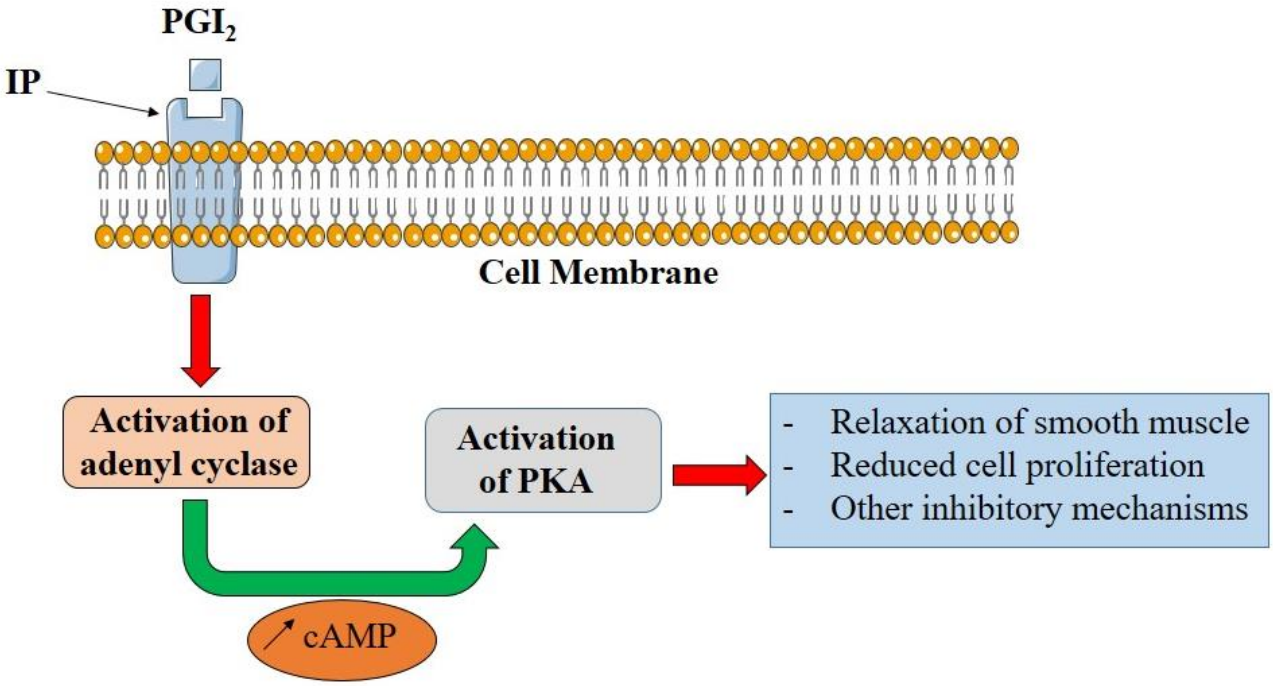


Figure 13: Prostacyclin (PGI_2) receptor signaling

When activated by PGI_2, the G-protein-coupled receptor termed IP stimulates adenyl cyclase leading to increased intracellular cyclic AMP (cAMP) in the vascular smooth muscle. Increased cAMP then leads to activation of protein kinase A (PKA) that reduces intracellular Ca^{2+} by decreasing Ca^{2+} release and stimulating its uptake from the endoplasmic reticulum. These actions terminate in relaxation of smooth muscle. In addition, it has been reported that PGI_2 can enhance the release of NO in endothelial cells.

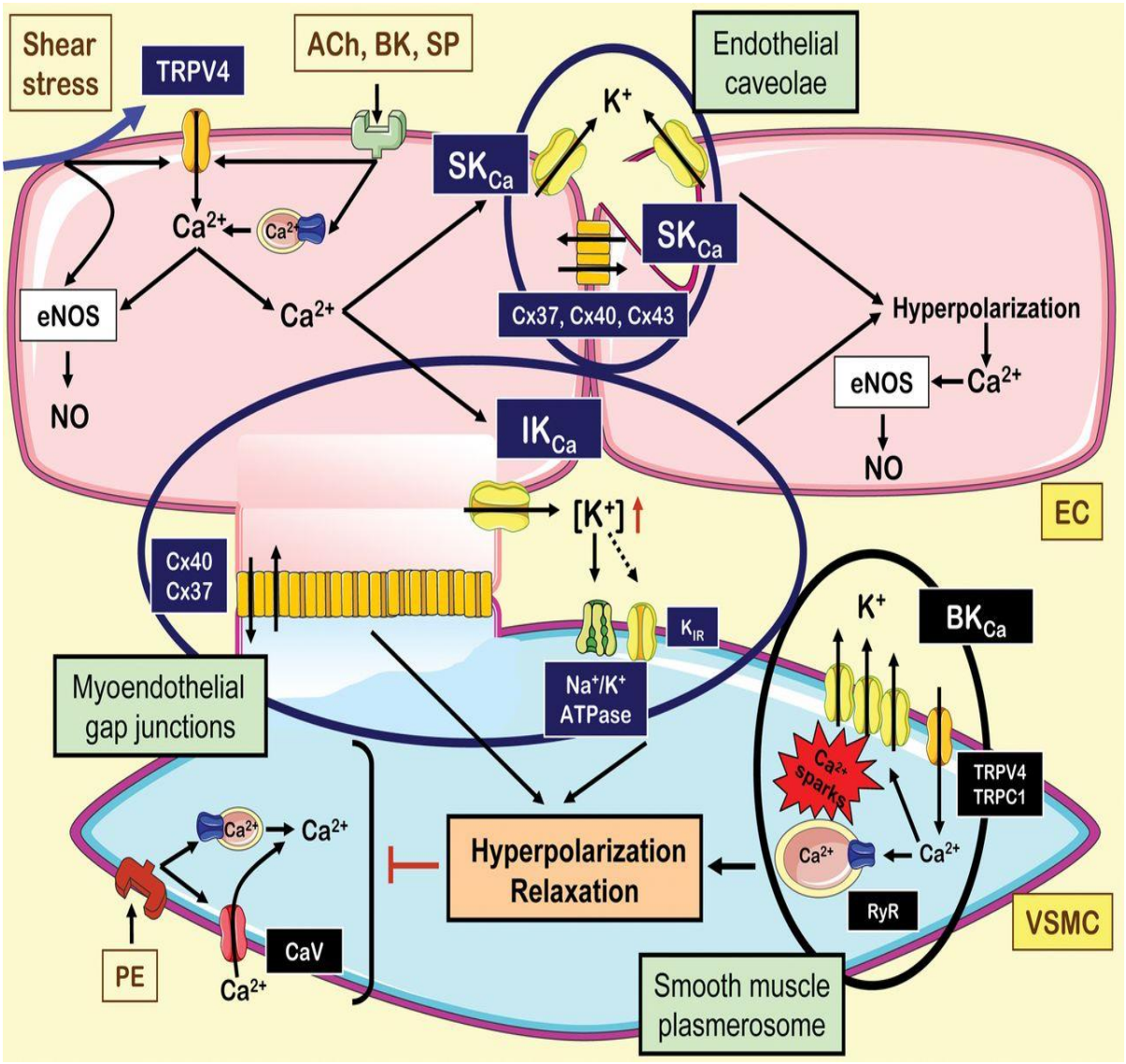


Figure 14: EDHF-mediated responses (Feletou M. and Vanhoutte M., Clin Sci (Lond), 2009)

The activation of endothelial receptors and the shear stress exerted by the flowing blood increase endothelial intracellular Ca_2^+ concentrations and activates eNOS, as well as the small conductance (SKCa) and the intermediate conductance (IKCa) channels. The subsequent endothelial hyperpolarization favours the entry of Ca_2^+ as a positive-feedback loop. The hyperpolarization can be conducted through myoendothelial gap junctions composed of Cx40 and possibly Cx37 to the underlying vascular smooth muscle. Hyperpolarization of VSMC leads to the decrease of cytosolic calcium concentration following closure of voltage-activated calcium channels which cause relaxation. Additionally, accumulation of K^+ ions in the intercellular space can hyperpolarize the SMCs by activating Na^+/K^+-ATPase and KIR channels. ACh, acetylcholine; BK, bradykinin; PE, phenylephrine; RyR, ryanodine receptor; SP, substance P.

arachidonic acid into endoperoxides. This product diffuses to the underlying vascular smooth muscle cells or are metabolized by individual synthases, essentially into either thromboxane A_2 or prostacyclin (PGI_2) [172]. Thromboxane A_2 (TXA_2) is a powerful stimulator of platelet activation, aggregation and vascular constriction [173]. PGI_2 acts on PGI_2 receptors (IP) in vascular endothelium to mediate vasodilatation and protect vessels from the development of diseases. Conversely, in some cases, under disease conditions, such as hypertension, IP receptors become dysfunctional and the role of PGI_2 is converted to be as an EDCF [171]. In normal vessels, the regulation of TxA_2 and PGI_2 is very important and has a crucial but opposite functions in the maintenance of vascular homeostasis.

V.3.2.b Angiotensin II (Ang II): a key element in ECs function and survival

The liver-synthetized precursor peptide angiotensinogen is cleaved by the kidney-produced enzyme, renin to Angiotensin I, which is converted to Angiotensin II (Ang II) by removal of two C-terminal residues to form an octapeptide by the enzyme angiotensin-converting enzyme (ACE). Angiotensin II is a potent vasoconstrictor hormone of the renin-angiotensin system (RAS) that appears to exert a central role in both the pathophysiology of essential hypertension, arteriosclerosis-associated hypertension [174] and insulin resistance [175].

The classical effects of Ang II on its target organs are mostly mediated through two pharmacologically distinct seven trans-membrane G protein-coupled receptors, the Ang II type 1 receptors (AT1R) and type 2 receptors (AT2R). Both mediate tissue-specific functions [176]. AT1R are expressed in all organs, including the heart, kidney, liver, adrenal glands, brain, lungs and in all cells of the cardiovascular system. In the latter, it specially acts on endothelial cells and smooth muscle cells. On the other hand, AT2R is highly expressed in the fetal heart and fetal aorta, lung and liver [177]. Ang-II signaling via G protein-coupled AT1 receptors (AT1R), promotes vasoconstriction where as it promotes vasodilatory effects when interaction with AT2R through the release of NO [178]. Within the vascular wall, studies have found that Ang II is also known to be a potent stimulant for ROS production via the AT1R through NADPH oxidase activation [179] (Figure 15).

V.3.2.c Reactive oxygen species (ROS)

ROS are chemically reactive species containing oxygen. In a biological environment, ROS are formed as a natural byproduct of the normal metabolism of oxygen and have an essential role in cell signaling and homeostasis [180]. They are produced and released from endothelial cells under physiological conditions and participate to vascular homeostasis. Moreover,

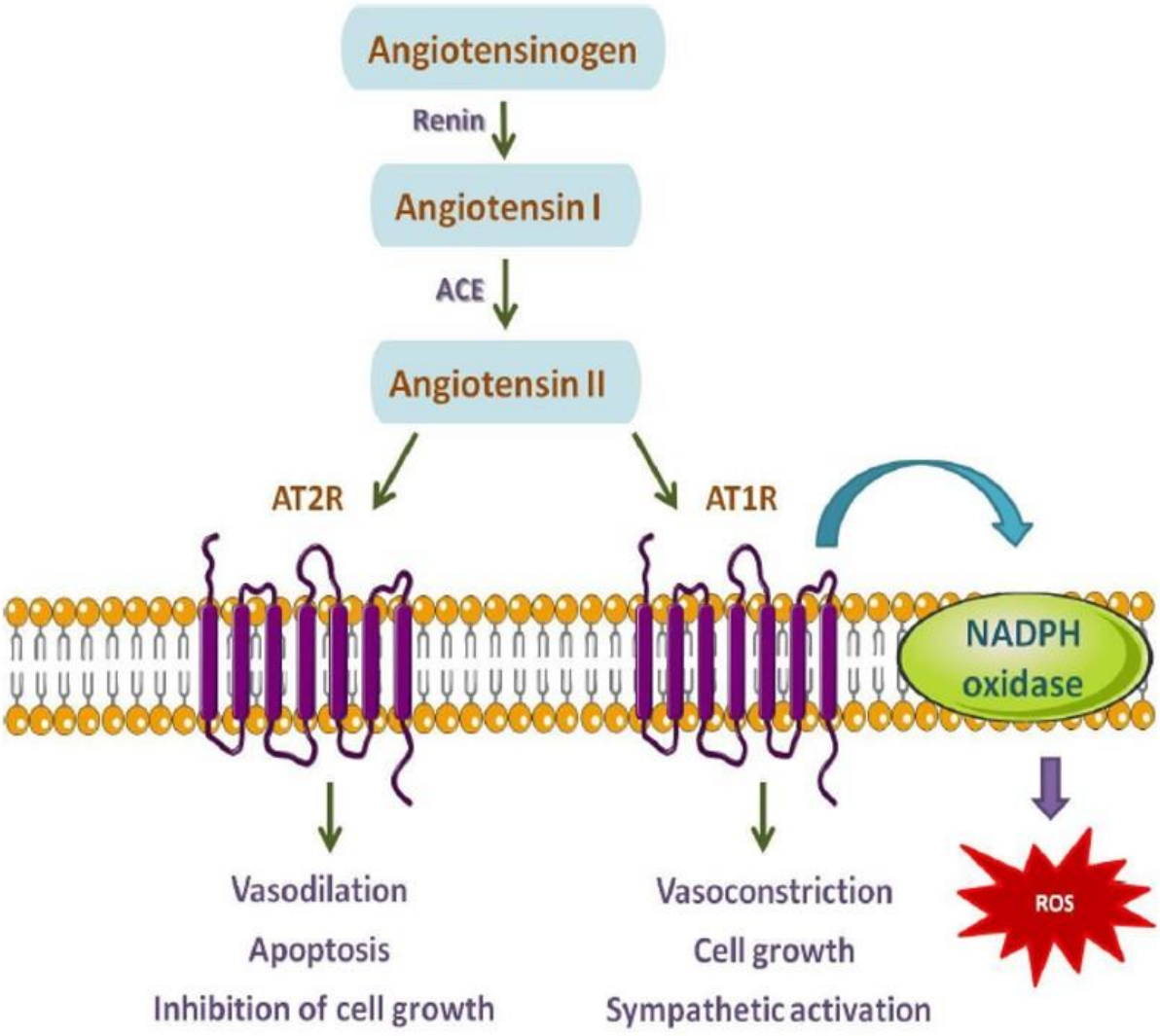


Figure 15: RAS (renin-angiotensin system)

ACE = angiotensin-converting enzyme; AT1R= angiotensin II type 1 receptor; AT2R= angiotensin II type 2 receptor. Ang II stimulates NADPH oxidase, via the angiotensin type 1 (AT1) receptor, which leads to the ROS generation.

mitochondrial electron transport chain was demonstrated as an important source of ROS within endothelial cells under physiological or pathological conditions [159, 181].

It is also well known that the normal endothelium produces a number of ROS that act as physiologic redox signals [159]. However, over-production of ROS is observed in different pathological settings such as hypertension, diabetes mellitus, acute and chronic inflammatory diseases and ageing. Essentially, ROS contain species, like the hydroxyl radical ($^{\bullet}OH$), which reacts near the site of formation due to its high reactivity. Other species are also formed, but are less reactive such as superoxide ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) [182].

ROS, in particular superoxide anions ($O_2^{\bullet-}$), can potentiate the contractile responses directly or indirectly via the reduction of the NO bioavailability [183]. This leads to the impairment of endothelium-dependent relaxation [184]. In addition, regardless of the source, ROS may have direct vasoconstriction effects by facilitating the mobilization of cytosolic Ca^{2+} or promoting Ca^{2+} sensitization of the contractile elements [185]. Furthermore, ROS can impair EDH-mediated endothelium dependent relaxations by the reduction of the activity of calcium-dependent potassium channels (SKCa and IKCa) [186] and by the modification of the passage of hyperpolarization factor from endothelial cells to underlining smooth muscle cells through myo-endothelial gap junctions [187]. Many studies on the effect of oxidative stress on the endothelial function have demonstrated the beneficial effects of using antioxidant-therapies to avoid the deleterious effects of ROS [184, 188].

V.3.3 The endothelium: sites of coagulation

Endothelial cells form the luminal vascular surface and contribute strongly in the regulation of coagulation, by controlling the clotting system. The regulation is done by regulating the expression of binding sites for anticoagulant and procoagulant factors on the cell surface [189]. Therefore, coagulation-related receptors on the surface of vascular cells and circulating coagulation proteins are strongly controlled. Endothelial and smooth muscle cells express a diversity of proteins directly participating in hemostasis. These cells are activated by the engagement of activated coagulation proteins by their specific receptors on the vascular cell surface leading to the expression of genes that play a role in coagulation, angiogenesis, leucocyte adhesion, regulation of the vascular wall tone [142]. The fact that blood coagulation is essential and should be highly effective, so the tight regulation of the blood coagulation system is crucial to avoid unnecessary clot formation.

V.4 Endothelium dysfunction: role in islet transplantation outcome

Endothelial dysfunction is a systemic pathological state of the endothelium characterized by impaired endothelium-dependent relaxation and vascular responses. It includes an imbalance between the formation of the endothelium derived vasodilatation and vasoconstriction substances produced by (or acting on) the endothelium [190]. It is characterized by the decrease of the bioavailability of vasodilators, mainly nitric oxide (NO) and/or an increase in endothelium-derived contracting factors [191]. Endothelial dysfunction can result or contribute in many diseases like hypertension, hypercholesterolemia, diabetes and aging (pre-mature senescence). In addition, environmental factors like smoking tobacco products and exposure to air pollution can contribute to endothelial dysfunction [192].

In the case of hypertension and cardiovascular diseases, endothelial dysfunction is relatively dependent on the production of ROS. AT1R-induced ROS not only destroys nitric oxide (NO), but also decreases NO formation in the endothelium by oxidizing tetrahydrobiopterin, an essential co-factor for endothelial NO synthase [193].

V.4.1 Endothelium dysfunction in diabetes

Diabetes mellitus (DM) is a global disorder, which carries a risk of vascular damage [194]. Endothelial dysfunction is linked with both insulin-dependent (T1DM) and independent diabetes mellitus (T2DM) [195]. Studies have shown evidence of both microvascular and macrovascular endothelial dysfunction [196].

Hyperglycemia was considered by the clinical trials as the key contributing factor in the development of chronic diabetic complications [197]. In hyperglycemia there is an increase in ROS generation by activating protein kinase C mediated NADPH oxidases [198] and peroxynitrite-mediated eNOS uncoupling [199], which leads to decreased in NO bioavailability. Depending on its concentration, ROS can impair β-cell functions [200]. In type 1 diabetes, ROS contribute in β-cell dysfunction starting by inflammatory cytokines and autoimmune reactions [201]. However, in type 2 diabetes, too much ROS impairs insulin production and activates β-cell apoptotic pathways [202, 203]. Therefore, in pancreatic islets, the brief exposure to oxidative stress can induce an alteration in glucose-stimulated insulin secretion. In addition one of the ROS targets is attacking the β-cell, which lead to mitochondrial inactivation and by consequence disturbing the transduction of signals that normally coupling glucose metabolism to insulin secretion [204].

Hyperglycemia also increase pancreatic islet endothelial cell apoptosis by higher expression of iNOS and iNOS derived NO which leads to the increase in ROS levels [205]. In addition, hyperglycemia stimulates platelets aggregation by increasing expression and circulating levels of endothelial adhesion molecules [206] and increases endothelial apoptosis [207].

In many diseases, insulin resistance has been shown to increase cardiovascular risk and mortality such as diabetes, obesity, hypertension, metabolic syndrome and heart failure [197]. Insulin resistance is defined by the decrease in the ability of insulin to stimulate glucose uptake by skeletal muscle and adipose tissue in order to suppress hepatic glucose output [208]. It is often preceded by impaired glucose tolerance and hyperglycemia [209] (Figure 16).

Many studies have also shown that diabetes and insulin resistance cause a combination of endothelial dysfunctions that may reduce the anti-atherogenic role of the vascular endothelium [197]. Furthermore, in diabetes, the balance between the endothelial-derived relaxation and contraction factors is altered. This leads to the endothelial dysfunction that in turn, participates in the pathogenesis of the vascular disease in diabetes [210]. In T2DM, many studies have shown that NO level was abnormal. This might explain the endothelial dysfunction [211].

A Study done in a rat model, suggested that changes in EDHF also participate to endothelial dysfunction, particularly in T2DM, where an impaired EDHF-mediated vasorelaxation was observed before marked alteration in NO-mediated responses [212].

The impact of ageing, hypertension and other factors may aggravate endothelial dysfunction. However, it is more complicated in type 2 diabetes than in type 1 [197]. At experimental level, our laboratory proved that hyperglycemia induces the pre-mature endothelial senescence *in vitro*. Furthermore, Sabatier *et al.* showed that circulated and pro-coagulant endothelial microparticles (MPs) increase in diabetic patients [213].

Many studies were still needed to elucidate the hidden part of the iceberg concerning the pathological process of the endothelial dysfunction in diabetes mellitus.

V.4.2 Progressive endothelium dysfunction with age and premature senescence

Aging comes with structural and functional modifications of the vasculature that leads to endothelial dysfunction and smooth muscle cells [214]. Endothelial cell aging is characterized by a decrease in NO synthesis [215]. In a study done with old rats, the loss of PI3K/Akt-dependent eNOS phosphorylation appeared to be an essential mechanism explaining the decrease in NO production [216]. With aging, many structural and functional alterations occurred gradually in blood vessels [214], where smooth muscle cells and intima layers are

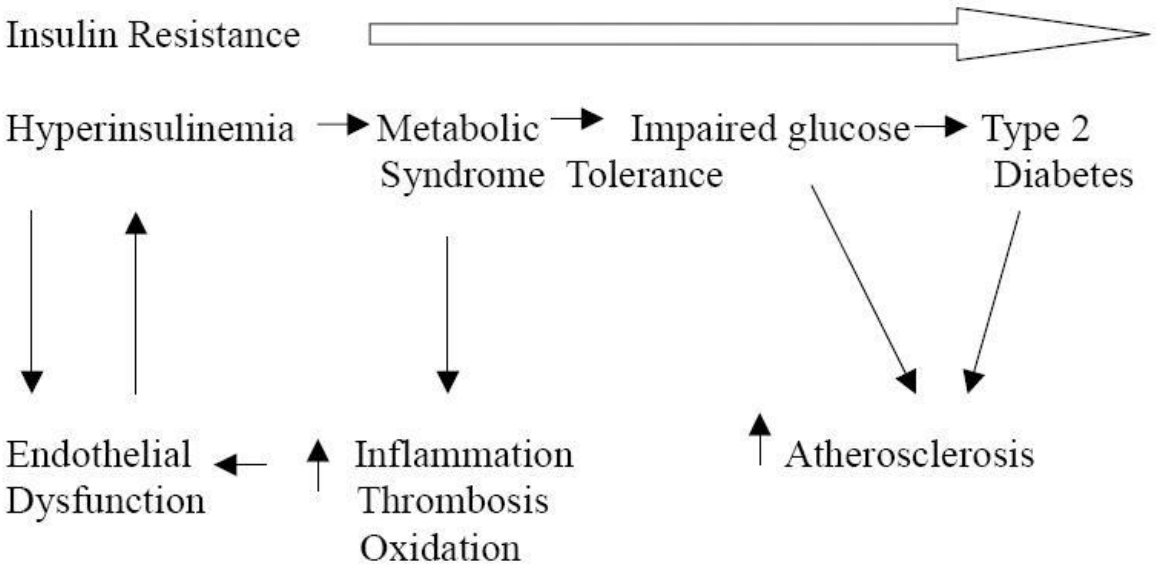


Figure 16 : Progression of endothelial dysfunction in relation to the progression of insulin resistance (Hsueh *et al.*, Am J Med, 2004)

affected [217]. All these changes lead to the reduction of the lumen diameter of the vessel and endothelial dysfunction. In addition, they are accompanied by structural changes like increase in media thickness, wall collagen content and size or number of smooth muscle cells and by functional modifications which contribute to the imbalance between endothelium-derived relaxing and contracting factors [218]. Endothelial dysfunction related to aging has been observed in several types of arteries such as the aorta and mesenteric artery of the rat and the brachial artery in humans [219]. Regardless of ageing, Angiotensin II (Ang II) has been identified as a major inducer of endothelial dysfunction and vascular oxidative stress via the induction and activation of NADPH oxidase. At the molecular level, experimental work have underscored that Ang II through AT1 receptor stimulation leads to cellular senescence via Ras signaling and the activation of the p53/p21 pathway [220].

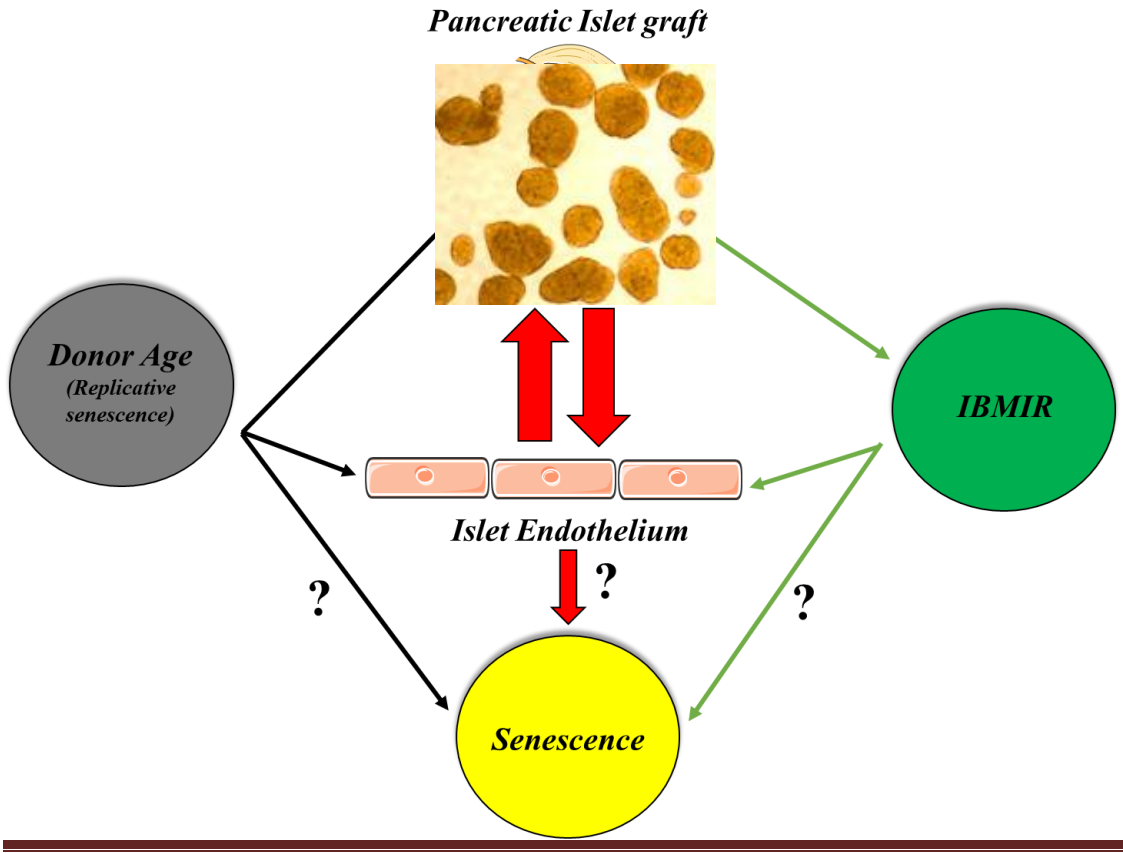
During islet transplantation and especially in every step of the protocols, the stress resulting from the mechanical isolation or the enzyme used lead to an alteration in the islet endothelium. This may lead to what we call the pre-mature senescence (chapter 6). In addition, after transplantation and directly after first contact with the portal blood of the recipient, the IBMIR affects donor islet's and liver recipient's endothelial cells. Interestingly, endothelial cells phenotype varies with the vascular territory. Whether the state of liver capillary endothelial cells contribute to exaggerated ischemia-reperfusion owing to reduced oxygen pressure remains to be demonstrated (see Figure 35 & discussion).

Nevertheless, recipient endothelial cells are pivotal for the revascularization process, eventually through the homing of endothelial progenitors possibly by MPs shedding [221]. Because islet revascularization is essential for its function and survival, the limitation of the islet or host endothelial dysfunction seems to be a crucial step for a better outcome of islet transplantation.

Therefore, the understanding of the molecular networks underlying the communication between the islet endothelium and β-cell surface receptors may help explaining how the mass of functional insulin-secreting β-cells can increase. In addition, the controlling of the side effects of aging or pre-mature senescence during islet transplantation seems to be crucial for the success of this technique. From here, the need for a better understanding of the mechanism of normal aging (replicative senescence), or the induced aging (pre-mature senescence) that can be induced by many factors especially by the stress and endothelial dysfunction, is crucial for a better transplantation success. Finally, the most important point is to clarify the link between the islet graft, endothelium dysfunction and senescence, where the effect of endothelial MPs can be one explanation.

Chapter VI

The impact of senescence on islet β-cells & transplantation



VI The impact of senescence on islet β-cells and transplantation

VI.1 Definition and physiopathological impact of cellular Senescence

Aging is a biological process that worsens the structure and function of human body at multiple levels, which touch all organs over the time. According to the United Nations report (the world population ageing 1950-2050), the number of people aged ≥60 years will go above that of younger people aged <15 years by 2050 and that older people (≥60 years) number is estimated to increase from 11% (760 millions) in 2005 to 22% (2 billion) in 2050 which will negatively affect the country's economy. The lifespan increasing in industrialized countries and aging creates new challenges for the public health care. Indeed, prevalence of neurodegenerative and metabolic disorders increase with age. Therefore, research on the specific mechanisms of cellular senescence as key associated mechanisms can lead to the development of specific pro-healthy aging therapies and the development of senolytic drugs [222].

Before a half century, cellular senescence was first revealed by Leonard Hayflick, while cultivating primary human fibroblast. He found that primary cell culture after about 50 to 70 population replications are irreversibly arrested in G1 phase of the cell cycle and become unresponsive to proliferative mitogenic stimuli [223-225]. In the recent years, cellular senescence was recognized a complex biological process raising high interest in the field of cell biology and more recently vascular biology [226]. Studies showed that cells respond to damaged or inappropriate environment not only by dying or apoptosis but also by acquiring a cellular senescent state characterized by irreversible proliferation arrest [227].

Aging is associated with endothelial dysfunction and silent clinical outcome at least in the early stages. Endothelial senescence mediated dysfunction was primary investigated in cardiovascular disease [214]. In the vascular compartment, cellular senescence is considered as an essential mechanism of homeostasis and a target of the immune system that eliminates the senescent cells, thereby limiting tumorigenesis and tissue damage [228]. In physiological conditions, senescence can be a double-edge weapon, from one side it is crucial to stop the proliferation of damaged cells before they enter into oncogenic transformation [222]. On the other side, cells that have undergone permanent proliferative arrest or apoptotic cells can both be very harmful if not eliminated by immune system, their accumulation initiating premature aging or cancer [229]. The long-term existence of senescent cells in tissues has the potential to endorse age-related diseases, which can explain the decline of an organ function with aging [230].

VI.2 Physiopathological impact at cellular level of endothelial senescence

During the last years, studies have shown that cellular senescence could induce by different stimuli telomere shortening that constitute the main cause of the proliferation arrest of primary cell cultures discovered by Hayflick, in addition to DNA damage, oxidative stress and cell-cell fusion [231-233] (Figure 17). Moreover, the activation of oncogene also induces cell senescence in a process termed oncogene-induced senescence (OIS) [234], by inducing DNA damage, leading to the persistent activation of DNA damage response (DDR) [235]. Other pathways induce endothelial senescence, independently of the DNA damage, possibly through p38 and NF-κB pathways activation or by the activation of extracellular signal-regulated kinases (ERK) pathways that promotes the degradation of proteins required for cell cycle progression [236]. Studies on cultured endothelial cells have shown a pro-inflammatory and pro-thrombogenic phenotypes changes associated with the induction of senescence. As a direct result of the permanent growth arrest, endothelial senescence could also obstruct the motility of the cells and may alter their angiogenic capacity, thereby the restoration of a functional and perfused tissue. The incapability of senescent cells to proliferate alone can possibly impair tissue regeneration, especially if stem or progenitor cells become senescent.

Senescent cells have three main characteristics that are associated to their noxious effects [228]. First, an efficient tumor suppression mechanism that limit cell proliferation, which in turn delays the accumulation of mutations. Indeed, senescence is tightly regulated by a set of tumor suppressors like p53, p21, p16, pRb and Arf [237]. In addition, senescence cells display essential role by limiting tissue fibrosis in non-cancer pathologies. In conclusion, it appears that senescence is pivotal for tightly controlled tissue remodeling. The second characteristic of senescence is the cellular dysfunction, which terminates normal physiological functions. This cellular dysfunction leads to tissue dysfunction either by altering tissue structure or function, thereby promoting diseases. There are many examples of senescent cells dysfunction in a variety of organs. For instance, senescent pancreatic β-cells, lead to progressive reduced insulin release during age-related diabetes [238]. Senescent endothelial cells show a decreased expression of eNOS [239] and NO formation which is required for vasoprotection [240] and may lead to the local accumulation of thrombogenic endothelial areas at sites of flow disturbance. Indeed, only laminar blood flow stimulates NO production in the endothelial cell. The third important characteristic is the secretion of soluble factors like cytokines, growth factors and proteases that impact senescent cells microenvironment [241]. These soluble factors

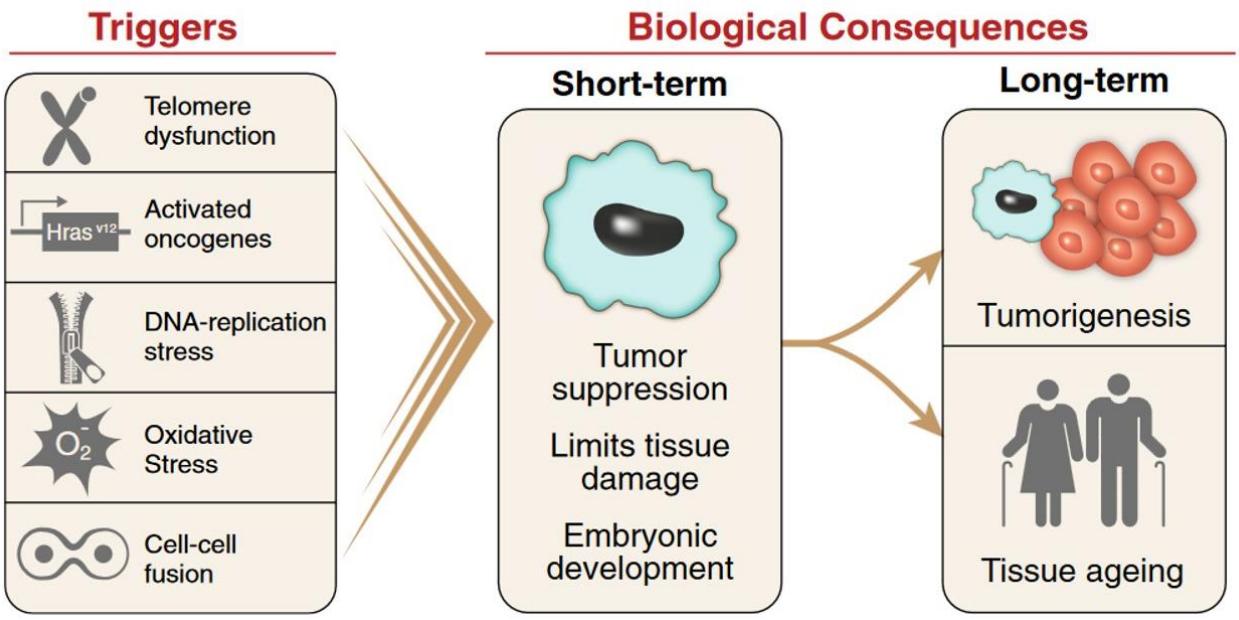


Figure 17: Biological causes and consequences of cellular senescence (Burton and Krizhanovsky, J Cell Mol Life Sci, 2014)

facilitate cellular proliferation and tumorigenesis in neighboring cells. The permanent crosstalk between senescent cells and neighboring cells through production of various factors or through cell-cell contact offers a new insight for a better understanding of the pathophysiology of various chronic diseases.

VI.3 The different senescence types

VI.3.1 Replicative senescence

In the last 30 years, studies prove that replicative senescent is largely due to telomere shortening. Telomeres are progressively shortened with the propagation of human cells in culture and finally lead cells to reach their "hayflick limit" and this state is called replicative senescence. The ends of eukaryotic chromosomes are capped by protective structures called telomeres, which are of long double stranded DNA sequences consist of hundreds to thousands of repetitive sequences of TTAGGG averaging from 5 to 15 kb in humans [242].

To ensure the appropriate functioning of telomeres and continuance of chromosome integrity and stability, the presence of the telomeric repeats at the chromosome ends is crucial [243]. Telomeres are associated with complex proteins, named shelterin composed of six capping proteins: telomere repeat-binding factor 1 (TRF1), telomere repeat-binding factor 2 (TRF2), repressor activator protein 1 (Rap1), TRF1 interacting nuclear protein 2 (TIN2), TIN2-interacting protein (Tripeptidyl-peptidase 1) (TPP1) and protection of telomere 1 (POT1) [244]. During the DNA-repair process, there telomere specific proteins protect chromosome from degradation or chromosomal end-to-end fusion [245]. In telomere shortening, DNA polymerase fails to completely replicate the lagging strands, when the length of one or more telomeres is below a certain minimal threshold [227]. Telomerase, is a ribonucleoprotein complex that is composed from a RNA template (TERC) and the reverse transcriptase catalytic subunit (TERT) [246]. With the development in senescence research, especially in the telomere
shortening it was proved that re-introducing telomerase expression in normal primary cells delayed or eliminated senescence thereby favoring the immortalisation state of various human cells types [247, 248].

Additionally, replicative senescence is also associated to the Retinoblastoma (RB) tumor suppressor, which includes p16 that is a cyclin dependent kinase inhibitor. Certainly, both p53 and p16 activation seem to be mandatory for senescence induction in a variety of human cell strains [227].

VI.3.2 Pre-mature cellular senescence

Studies have shown another type of senescence that can be induced in the absence of any noticeable telomere loss or dysfunction. This type of senescence is called premature senescence [249] which can be induced by a variety of stress conditions (Figure 18).

VI.3.2.a Tumor suppressor loss-induced senescence in vitro

Serrano *et al.* showed that in response to an oncogenic form of Ras, the primary cells acquire a premature senescence state [250]. Studies revealed that loss of tumor suppressor that restrains the activity of oncogenic pathways, such as phosphatase and tensin homolog (PTEN) that is a transcription factor that regulates p53 stability was showed to activate senescence in mouse and human cells through p53 induction. In addition, depletion of neurofibromin 1 (NF1) induced senescence *in vitro* by decreasing ERK and AKT activities. The loss of von Hippel-Lindau tumor suppressor (VHL), which triggers senescence in a RB- and p400-dependent manner is another example of tumor suppressor [251, 252].

VI.3.2.b Stress-induced premature senescence (SIPS) in vitro

Stress is a major contributing factor to cellular senescence [253]. When cells explanted from organisms and cultured *in vitro*, they generally undergo stress shock, owing to a different environment characterized by abnormal nutrient concentration and abnormal growth factors, absence of neighboring cells and extracellular matrix components [249, 254]. In addition, SIPS can be caused by physical, chemical and cellular stressors such as mitomycin C and ionizing radiation [255]. Oxygen is one of the main factors of SIPS, because excessive O_2 leads to ROS formation [253], now a proven cause of cellular senescence. In addition, in islet transplantation and with the first contact with blood, appears the vascular stress resulting from the IBMIR characterized by acute inflammatory and immune responses that affect grafted islet and aggravate the ischemia associated with islet isolation. Besides IBMIR, all isolation steps may by themselves constitute a shock stress possibly contributing to the induction of senescence, both in endothelium and other cells constitutive of the islets.

VI.4 Senescence features and biomarkers

VI.4.1 Morphological transformation

Generally, senescent cells lose their original morphology, where morphological changes happen at the cellular and organism level and appear as specific features of the senescent phenotype [256]. According on the senescence causes, cells show different characteristic such

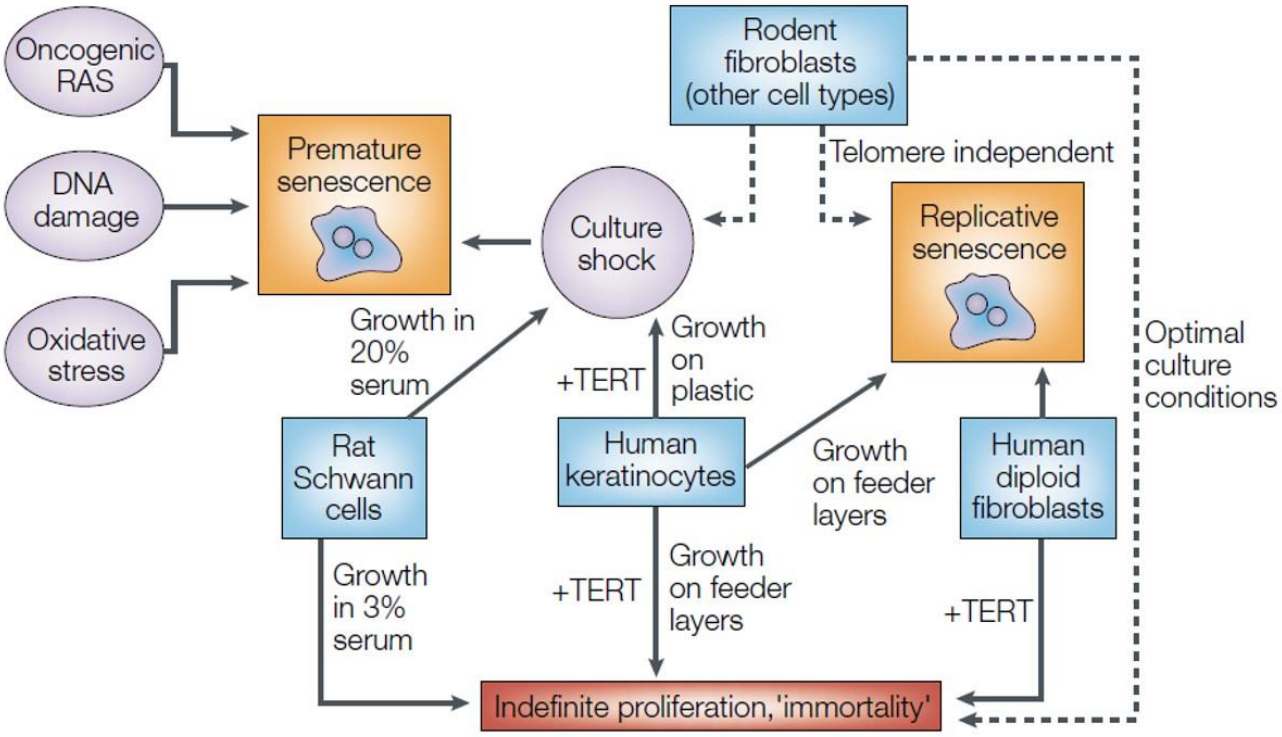


Figure 18: Pathways of senescence leading to either replicative senescence or premature (Nicole F. *et al.*, Nature Reviews Cancer, 2001)

as flattened and enlarged cell shapes, with larger cytoplasm, which may contain many cytoplasmic filaments and vacuoles [257, 258]. In addition nucleus is bigger and multinucleated in certain cases [259, 260].

VI.4.2 Growth arrest

Growth arrest constituted for long time one of the most observable features of cellular senescence, it is essentially stable and irreversible by known physiological stimuli [261]. Studies showed that senescent cells are usually blocked in the G1 phase of the cell cycle [262]. However, several ways to reverse the cell cycle arrest have been reported. For example, inactivation of the P53 pathway allows senescence reversal while some of the interleukin may abolish the growth arrest [263]. In addition, the entering into irreversible senescence depends on p16 expression [264]. p16 and p21 which are a tumor suppressors are in the same time mediators of cell cycle arrest and senescence (Figure 19).

VI.4.3 Induction of senescence associated -beta-galactosaidase activity (SA-βgal)

Researcher tried for long time to obtain specific markers to identify senescent cells. Besides the ordinary markers like alteration of cell morphology, growth arrest state and a specific gene expression like p53, p21 and p16, SA-βgal is the most commonly used senescence biomarker which is detectable at pH 6.0 where it gives a blue green color [265, 266]. The increase of SA-βgal activity in senescent cells was related to an enlargement of the lysosomal compartment, leading to an increase in β-galactosidase activity [267-269]. But it should be noted that the increase of this enzyme is not fully specific of senescence since there is no evidence pointing at its actual involvement in senescence responses, besides the fact that it is also detectable *in vitro* after prolonged cell culture, serum withdrawal, TGF-β, or heparin treatment [269].

VI.4.4 Senescence-associated heterochromatic foci (SAHF)

At a chromatin level, a specific feature of senescent cells, which are the condensed heterochromatin regions were discovered and termed senescence-associated-heterochromatin foci (SAHF) [270]. These structures, which are regulated by the Retinoblastoma gene (RB), participate in E2F target genes suppression and maintain the cell cycle arrest. Bypass of senescence can achieved by avoiding SAHF formation by interference with p16 ^{INK4A}–RB pathway signaling [227]. DNA SAHF structure are specially enriched in methylated Lys 9 of histone H3 which are modifications catalysed by the histone methyltransferase Suv39h1, while histone H3-lys9 acetylation and Lys-4 methylation, which are euchromatin markers are

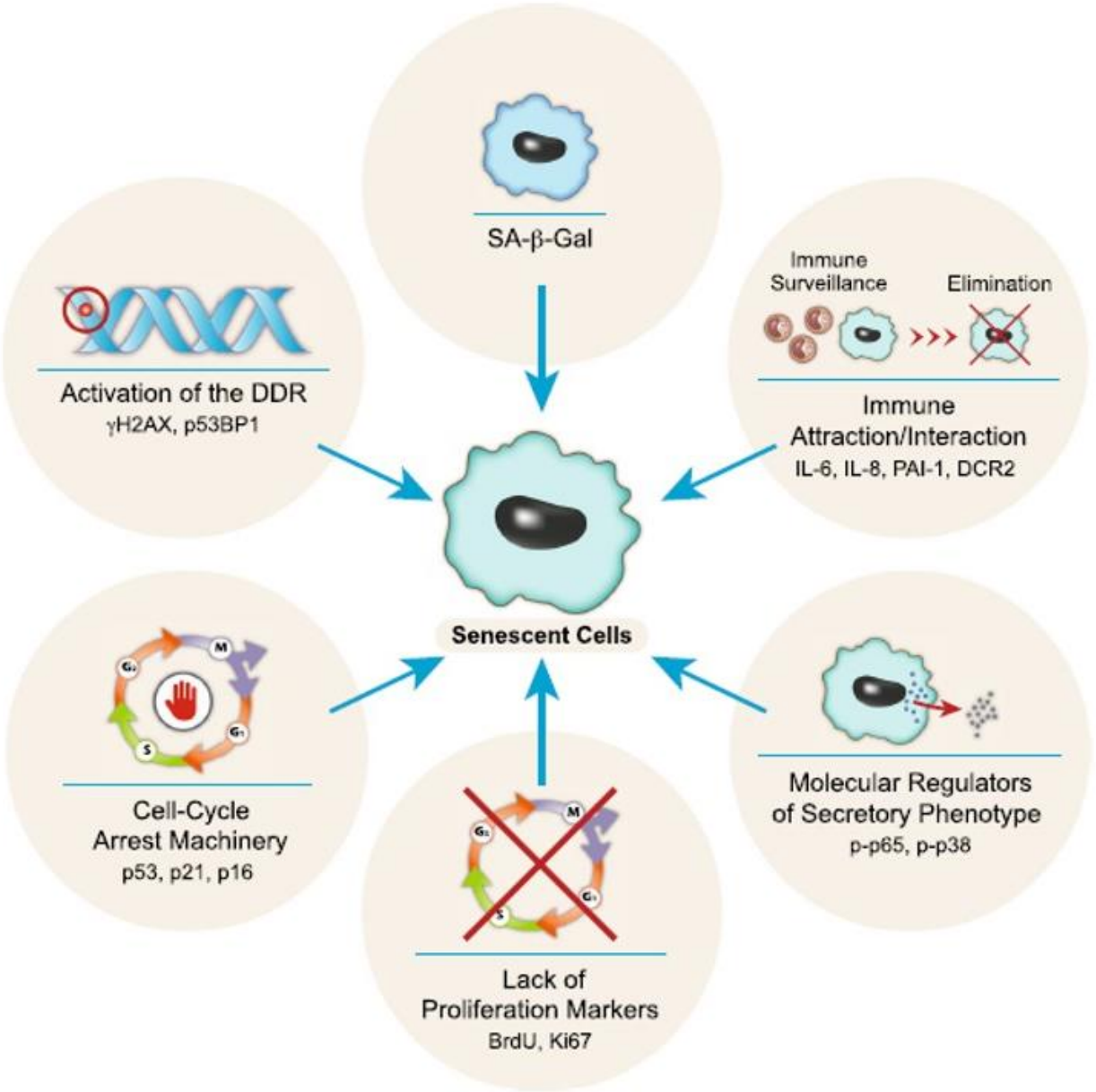


Figure 19: Biochemical and Morphological characteristics of Senescent Cells (Burton and Krizhanovsky, J Cell Mol Life Sci, 2014)

debarred from SAHF. SAHF comprise markers of heterochromatin like hypo-acetylated histones, methylated histones (H3K9Me) and the presence of heterochromatin protein 1 (HP1) [271]. A crucial step in the launch phase of chromosome condensation is the translocation of histone chaperone to proteolytic leukaemia nuclear bodies. In addition, chromosome condensation that is mediated by the histone chaperone ASF1a, depends on its binding to histone H3, as well as HIRA [271]. Indeed, histone acetylation favors chromatin de-condensation allowing access to transcription factors, co-activators and regulators controlling the RNA polymerase binding to selective DNA sequences and the transcription of downstream genes involved in senescence control [272].

VI.4.5 Secreted factors in senescence

Cells experiencing senescence in response to many factors such as DNA damage, telomere malfunction and oncogenic alterations, which show deep changes in the cells transcriptomes. Senescent cells secrete many factors that have strong autocrine and paracrine activities, like extracellular proteases (matrix metalloproteases (MMPs)), plasminogen activator inhibitor 1 (PAI-1) [273], growth factors (transforming growth factor β (TGFβ), basic fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and vascular endothelial growth factor (VEGF)), cytokines (interleukin (IL)-1β, (IL)-6), chemokines (IL-8 and monocyte chemoattractant protein-1 (MCP-1)) [274, 275]. In addition, cells exhibiting either replicative or premature senescence display profound changes in their secretome, a feature termed the senescence-associated secretory phenotype (SASP) [276], which enables the diffusion of senescent signals from senescent cells to the surrounding healthy tissue.

In general, it is supposed that the main pro-inflammatory component of SASP is represented by several of the secreted cytokines and chemokines, like IL-6 and IL-8 that are highly up regulated during the transition phase in Ras-senescent cells [273]. Because senescent cells progressively accumulate with aging, SASP may be one of the players of the so-called inflammaging (the age associated pro-inflammatory phenotype), that has a major role in the development of age-related diseases [277]. Besides, it is also likely that various inflammatory cytokines and chemokine may have an indispensable role in the establishment and maintenance of the senescence arrest. For example, it is proved that to induce cell senescence in response to oncogenic BRAF or to replicative exhaustion signaling through IL-8 (CXCR2) and IL-6 receptors [278, 279] is mandatory.

VI.4.6 Reactive oxygen species (ROS)

The degree of oxygen saturation in culture affects the replicative potential of primary cells and it is also well known that is not oxygen itself, but rather ROS from the mitochondria that play a major role in cellular senescence [280, 281]. Replicative and pre-mature (stress and oncogene-induced) senescence are both correlated with elevated ROS levels [282].

DNA damage responses (DDR), starting when the chemical oxidants caused oxidative stress, which stimulate telomere shortening and accelerates the starting senescence process [283]. Study revealed that mitochondria produce the majority of ROS during the induction of senescence [284], as a consequence of oxidative phosphorylation [285]. As a proof of concept, treatment with antioxidants, inhibitors of cellular oxidant scavengers and hypoxia suppression preserve telomere length and prolongs the lifespan of cells [283, 286]. While it is well proved that ROS plays an important role in cellular senescence, the nature and machineries of this involvement remain unclear [281] and a lot of questions still without answer such as how the increased levels of ROS are produced and what are the cellular targets for ROS in senescence [227].

VI.4.7 Autophagy

Advancement in the autophagy domain led to the discovery of its mechanisms by Yoshinori Ohsumi, who obtained Nobel prize in Physiology 2016 for this achievement. Increasing evidences that autophagy participates in cell cycle arrest and to the production of senescence-associated interleukins are now accumulated [287]. Autophagy is a genetically regulated program, which constitutes a very important evolutionarily conserved process. It is characterized by the formation of cytosolic membrane vesicles termed autophagosomes, which isolate the cytoplasmic content and delivere it to lysosomes [287]. Autophagy plays an important role in the regulation of proteins/organelles, maintenance of homeostasis and human health [288]. Furthermore, recently senescence and autophagy have been connected more tightly, because autophagy modulates cell viability under stress conditions, including cellular senescence [273].

VI.5 Molecular mechanisms of cellular senescence

The retinoblastoma protein (pRb) and its family members are often mentioned to as the start signal of cellular senescence and the master brake of the cell cycle where its central function is to inhibit E2F transcription factors [289] (Figure 20). These pRB family are controlled by

phosphorylation of protein-protein interaction and proteins modifications [290]. During proliferation when cells are promoted towards cell division, a series of cyclin-dependent kinases (CDKs) are able to phosphorylate RB family proteins, which lead to loss its ability to bind to and inhibit the function of the E2F family, thus leading to gene transcription and beginning of DNA replication and cell cycle evolution [291-293]. When cells enter in senescence, they reach a state of non-proliferation by stopping at G1 phase of cell cycle. CDKs and their inhibition by CDKI play an important role in these mechanisms. The first members of the CDKI family are the INK4 proteins (p16, p15, p18 and p19), which block the progression of the cell cycle by binding to Cdk4 and Cdk6 [294]. The second class of CDKI is the KIP/CIP family (p21, p27 and p57), known to inhibit a wide range of CDK [295]. In normal physiological conditions, CDKI are expressed at low level in proliferating cells. The expression level of CDKI (p21 and p16) genes are up-regulated after stimuli from a diversity of oncogenic factors responsible for DNA damage, this up-regulation happens in a p53-dependent or p53-independent manner [225, 296]. When CDKI are activated all CDK are disabled, thereby inhibiting pRB family phosphorylation and causing ultimately arrest of the cell cycle [297, 298]. The activation of p16-RB pathway also induces in cooperation with mitogenic signals an elevation of the intracellular levels of reactive oxygen species (ROS), thus causing PKC activation, an important downstream mediator of the ROS signaling pathway, which leads to cytokinetic cell cycle block. As a result, ROS production is enhanced and sustained ROS-PKC complexes formation that is enabled in senescent cells. ROS-PKC complexes irreversibly block cytokinesis by decreasing the level of Mitotic Exit Network (MEN) kinases known as WARTS [299] (Figure 20).

VI.6 p53/p21 pathways

p53 is a trancription factor and a tumor suppressor which is widely known as the guardian of the genome because of its efficacy in avoiding genome mutation. Indeed, p53 is mutated in about 50% of the human cancers [300]. p53 is a tetrameric transcription factor regulated by post-transcriptional modifications [301]. It is well proved that physiological p53 activity prevents cancer and protects from premature aging, while uncontrolled and excessive p53 activation still protects from cancer, but is harmful to healthy aging [302].

In response to cellular stress signals like DNA damage, oncogene activation, hypoxia and viral infection, p53 is an essential regulator used by cells [303]. A number of E3 ligases including MDM2, MDM4, TOPORS and COP1 are produced in the absence of stress and favor the

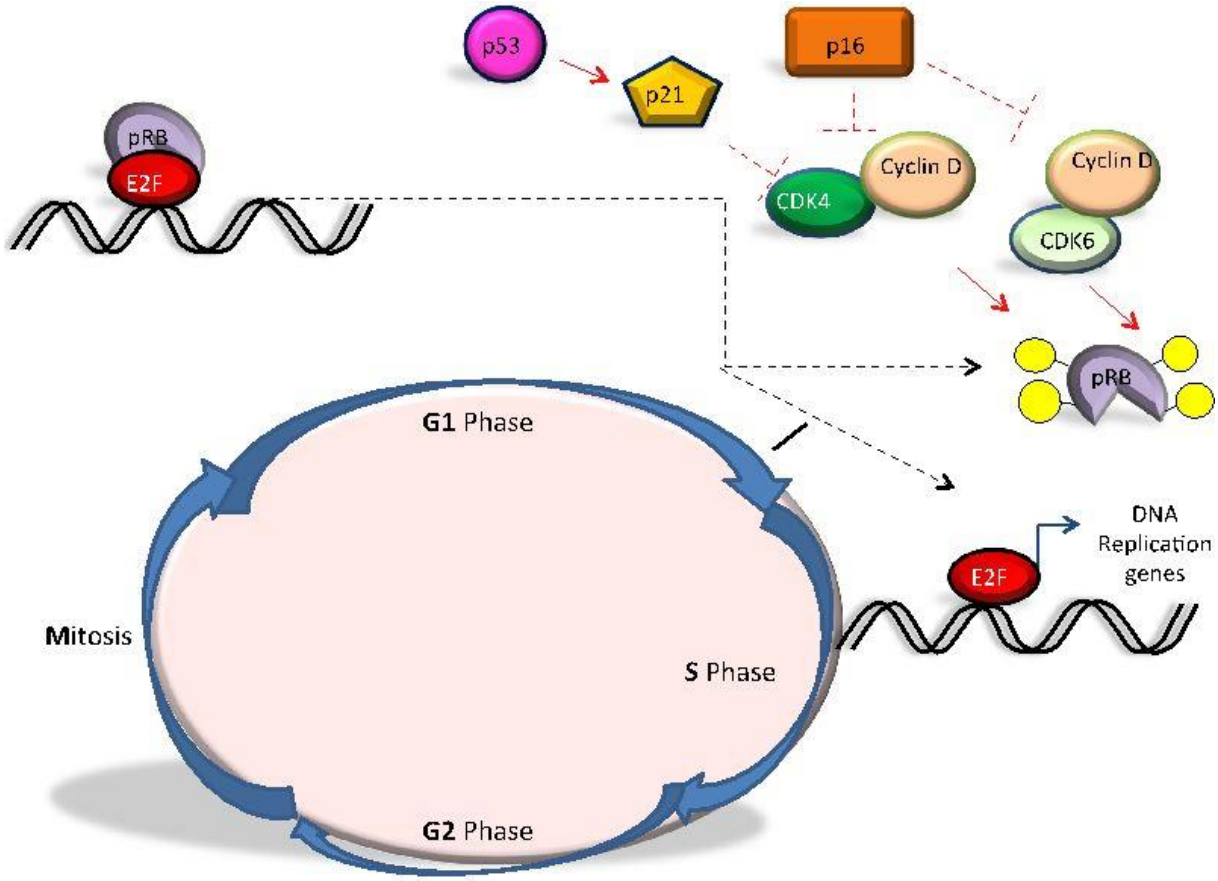


Figure 20: pRb in cell cycle regulation (Becker and Haferkam, 2013)

ubiquitinattion of p53, which is then degraded by the proteasome [304, 305]. The interaction between p53 and the E3 ubiquitin ligases can be altered by stress signals, that modify the covalent bounds and the efficient ubiquitination.

The stimulation of senescence after DNA damage responses is mainly conducted by the activation of p53 [306]. One of the most p53 targeted gene is its downstream effector p21.

p53 and p21 overexpression play an essential role in the regulation of cell cycle arrest and senescence. E2F is considered as a new target involved in p53 in cell-cycle arrest and senescence [270]. In addition, studies have shown that inactivation of p53 avoids senescence in mouse embryonic fibroblast [307] and that human fibroblast lacking p21 can prevent the senescence induced growth arrest [308]. Furthermore, p53 also interacts with the mTOR pathway and activates the expression of the AMP-activated protein kinase (AMPK) which in turn activates mTOR signaling that promotes cell and tissue aging [309]. Besides that p53 upregulates PTEN, which is an inhibitor of the PI3K pathway [310]. Altogether, show that p53 is controlling a network of tightly connected biological processes that affect senescence or reduce the cellular growth-dependent pathways [311].

VI.7 Impact of senescence on pancreatic β-cell function and in diabetes

Aging is accompanied by the accumulation of senescent cells, also noticed in diabetes and obesity. Worldwide, recent data show that diabetes is a risk to human health because it constitutes a main risk factor for many age-related diseases like cardiovascular diseases, renal dysfunction, stroke, infection and abnormal wound healing [312, 313]. In the American population, the diabetes prevalence rises with age to more than 25.9 % of people ≥65 years [314]. Senescence and diabetes may share a complex link, because from one side it appears that a diabetic environment allows the premature accumulation of senescent cells. On the other side, tissue and cell dysfunction seen in T2DM may be aggravated by senescent cells. Increasing interest in the cellular mechanisms responsible for the age-related decline in β-cell proliferation, especially in T2DM lead to the observation of increasing in insulin resistance possibly due to the drop of the β-cell mass to increase, a phenomenon that can be seen in young healthy individuals. The unbalance between insulin needed and the capability of the β-cell to respond would therefor favor age-related hyperglycemia as a result of β-cell failure and of the loss of a functional β-cell mass [315].

The residual metabolic activity of senescent cells favor the release of senescence associated secretory proteins gathered under the term of SASP (Senescence Secretory Associated

Phenotype) [276, 316] (Figure 21 A). Typically only a small portion (≤20%) of senescent cells from one organ would be able to produce a systemic effect [261]. In T2DM, senescent cells can play a major role in the disease pathogenesis by directly altering pancreatic β-cell function by promoting SASP-mediated tissue damage and adipose tissue dysfunction. Senescence in diabetes is promoted by the metabolic and signaling changes that accompany the progressive establishment of the disease like high glycaemia, perturbation of growth hormone and lipid metabolism [317]. Furthermore, diabetes and insulin resistance are associated with inflammation that is prompted by adipocyte activation [317]. In aging and obesity, adipose tissue dysfunction may be linked to the accumulation of senescent cells [318]. Adipognesis that was found to interfere with the expression of insulin receptors and GLUT4, receptors may also interfere with SASP through released inflammatory cytokines [319]. The rise of cellular dysfunction in diabetes can be reinforced by the damaging influence of senescent cells on adipose tissue that decrease its capacity for fat storage [320]. Studies on diabetes show that high glucose causes premature senescence *in vitro* in endothelial cells. In addition, heart disease is a major complication of diabetes and the increased prevalence of senescence in diabetes could also favor, at least at the initiation of the process the recruitment of cardiac progenitor [321], besides the senescence-induced endothelial lipotoxicity [317]. Until now, glucose-induced senescence is still poorly understood. Mitochondrial dysfunction and increased reactive oxygen species are possible inducers [322].

In diabetes one probable mechanism contributing to the decrease in β-cell function and mass is pancreatic β-cell senescence, especially in T2DM [238]. Indeed, the reestablishment of insulin secretion and islet mass after deletion of the cell cycle inhibitor p27 that is a marker of senescence in mice models is a strong evidence of the involvement of senescence in T2DM [323]. In addition, one of the key element of the mechanisms that may increase the risk of having T2DM, especially in the elderly might be the important shortening of the telomeres in β-cells, that may above a certain shortening threshold promote cell cycle arrest [324]. In a recent study, Tamura *et al.* found that the telomere length in the β-cells of aged patients with diabetes was shorter compared to age matured non-diabetic patients. The exact reason was unclear but is might be explained by that hyperglycemia known to induce oxidative stress-induced β-cell telomere shortening, thereby promoting β-cells premature senescence and hyperglycemia [325]. The β-cell dysfunction caused by the cellular senescence can be avoided by new treatment called senolytic agents [326] (Figure 21 B). When this treatment is taken at prediabetes diagnosis it might delay or avoid the progression to overt diabetes and may partly conserve β-

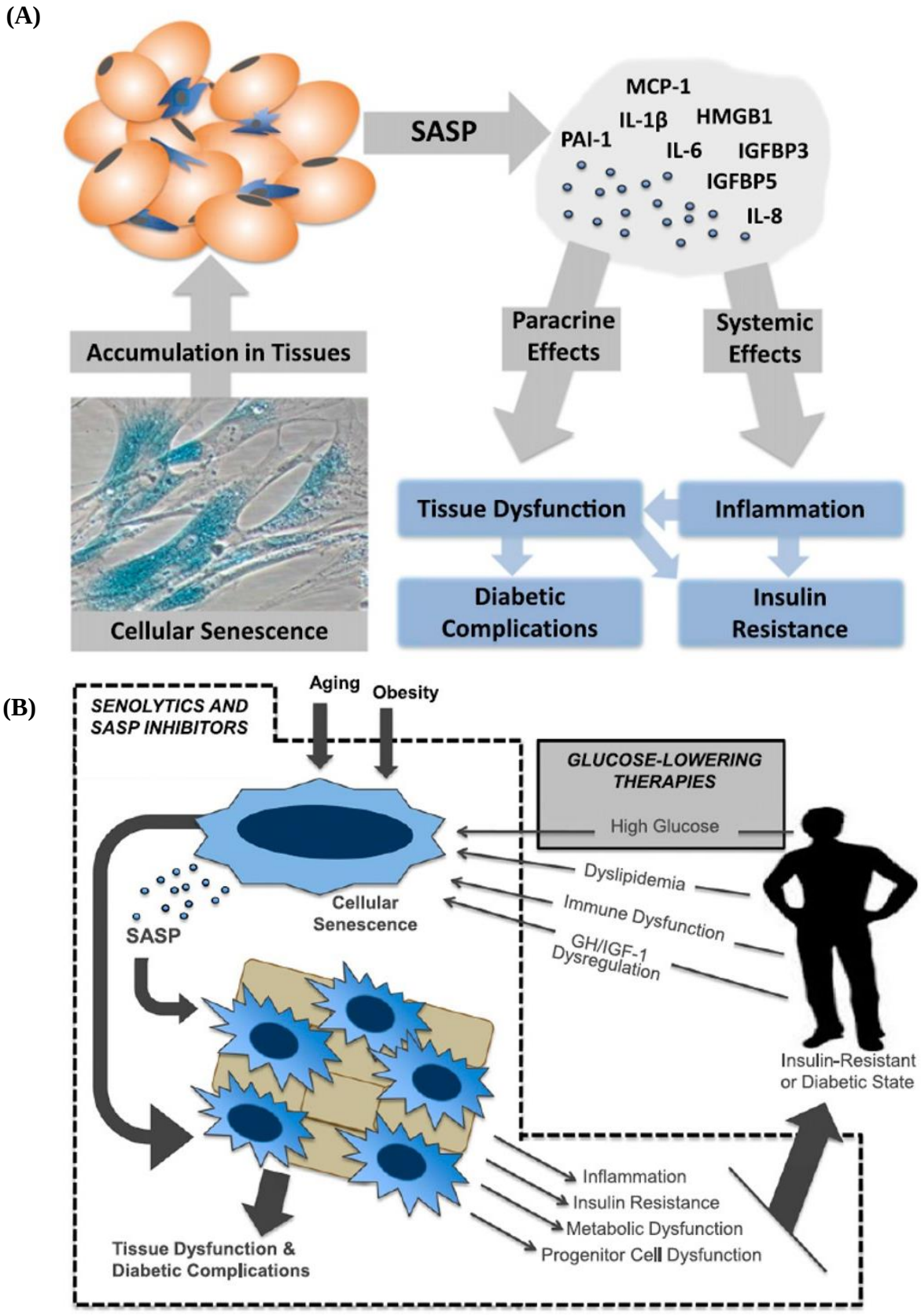


Figure 21: Senescences and diabetes (Palmer *et al.*, Diabetes, 2015)

(A) SASP mechanisms and effects, (B) Senolytics and SASP inhibitors effect on diabetes

cell function to escape insulin-dependent diabetes [317]. Besides participating to diabetes progression, the senescent cells may accelerate tissue injury making it an essential mechanism of diabetes complications [317].

p16 was known for long-time a tumor suppressor protein and a CDKI before it was identified as a driving force to senescence when overexpressed. Using p16 induced expression in juvenile mouse β-cells *in vivo,* Helman *et al.* studied how senescence affects β-cells function and structure. They found as anticipated that β-cells forced to enter cell cycle arrest by p16 over expression become senescent. In addition, β-cells were ~1.3-fold larger in volume and SA-βGal activity, considered as a reliable indicator of senescence, was detected, besides other markers amplified lysosomal activity. Helman *et al.* findings indicate that p16 expression alone is able to stimulate senescence in β-cells. This raises the question of p16 expression as an inducer of T1DM and T2DM [327]. In T2DM telomere shortening could accelerate β-cells senescence and thereby reduce functional β-cell mass [324].

VI.8 Senescence and transplantation outcomes

VI.8.1 Impact of donor age

In organ transplantation and particularly in pancreas and pancreatic islet transplantation, donor age is determinant for the efficacy of graft function and survival [328]. Aging is a determinant factor for the progressive post-maturational deterioration of all organs, leading to chronic and metabolic diseases including obesity, impaired glucose tolerance and type 2 diabetes (T2D) [329, 330].

During the last decade, the main achievement in the medical field is the prolonged duration of life and the increase proportion of elderly patients developing end-stage organ disease [331]. As a result, patient needing organ transplantation and donors are older (>50 years) [332]. In addition the number of old transplant recipients increases significantly which puts transplantation medicine to new challenging issues in patients monitoring [331].

The main difficulty of old donors with organs is that they may impair early and late graft function [333, 334]. For example, kidney transplantation from older donor (>60 years) show a mean life of 5 years success of graft in comparing with 10.2 years when the transplanted organ is from a younger donor [335]. In addition, kidneys extracted from old donors show a higher susceptibility to age-related diseases like hypertension, diabetes and arterial deterioration which contribute to the delay in graft function and to the fast rejection of graft [336]. With aging the

immune system undergoes a continuous complex and progressive change which plays an essential role for engraftment and graft survival [331]. So, as a consequence organ isolated from old donors were initially considered as better discarded [337]. One of the principal concerns about organs from old donors is the somatic cells senescence. However, each tissue has a limited and specific capability for survival, repair and replication, which decreases with age and stress, leading to the early failure of graft [338, 339]. Transplantation researchers have created mathematical formulas or scores to predict the graft outcome based on a combination of the number of risk factors, in conclusion all this formulas found that donor age contribute to worsened graft outcome [340].

VI.8.2 Immunosenescence of recipient and organ transplantation

Aging touches all sections of the immune response, the innate and the adaptive immunity, which have a crucial impact on organ transplantation success. Until now, the clinical trials and the experimental data on immunosenescence are still limited. Consequences of the immunosuppression on immunosenescence remain to be assessed and established in appropriate models in order to propose personalized therapies to younger patients or to those receiving old grafts. Several studies about the clinical implications of immunosenescence showed increased risks of infections, malignancies, autoimmune disorders, atherosclerosis and neuro-degenerative changes [331]. In aged recipients after post-transplant malignancies are increase 5 times after 65 years [331, 341].

In clinical trials with kidney, heart, liver and lung transplantation, the incidents of acute rejection was at lower rates due to the immunosenescence of the recipient [328], for example in renal transplantation, Cecka *et al.* showed that the rejections in old recipients was less than 25% by comparison with 50% in recipients aged less than 45 years [342].

However, the immunosenescence impact affects the hematopoietic stem cells (HSC). Aging may disturb HSC proliferation and regeneration capacity [343]. In addition, immunosenescence affects the functionality of all the immune cells such as NK, B, T, memory T, AB secreting and dendritic cells, macrophages and neutrophils [331] (Figure 22).

Experimental models of transplantation of old organs was associated with more powerful early immune responses that is caused by the augmented immunogenicity of these organs (Figure 23). These experimental works was confirmed by an increased incidence of acute rejection after transplantation of old kidneys [344]. Regarding the effect of the immunosenescence, immunosuppression in the elderly patients might have to be adapted with lower and optimized

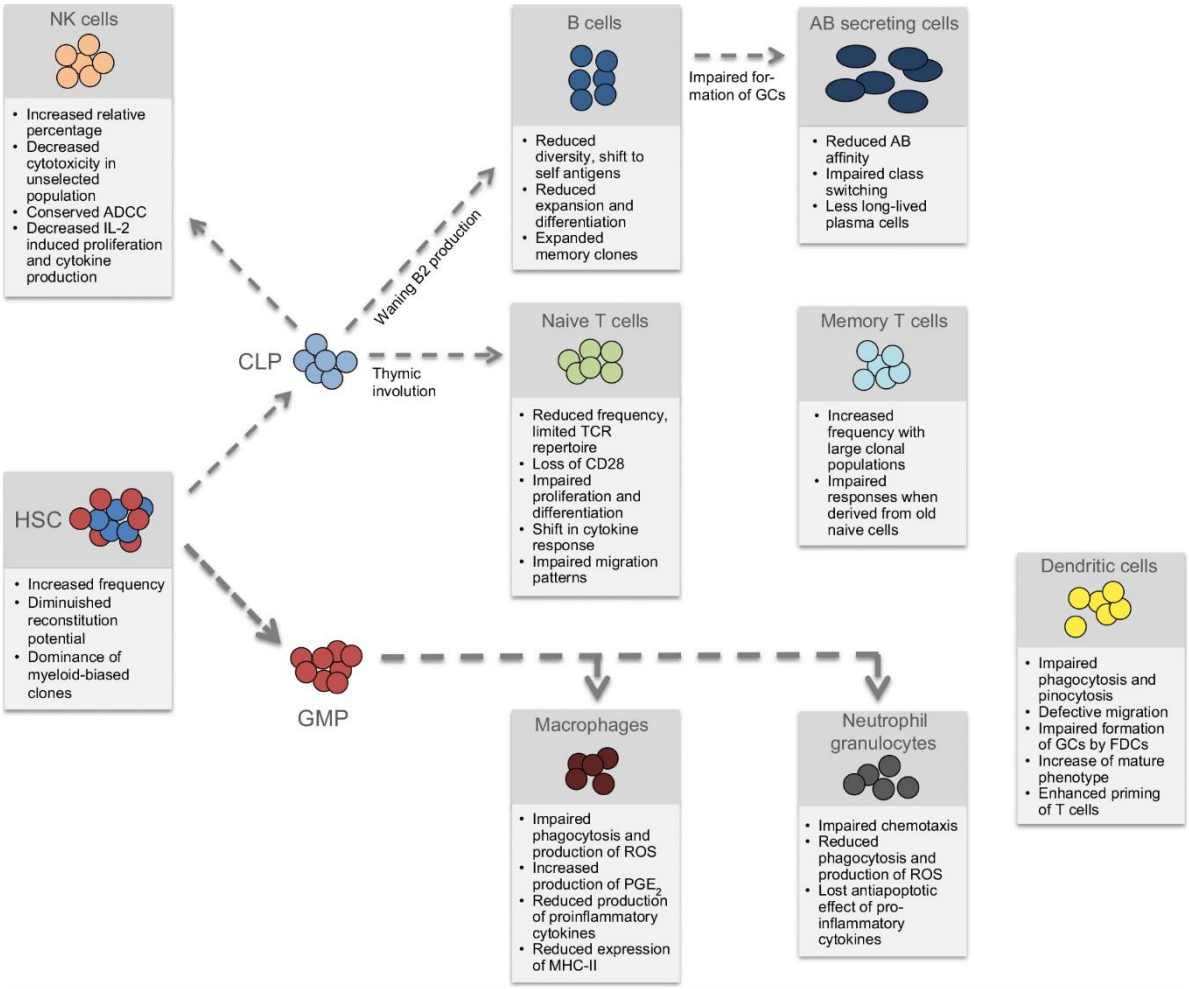


Figure 22 : Immunosenescence and accompanying changes of all innate and adaptive immune cells (Heinbokel T. *et al.*, Transplant international, 2012)

HSC, hematopoietic stem cell; CLP, common lymphoid progenitor;ADCC, antibody-dependent cell-mediated cytotoxicity; GC, germinal center; AB, antibody;

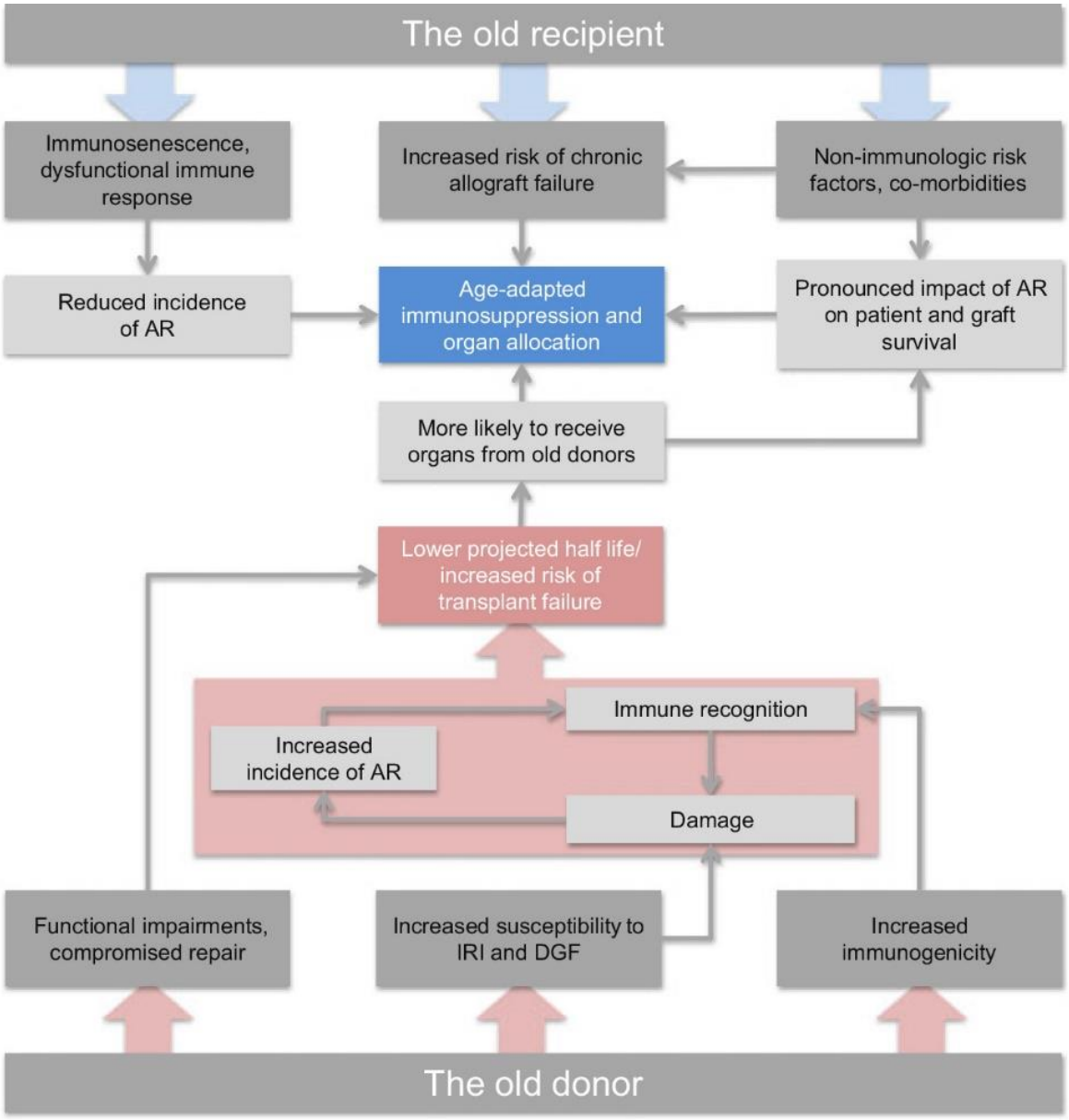


Figure 23: Impact of old donor and recipient on transplantation outcome (Heinbokel T. *et al.*, Transplant international, 2012)

AR: acute rejection, IRI: ischemia/reperfusion injury, DGF: delayed graft function

doses of the immunosuppressive drugs to minimize the side effects of the immunosuppression treatment since immunosenescence reduces the role of rejection [328].

So taken all these data in consideration, the immune system is affected by extrinsic and intrinsic factors caused by aging, which leads to imbalance of the immune control. It is therefore of vital importance to understand the mechanisms targeted by cellular senescence within the organ and by systemic and local immunosenescence. With the improved longevity, the transplantation of older organs to aged recipients will increase continuously [331]. Because, the aged recipients show a lower transplant outcome owing to higher complication issues and to the risk of chronic allograft failure, the clinical implications of immunosenescence is an open field for age-impacted processes related to immune responses and may bring new hints in other pathological issues like autoimmunity [331].

VI.8.3 Accelerated endothelial senescence induced during islet transplantation

During the different steps of the pancreatic islet transplantation, the generated oxidative stress may lead to accelerated senescence. Pancreatic islets are isolated from cadaveric donors and a variety of factors that affect islets before any isolation step such as cytokine release after death, or health complications from the donor and his/her age [4]. Directly after the pancreas withdrawal mechanical and enzymatical processes (collagenase, shaking, centrifugation) reinforce the ischemia and redox-sensitive stress. Ischemia will persist until islet revascularization that will further undergo reperfusion injury. Islet isolation itself leads to a high amount of β-cell death resulting from the necrosis, apoptosis and the production of the pro-inflammatory molecules [345]. During the culture period prior to transplantation islets also loose a considerable amount of endothelial cells, compared to the islets transplantation without culturing [128, 129]. After portal transplantation, islets directly suffer from the liver capillary environment and from the first contact with the portal blood leading to IBMIR (see Figure 35). All these conditions contribute and may amplify the generation of the oxidative stress that accelerate senescence. Therefore, the limitation of the causes of islet stress and ischemia / reperfusion injury is mandatory for a better transplantation outcome.

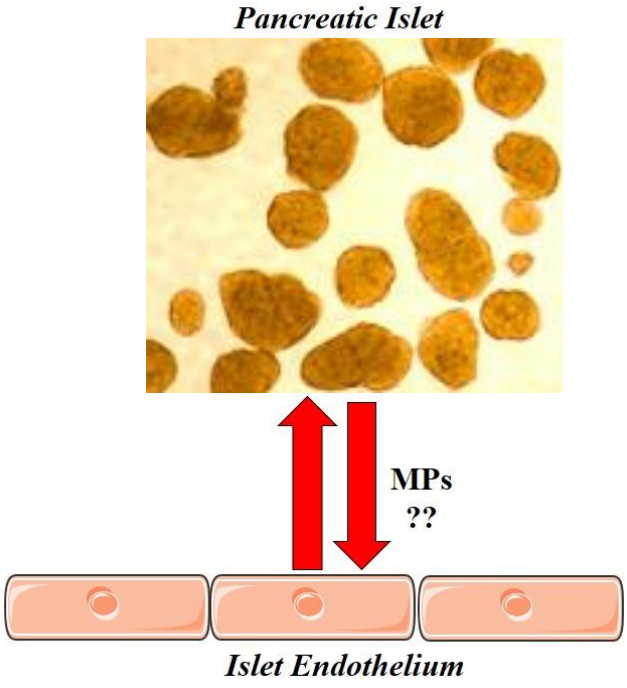
In conclusion, one of the central features of cellular senescence is the so-called snowball effect that accelerates and amplifies the accumulation of senescent cells within, nearby the islet. Altogether, the targeting of cellular senescence should prove an active area of research in the future decade that may have promising chance to improve transplantation outcome. Senescence can be one of the main contributor of the chronic dysfunction of grafted organ. The study of the

therapeutic clearance of senescent cells and their remote effects through the dissemination of pro-senescent MPs might bring major breakthrough in our understanding of the mechanisms involved in human healthy aging and lifespan [317, 346]. Recent work from our laboratory showed that senescence can be induced in the young endothelial cells by the effect of the pro-senescent endothelial MPs [347]. This suggests that pro-senescent MPs can affect the pancreatic islets as shown by our preliminary data *in vivo*. Pro-senescent MPs seem to circulate in patients with organ injury [347] like acute coronary syndrome and could be responsible for the local thrombogenic endothelial phenotype at vascular sites of flow disturbance.

Finally endothelial MPs seem to be a signature of the endothelial damage in patients with immunosuppressive therapy thereby suggesting their diagnosis value for the monitoring of thrombotic complication in transplanted patients [348].

Chapter VII

Microparticles: Important biomarkers & effectors in graft



VII Microparticles: Important biomarkers & effectors in graft

VII.1 Microparticles: stress markers and cellular effectors

MPs are submicron plasma membrane vesicles (0.05 to 1 μm in diameter) released into the extracellular space by stimulated or apoptotic cells after plasma membrane remodeling [349]. It express on the external leaflet of their membrane an anionic phospholipid, the phosphatidylserine (PhSer), which is procoagulant because it constitute the catalytic surface for the assembly of blood coagulation complexes (Figure 24). At physiological state, MPs circulate at low concentration in blood. In pathophysiological condition, MPs circulate at high concentration and constitute an additional procoagulant surface for blood coagulation and the generation of thrombin. In the vascular compartment, thrombin and other mediators activate the vascular cells and contribute to the disruption of hemostatic balance favoring procoagulant and prothrombotic state. Any cell can release MPs, the most extensively studied are those issued from vascular cells: platelets, leukocytes, erythrocytes and endothelial cells with differences in kinetics release depending on the lineage and the stress. Latest data show that MPs could also be released by senescent endothelial cells [350]. The amount of MPs released in the systemic circulation is directly correlated with the disease severity [351] and was shown *in vitro* directly proportional to the degree of cell apoptosis or stimulation, so MPs appear as real markers of cellular and tissular stress [352]. MPs express at it surface antigens and active biomolecules characteristics of cells origin and initial stress or agonist (Figure 25).

Besides being a cell damage marker, MPs act as cellular effectors by their ability to interact with the plasma membrane of target cells and transfer MPs content such as active proteins, lipids or membrane receptors [353]. These interactions can be autocrine or paracrine especially in the case of circulating MPs. Studies show that MPs can also carry and transfer genetic material like DNA, RNA and miRNAs [354, 355].

MPs participate to cell survival, apoptosis, inflammatory responses, vascular remodeling and angiogenesis [349] and contribute to the delicate balance of homeostasis. When they are in excess they can be deleterious [356]. The composition of MPs determines their specific action for instance MPs of tumoral origin, which are carrier of TF deliver it to endothelial cells and stimulate the recycling of MPs-associated tissue factor (TF) to the surface of the target cell with enhanced procoagulant activity within 4 hours under the dependence of rab G proteins [357]. The variety in MPs protein content suggest that they contribute to amplification and retrocontrol of cell activation. A very important notice is the distinction between MPs and other types of

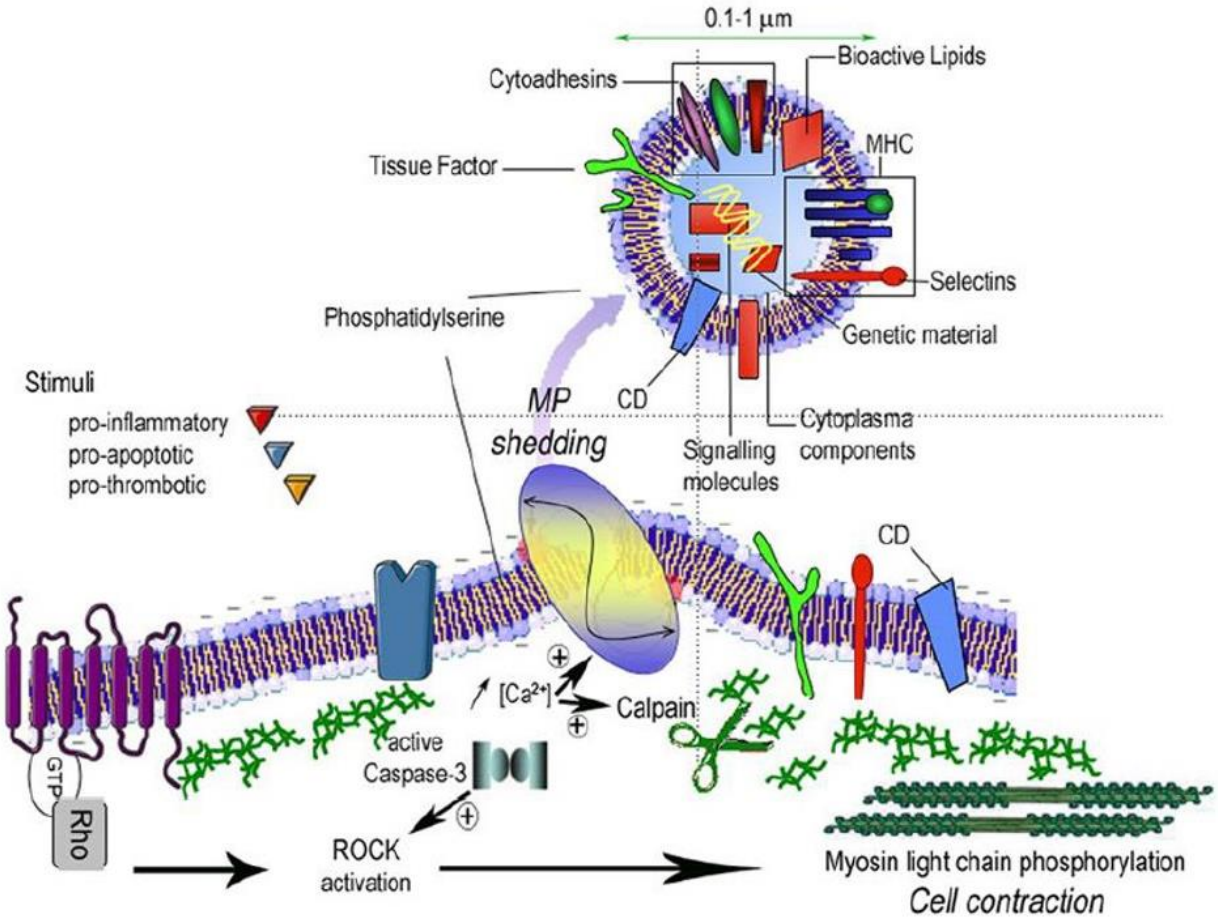


Figure 24: Membrane remodeling and MP generation (Bakouboula B, Frontiers in Biosceinces 2011)

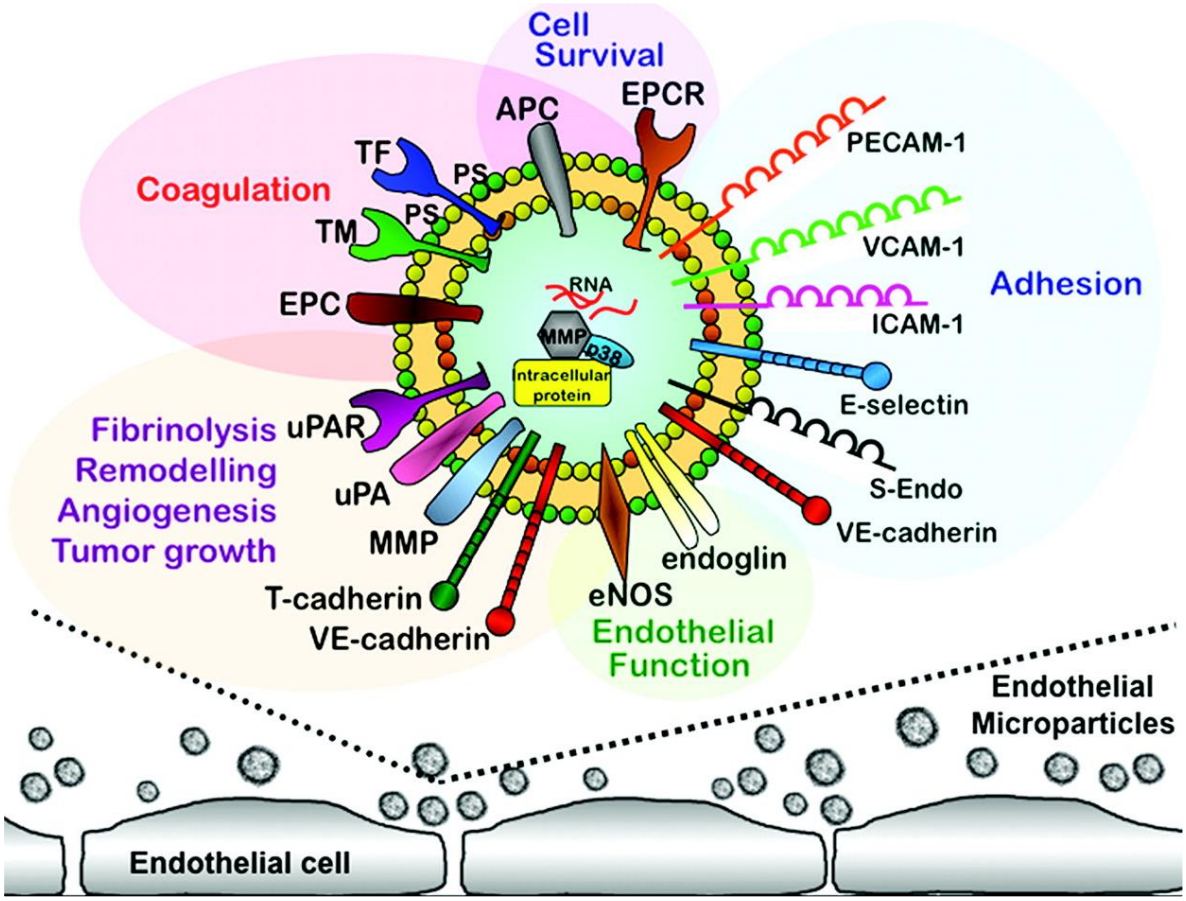


Figure 25: MPs a circulant pool of bioactif effectors (Dignat-Georges F and Boulanger C, J Arterioscler Thromb Vasc Biol, 2011)

EPCR : endothelial protein C receptor; PECAM-1 : platelet endothelial cell adhesion molecule-1; VCAM-1 : vascular cell adhesion molecule-1; ICAM-1 : intercellular cell adhesion molecule-1; E-selectin : endothelial selectin; S-Endo : CD146/melanoma cell adhesion molecule; VE-cadherin : vascular endothelial cadherin; eNOS : endothelial NO synthase; MMP : matrix metalloproteases; uPA : urokinase plasminogen activator; uPAR : urokinase plasminogen activator receptor; EPC : endothelial protein C; TM : thrombomodulin.

vesicles (<50 nm in diameter), like exosomes, which are of endosomal origin and released by exocytosis. In exosomes, there are small amounts of PhSer and conversely an enrichment in tetraspanins. Their surface markers and their properties are different from those of MPs [358].

VII.2 MP formation mechanisms

Following cell activation or an apoptotic stress, the phosphatidylserine randomization through the two leaflets of the plasma membrane initiates vesiculation.

VII.2.1 Plasma membrane: a central player in vesiculation

VII.2.1.a Asymmetry of the plasma membrane

The function of the cells is supported by the fluidity of the plasma membrane, which depends on lipid composition and temperature. The composition of phospholipids, cholesterol and glycolipids ensures the membrane barrier selectivity. The plasma membrane bilayer is asymmetric: the anionic phospholipids like phosphatidylethanolamine (PhEth) and phosphatidylserine (PhSer) are specifically sequestered in the inner leaflet while phosphatidylcholine (PhChol) and sphingomyelin (SM) are enriched in the outer one [359]. The local thickness and the flexibility of the membrane depends on the nature of the fatty acids, specifically their chain length and number of unsaturated links.

The fluidity of the membrane of the lateral movement of proteins and phospholipids within the same monolayer depends on the membrane curvature and on its tension. The lateral diffusion allows transitorily a particular reorganization of the plasma membrane into functional platforms called "lipid rafts" (or raft) that stimulate the local concentration of specific receptors and phospholipids and allows the initiation of intracellular signaling [360]. Of note, PhSer was found together at the edges of rafts [360].

VII.2.1.b The phospholipids transporters

In many biological processes, studies showed that the transport of phospholipids across the plasma membrane plays an essential role [361]. Normally, at rest the flip-flop or also called the transverse diffusion of PhSer from inner to outer leaflet of the plasma membrane is rare and slow. On the other hand, the transmembrane transport of phospholipids is important in causing lipid imbalance between the two leaflets, which participates to membrane tension and curvature. Furthermore, PhSer is a recognition signal for cell clearance by phagocytosis that should be tightly controlled. Due to this imbalance and lipid asymmetry, leaflet transport of phospholipids play an central role in various cellular processes like the synthesis of the plasma membrane,

phagocytosis, fertilization, membrane budding, regulating the functions of membrane proteins, coagulation and apoptosis differentiation [361].

Studies prove that the phospholipid bilayer works as an energy barrier for the free movement of hydrophilic molecules into and out of cells and subcellular organelles. Therefore, to transport hydrophilic molecules through cell membranes particular transporters and channels are required. Actually, there are three types of transporters: floppases, flippases and scramblases transporters. ABC transporters (ATP binding cassette) like ABC A-1 are occasional ''floppases''. However they are not mutated in scott syndrome, a rare pathology with defective microvesicles (MV) shedding [362]. P_4-ATPases are flippases and constitute a class of P-type ATPases which uses the energy from ATP hydrolysis to transport aminophospholipids from the outer layer to the inner layer of cellular membranes [363]. Scramblases, which are energy independent but calcium-dependent, bi-directional non specific lipid transporters. It ensures a homogeneous distribution of newly formed lipid in the endoplasmic reticulum (ER) or in the plasma membrane of an activated cell [364] (Figure 26).

Under conditions of stress and calcium influx, inactivation of flippase and activation of scramblases or floppases, cause an alteration of membrane asymmetry and exposing PhSer in the outer leaflet of the membrane [365]. This stimulus leads to major changes within the surface properties of the cells. Finally, it causes a shift to a procoagulant and pro-adhesive state.

Recent studies put in evidence others types of floppases. The first one is an eight transmembrane containing protein TMEM16F. It is an anoctamin that favors scramblity through Ca^{2+} influx-mediated mechanisms and seem dependent on longterm exposure to calcium [366]. When TMEM16F is mutated in scott patients, platelets or red blood cells are enable to produce scrambling and MP shedding. However in lymphocytes from scott cells, long-term exposure to calcium enables PhSer exposure and MP shedding most probably through oligomerization [367] in stimulated murine platelets. Recently, six transmembrane-containing protein Xk-related family protein 8 (XRP8) was shown to stimulate phospholipid scrambling in kell system in response to the activation by cleavage of caspase-3 initiated by apoptosis [368] (Figure 27).

VII.2.2 Membrane remodeling and vesiculation

VII.2.2.a Calcium influx and rupture of membrane asymmetry

There are different stimuli that can cause cell activation and emission of MPs directly or after longer stimulation such as thrombin, growth factors, hypoxia and inflammatory molecules (IL-

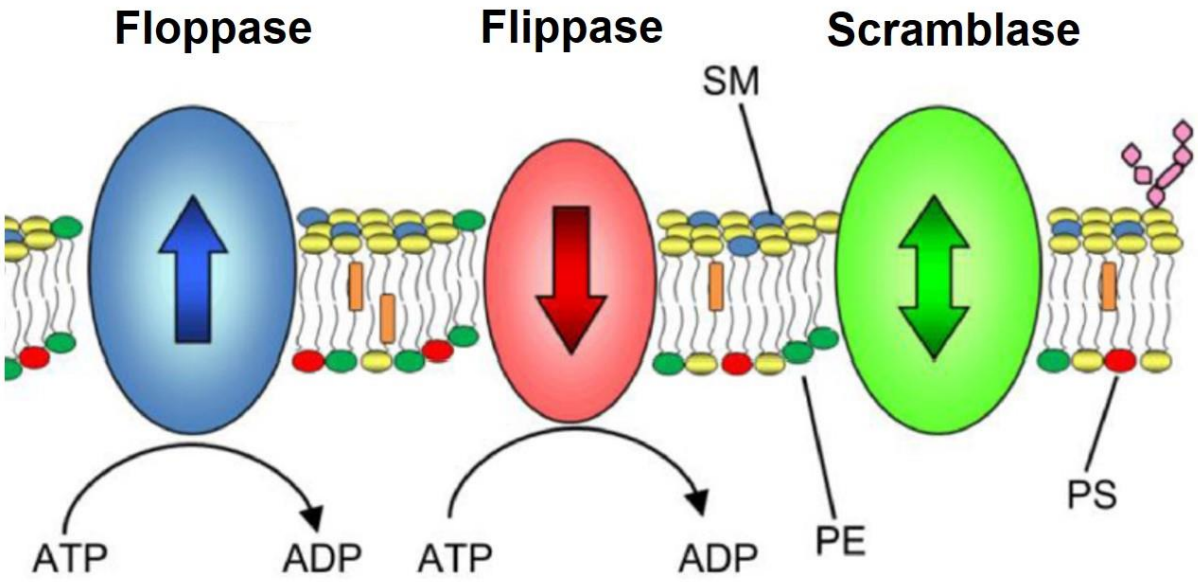


Figure 26: Function of the phospholipid transporters (Coleman J. *et al.*, J Biochim Biophys Acta, 2013)

Most floppases transporters catalyze the ATP-dependent transport of lipids from the cytoplasmic leaflet of the bilayer to the extracellular (lumenal) side of the membrane while flippases transport in the opposite direction. Scramblases are energy independent transporters and calcium-dependent, which ensures the movement of the non-specific phospholipids according to their concentration gradient.
Abbreviations used: SM sphingomyelin. PE (or PhEth): phosphatidylethanolamine; PS (Or PhSer) phosphatidylserine.

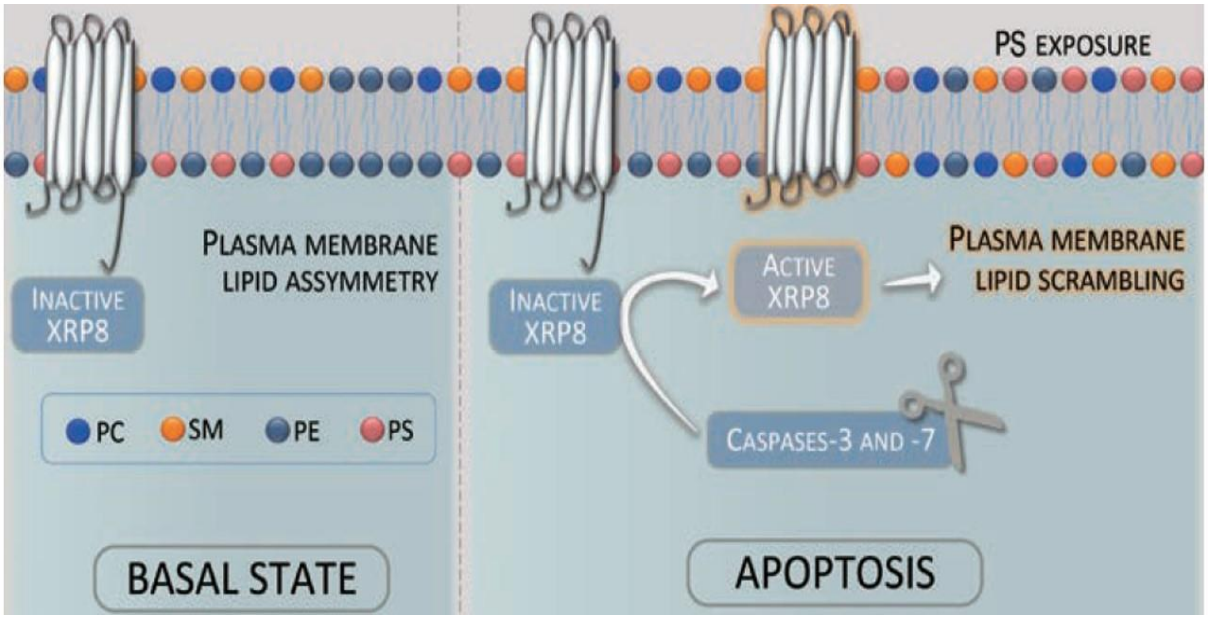


Figure 27: Involvement of scramblase activity in XRP8 PhSer exposure following an apoptotic stress (Marino G., Kroemer G., J Cell Res, 2013)

Under normal conditions, the combined action of multiple mechanisms, including the activity of flippases and floppases, maintains lipid asymmetry between the outer and inner leaflets of the plasma membrane. Once apoptotic program is activated, caspases-3 and -7 are able to cleave and activate Xrp8 protein, which acts as a lipid scramblase and leads to the loss of lipid asymmetry, resulting in PS exposure to the extracellular space. This acts as the "eat-me" signal that will allow phagocytosis of post-apoptotic cell corpses. Abbreviations used: PC (PhChol) phosphatidylcholine; SM, sphingomyelin; PE (or PhEth): phosphatidylethanolamine; PS (Or PhSer) phosphatidylserine.

1β, TNF-α, Fas). Intracellular calcium is highly increased after cellular activation or apoptosis from 1 μM at baseline to 30-350 μM due to opening of the ion channels as well as the release of intracellular [369]. Besides that, the opening of the mitochondrial pores resulting from the depolarization of the external membrane of the mitochondria rises the intracellular calcium concentration. High concentration of intracellular calcium finally lead to activation of both the floppase activity and scramblase, besides the inhibition of flippase [370] (Figure 28). During cell activation, a faster transportation of the PhSer to the outer leaflet compared to inverse transport that occurs slower leading to a lipid overload of the outer layer, which is finally resolved by vesiculation. Therefore, the rupture of membrane asymmetry contributes to the emission of MPs.

VII.2.2.b Calcium influx and degradation of the cytoskeleton

Cytoskeleton integrity is supposed to contribute in the maintenance of cell shape and membrane asymmetry, where its degradation is considered a facilitator of membrane budding in stimulated cells. Studies demonstrated that rise of intracellular calcium is directly related with a proteolytic activity of calpains and cysteine proteases like caspase. Both calpains and caspases are Ca^{2+}-dependent thiol proteases that contribute to cytoskeleton reorganization by stimulating the degradation of filamin-1, gelosine, talin and myosin [371]. In addition, caspase activation lead to the vesiculation of MPs under apoptotic stress. Indeed, activated caspase-3 is also demonstrated by the studies to be a major factor in cytoskeleton reorganization by cleaving Rho-kinase-1 (ROCK-1) that is one of many effectors for the Rho GTPases [372]. In endothelial cells, this then leads to the phosphorylation of myosin light chain that promotes destabilization of the cytoskeleton cell membrane contraction, shrinkage and MPs release [373] (Figure 29). Remarkably, ROCK-1 inhibition, in activated endothelial cells, blocks the emission of MPs enriched in caspase-3 and causes the detachment and cell death. This suggests that the emission of MPs, at least in the early stage of apoptosis is a cell survival mechanism to limit the intracellular accumulation of cytotoxic molecules by their release in soluble form in the extracellular medium [374].

VII.2.2.c Membrane remodeling and rafts

Membrane remodeling is partly based on the formation of so-called ‘’lipid rafts’’ that are dynamic assemblages of proteins and lipids that float easily within the liquid-disordered bilayer of cellular membranes. Normally, rafts are small platforms, enriched in sphingolipids and cholesterol. Association of rafts forms larger and ordered platforms after cell activation [375].

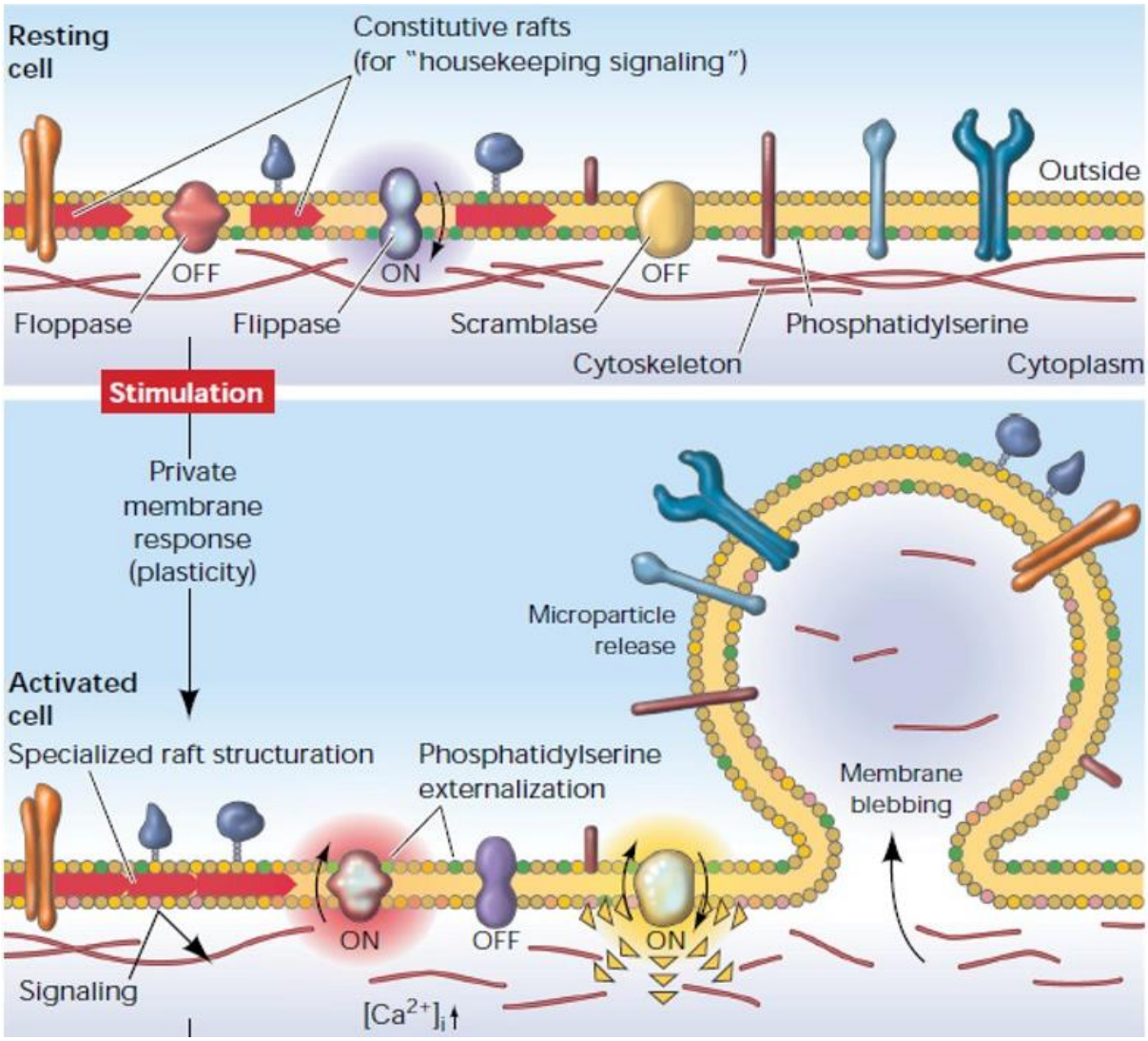


Figure 28: Membrane mechanisms responsible for the release of MPs (Hugel *et al.*, J Physiology, 2005)

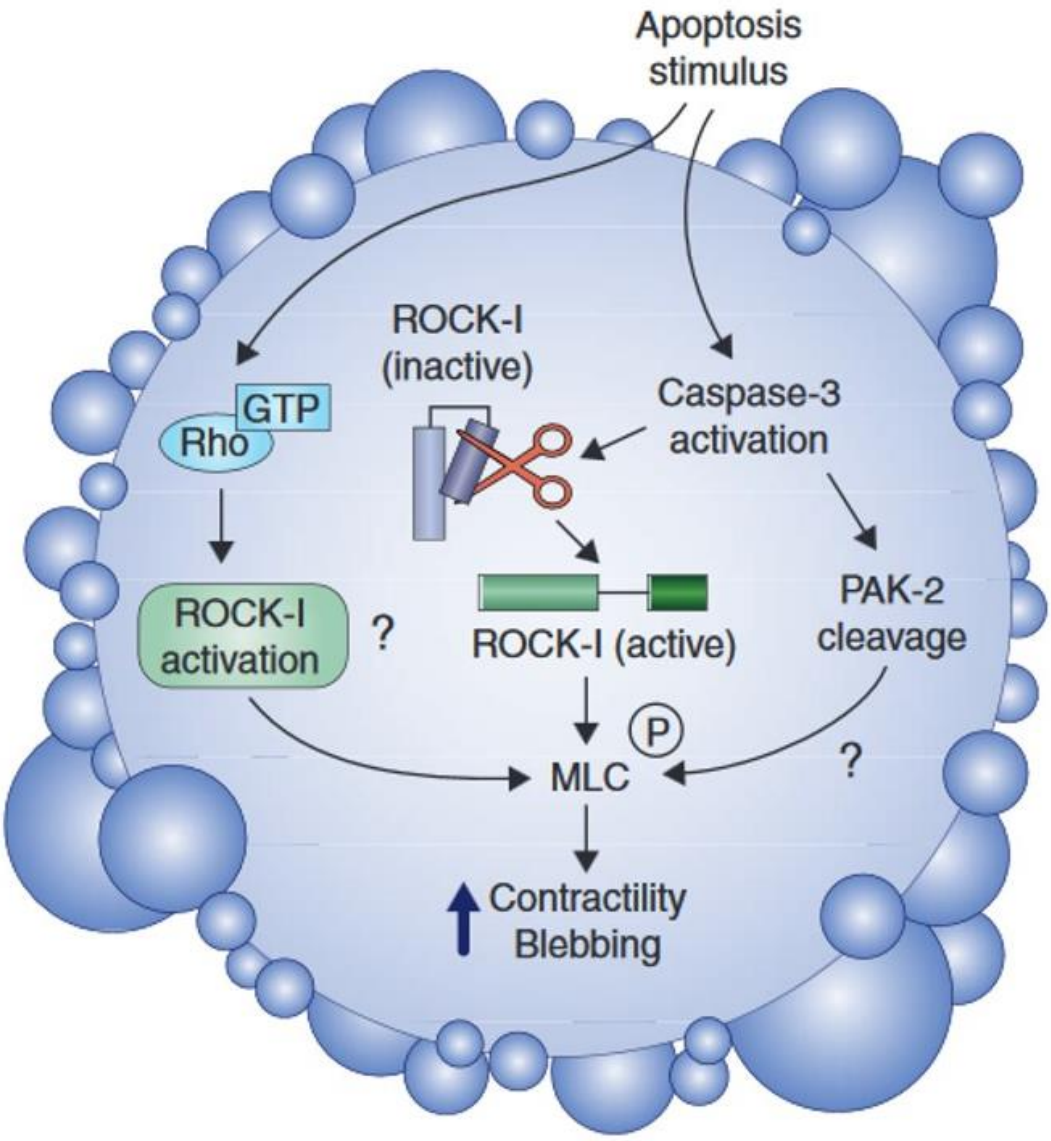


Figure 29 : Reorganization of the cytoskeleton during apoptosis (Leverrier Y and Ridley AJ, J Nat Cell Biol, 2001)

Membrane fluidity and their lateral mobility allows transitorily a local compartmentalization of the plasma membrane. This dynamic is reflected by the transient interaction of lipids with saturated acyl chains and ensures compaction with other components of rafts especially with the transmembrane proteins that have a particular affinity for these lipid components such as glycosylphosphatidylinositol (GPI) anchor proteins (Figure 30).

Rafts are located in different membrane situation: 1) planar lipid rafts are continuous with the plane of the plasma membrane and lack characteristic morphological features. whereas 2) caveolae are small and flask shaped plasma-membrane invaginations that can be observed as a subgroup of lipid rafts that contain caveolin proteins and are the most readily-observed structures in lipid rafts. However, the main characteristic of such lipid rafts is the presence of the cholesterol-binding protein caveolin-1 [376].

Actually, lipid rafts structure has been linked with a specific biological role needed for the function of the cell, mainly to provide protein sorting and to stabilize privileged interactions between raft constituents, besides playing an important role in the process of signal transduction [377]. These areas constitute a proteolipidic platforms where the receptor activity and the downstream metabolic pathways is modulated, allowing the coupling between signal extracellular and intracellular signaling pathways. Lipid rafts are highly involved in cell adhesion and the phenomena of vesiculation. For instance, it was demonstrated that the emission of monocytes MPs, which have TF on their surface, merge from rafts enriched in TF and P-selectin glycoprotein ligand-1 [378].

VII.3 MPs clearance

Low levels of circulating MPs identified in healthy individuals, which suppose that MPs may have a physiological function for example to remove stress-induced cellular by-products [379]. In pathology like in cardiovascular disease, the failure of MPs clearence could increase the circulating levels of proatherogenic factors [380]. Although MPs express PhSer, which in turn helps recognition by phagocyte through the exposing of ‘’eat me’’ signals, they escape to quick clearance due to their small size (0.05-1 μm in diameter) that do not allow the characterization of sufficient and various antigens (Ag) necessary for optimized phagocytosis.

The time needed for the clearance of MPs depends on many factors such as the cellular origin of the MPs, the animal species and the stimulus causing vesiculation. Previous works have suggested that the half-life of MPs may vary from minutes to several hours. In humans it has been proved, following injection of MPs platelet, that 70% of MPs were found 3h after injection

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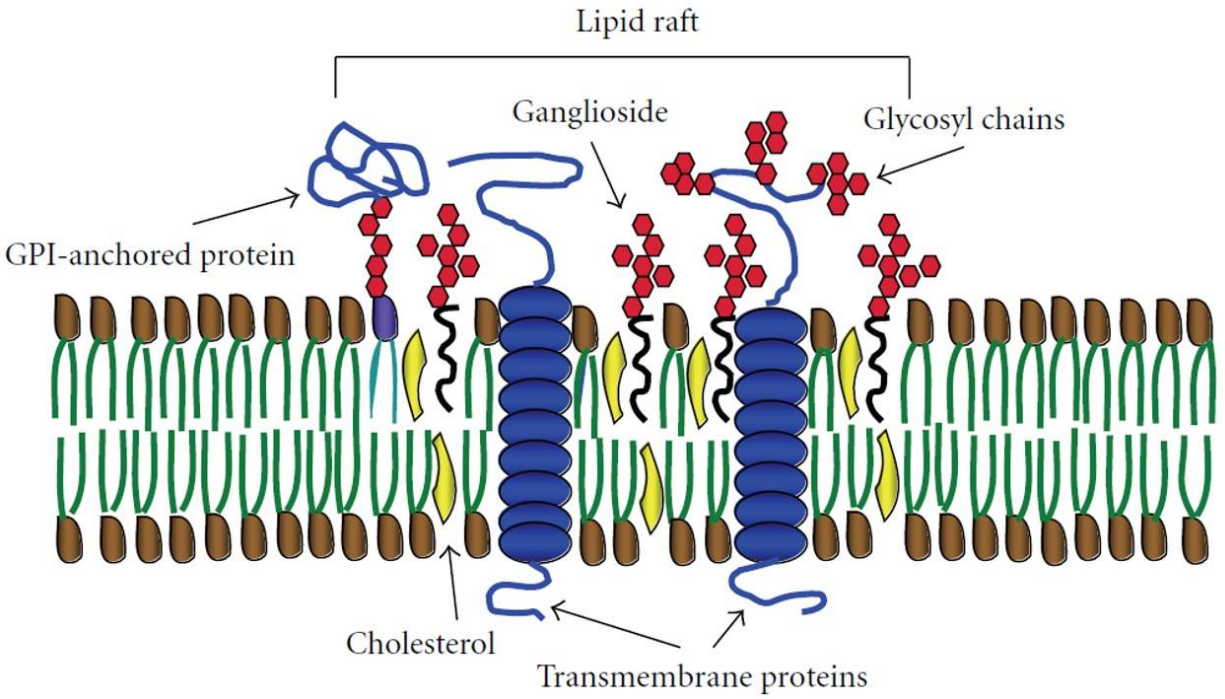

Figure 30: Lipid rafts compositions (Malchiodi-Albedi F. et al, International Journal of Alzheimer's Disease, 2011)

[381], while they would be removed in less than 30 minutes in mice [382] and 10 minutes in rabbits [383].

In 2009, Dasgupta *et al.* were described a route of elimination of circulating MPs, which is based on the relation between lactadherin and PhSer of apoptotic cells and MPs. Lactadherin also known as milk fat globule–epidermal growth factor 8 (EGF-8) is secreted by macrophages [384]. It is a bi-functional glycoprotein that has a discoin I-like domain that binds to the PhSer tripeptide motif Arg-Gly-Asp (RGD), which is characteristic of integrins such as αvβ3 [385] (Figure 31). Lactadherin is secreted by macrophages and immature dendritic cells and play an important role in the clearence of MPs by splenic macrophages. *In vitro*, Dasgupta *et al.* showed that lactadherin improves the adhesion of macrophages to MPs and stimulating their phagocytosis [385]. Clinically, lactadherin is not detected in healthy patients, but is expressed on the surface of platelet MPs. In addition, mice deficient for the gene of lactadherin have a higher level of circulating MPs leading to hypercoagulating state, which was reversible by supplementation of lactadherin [385]. In 2012, the same team described a new pathway of MPs clearance that imply glycoprotein developmental endothelial locus-1 (Del-1) [386]. Del-1 is secreted by the endothelium. As lactadherin, it has an I-like domain discoin an RGD motif, which allows it to perform it function by binding to the PhSer of MPs and integrin αvβ3 cell (Figure 31). Conversely to lactadherin, Del-1 was detectable in healthy patients and that Del-1 deficient mice show no increase in circulating MP rates compared to the control group [386]. The clearance of MPs mediated Del-1 is activated under stress conditions. This is suggested and proved by the application of endotoxic stress on mice deficient in Del-1, which leads to a higher rate of circulating MPs. Clearance of MPs mediated by Del-1 is dependent on the phenotype of MPs, because it does not allow the removal of endothelial and platelet MPs. This can be explained by that Del-1 is only expressed by the endothelium of certain tissues like the brain and lungs but not from the spleen and liver in mice.

Interestingly, the clearance of MPs is performed through the involvement of many cells depending on the cellular origin of the MPs. For instance platelet MPs are cleared by the macrophages and the endothelial cells, endothelial MPs by monocytes and macrophages, erythrocyte MPs by Kupffer cells in the liver [387].

VII.4 Microparticles isolation and measurement

Because chronic diseases habitually take years to appear, research focused on the importance of having biomarkers that allow the early detection of pathology and that may help to facilitate

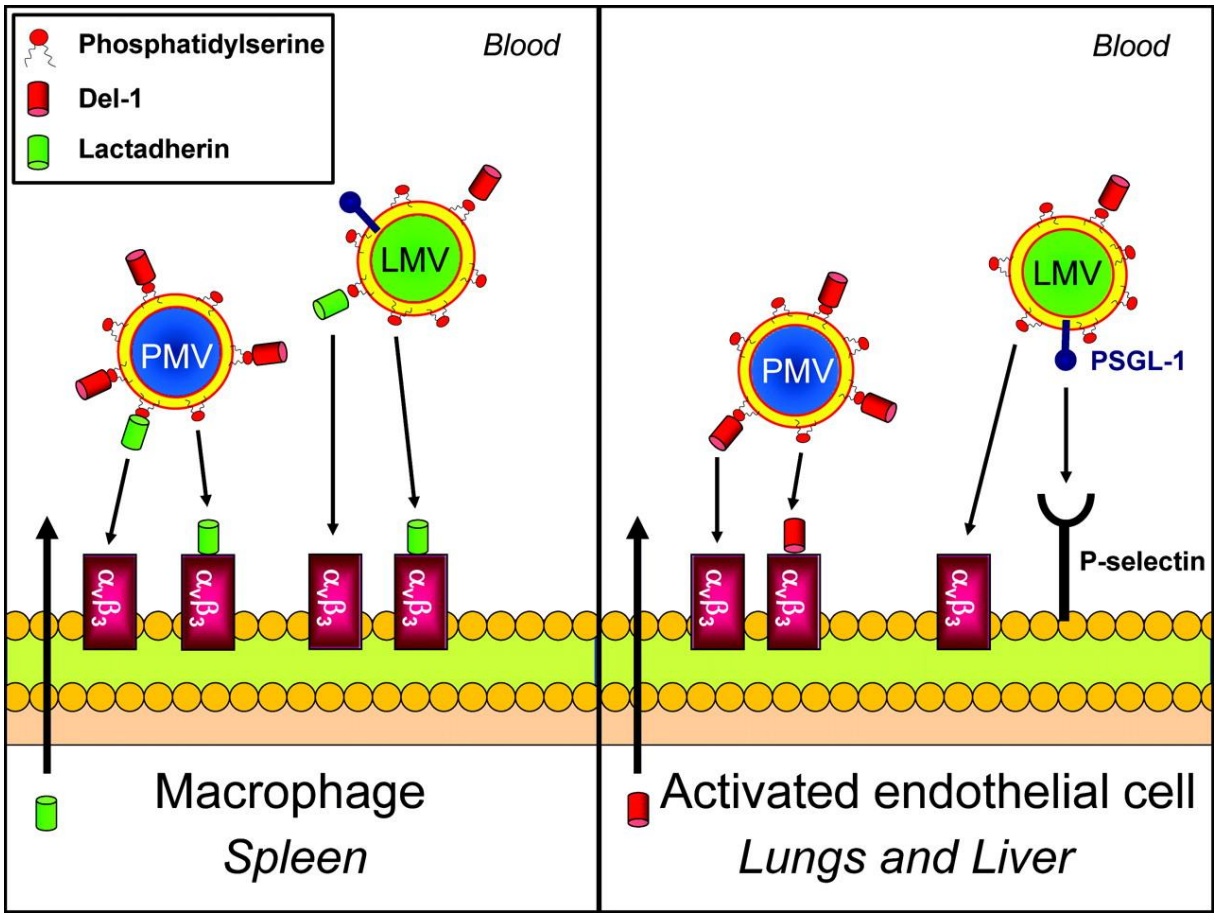

Figure 31: Mechanisms involved in clearance of circulating MPs (Rautou PE and Mackman N., J circulation, 2012)

In normal conditions, the lactadherin pathway mediates clearance of microvesicles. Macrophages in the spleen secrete lactadherin and this facilitates capture of circulating microvesicles. In endotoxemia, the developmental endothelial locus-1 (Del-1) pathway mediates clearance of microvesicles. We speculate that this pathway is turned on by the increased *αvβ3* expression on the surface of endothelial cells. Activated endothelial cells also express adhesion molecules, such as P-selectin, which can capture leukocyte microvesicles that contain P-selectin glycoprotein ligand-1 (PSGL-1). LMV indicates leukocyte microvesicle; PMV, platelet microvesicle

earlier therapeutic intervention. Because MP are procoagulant and remains in the circulation long after the activated cell, much research has focused on the potential of MPs as biomarkers of coagulation, endothelial dysfunction, inflammation [388]. To isolate and obtain pure MPs from blood, tissues of cells supernatant, a differential centrifugation method that consists of successive centrifugation steps can be used. It is based on increasing centrifugation forces and times, which help to isolate smaller from larger molecules (Figure 32) [389, 390]. For measurement, MPs from blood plasma samples or from the supernatant of cells in culture need a low centrifugation step to eliminate cells and debris. In the literature there are many approaches used for the detection and characterization of MPs, the main methods are the flow cytometry and the use of capture-based enzymatic assay. All methods used have limitations, advantages and disadvantages, where they are described in detail in table 1, these methods should be chosen in adequation with the MP source or tissue.

VII.5 Microparticles in diabetes and its related vascular diseases

Besides to their biomarker potential in cardiovascular diseases, MPs act as true diffusible conveyors and cellular effectors by transfer of bioactive molecules to target cells [391]. They are studied mainly in vascular pathologies such as hypertension, heart failure but also in diabetes where the study of MPs is seems relevant, because diabetes is being associated with endothelial dysfunction, atherosclerosis, inflammatory conditions, insulin resistance, associated vascular diseases such as macrovascular and microvascular complications that participate to reduced quality of life and even death and finally with hyperglycemia. Indeed, a prothrombotic, pro-inflammatory, pro-angiogenic state and dysfunction of the endothelium [392, 393]. Recently Kurtzman *et al.*, showed in T2DM groups a significant increase in circulating amounts of total, platelet and monocyte MPs in comparing with control group [394]. Interestingly, the type 1 and type 2 diabetes patients have different amounts and phenotypes of MPs. Another study by Sabatier *et al.* showed that type 1 and 2 diabetic patients, have higher circulating levels of MPs than healthy patients. But when comparing the MPs phenotypes they found that in type 2 diabetic patients only the total MPs are significantly increased while total, endothelial and platelet MPs were higher in type 1 diabetes [213] indicating a modification in the cellular source of MPs in T1D compared to T2D.

Many studies about the evaluation of endothelial function in diabetes related vascular diseases showed that endothelial microparticles (EMPs), shed from activated or apoptotic endothelial cells vary from 5 to 15% of the total MPs and could constitute a reliable biomarkers in different

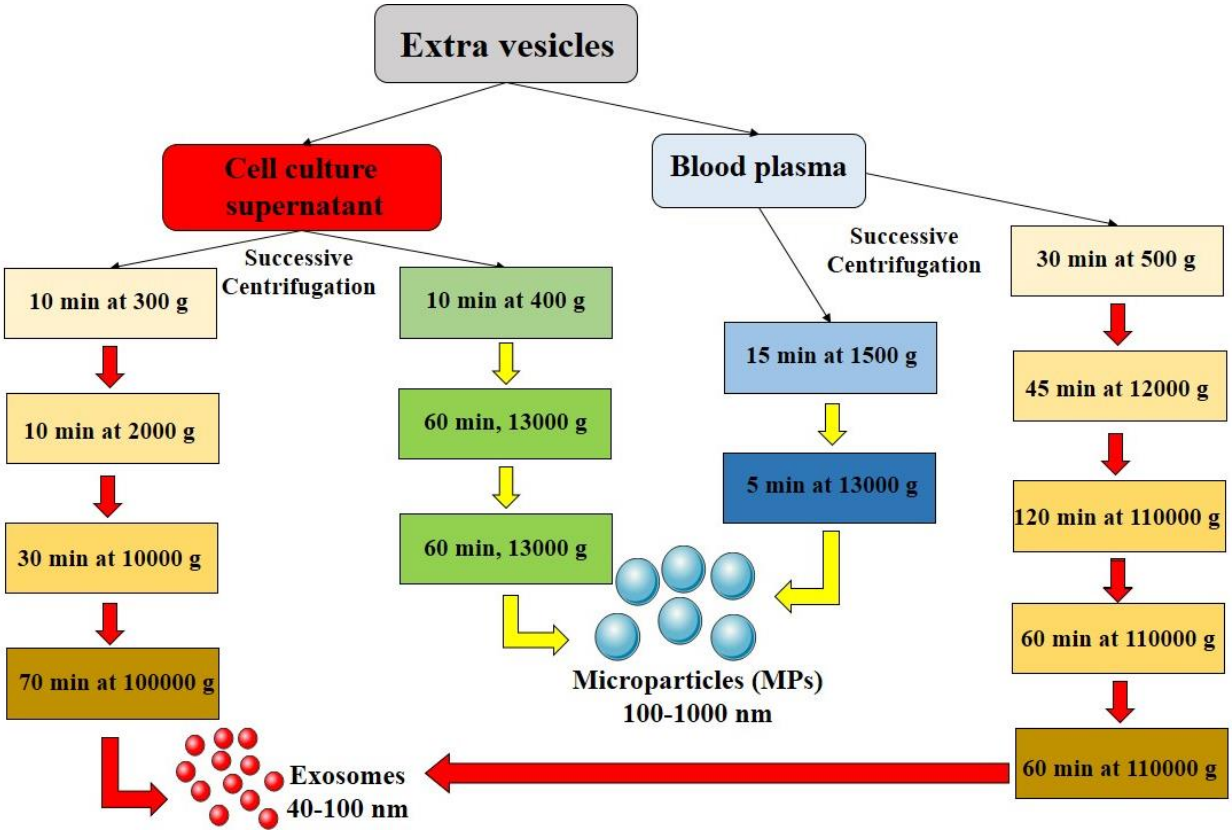

Figure 32 : Isolation of Exosomes & MPs

From the extra vesicles of different biological fluids (Cell culture supernatant and blood plasma), Exosomes & MPs were isolated by a serial of successive centrifugation.

Table 1: Summary of methods of MP quantification (Burger *et al.*, J Clinical Science, 2013)

Protocol	Method of Quantification	Advantages	Disadvantages
Flow cytometry	Fluorescence and light-scattering properties MPs in suspension	Available to most research Facilities, rapid, multiple antigens may be analysed in a single sample and MPs analysed on an individual basis	Quantification of 100–400 nm may be imperfect, Cell origin identification is antibody-dependent
Elisa	Immunocapture of MPs and quantification based on the presence of surface antigen	Available to most research Facilities, no size restrictions	Quantification is done in bulk, quantifies based on a single antigen, does not allow for size determination
Functional assays	Procoagulant or prothrombinase activity of MPs	Available to most research facilities Provides an indication of biological activity	Quantification is done in bulk, measures only a single biological activity, does not allow for size determination
Atomic force microscopy	Cantilever is used to scan the surface of MPs and tip displacement is related to surface properties	Allows for very accurate sizing of MPs, allows for three-dimensional view of MP structure, may be used for quantification	Non-universal technology, determination of cell origin requires development of specialized antibody-coated surfaces, not conducive to large sample numbers
Nanoparticle tracking analysis	MPs are visualized by light microscopy and light scattering is observed; Brownian motion of individual particles is tracked by video	Clear idea of MP size Allows for quantification	Utility of assay for quantification is unclear, non-universal technology, may be time-consuming

pathophysiological states [395-397]. In patients with diabetes, Tramontano *et al.* showed that the plasma level of EMPs is significantly higher than in control healthy group [398]. In addition, other studies revealed that when diabetes is associated with other complications, the plasma level of EMPs is greater than diabetes without complications [399, 400].

VII.5.1 Pathogenic effects of MPs in some vascular complications related to diabetes

VII.5.1.a MPs and endothelial cell dysfunction

Endothelial dysfunction is an essential pathogenic mechanism in the developpement of the vascular complications related to diabetes. Recent studies have highlighted the potency of MPs as cellular effectors of oxidative stress in blood vessels, in addition to being a biomarker of vascular damage. Tual *et al.* showed that hypoxic circulating MPs stimulate endothelial dysfunction in rat aorta and pulmonary arteries by reducing NO production. Furthermore, MPs display tissue specificity with respect to increased oxidative stress that occurs only in pulmonary ECs [401]. Another *in vitro* study by Cheng F *et al.* proved that circulating EMPs cause endothelial dysfunction by increasing oxidative stress and that for the first time EMPs induced up-regulation of Nox4 expression may increase ROS production in HUVECs [402]. *Ex- vivo,* treatment of ECs with MPs from patients with metabolic syndrome led to a decreased in eNOS expression and as a result decreased NO production. In addition, *in vivo* injection of MPs from patients with metabolic syndrome into mice impaired endothelium-dependent relaxation and decreased eNOS expression [403]. Other studies prove that MPs from apoptotic T lymphocytes decrease the NO production and increase oxidative stress in endothelial cells via xanthine oxidase or through NADPH oxidase [404, 405] . In the same manner, MPs of T lymphocytes derived from diabetic patients reduced endothelial NO synthase expression [406]. In addition, monocyte derived MPs were also demonstrated as an important source of ROS generation in the vessel. Monocyte MPs can induce ROS production, essentially anion superoxide, in endothelial cells, with a rapid expression of von Willebrand factor at the cell surface, which thereby enables the expression of functional TF by the endothelial cells. ROS production from stimulated EC by monocyte MPs is induced via NADPH oxidase, mitochondria, xanthine oxidase, cyclooxygenase and uncoupled nitric oxide synthase [407, 408]. Altogether, the MPs-mediated endothelial dysfunction resulting from oxidative stress appears pivotal in diabetes and could contribute to many associated cardiovascular complications.

VII.5.1.b Microparticles disseminated coagulation and thrombosis

Studies showed that the amount of circulating MPs and in particular platelet- and endothelial-derived MPs (PMPs and EMPs) is link to the severity of the disease, its progression and the thrombotic outcome. The procoagulant properties of MPs, mainly due to exposure to the PhSer or TF, play a central role in the prothrombotic effect of MPs. In patients with T2D, a higher level of MP, like TF^+ MPs, have been shown to be one of its procoagulant determinants [409]. In addition, *in vivo* studies in mice and primates have shown that TF^+ MPs incorporate into the growing thrombus [410]. Innovative studies showed that TF carried by MPs is the key in the growth of the thrombus [411]. This particular population of MPs represent a TF reservoir designated as the ‘’blood-borne TF’’ [409].

Different study show that the level of procoagulant MPs is higher among diabetic patients. Cimmino *et al.* showed that the amount of MPs that carry TF is elevated in patients with diabetes type 2 compared to healthy subjects and that this increase is correlated with a raise in coagulation markers [412]. The procoagulant activity of MPs in type 1 diabetic patients is correlated with HbA1c, suggesting that the procoagulant potential of MPs is associated with the glycemic balance [213].

VII.5.1.c MPs levels in micro- and macro-vascular complications

In the development of diabetes related micro- and macro-vascular complications, the endothelial dysfunction constitutes an essential pathogenic mechanism and endothelial microparticles (EMPs) would facilitate micro- and macro-vascular complications [397]. The micro-vascular complications mainly affect type 1 diabetes patients while macro-vascular complications appear later for these patients and are mainly observed in patients with diabetes type 2. In diabetic patients, plasma levels of $CD31^+/CD42b^-$ EMPs and $CD31^+/AV^+$ EMPs were significantly higher in macro-vascular complications in comparing with patients displaying micro-vascular complications [413]. While EMPs levels could distinguish macro- and microvascular complications, other MPs with pro-inflammatory properties could also contribute. Whether these MPs may prove better indicators than traditional vascular risk factors and blood glucose levels remains to be demonstrated.

VII.6 Microparticles as a biomarkers in transplantation

One of the most used technics for monitoring of transplanted organs is graft biopsies. However, in certain case and especially in the case of the transplanted islets of Langerhans, this invasive technique is generally uninformative. Owing to the embolization of the islets at the end of

hepatic capillaries, therefore detecting circulating molecules or cellular components, which normally are not present and reflect pathophysiological processes, can be considered as an alternate and non-invasive approach with limited drawbacks. In this case, MPs shed by activated cells constitute a signature of cell stress that meet the definition of biomarkers and are more specific to the activated cell from which it reflects the state. Currently the use of MPs as biomarkers in post-transplant monitoring is still limited and focused mainly in pancreatic islets, renal, heart, lung and hematopoietic stem cell transplantation.

VII.6.1 Pancreatic islets transplantation

In the case of pancreatic islet transplantation, the monitoring of the graft showed that blood C-peptide is significantly reduced when 95% of the graft is destroyed and therefore it constitutes a late marker of the insular dysfunction. When transplanted, islets are scattered in the hepatic vascular tree, which make it very difficult to have informative biopsies of the situation of grafted islets, effective monitoring of islet transplantation is challenging. MPs appear particularly attractive as a noninvasive marker of the status of the graft that avoid the limitations of biopsies. The laboratory team has studied the changes in the C-peptide, total MPs level and the need for insulin in patients submitted to islets transplantation [77]. Toti *et al.* showed that in the case of patient 1, injection of islets is immediately followed by a simultaneous increase in plasma C-peptide and in total MPs, which reveal the functionality of the transplanted islets and the intense membrane remodeling during the first post-transplant week. In the case of patient 2, the injection of islet allows a return to baseline C-peptide stabilized after 6 weeks. Then, in a possible rejection case, the amount of total MPs and C-peptide vary oppositely. Indeed, a fall of C-peptide is associated with increased insulin need, while our team show that a peak of total circulating MPs was detected a month ago before fall of C-peptide. In the third patient with early islet dysfunction, steroids restore C-peptide values and MP levels diminished thereafter. These results suggest that the peak of MPs could have prognostic value of acute rejection and give evidence of apoptosis in the transplanted islets. The loss of functional graft could be corrected by a further injection of islets like in case of patient 2 in this study, where it allows the reestablishment of the amount of total MPs and C-peptide basal value which indicate the normal restoration of grafted islets function [77] (Figure 33).

VII.6.2 Kidney transplantation

In patients with renal failure, Al-Massarani G *et al.* used MPs as biomarkers of renal function and showed that kidney transplant allowed a significant decrease of the amount of circulating

procoagulant MPs. After transplantation, the procoagulant activity of MPs measured by a two-step amidolytic assay based on the capability of the tissue factor to produce Xa in the presence of an excess of VII and X Factors, remained high and decreased progressively to reach a lower and stable level after 1 year. In addition, 12 months after transplantation the decrease in the amount of circulating MPs and their procoagulant activity was more important in the patient with no history of heart issues [414]. Another study, Qamri Z *et al.* showed that a significant decrease in EMPs is observable two months after transplantation, only in patients with end stage kidney disease (ESKD) whose kidney failure was secondary to diabetes [415]. In addition, Al-Massarani G *et al.*, in a second study showed that EMPs are also an indicator of the impact of immunosuppressive treatment. In this study they showed that EMPs return to baseline after 9 months when patients were treated by immunosuppressive microemulsion of azathioprine and cyclosporine and after 12 months using tacrolimus/ mycophenolate mofetil [348].

VII.6.3 Heart transplantation

Morel *et al.* showed that in a group of 64 patients including 23 acute rejection, that procoagulant MPs conveying Fas and TF were significantly increased in acute rejection [416]. These results suggest that the acute rejection is associated with apoptosis via Fas and is characterized by significant production of TF^+-MPs, possibly of monocyte origin. In addition, a correlation was observed between the time of ischemia and circulating procoagulant MPs two days after a heart transplant, where with more important ischemia time a less amount of procoagulant MPs was issued two days after the transplant suggesting earlier platelet activation [417]. Garcia *et al.* showed that the ratio of endothelial E-selectin$^+$-MPs / platelet PECAM$^+$-MPs would be an indicator of cellular activation in the case of high apoptosis [418].

VII.6.4 Lung transplantation

In lung transplantation, primary graft dysfunction happens at least in one third of the death case reported in the first post-operative month. Although increasing experience and development of knowledge on the cellular events of ischemia-reperfusion graft injuries, there is still a lack of an early marker. Our laboratory team showed that alveolar MPs is a surrogate marker of strong ischemia injury in *ex vivo* reperfusion experimental models. In this study, the rat lungs submitted to warm reperfusion following longer cold ischemia duration produce higher amounts of circulating MPs, whereas lungs that are not submitted to cold ischemia do not produce noticeable amount of MPs. These data suggest that MPs may be helpful to evaluate the extent of lung ischemia-reperfusion injuries and predict the occurrence of primary graft

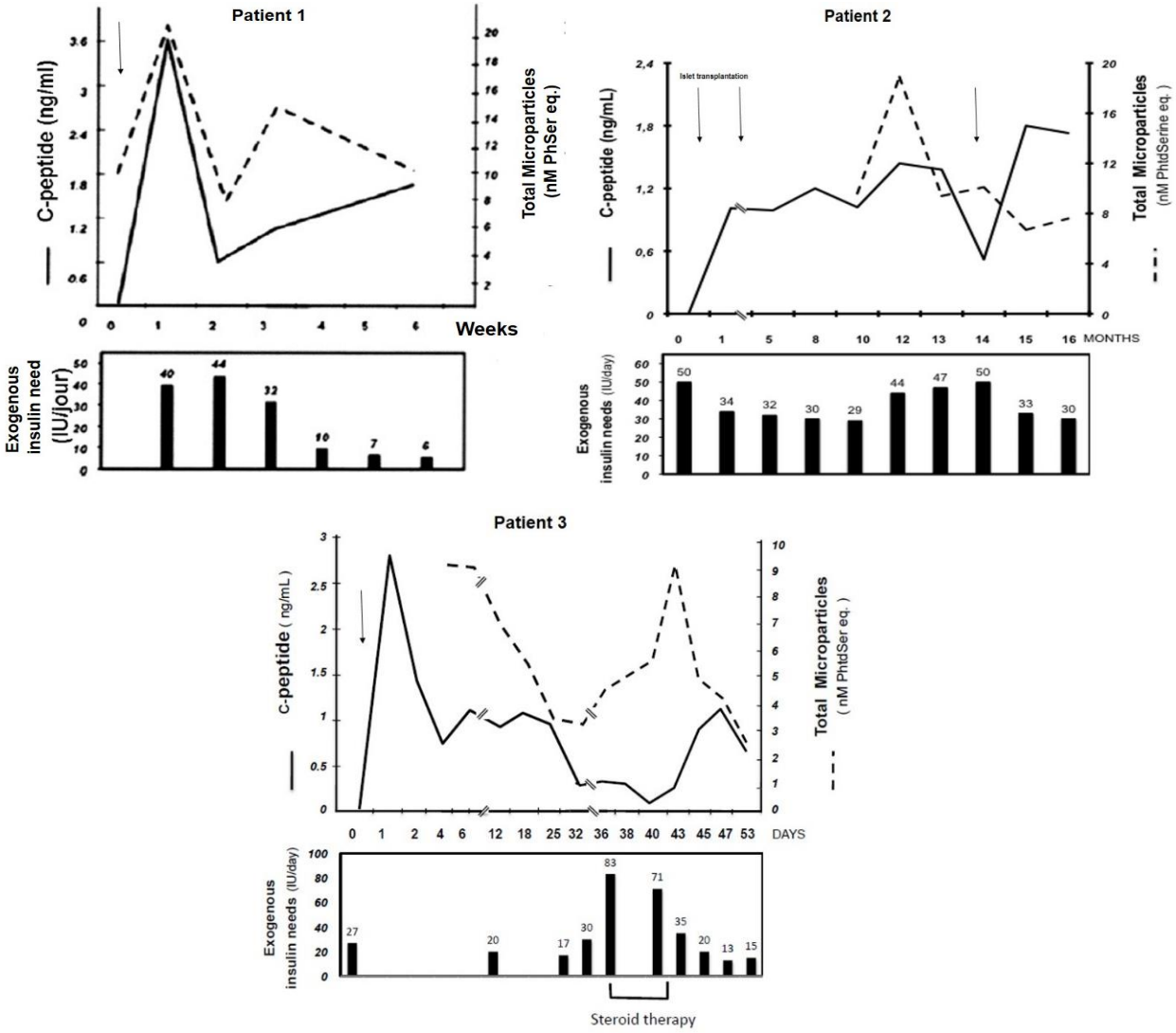

Figure 33: Variations of the C-peptide, the need for insulin and circulating microparticles during islet transplantation (Toti F *et al.*, J Transplantation proceedings, 2011)

C-peptide (dashed lines), MPs (solid line) and the need for exogenous insulin (bottom panel) were measured in three islets transplant patients: patient 1 in the 6 weeks post-transplantation, patient 2 within 12 months post-transplantation, patient 3 with graft dysfunction and restored C-peptide after steroid treatment. The arrows represent the islet injection.

dysfunction [419].

VII.6.5 Hematopoietic Stem Cell Transplantation

The use of MPs levels alteration in the domain of hematopoietic stem cell transplantation to investigate the graft dysfunction is a new strategy to elucidate the thrombotic complications that may lead to this dysfunction. Zhou LL *et al.* showed that the levels of TF^+-MPs and EMPs at different times of the graft conditioning treatment and at the early stage after transplantation were significantly different from those in other subsets namely acute graft-versus-host disease (aGVHD), infection and non-complication groups ($p<0.05$). This result showed that the dynamically monitoring levels of TF^+-MPs and EMPs participate to early detection of thrombotic complications [420, 421].

VII.7 Microparticles role in senescence and islet survival

MPs have become established cellular biomarkers of vascular injury or dysfunction in many pathologies. Recently, several studies have suggested a relationship between endothelial senescence and MPs. Burger *et al.* showed that treatment with MPs or H_2O_2 was associated with a shift from a proliferating to a non-proliferating phenotype, which means that MPs induce premature senescence in cultured ECs. Senescence is assessed by the increase in the expression of the cyclin-dependent kinase (CDK) inhibitors p21 and p16 and probed in positive cells using a specific chromogenic substrate of β-galactosidase activity revealed at pH 6 [226]. In their murine cell model, Burger *et al.* found that MPs promote EC senescence through nicotinamide adenine dinucleotide phosphate oxidase (NADPH oxidase) and mitochondrial-derived reactive oxygen species (ROS). Our laboratory showed that EMPs from senescent cells are pro-inflammatory, pro-senescent and pro-thrombotic. In addition, we demonstrated that MPs released by senescent ECs promote premature endothelial senescence associated with an impaired NO formation and oxidative stress [347]. Another study Brodsky *et al.* showed that in Zucker diabetic rats the development of accelerated vasculopathy was characterized by 6 fold increase in the number of senescent endothelial cells, increased MP levels, diminished NO production and inducing vasorelaxation [422]. Our laboratory also proved that hyperglycemia induce the pre-mature endothelial senescence *in vitro.* Besides, Turner M *et al.* showed that high glucose increase EMPs formation [423]. Therefore, senescent MPs may be anticipated as one of the mediators of high glucose induced senescence.

During islet transplantation, ischemia-reperfusion may cause endothelial senescence and the

generation within the islets or at its vicinity at the end of the host recipient capillary of pro-senescent endothelial MPs from the donor and recipient. However, the exact mechanisms of MPs induced senescence is still a matter of debate.

Nevertheless, studies showed that MPs could have beneficial effect in islet transplantation. Cantaluppi V *et al.* showed that a suspension of extracellular vesicles constituted of endothelial progenitor cells and exosomes, improved human islet vascularization when integrated into pseudo-islets made of endothelial and β-cells. Furthermore, the extracellular vesicles stimulated insulin secretion, islets revascularization and survival in mice [424]. In a more recent study Figliolini F, Cantaluppi V *et al.*, isolated extracellular vesicles (MPs and other exosomes-like components) released from human islets to assess their possible role in beta cell-endothelium crosstalk and in islet angiogenesis. In this study, they demonstrated that extracellular vesicles are incorporated by islet endothelial cells, which causes a cell reprogramming toward a pro-angiogenic and anti-apoptotic cell phenotype. Therefore, they concluded that islet derived extracellular vesicles may have an important role in the preservation of endothelial integrity and function [425]. These data are in accordance with our laboratory results that also indicate MPs as effectors in islet cell cross talk.

In conclusion, elevated amounts of endothelial and monocytes MPs circulate in T2DM patients without associated complications and with good glycemic control. These results suggest that despite effective glycemic control, there is still a hidden cellular stress in diabetic patients, which causes the release of MPs and may contribute to the endothelial senescence phenotype with time. In addition, vascular alteration in T1D leads to tissue stress that in turn stimulates the release of significant amounts of circulating procoagulant MPs that can participate to the progression of diabetes complications. This potentially pathogenic pool of MPs could be a harmful environment for transplanted organ and may amplify cytotoxic phenomena during IBMIR that contribute to the senescence phenotype.

In the domain of transplantation, all evidences described below show clearly that measurement of circulating MPs in blood patients might prove useful by providing a biomarker for the functionality of the graft. According to various clinical studies, these MPs could be a valuable tool for diagnosis and assessment of early loss of graft function.

The other procedure possibly interesting in graft preconditioning is the pharmacological targeting of excessive MP release when they are prosenescent or procoagulant.

Hypothesis and Objectives

With time, the need of functional organ for transplantation increases in the same time with an increase in the half-life of human, which mean that most of organ donor are aged. We hypothesize that endothelial senescence of islets that is related to the physiological or induced aging by the initial disorders of the graft vascularization, is present in the endothelium of the islet and participates in its post-transplant dysfunction. Indeed, in aged subjects, senescence can affect islet function either by promoting intra-islets damages (donor effect) or by prompting endothelial dysfunction and prothrombotic propensity at sites of islet engraftment (recipient effect). The IBMIR induced cellular stress can also cause early endothelial thrombogenicity and senescence, both a putative cause for the drastic release of noxious MPs contributing to ischemia-reperfusion, cell dysfunction and damage (Figure 34). Although MPs concentrations varies with the immunosuppressive therapy and are surrogate markers of graft rejection. In addition, little is known on the role of MPs in graft survival, function or engraftment and their underlying mechanisms of their action.

The present work aims at studying the impact of aging on pancreatic islets function and survival. In the frame of islet transplantation, the study focused on MPs as effectors of thrombogenicity and senescence in the islets. For these purposes, we studied the early signs of senescence in middle-aged rats and set a model of MPs-mediated cell cross talk in the islets.

In our work, we have two essential objectives, which are the study of:

1- The impact of pancreatic aging on the morphology, the fate and function of pancreatic islets by comparing between young rats and middle-aged.

2- The role of pro-senescent endothelial MPs in the accelerated senescence of isolated pancreatic islets.

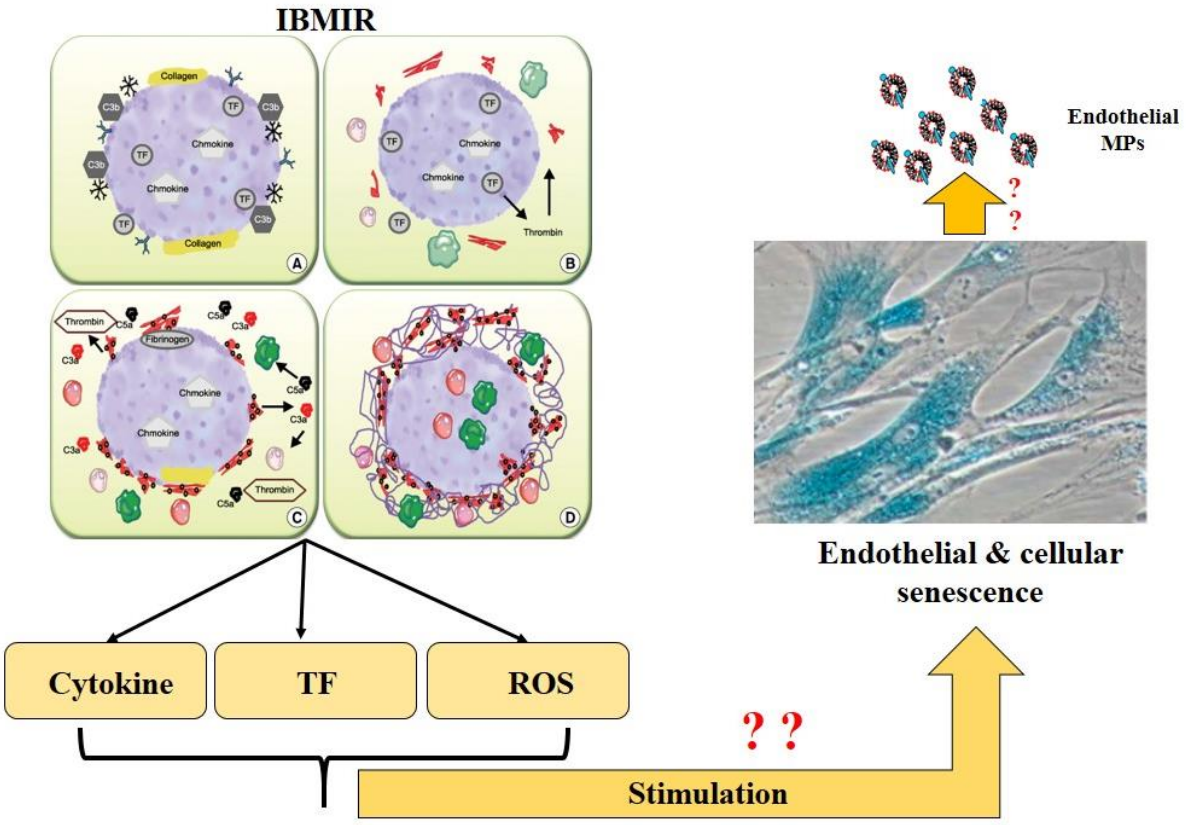

Figure 34: Possible relation between IBMIR, Senescence & MPs shedding.

TF : tissue factor, ROS : reactive oxygen species

Scientific Publications

Article I

Senescence of Pancreas in Middle-aged Rats with Normal Vascular Function

Introduction

In organ transplantation and particularly in pancreas and pancreatic islet transplantation, donor age is determinant for the efficacy of graft function and survival. Aging is a determinant factor for the progressive post-maturational deterioration of all organs, leading to chronic and metabolic diseases including obesity, impaired glucose tolerance, and type 2 diabetes (T2D). Senescence arises from age or from organ premature dysfunction as a consequence of altered cell function in response to stress. Cellular senescence is characterized by an irreversible arrest of cell division that occurs in response to various cellular stressors, such as telomere erosion, DNA damage, oxidative stress, or oncogenic activation. Pancreatic islet, is an endocrine structure with an organized vascularization, undergoes morphological changes and functional decline during normal aging.

Based on the hypothesis that donor age has an impact on organ transplantation and specially in pancreas transplantation, we compared in rat model the senescence and function features of pancreas and vascular tissues. We take 2 groups of rats young (YR) and middle-aged rats (MAR), where we investigated the change in islet morphology and the expression of senescence marker like p53 and its downstream target p21 and p16, we also measured the expression oxidative stress marker (ROS). The Circulating microparticles (MPs) was measured as surrogates of vascular cell injury. Vascular function was studied in mesenteric arterial rings.

We could demonstrate that pancreatic islet alterations were detected in middle-aged rats before any measurement of macro-vascular dysfunction. The data indicate a pancreatic senescence in the process of aging associated with uncontrolled accumulation of oxidative species that suggests a determining role of donor age in transplantation.

Limitations and further perspectives

Because vascular dysfunction was not evidenced by vascular reactivity nor by MPs signature, we examined the eventual presence of senescence in the aorta using X-gal blue coloration of the SA-βgal activity in only two individuals. Results of these complementary experiments are suggestive of a higher degree of senescence appearing only at the vicinity of branched vessels, where flow turbulence is elevated in middle-aged rats (see figure 1, complementary data).

Our data were raised in a small animal model with short lifetime. Elder wistar rats reach 24 months and show elevated body mass (up to 1 kg) compared to young rats (463 g). It is therefore difficult to analyze the effect of aging in such a reduced time duration. Moreover, we

experienced in this cohort of middle-aged rats (739 g), the impact of the environment on the degree of the aging related dysfunction. Indeed, those rats were old breeding male.

In another preliminary cohort, middle-aged rats showed already vascular dysfunction of 13 months rats.

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Senescence of Pancreas in Middle-Aged Rats with Normal Vascular Function

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Background: In organ transplantation, particularly pancreas transplantation, donor age is a determinant factor for graft survival. Physiological aging is crucial in the progressive deterioration of organs in adulthood. We compared the senescence and function features of pancreas and vascular tissues in young rats and middle-aged rats.

Material/Methods: Islet morphology and the area of cells secreting insulin or glucagon was investigated using immunohistology in young rats (12 weeks) and middle-aged rats (52 weeks) (n=8). Senescence markers, oxidative stress (ROS), and tissue factor (TF) were measured in the rat pancreases. Circulating microparticles (MPs) were measured as surrogates of vascular cell injury. Vascular function was studied in mesenteric arterial rings.

Results: Larger islets were twice as frequent in young rats versus middle-aged rats. In middle-aged rats there was a significant decrease of the β-cells/islet area ratio. Western blot analysis showed an increased expression of p53, p21, and p16 senescence markers (2-, 7- and 3-fold respectively) with no modification in caspase-3 activation. A 30% decrease of endothelial nitric oxide synthase (eNOS) was observed together with a 4-fold increase in TF expression. ROS formation increased significantly (2-fold) in middle-aged rats and their main source, determined by pharmacological inhibition, was NADPH oxidase and uncoupled nitric-oxide (NO) synthase. No sign of vascular injury (microparticles) or dysfunction was evidenced.

Conclusions: Modification in islet morphology and function were detected in middle-aged rats before any measurement of macro-vascular dysfunction. The data indicate a pancreatic senescence in the process of aging associated with uncontrolled accumulation of oxidative species that suggests a determining role of donor age in transplantation.

MeSH Keywords: **Cell Aging • Cell-Derived Microparticles • Islets of Langerhans • Reactive Oxygen Species • Tissue Factor • Transplantation**

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Background

In organ transplantation, and particularly in pancreas and pancreatic islet transplantation, donor age is a determinant for the efficacy of graft function and survival [1]. Physiological aging is crucial in the progressive deterioration of all organs, and is associated with chronic and metabolic diseases, including obesity, impaired glucose tolerance, and type 2 diabetes (T2D) [2,3].

Cell senescence arises progressively with age or may arise because of organ premature dysfunction that consecutively alters cell function promoted by acute stress. Cellular senescence is characterized by an irreversible arrest of cell division that occurs after telomere erosion, DNA damage, oxidative stress, or oncogenic activation. It is supported by a shift in optimum pH of lysosomal β-galactosidase (senescence-associated β-galactosidase [SA-βgal] activity), which is the gold standard for the detection of senescence cells [4,5]. The proteins p53, p21, and p16 are cell cycle inhibitors contributing to the senescence process. It would seem that β-cell proliferation and/or ROS (reactive oxygen species) generation accelerates cellular senescence [6,7].

Pancreatic islet, an endocrine structure with an organized vascularization, undergoes morphological changes and functional decline during normal aging [8], thereby suggesting a possible interaction between islet cells and the vascular endothelium.

Endothelial cells contribute to many vital physiological functions such as the control of hemostasis and blood pressure [9,10]. Endothelium-mediated vasodilatory function progressively declines with age [11]. It is associated with decreases in endothelial nitric oxide synthase (eNOS) expression and in nitric oxide (NO) formation, (a major vasoprotector of aging endothelial cells), leading to a progressive deterioration of cell function owing to cell cycle arrest and senescence [12,13].

Oxidative stress causes endothelial aging and dysfunction through uncontrolled production of ROS [14]. Inflammation, which increases with age, is another major cause of cell dysfunction. At the surface of the inflamed endothelium, the expression of tissue factor (TF), which is the membrane initiator of coagulation and an early responsive gene, is upregulated as are pro-adhesive membrane proteins involved in leucocyte recruitment [15].

Microparticles (MPs) are submicron plasma membrane vesicles shed by stressed or apoptotic cells. They are circulating markers of vascular cell damage and behave as cellular effectors. In blood flow, MPs are procoagulant and convey the active form of TF. MP cell origin can be determined by detection of specific membrane proteins. An inflammation driven β-cell membrane remodeling can lead to insulin impairment and the release of TF-bearing MPs able to induce β-cell apoptosis [16,17]. Furthermore, MPs are circulating surrogate markers of endothelial damage and associated with cardiometabolic risk factors [18].

These findings raise the question of the contribution of pancreas senescence to the pancreas or islets graft failure. The aim of the present study was to: (1) analyze islet morphological or functional changes, and determine whether alteration of the pancreas within the process of aging could sense pancreatic senescence-driven dysfunction, and (2) compare the variations of markers of senescence and oxidative stress with age in the pancreas and vessels using plasma MPs as a surrogate of vascular tissue damage.

Material and Methods

Ethics statement

Male Wistar rats (Janvier-Labs, Le Genest-St-Isle, France) were housed in a temperature-controlled (22°C) room and maintained on a standard 12-hour light/dark cycle (lights on at 07: 00 am) with free access to food and water. Experiments conform to the Guide of Care and the Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and were authorized by the French Ministry of Higher Education and Research and by a local ethic committee (Comite d'éthique en Matière d'expérimentation animale de Strasbourg, authorization 03799.01). All animal experiments were done in a registered animal yard within Faculty of Pharmacy (Authorization number E-67-218-26).

Animal model, blood and tissue sampling

Young, 12-week-old male Wistar rats (n=8) with average body weight (463.6±9.49 g) and 52-week-old middle-aged male Wistar rats (n=8) with average body weight (739.4±46.29 g) were investigated. Rats were anaesthetized by sub-lethal IP injection of 50 mg/kg sodium pentobarbital (Ceva Animal Health, Libourne, France), and euthanized by exsanguination. The pancreas and mesenteric artery were removed and weighed. The pancreas was cut into three parts. One part was fixed in freshly depolymerized paraformaldehyde 4% (Electron Microscopy Sciences 15710, Hatfield) and subsequently embedded in paraffin for histological analysis. The second part was embedded in Tissu-Tek O.C.T. Compound (Sakura 4583, Leiden, Netherlands) and snap-frozen for immunofluorescence studies and the determination of the formation of ROS. The third part was frozen in liquid nitrogen for Western blot analysis. Blood samples were withdrawn by heart puncture and harvested on 13 mM sodium citrate or 1.8 mg/mL EDTA. Citrated plasma was obtained by sequential centrifugation as recommended by the SSC committee of the International society for Thrombosis and

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Haemostasis (ISTH) for the measurement of MPs in plasma. Plasma was stored at –80°C for subsequent analysis.

Histological and morphological analyses

Four micrometer (µm) thick paraffin sections of pancreas samples were stained with hematoxylin-eosin and Gomori's trichrome stain. Four sections were taken from each sample every 100 µm. For each section, the surface of the islets was measured using ImageJ software. The mean islet surface area was calculated by adding the values measured for each of the four sections to cover the whole islet area.

Immunohistochemistry and immunofluorescence determinations

For immunohistochemistry, paraffin-embedded pancreas sections were subjected to microwave antigen retrieval in 10 mM sodium citrate buffer (pH 6.0). After incubation with the primary antibody, mouse monoclonal anti-PSA-NCAM (1/500) (Millipore, clone 2-2B, MAB5324, Darmstadt, Germany) overnight, labeling was revealed by anti-mouse (two hours) and ABC complex formation (30 minutes) (VECTASTAIN Elite ABC Kit (Standard), California, USA); VIP (VECTOR VIP Peroxidase (HRP) Substrate Kit, California, USA) as a chromogen.

Double labeling of insulin and glucagon was performed by immunofluorescence on paraffin-embedded pancreas sections by overnight incubation with rabbit polyclonal anti-insulin (1/200, 4590S Cell Signaling, Danvers, USA) or mouse monoclonal anti-glucagon primary antibodies (1/5,000, G2654 Sigma, Missouri, USA), followed by two hours incubation at room temperature with an Alexa Fluor 488 FITC-goat anti-mouse IgG (H+L) and Alexa Fluor 633 goat anti-rabbit IgG (H+L) (Life technologies, USA antibodies). Nuclei were stained with DAPI (Roche Diagnostics, Meylan, France), and sections were cover-slipped with fluorescence-mounting medium (Dako S3023, Carpinteria, USA). For negative controls, primary antibodies were omitted.

Intra peritoneal glucose tolerance test (IPGTT)

Rats were fasted for eight hours before intraperitoneal injection of glucose (2 g/kg). Glucose plasma concentrations were measured in the blood of the tail vein using a glucometer (BG STAR, Agamatrix, USA) at different times 0, 15, 30, 45, 60, 90, and 120 minutes.

Biological markers

Glucose was measured in plasma collected at the moment of sacrifice by the hexokinase apectrophotometry method, insulin by ELISA kit (Milllipore cat# EZRMI-13K, Billerica, MA, USA).

Western blot analysis

Protein lysates from frozen pancreatic tissue were incubated with extraction buffer (Tris/HCl 20 mM (pH 7.5; Q-Biogene, California, USA), NaCl 150 mM, Na_3VO_4 1 mM, sodium pyrophosphate 10 mM, NaF 20 mM, okadaic acid 0.01 mM (Sigma-Aldrich, Missouri, USA) containing a tablet of protease inhibitor (Complete Roche, Basel, Switzerland) and 1% Triton X-100 (Euromedex, 2000-B, Souffelweyersheim, France). Total proteins (30 µg) were separated on 10% SDS-polyacrylamide gels at 100 V for two hours and transferred by electrophoresis onto polyvinylidine difluoride membranes (Amersham, Life Sciences, Germany) at 100 V for 120 minutes. Non specific binding was blocked by incubation in TBS buffer (Biorad) containing 5% bovine serum albumin (BSA) (Euromedex, 04-100-812-E, Souffelweyersheim, France), and 0.1% Tween 20 (Sigma) (TBS-T) for two hours.

Membranes were then incubated overnight at 4°C in TBS-T containing 5% BSA and polyclonal antibody raised against human p53 (1/1,000) (Santa Cruz Biotechnology FL-393 SC-6243, Dallas, Texas, USA), human p21 (1/1,000) (Santa Cruz Biotechnology C-19 SC-397, Dallas, Texas, USA), human p16 (1/1,000) (Abcam ab51243, Cambridge, UK), rat eNOS (1/1,000) (BD Transdaction Laboratory cat: 610297), human TF (1/1,000) (Sekisui diagnostics, Sekisui Wiretech Gbmh, Russelsheim, Germany), and rat cleaved caspase-3 (1/1,000) (Cell Signaling Technology, Danvers, USA). After three washings with TBS-T, membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and density signals of each band detected using ECL Substrate (170-0561, BIO-RAD, United States). Membranes were incubated with a mouse polyclonal anti-glyceraldehyde-3-phosphate deshydrogenase (GAPDH) antibody (Millipore MAB 374, Darmstadt, Germany), or monoclonal anti-β-tubulin I mouse antibody (Sigma-Aldrich, T7816, Missouri, USA) for normalization purposes.

Determination of mitochondrial ROS formation and sources

In situ formation of ROS was assessed as previously described [19]. The redox-sensitive red fluorescent dye dihydroethidium (DHE, 2.5 µM) was applied to 25 µm unfixed cryosections of the pancreas for 30 minutes at 37°C in a light-protected humidified chamber. Sections were washed three times with PBS, mounted in DAKO, and cover-slipped. The sections were examined under a confocal microscope (Confocal Leica TSC SPE-Mannheim, Germany). To determine sources of ROS, sections were incubated 30 minutes at 37°C before DHE staining with the specific inhibitors VAS-2870 (VAS, NADPH oxidase 10 µM), N-nitro-L-arginine (L-NA, NO synthase inhibitors 300 µM), Indomethacin (Indo, cyclooxygenases COX 10 µM) and MRK (inhibitors of the mitochondrial respiration chain, myxothiazol 0.5 µM + rotenone 1 µM + potassium cyanide (KCN) 1 µM).

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Vascular reactivity

Vascular reactivity studies of secondary mesenteric artery rings were performed as previously described [20]. Briefly, the secondary mesenteric artery was cleaned of connective tissue, cut into rings (2–3 mm in length) and suspended in organ baths containing oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (composition in mM: NaCl 119, KCl 4.7, KH_2PO_4 1.18, $MgSO_4$ 1.18, $CaCl_2$ 1.25, $NaHCO_3$ 25, and D-glucose 11, pH 7.4, at 37°C) for the determination of changes in isometric tension. After equilibration and functional tests, rings were pre-contracted with phenylephrine (PE, 1 µM) before construction of concentration-response curves to acetylcholine (ACh). In some experiments, rings were exposed to an inhibitor for 30 minutes before contraction with PE. Relaxations were expressed as percentage of the contraction induced by PE [21].

Microparticles measurement and characterization

Platelet-free rat plasma (PFP) was obtained from blood by sequential centrifugation steps: 15 minutes at 500 g, and supernatant further submitted to 13,000 g for five minutes. The PFP was kept at –80°C. MPs measurement was performed using prothrombinase assay after capture onto annexin-5 as previously described. The assay allows extensive washing of insolubilized MPs, taking advantage of the high affinity of annexin-5 for the phosphatidylserine (PhtdSer) exposed at MPs surface. MPs concentration was referred to as PhtdSer equivalent, by reference to a standard curve made with synthetic vesicles of known amounts of PhtdSer [22].

In addition, MPs cell origin was determined by replacing annexin-5 with specific biotinylated monoclonal antibodies, each directed against one typical CD: mouse IgG against rat CD61 for platelet (Biolegend, San Diego, CA, USA), anti-rat CD45 for leucocytes (Biolegend, San Diego, CA, USA), anti-rat CD54 for endothelial cells (Biolegend, San Diego, CA, USA), anti-rat CD245 for erythroid cells (BD Pharmingen, New Jersey, USA).

Image analysis

For immunohistochemistry and immunofluorescence analysis, four sections from each pancreas sample were taken every 100 µm, and the mean value of 400 µm tissue labeling was calculated for each sample. Images were analyzed by the NIH ImageJ software.

Statistical analyses

Data are expressed as mean ±S.E.M. Mean values were compared using unpaired Student's *t*-test for the comparisons of groups using GraphPad Prism version 6.01 for Windows™ (GraphPad Software, San Diego, USA). A value of $p<0.05$ was considered statistically significant.

Results

Size distribution and morphology of islets are altered in middle-aged rats

Histological examination of pancreas sections from young rats showed a normal islet morphology with a regular, spherical shape surrounded by a thin collagen capsule, whereas middle-aged rat islets showed an altered shape with high fragmentation of the biggest islets into small irregular units surrounded by dense fibrous tissue (Figure 1A, 1B). Regardless of age, the size distribution ranged from 300 µm² to >80,000 µm², although small islets (300 µm² to 20,000 µm²) were significantly more abundant in middle-aged rats compared to young rats (92.7%±1.9 *vs.* 86.8%±1.4, $p<0.05$). The larger islets (20,000 µm² to 40,000 µm²) appeared to be characteristic features of young rats compared to middle-aged rats (8.6%±0.9 *vs.* 4.3%±1, $p<0.01$) (Figure 1C). There was no modification of exocrine tissue regarding the age of rats.

Islet cell populations, plasticity, and activity

Because PSA-NCAM was shown by Karaca et al. [23] as a specific marker of β-cell function and activity, we investigated its expression in pancreatic tissues of both rat subsets by measuring the PSA-NCAM surface area. PSA-NCAM staining remained restricted to the islet endocrine tissue in both young and aged pancreases, and the labeling area was identical between young rats at compared to middle-aged rats (0.42±0.01 µm² *vs.* 0.41±0.01 µm²). Double insulin/glucagon immunolabeling showed a similar distribution of both β and α cells in the islets of young rats and middle-aged rats (Figure 1D). However, in middle-aged rats, the glucagon/islets surface ratio increased significantly from 6.04%±0.01 to 10.40%±0.01 ($p<0.05$) (Figure 1E), while the insulin area/islet surface ratio significantly decreased from 93.96%±0.01 to 89.60%±0.01 ($p<0.05$) (Figure 1F).

IPGTT and insulin levels were modified in middle-aged rats

At the beginning of the IPGTT test after fasting for eight hours, young rats compared to middle-aged rats showed higher (but not significantly different) glycaemia level. After fasting, a shift in glucose response was shown at 15–30 minutes: 250 mg/dL after 15 minutes shifting to 267.3±16.7 in middle-aged rats *vs.* 211±6.7 mg/dL in young rats after 30 minutes) (Figure 2A). In addition, values of glycaemia in middle-aged rats remained elevated at all test points starting from 30 minutes when compared to young rats. However, this elevation was significantly higher only after 30 minutes (211±6.7 increased to 267.3±16.7; $p<0.01$) and 120 minutes (156±5.4 increased to 199.8±16.7; $p<0.05$) of intraperitoneal injection of glucose in young rats and middle-aged rats, respectively. Therefore, it was clearly shown that plasma glucose remained unchanged,

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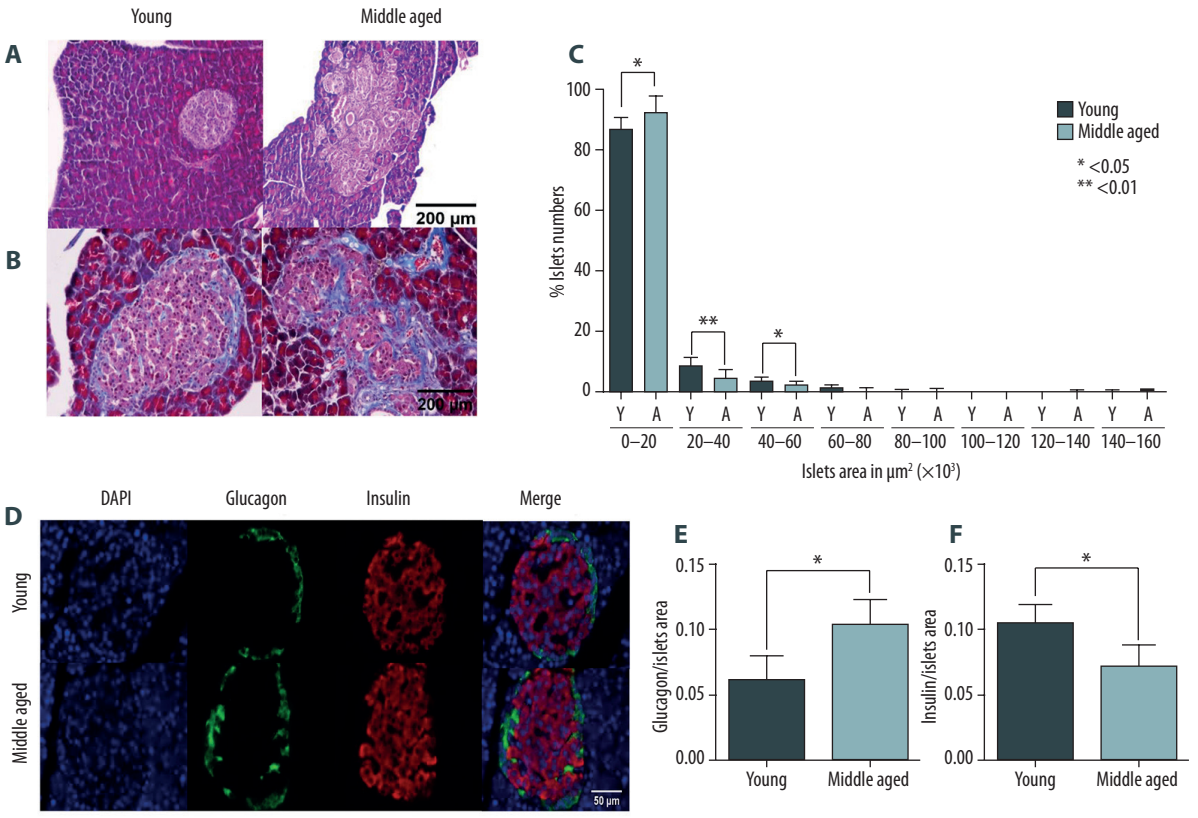

Figure 1. Islet morphology, size distribution, and glucagon and insulin-secreting cell area are altered in middle-aged rats: (**A**) Morphological analysis of islet structure was determined on formaldehyde-fixed 4 µm thick paraffin sections after hematoxylin-eosin staining (scale bar=200 µm). (**B**) Accumulation of collagen was evidenced using Gomori's trichrome (scale bar=200 µm) at 10×. (**C**) Size range distribution was determined by measurement of total islet area every 20,000 µm². Four sections for each sample were taken (section every 100 µm). (**D**) The distribution of glucagon and insulin-secreting cells was analyzed on 4 µm paraffin sections following double-labeling with fluorescent anti-insulin and anti-glucagon antibodies; cell nuclei were revealed with blue DAPI staining (scale bar=50 µm), (magnification 40×). The ratio of glucagon (**E**) and insulin (**F**) areas per islet area and quantification was performed using ImageJ software; results expressed as mean ±SEM of eight rats per group, * $p<0.05$, ** $p<0.01$.

whereas insulin levels significantly increased in middle-aged rats from 5.69±0.47 to 7.05±0.35 ng/mL ($p<0.05$) (Figure 2B).

Senescence markers were overexpressed and oxidative stress increased with aging in rat pancreatic tissue

Because ROS production was reported to promote senescence, we measured ROS in pancreas cryosections and detected a 2-fold increase in middle-aged rats compared to young rats (Figure 3A). ROS staining was homogenous and detected in whole pancreatic tissue. Pharmacological inhibitors applied on pancreatic sections revealed that the major sources of ROS in middle-aged rats were the NADPH oxidase and uncoupled NO synthase (Figure 3A). Furthermore, a 30% decrease in eNOS expression was demonstrated by Western-blot in the pancreas lysates from middle-aged rats compared to young rats (Figure 3B). Interestingly, no modification in activated caspase-3 levels could be established between young and middle-aged samples, thereby excluding apoptosis induction in the older pancreases (Figure 3C). In addition, a significant 4-fold upregulation of TF expression was evidenced, suggesting enhanced thrombogenicity and inflammation (Figure 3D).

Senescence markers were significantly upregulated among aged individuals with a doubled p53 expression, along with a seven-fold increase in cyclin-dependent kinase inhibitor protein p21, and a three-fold rise in its downstream p16 effector, as previously demonstrated [24] (Figure 4).

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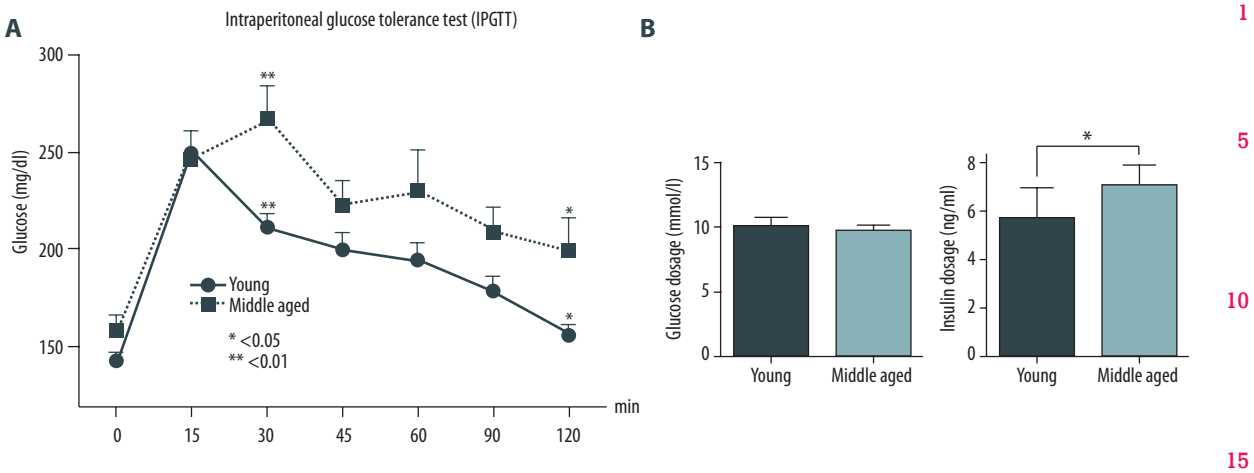

Figure 2. IPGTT, plasma glucose and insulin concentrations: (**A**) Intraperitoneal glucose tolerance test IPGTT: glucose was measured in blood samples obtained from the tail vein using a glucometer in fasting rats. (**B**) Plasma glucose concentration was assessed by hexokinase spectrophotometry method. Insulin concentration was established by ELISA kit (Millipore cat# EZRMI-13K) using samples drawn by heart puncture at the time of sacrifice. Results expressed as mean ±SEM of eight rats per group, * $p<0.05$, ** $p<0.01$.

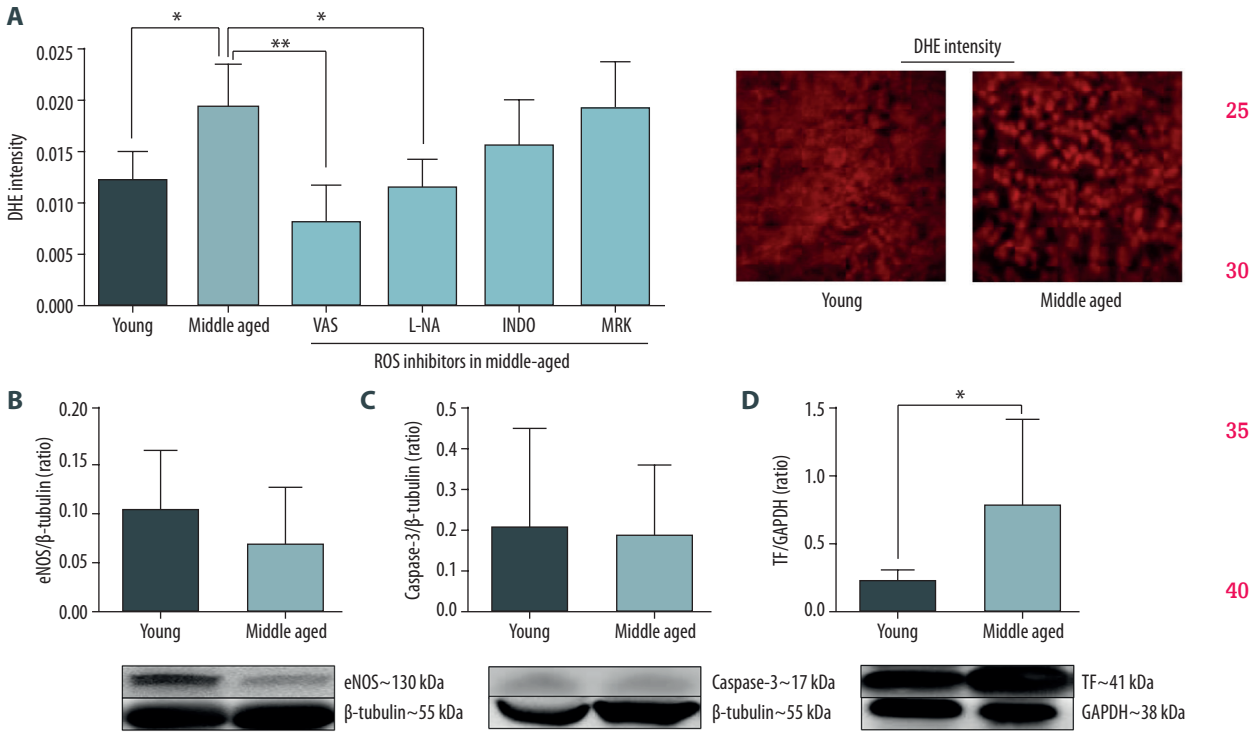

Figure 3. Expression of oxidative stress in pancreas tissues: (**A**) *In situ* ROS accumulation measured by fluorescent immunohistochemistry using the redox-sensitive probe dihydroethidium and sources of ROS investigated by pharmacological inhibition for 30 minutes using VAS-2078 (NADPH oxidase), L-NA (NO synthase inhibitor), indomethacin (cyclooxygenases COX), and MRK (inhibitors of mitochondrial respiration chain) incubated on sections, using confocal laser-scanning microscope (Confocal Leica TSC SPE) magnification 20×. (**B**) Markers of oxidative stress, eNOS; (**C**) markers of apoptosis, cleaved caspase-3; and (**D**) markers of inflammation, TF, were measured by Western blot (**lower panel** representative blot, **upper panel** cumulative data). Quantification of red fluorescence as well as that of chemiluminescence as a ratio of protein of interest/housekeeping protein levels (GAPDH or β-Tubilin) performed by ImageJ software. Results expressed as means ±SEM of eight rats per group, * $p<0.05$, ** $p<0.01$.

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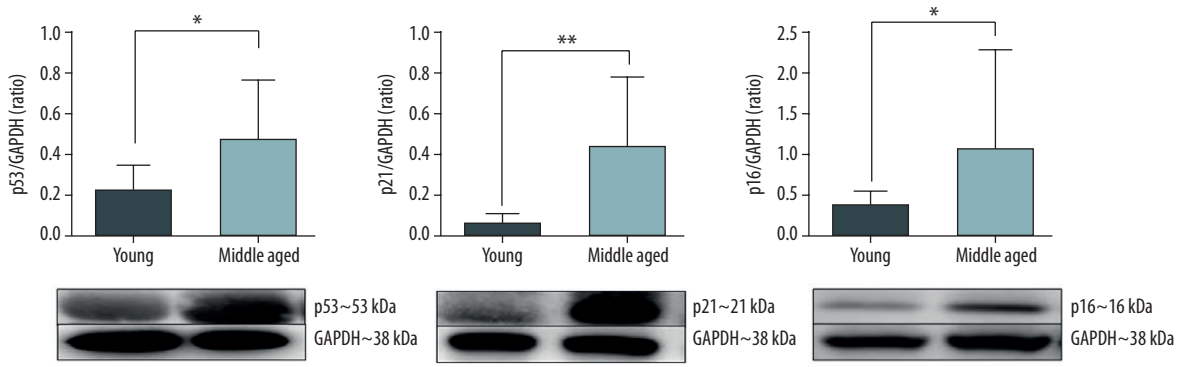

Figure 4. Expression of senescence markers in pancreatic tissues. The senescence markers p53, p21, and p16 were measured by Western blot (lower panel representative blot, upper panel cumulative data). Quantification of chemiluminescence as a ratio of protein of interest/housekeeping protein levels (GAPDH) performed by ImageJ software. Results are expressed as means ±SEM of eight rats per group, * $p<0.05$, ** $p<0.01$.

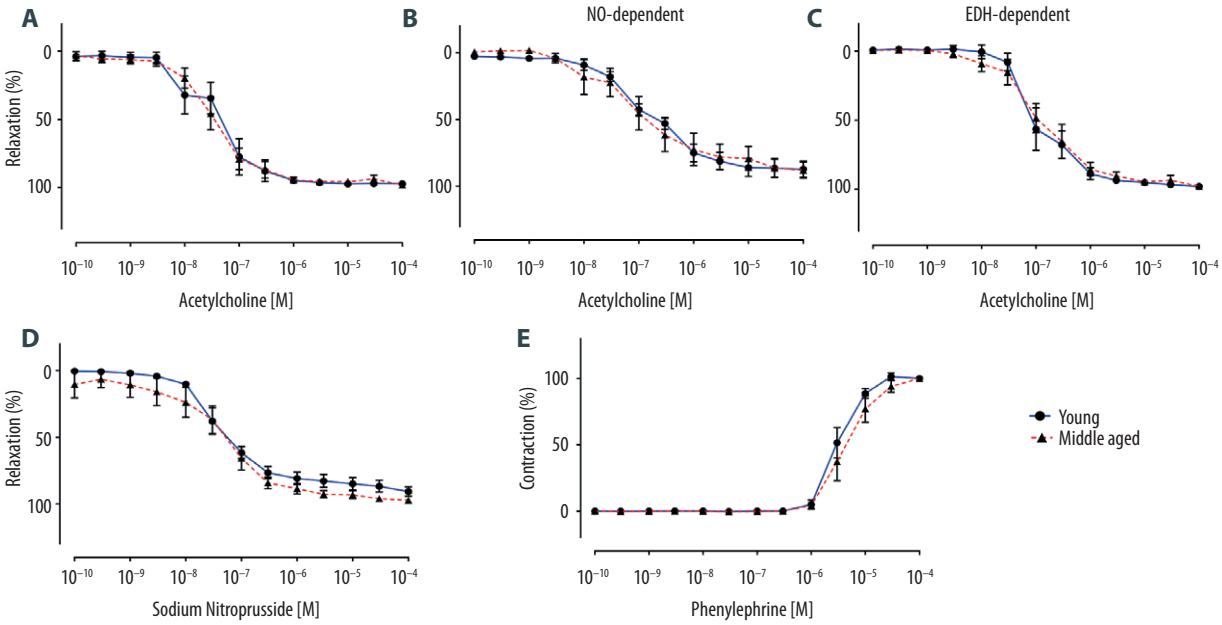

Figure 5. No variation of vascular reactivity of the secondary mesenteric artery. Concentration-relaxation curves to acetylcholine in mesenteric artery rings with endothelium in young rats and middle-aged rats. (**A**) Global relaxation in presence of acetylcholine. (**B**) NO component of relaxation assessed in the presence of indomethacin (10 µM) and apamin plus charybdotoxin (100 nM each). (**C**) EDH component in the presence of indomethacin and L-NA (300 µM). (**D**) Relaxation resulted from sodium nitroprusside (an exogenous donor of NO). (**E**) Contraction occurred in presence of phenylephrine. Results are shown as mean ±SEM of eight different rats.

Endothelial-dependent vascular reactivity and circulating MPs level were unmodified in middle-aged rats

Acetylcholine caused similar concentration-dependent relaxations in mesenteric arterial rings from young rats and middle-aged rats (Figure 5A). The NO component of the relaxation was assessed in the presence of indomethacin and charybdotoxin plus apamin to prevent the formation of vasoactive prostanoids and endothelium-derived hyperpolarizing (EDH) (Figure 5B); the EDH-mediated component of relaxation was assessed in the presence of indomethacin plus L-NA (an inhibitor of eNOS) (Figure 5C). In both cases, no variation between the two groups could be established. In addition, the sodium nitroprusside, which is an NO donor, induced similar endothelium-independent relaxation in both groups (Figure 5D). Phenylephrine-induced contraction reached identical ranges

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Table 1. Characterization of circulating MPs in young and middle-aged rats.

Plasma MPs concentration (nM Phtdser EQ.)	Young	Middle-Aged
CD61 +	1.23±0.11	1.03±0.18
CD45 +	0.68±0.09	0.61±0.13
CD54 +	0.43±0.04	0.36±0.06
CD245 +	0.36±0.05	0.26±0.08
Total MPs	5.08±0.23	4.53±0.43

CD 61 (platelet); CD 45 (leucocyte); CD 54 (leucocyte and endothelial); and CD 245 (erythrocyte). Results expressed as means ±SEM of eight rats per group.

in mesenteric artery rings from young rats and middle-aged rats (Figure 5E). Because premature senescence is not a feature of vascular tissues under condition of protective laminar flow, we assessed circulating MPs as plasma surrogates of an eventual vascular damage. In young rats and middle-aged rats, total MPs levels remained similar 5.08±0.23 *vs.* 4.53±0.43 nM PhtdSer EQ. respectively and no variation in the concentration of each MPs subpopulation from platelet, leucocyte, erythrocyte, or endothelial cell origin could be observed (Table 1).

Discussion

In this study, we report the first experiment aiming to simultaneously compare the effect of age in the pancreas and in vascular tissues. By studying islet morphology and function, we found that larger islets were twice as frequent in young rats versus middle-aged rats. In addition, β-cells/islets area ratios were decreased significantly with age. The latter observation is in accordance with other studies that showed a decrease in β-cell mass and proliferation simultaneous to an increase in β-cell apoptosis [8,25–28]. A shift in glucose response was shown by IPGTT at 15–30 minutes with age, together with a significant increase in plasma insulin concentration. Altogether, our morphological and histological data were in accordance with other reports showing the impact of age on the ratio of α-cells to β-cells area [29].

Interestingly, our data demonstrated that in pancreatic tissue p53, p21, and p16 senescence markers were overexpressed, oxidative stress increased by ROS production, and eNOS down-expression, in middle-aged rats. Although a significant over-expression of TF was measured in the pancreas, no sign of vascular injury by MPs measurement, or of vascular dysfunction by contraction or relaxation studies in mesenteric artery rings, could be detected.

Our findings showed no difference in PSA-NCAM expression by β-cells between young rats and middle-aged rats, in accordance with the report of Gu et al. who could detect a significant 50% decrease in β-cell proliferation only in two years old rats by PCNA (anti-Proliferating Cell Nuclear Antigen) labeling [29].

Oxidative stress as an early inducer of senescence in pancreas of middle-aged rats

Although our data showing early senescence in the pancreas of middle-aged rats were obtained by global assessment in the pancreas lysates, it is tempting to speculate that islet dysfunction is at least in part driven by senescence. Our data indeed confirm and extend the previous observation by Liu et al. [24] showing an upregulation of p16 in the isolated islets from adult rats and the presence of SA-βgal activity only in the endocrine pancreatic tissue. Since no apoptosis could be detected by active caspase-3 labeling in the pancreas lysates, senescence appears a mechanism specifically prompted in the middle-aged rat pancreas.

Our data suggests an excessive production of ROS in middle-aged rat pancreas is driven by NADPH oxidase that is not counter-balanced due to the reduced expression of functional eNOS. Therefore, excessive oxidative stress seems to trigger early senescence in the pancreas while no evidence of oxidative stress-driven endothelial dysfunction could be evidenced in macro-vessels such as mesenteric arteries.

The pancreas served as an early sensor of oxidative stress and senescence, before any sign of vascular injury in middle-aged rats

Our data on pancreatic function correlated with modified islet morphology occurring in middle-aged rats. In accordance with a previous report by Liu et al. [24], IPGTT assays showed an early alteration in glucose control, which we found concomitant with the occurrence of smaller, collagen-surrounded islets characterized by lower insulin and higher glucagon labeling, yet occurring to a smaller but significant extent. Furthermore, variations of glucose and insulin blood concentrations were highly suggestive of an early insulin resistance in middle-aged rats.

Most interestingly, while pancreas function was initially altered, vascular damage could not be evidenced in middle-aged rats when assessed in mesenteric arteries. In particular, accumulation of oxidative stress and the higher expression of thrombogenic and senescence markers, all widely recognized as a pattern of vascular dysfunction, were solely detected in the pancreatic tissue. Because early endothelial alteration could have remained limited and undetectable by vascular reactivity assays or Western blot analysis, we also investigated circulating MPs as surrogates of vascular damage or thrombogenicity.

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Again, no significant variation in MPs levels from endothelial platelets or leucocyte origin could be shown between young rats and middle-aged rats.

This observation is of prime importance related to pancreas transplantation and in the context of islet transplantation, owing to the limited availability of organs. Indeed, in the latter case, allocated islets are generally isolated from elder grafts with presumed higher tissue damage and oxidative stress, with possible impact on three years post-transplantation insulin independence [30] and on delayed graft function in several cases.

Our data suggest that oxidative stress and consecutive senescence may become a therapeutic target in islet graft pre-conditioning, where multiple cellular lineages, like endothelial progenitor cells, contribute to islet protection [31] or constitute a local immunoprivileged site, like mesenchymal stem cells [32]. Because a variety of studies have shown that senescent cell burden is increased in aged tissues, further studies on the underlying mechanisms specifically favoring the early development of senescence in the pancreas of middle-aged individuals and to link cellular senescence to the pathogenesis of diabetes are needed [33].

In islet and pancreas transplantation, senescence would favor the progression of age-related dysfunction [34,35]. Therefore senescent cell clearance could have major benefits to human health span and lifespan [36]. The development of new protective strategies during pancreas or islet transplantation will further rely on the identification of senescence pathways and on the characterization of reliable sensors of pancreas and islet senescence.

Conclusions

The novelty of the present work is that it indicates the pancreas as an early sensor of age-related organ dysfunction before any sign of vascular disease or dysfunction in middle-aged individuals, and related to senescence-associated organ dysfunction. This observation may be of importance for a better outcome in pancreas or islet transplantation and suggests further prospects in islet preconditioning.

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Article II (in preparation)

Impact of Pro-Senescent Endothelial Microparticles on Rat Pancreatic Islet

Introduction

By 2030 diabetes is foreseen the seventh leading cause of death worldwide. Islet transplantation is a cell therapy proposed in patients with unstable T1D and life threatening issues. Indeed, the grafted islet's α and β cells dramatically contribute to the restoration of a glycemic control and insulino-independence is observed in 50% of transplanted patients after 1 year and decreases to 50% after 5 years. Aside this progressive graft dysfunction, the donor age is another possible cause contributing to the limited graft effectiveness. Aging is characterized by cellular senescence. As evidenced in our first article, senescence is associated with altered pancreas morphology and function.

In the laboratory, evidences of altered vascular function have been observed in aged rats and senescence could be detected at sites of flow disturbance by probing SA-βgal activity in aortic cross using the colorimetric X-βgal substrate. Taken together these data raise the question of the role of an altered endothelial function on the graft revascularization and survival peri and post-transplantation. Indeed endothelial MPs shed from senescent endothelial cells act as pro-thrombotic and pro-senescent effectors promoting the up-regulation of the local Ang II system of tissue factor and favoring oxidative stress-mediated accelerated endothelial dysfunction.

In islet-transplanted patients, an early peak of circulating MPs was observed before graft dysfunction and associated with increased insulin needs suggesting that MPs could prove useful as a noninvasive and early biomarker of the loss of islet graft function [1]. In France, the youngest donors are allocated to whole pancreas transplantation, making donor age a factor with possibly exaggerated impact on the success of islet transplantation.

In the present study, we sought to determine the role of pro-senescent endothelial MPs in the premature senescence of isolated pancreatic islets. Pro-senescent and young MPs were harvested from the supernatant of porcine coronary artery endothelial cells. Senescent cells were maintained until P3 and young until P1 passage. Pancreatic islets were isolated from male wistar rats and treated after 24 hours with MPs and pharmacological agents. MPs integration into the islets was probed by fluorescent microscopy, islets viability and functionality were measured and senescence markers expression assessed.

Our data show that pro-senescent endothelial MPs induce cellular senescence in pancreatic islets and the alteration of their function without affecting viability after 24h incubation. MPs probing indicated that after 24h the optimal MPs integration was obtained and that MPs targeted 56 ± 2% (P1-MPs), 27 ± 1.8% (P3-MPs). This indicates that such MPs are early bio-

effectors of isolated islets survival and function. Whether their pro-senescent, pro-coagulant action will impair the islet engraftment remains to be determined in transplantation models. However, the fact that pro-senescent MPs already reduce the function of the isolated islets is highly suggestive of an altered graft function. Furthermore, pro-senescent endothelial MPs are also highly pro-coagulant because they convey tissue factor, another mediator of the early instant blood mediated inflammatory reaction (IBMIR) known to worsen the outcome of islet transplantation.

Limitations

The generation of a sufficient amount of pro-senescent P3-MPs in a rat replicative model of endothelial senescence was technically challenging owing to the small size of the animals. Therefore, we chose to use primary cultures of endothelial cells derived from porcine coronary artery, as a convenient and unlimited source of endothelial cells. The model was developed at the UMR CNRS 7213 using porcine hearts from the slaughterhouse.

Other possible sources of primary endothelial cells, more related to the pancreatic islet transplantation background would have been the hepatic portal vein from the rats or from the mesenteric vasculature.

Although, enabling an autologous model, these two latter sources remained less convenient owing to the small size of the animal.

Other teams, have published MPs driven cross-talk studies using two different species in in-vitro models, where the immune response are negligible.

Our data also question the role of the paracrine interaction between endothelial cells and the islet especially with the respect to the age of donor and recipient. These preliminary elements have to be confirmed in a larger number of rats.

Impact of Pro-Senescent Endothelial Microparticles on Rat Pancreatic Islet

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Running title: Microparticles impact on pancreatic islet

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Abstract

Background: Pancreatic islets transplantation is cell therapy for patients with brittle type 1 diabetes, the effectiveness of which remains limited due to the progressive graft dysfunction. Aging is one of the limiting cause of graft success characterized by cellular senescence. We investigated whether pro-senescent endothelial microparticles prompt islet senescence and dysfunction *in vitro*.

Material and Methods: Pancreatic islets were isolated from male Wistar rats (200-250 g) (n=3-5). The microparticles (MPs) were isolated from primary porcine coronary artery endothelial cells after one passage (P1) or after three passage (P3, corresponding to the appearance of senescence). After concentration, 5 nM of MPs P1 and P3 and 100 µM of H_2O_2 were applied for 24 hours on the pancreatic islet. Viability was assessed by fluorescence microscopy (Propidium iodide (PI) / Fluorescein diacetate double labeling), apoptosis by flow cytometry (PI /Annexin V double labeling). Islet function was assessed by calculating the stimulation index defined as the ratio of the secreted insulin measured by Elisa after incubation in low 2.5 mM compared to high 25 mM glucose media. Senescence markers p53, p21 and p16 were measured in islet by western blot. The integration of MPs stained by the lipid fluorescent PKH26 probe was assessed by microscopy and flow cytometry.

Results: The stimulation index that show the ability of islets to secrete insulin in response to glucose significantly decreased after treatment by P3 ECs derived MPs (1.7 ± 0.2 vs. 2.7 ± 0.2 untreated islet, $p < 0.05$) without altering the viability (89% ± 1.7 vs. 93 ± 1% for untreated islet) and with no evidence of significant apoptosis. P3 MPs treatment induced a significant 2 fold increase of the expression of p53, p21 and p16 ($p < 0.05$), whereas P1 MPs had no significant effect. Microscopy and flow cytometry data showed that ECs MPs are well incorporated in pancreatic islet cells.

Conclusion: Pro-senescent endothelial induces cellular senescence in pancreatic islets and alters their function without modifying viability. These data suggest an impact of the donor age on islet graft success and question relevance of the pre-conditioning of the graft to limit the aged-induced senescence of the isolated islets prior transplantation and the effect of IBMIR one of the earlier cause for graft failure.

Background

The World Health Organization (WHO) currently reported 630 million people with diabetes worldwide, it predicts also that until year 2030 diabetes will be the seventh leading cause of death worldwide [2]. T1D is due to the autoimmune destruction of pancreatic islets leading to insufficient insulin production in young adults and children [3]. Treatment of T1D necessitates the daily monitoring of glycaemia and the injection of insulin [4].

Islet transplantation is a cell therapy proposed in patients with brittle T1D, with life threatening issues. Indeed, the grafted islet's α and β cells dramatically contribute to the restoration of a glycemic control [5]. Pancreatic islets are injected in the portal vein as a cell suspension, which will take place in liver microvessels.

Despite improvements in immunosuppression protocols, and improved insulin independence rates, reaching up to 50% at 5 years, a gradual loss of islet function that marks chronic graft dysfunction is reported [6].

Currently, the pancreas of younger donors preferentially allocated to whole pancreas transplantation and those of older donors to islet transplantation. Moreover, aging is accompanied by a loss of vasoprotective capacity of the endothelium due to an endothelial senescence [7] that may constitutes another cause of altered vascularization and dysfunction of the graft.

Cellular senescence is defined by an irreversible cell cycle arrest of the cell accompanied by gene and chromosomal changes [8]. It occurs in response to various signals such as cellular stress, telomere erosion, DNA damage, oxidative stress, or oncogenic activation. Senescence is characterized by a shifted pH of the lysosomal β-galactosidase (Senescence-Associated β-galactosidase (SA-βgal) activity) [9, 10].

The senescence process involves different actors of cell cycle regulation like tumors suppressor and cyclin dependent kinases. The antioxidant capacity of the pancreas is weak, suggesting a particular susceptibility to oxidative stress eventually leading to endothelial dysfunction [11].

Microparticles (MPs) are submicron plasma membrane fragments (0.05 to 1 μm in diameter) released into the extracellular space by stimulated or apoptotic cells after plasma membrane remodeling [12]. MPs express bio-active molecules and surface antigens that are characteristics of cells origin. In islet-transplanted patients, an early peak of circulating MPs was observed before graft loss and associated with increased insulin needs suggesting that MPs could prove useful as a noninvasive and early biomarker of the loss of islet graft function [1]. Data from the literature clearly demonstrate that endothelial MPs contribute to vascular responses and that

when released from senescent cells promote thrombogenicity and endothelial dysfunction [13-15]. The amount of MPs released in the systemic circulation is directly correlated to disease severity [16]. Besides their role as stress markers, MPs act as cellular effector by delivering the content of harbored active molecules to target cells [17].

In the present study, we sought to determine the role of pro-senescent endothelial MPs in the premature senescence of pancreatic islets. Pro-senescent and young MPs were harvested from the supernatant of porcine coronary artery endothelial cells and incubated with freshly isolated rats islets before measurement of islet function and identification of senescence markers.

Material and Methods

Ethics statement

Male Wistar rats (Janvier-Labs, Le Genest-St-Isle, France) were housed in a temperature-controlled (22°C) room and maintained on a standard 12-h light/dark cycle (lights on at 07:00 am), with free access to food and water. Experiments conform to the Guide of Care and the Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and were authorized by the French Ministry of Higher Education and Research and by a local ethic committee (Comite d'éthique en Matière d'expérimentation animale de Strasbourg, authorization 03799.01). All animal experiments were done in a registered animal yard within Faculty of Pharmacy (Authorization number E-67-218-26).

Endothelial primary cell culture

Pig hearts were collected from the local slaughterhouse (COPVIAL, Holtzheim, France) and endothelial cells were isolated from the left circumflex coronary arteries as described previously [18]. Briefly, left circumflex coronary artery was excised from fresh heart, cleaned of adhesive conjunctive tissues and the remaining blood was flushed with cold Phosphate Buffered Saline (DPBS) without calcium (Lonza, BE17-512F, Basel, Switzerland). Endothelial cells (ECs) were isolated by filling the artery with a 1 mg/ml, type I collagenase solution (Worthington, Serlabo Technologies, Entraigues sur la Sorgue, France) dissolved in MCDB131 medium (Invitrogen ; Carlsbad, CA, USA) with penicillin (100 U/ml), streptomycin (100 U/ml), fungizone (250 µg/ml) and L-glutamine (1mM, all from Lonza, Ozyme, St Quentin en Yvelines, France) for 15 min at 37°C. The ECs were collected after circular massages of the arteries and the remaining cells were flushed with medium. Collected cells were gently centrifuged at 600 g for 5 min, then medium was removed and cells were suspended in MCDB131 medium supplemented with 10% fetal calf serum, penicillin (100 U/ml),

streptomycin (100 U/ml), fungizone (250 mg/ml), and L-glutamine (1 mM). Extracted cells from arteries were seeded in a T25 flask and cultivated at 37°C in a humidified incubator with 5% CO_2. The ECs that quickly adhere to the bottom of the flask, were selected by removing non-adherent cells within the medium 6-8 hours after seeding. ECs were sequentially passaged from passage P1 (young cells) to P3 (senescent cells). The medium was changed every 48 h. The cells developed a senescent phenotype (circular shape, largest and better visible nucleus) from the third passage (cells P3).

MPs isolation and measurement

MPs were collected from the supernatant at P1 and P3 passage by sequential centrifugation under sterile conditions [19]. Briefly, cells and debris were discarded by centrifugation at 800 g for 10 min and the supernatant was further centrifuged at 13 000 g, 4 °C for 60 min. The final suspension was kept for less than 30 days at 4°C. MPs measurement was performed by prothrombinase assay after capture onto biotinylated Annexin-5 as previously described [20]. This procedure realizes on the high affinity of annexin V (Ann-V) for phosphatidylserine (PS) at MPs surface. The first step consist of the immobilization of biotinylated Ann-V (400 ng / mL) on a plate coated with streptavidin (Roche, France). After three washings with Tris buffered saline (TBS) to remove any excess of non-covalently linked Ann-V. 100 µl sample were added to each well and incubated at 37°C for one hour. After three washes with TBS, the amount of insolubilized MPs was measured by prothrombinase assay. In this assay, the degree of exposure of anionic phospholipids (PS) on the surface of MPs is the limiting factor of the prothrombinase reaction. PS in the presence of the coagulation factors (FII 4.2 µM, FVa 250 pM, FXa 53 pM and $CaCl_2$ 2 mM), catalyzes the assembly of the prothrombinase complex which converts prothrombin into thrombin. After 10 minutes at 37°C, the generated thrombin is measured using a chromogenic substrate, pNAPEP 0216 (1.52 mM). The cleavage reaction product is detectable at 405 nm. Absorbance is then converted into equivalent phosphatidylserine (eq PhtdSer) by reference to a standard curve obtained using a suspension of synthetic vesicles of known amounts of PS (33% (w/w) of PS and 67% (w/w) of phosphatidylcholine) [21].

Rats pancreatic islets isolation

Pancreatic islets were isolated from Wistar male rats (mean weight 250 g) as described elsewhere [22, 23]. Briefly, rat were anaesthetized by intraperitoneally injection of mixture of Ketamine and Xylazine. A 10 mL of 1 mg/mL Type-XI collagenase (C7657, Sigma-Aldrich,

Saint-Louis, USA) suspension in Hanks balanced saline solution (HBSS) (Lonza, BE10-527F, Basel, Switzerland) was injected into the common bile duct of the euthanized animals. Pancreas was excised within 30 min maximum, digested at 37°C for 10 min 30 seconds and submitted to strong hand shaking. Digestion was stopped by the addition of cold HBSS supplemented with 1 % bovine serum albumin. The digest was centrifuged at 300 g for 1 min and fat removed. The pellet was submitted to three successive cold washings (4°C) in FBS-supplemented HBSS. Isolation of islets was achieved using a three density gradient Ficoll PM-400 solutions (F4375, Sigma-Aldrich, Saint-Louis, USA) (densities: 1108, 1096, 1069 g/ml) and centrifugation at 800g for 18 min at 4°C. Islets were recovered from the interface between second and third density, and their purity was evaluated after dithizone (D5130, Sigma-Aldrich, Saint-Louis, USA) labeling by optical microscopy. Islets were re-suspended in RPMI 1640 (Gibco 21875-034, Saint Aubin, France) containing 11.2 mM glucose and supplemented with 1% penicillin, 1% streptomycin and 10% FBS. After manual handpicking 200 islets were cultured in non-adherent six-wells plates, and incubated at 37°C in a humidified incubator with 5% CO_2 for 24 hours before treatment.

Pancreatic islet treatments

Incubated pancreatic islets were treated for 24 hours by three different agents: 5 nM PhtdSer EQ P1 or P3 cell-derived MPs and hydrogen peroxide 100 µM (H_2O_2) (H1009, Sigma-Aldrich, Saint-Louis, USA as a positive control). The negative control corresponds to the addition of an equivalent volume of medium. In preliminary experiments the optimal 5 nM MPs concentration was determined by a dose curve response (0-20 nM PhtdSer EQ) established for a 24 hours incubation time.

Islet viability

After 24 hours of stimulation, islet viability was assessed by 2 different approaches. In the first one, we examined the double red PI / green fluorescein diacetate staining. Using an epi fluorescence microscope, red necrotic cells and green viable cells were counted within a suspension of 30-labeled islets. The viability of the islets was calculated as the ratio of green and red fluorescent area / total islet area.

In the second approach, 30 islets per condition were disassociated to isolated cell suspension, and studied by flow cytometry using double PI / Ann-V staining. For this purpose, islets were first centrifuged at 600 g for 10 minutes. After removal of the supernatant, the pellet was suspended in 500 µl of a solution 2x trypsin (340 000 IU/L) and then incubated for 8-10 minutes

at 37°C with gentle shaking. After checking under the microscope the complete dissociation of the islets, the reaction was stopped by addition of 700 µl of supplemented medium followed by a 10 minutes centrifugation at 1500 g. The pellet was resuspended in 500 µl fixation buffer and 5 µl of a 50 µg/ml annexin V-FITC solution (final concentration 0.5 µg/ml) and 4.5 µl of IP solution at 100 µg/l (final concentration 1 µg/ml) were added. After 10 minutes in the incubator, the cell suspension was analyzed using a flow cytometer set at logarithmic gain. 5000 events were recorded for each sample (Facs calibur, Becton Dickinson).

Measurement of islet function by Glucose-stimulated insulin secretion

Glucose-stimulated insulin secretion (GSIS) was measured in 40 islets in a KREBS solution supplemented with 0.5% albumin, 10 mM HEPES and 2.5 mM (0.45 g / L) glucose (basal), or 25 mM (4.5 g / L) glucose (stimulated). After MPs treatment (see below), islets were transferred for 1 hour into the KREBS solution supplemented with 2.5 mM glucose and then centrifuged at 1500 g for 10 minutes. The pelleted islets were incubated for another 1 hour in the KREBS solution 2.5 mM glucose, centrifuged at 1500 g for 10 minutes and their supernatant stored at -80°C until insulin measurement. Islets were finally incubated in the KREBS solution supplemented with 25 mM glucose for 1 hour 30 minutes, centrifuged at 1500 g for 10 minutes and their supernatant harvested and stored at -80°C. The amount of secreted insulin was determined by ELISA (Milllipore cat# EZRMI-13K, Billerica, MA, USA). The glucose stimulation index (SI) is the measure of the ability of the purified pancreatic islets to produce insulin when stimulated by an increase in the concentration of glucose. It was calculated by the ratio of insulin concentration after high glucose concentration stimulation (25 mM) to insulin concentration after low glucose concentration stimulation (2.5 mM).

Assessment of MPs integration into islets

Collected MPs from P1 and P3 cells were labeled by a red fluorescent lipid probe PKH26 (Sigma-Aldrich, Saint-Quentin Fallavier, France), washed twice by centrifugation (13 000 g, 60 min), and 5 nM PhtdSer EQ. MPs were incubated with 20 pancreatic islets for each condition for 24 h at 37°C. Accumulation of the probe on target cell surface was observed by fluorescent microscopy (emission maximum λ=567 nm) and quantified by flow cytometry using a FACS-scan cytometer set at logarithmic gain. 5000 events were recorded for each sample.

Assessment of Senescence markers by Western blot

The degree of senescence was evaluated by the expression of p53, p21 and p16 senescence markers in 200 islets per each condition. Following centrifugation of the islets, cellular proteins

were extracted after 15 min incubation at 4°C with a specific lysis buffer (Tris/HCl 20 mM (pH 7.5; Q-Biogene, California, USA), NaCl 150 mM, Na3VO4 1 mM, sodium pyrophosphate 10 mM, NaF 20 mM, okadaic acid 0.01 mM (Sigma-Aldrich, Missouri, USA) containing a tablet of protease inhibitor (Complete Roche, Basel, Switzerland) and 1% Triton X-100 (Euromedex, 2000-B, Souffelweyersheim, France).

Protein lysates (25 µg) were loaded on 10% SDS-polyacrylamide gels at 100 V for 2 h and transferred electrophoretically onto polyvinylidine difluoride membranes (Amersham, Life Sciences, Germany) at 100 V for 120 min. Aspecific binding was blocked by incubation in TBS buffer (Biorad) containing 5% bovine serum albumin (BSA) (Euromedex, 04-100-812-E, Souffelweyersheim, France), and 0.1% Tween 20 (Sigma) (TBS-T) for 2 h.

Membranes were then incubated overnight at 4°C in TBS-T containing 5% BSA and polyclonal rabbit antibody raised against human p53 (1/1000) (Santa Cruz Biotechnology FL-393 SC-6243, Dallas, Texas, USA), human p21 (1/1000) (Abcam ab7960, Cambridge, UK), monoclonal antibody to human p16 (1/1000) (Abcam ab51243, Cambridge, UK). After 3 washings with TBS-T, membranes were incubated with an appropriate horseradish peroxidase-conjugated secondary antibody and density signals of each band detected using ECL Substrate (170-0561, BIO-RAD, United States). Membranes were incubated with a mouse monoclonal anti-β-tubulin I mouse antibody (Sigma-Aldrich, T7816, Missouri, USA) for normalization purposes. In addition, to allow comparison between different experiments, all data were reported to the untreated control expressed as 100%.

Statistical analyses

All experiments were produced with n=3-5 repetitions. Data are expressed as mean ± S.E.M. Mean values were compared using Mann-Whitney test for the comparisons of groups using GraphPad Prism version 6.01 for Windows™ (GraphPad Software, San Diego, USA). $p < 0.05$ was considered statistically significant.

Results

MPs integration into the islet

Fluorescent PKH26 stained MPs confirmed the interaction between MPs and pancreatic islets. Indeed, after 24 hours incubation with 5nM eq PhtdSer MPs, PKH26 red probe labeled the islets observed by fluorescent microscopy, suggesting that MPs had integrated into islets (Figure 1A).

After complete dissociation of the islets, flow cytometry showed a significant increase in the proportion of fluorescent cells after treatment with both P1 and P3 labeled MPs compared to the untreated islets (P1-MPs 56 ± 2%, P3-MPs 27 ± 1.8% vs. untreated islets 9 ± 1.7%, $p<0.01$ and $p<0.001$ respectively). Interestingly, in P3-MPs treated islets, only 27% of the cells were labelled with a low mean fluorescence intensity of 10 A.U. In P1-MPs islets, labeled cells constituted 2 equal populations of low and high mean fluorescence suggesting that half of the P1-MPs have a better ability to interact with the cell membrane of the target islets cells after 24 hours (Figure 1 B).

Effect of pro-senescent endothelial MPs on islet viability

Treatment by P3-ECs derived MPs (P3-MPs) or by H_2O_2 showed a trend to decrease of pancreatic islets viability (89 ± 1.7% for P3-MPs; 87 ± 2% H_2O_2; $p>0.05$) compared to untreated islets after 24 hours. Similarly, P1-Ecs-derived MPs (P1-MPs) did not lead to any significant change in islet viability (95 ± 1% for P1; 93 ± 1% for untreated islets, $p>0.05$). In addition, no morphological change of the islets could be evidenced and central necrosis remained low in both P1- and P3-MPs treated islets (Figure 2).

Furthermore, apoptosis quantification by flow cytometry performed on cell suspensions after complete dissociation of the islets confirmed the absence of a significant viability loss in MPs-treated islets. P3-MPs led to a trend to increase in apoptosis (P3-MPs: 37.5 ± 3.5% vs. 24 ± 3.5%, $p>0.05$ in untreated islets). However H_2O_2 treatment was associated with a significant increase in apoptosis (42 ± 5% vs. 24 ± 3.5% in the untreated islets, $p<0.05$) indicating that the isolated islets indeed remained sensitive to oxidative stress under our conditions. Furthermore, the degree of apoptosis after P1-MPs treatment (23.5 ± 2.9%) was similar to that of untreated islets (Figure 3).

Pro-senescent endothelial MPs alter islet function

A significant 37% SI reduction was observed after treatment with the pro-senescent P3-MPs (1.7 ± 0.2 in P3-MPs vs 2.7 ± 0.2 in untreated islets, $p<0.05$). Treatment with H_2O_2 led to a 56% decrease in the ability of islets to secrete insulin in response to glucose (1.2 ± 0.2 vs. untreated islets; $p<0.01$). A non-significant trend to a SI decrease was observed after P1-MPs treatment (1.9 ± 0.3, $p>0.05$ vs. untreated islets) (Figure 4).

Pro-senescent MPs induce the up-regulation of senescence markers

P3-MPs significantly increased the expression of p53 after 24 hours compared to P1-MPs and untreated islets (P3-MPs: 1.83 ± 0.24 vs. P1-MPs: 1,09 ± 0,18 or untreated islets: 1, $p<0.05$).

A similar pattern was observed for p21 up-regulation (P3-MPs: 1.94 ± 0.33 for; vs. P1-MPs 0.87 ± 0.19 or untreated islets; $p<0.05$). The expression of p16, the p21 downstream effector, was solely and significantly increased by P3-MPs (2.1 ± 0.4 for P3-MPs vs. untreated islets, $p<0.05$), whereas no significant variation was observed after treatment by P1-MPs (1.2 ± 0.3). H_2O_2 induced a higher p53 up-regulation than P3-MPs (H_2O_2: 2.66 ± 0.57 vs. untreated islets, $p<0.05$). By contrast no significant effect of P1-MPs could be detected on the expression level of p53, p21 and p16, suggesting a specific pro-senescent action exerted by P3-MPs (Figure 5).

Discussion

The present study demonstrates that endothelial pro-senescent P3-MPs integrate into freshly isolated islets, prompt the up-regulation of senescence markers and alter islets' function assessed by glucose SI.

Endothelial MPs from young and senescent cells differentially integrate into the islet

In previous functional MP-mediated cross-talk studies, we had shown that PKH-26 labelled MPs released from insulin secreting cells after over-night cytokine stimulation or oxidative stress are early pro-inflammatory autocrine effectors that interact with the β-cell plasma membrane within 8 hours and reduce insulin secretion [19]. Similarly, MPs shed from exocrine pancreatic cells in response to 1 μg/mL LPS, also altered the β-cell function after 18 h treatment [24]. In the present study, fluorescence cytometry assays indicated that PKH26 labelled P1-MPs and P3-MPs have different abilities to interact with the cells of the target islets after 24 h. While 56% of the islet cells were targeted by P1-MPs only 27% had integrated P3-MPs. Indeed, the 5 nM Phtser EQ concentration applied was similar for both pro-senescent and non-senescent MPs, suggesting that proteins conveyed by MPs could account for the differences in MP integration through specific ligand counter-ligand interactions [14, 25, 26].

Pro-senescent endothelial MPs do not impair islet viability

After 24 hours incubation, P3-MPs did not induce significant changes in cell viability. Moreover, apoptosis measurements confirmed this observation, with a non-significant slight increase by flow cytometry, whereas H_2O_2 treatment led to a high apoptotic response. However, it has to be noted that P3-MPs may have not reached the core of the islet as efficiently as P1-MPs as suggested by the low proportion of cells probed by PKH26 stained P3-MPs (see above). Our data therefore do not exclude that cells from the external layer of the islet may have

contribute to the P3-MPs mediated effect through the secondary generation of islets-derived MPs of yet unknown cells origin.

Pro-senescent endothelial MPs decrease islet function

In contrast to P1 MPs, pro-senescent P3-MPs, prompted a 37% significant decrease of the stimulation index, close to the maximal reduction observed in response to H_2O_2 treatment, in spite of a lower islet integration. Those data are highly suggestive of a deleterious message specifically conveyed to endocrine cells by endothelial P3-MPs. Indeed, pro-senescent P3-MPs diminish the ability of insulin secretion of pancreatic islets without affecting their viability. It is therefore likely that the MPs influence other mechanisms that control the activity of the β-cells with limited necrosis or apoptosis after 24 h. In the context of pancreatic islet transplantation, our data question the impact of senescent endothelial cells and shed MPs within the islet graft and at the end of the hepatic capillary, at vicinity of the engrafted islets.

Pro-senescent endothelial MPs induce the senescence of the islet

Pro-senescent P3-MPs dramatically increased senescent markers upstream p53, and downstream p21, p16 by a 2 fold range, whereas P1-MPs remained not effective after 24 h. p53 is a cell cycle regulator involved in tumor suppression and controlling intricate pathways of cell death and survival [27]. The activation of p53 and p21 can lead to apoptosis or senescence [28], whereas the down-stream p16 is characteristic of cellular senescence [29].

In accordance with the reported of poorer recovery of the function of older islets that overexpress p16, our observation of the increased expression of senescent markers induced by pro-senescent P3-MPs is therefore highly suggestive of the senescence of the cells constituting the islets [30]. These data are consistent with previous works from our team and others describing the redox sensitive pro-senescent and thrombogenic effect of such MPs on the endothelium that are able to up-regulate tissue factor expression [14, 25]. Furthermore, in response to inflammatory or oxidative stress, MPs promote the expression of tissue factor by endothelial or insulin secreting β-cells. In the context of islet transplantation, IBMIR (Instant blood mediated inflammatory reaction) is an early inflammatory process initiated by the contact of islet with the portal blood that is associated with leukocyte and platelet activation, tissue factor expression at the vicinity of and within islets, and disseminated coagulation events associated with the negative outcome of clinical islet transplantation [19, 31].

Our data point at pro-senescent endothelial MPs as early contributors to islet dysfunction already present in the isolated islet suspension. Whether their pro-senescent, pro-coagulant

action will impair the islet engraftment remains to be determined in transplantation models. However, the fact that pro-senescent MPs already reduce the function of the isolated islet is highly suggestive of an altered graft function.

Conclusion

In conclusion, our study has shown a deleterious paracrine effect of the pro-senescent endothelial P3-MPs on islet function that is associated with enhanced senescence. These data confirm the presence of a noxious MPs mediated cross-talk between senescent endothelial and pancreatic islet cells. Our data, also point at MPs as a therapeutic target in the preservation of islets and are in favor of an early pre-conditioning of the graft. Because engraftment is conditioned by islet vascularization and the migration of the recipient endothelial cells. The endothelium of the recipient appears another source of pro-senescent MPs that might need pharmacological control.

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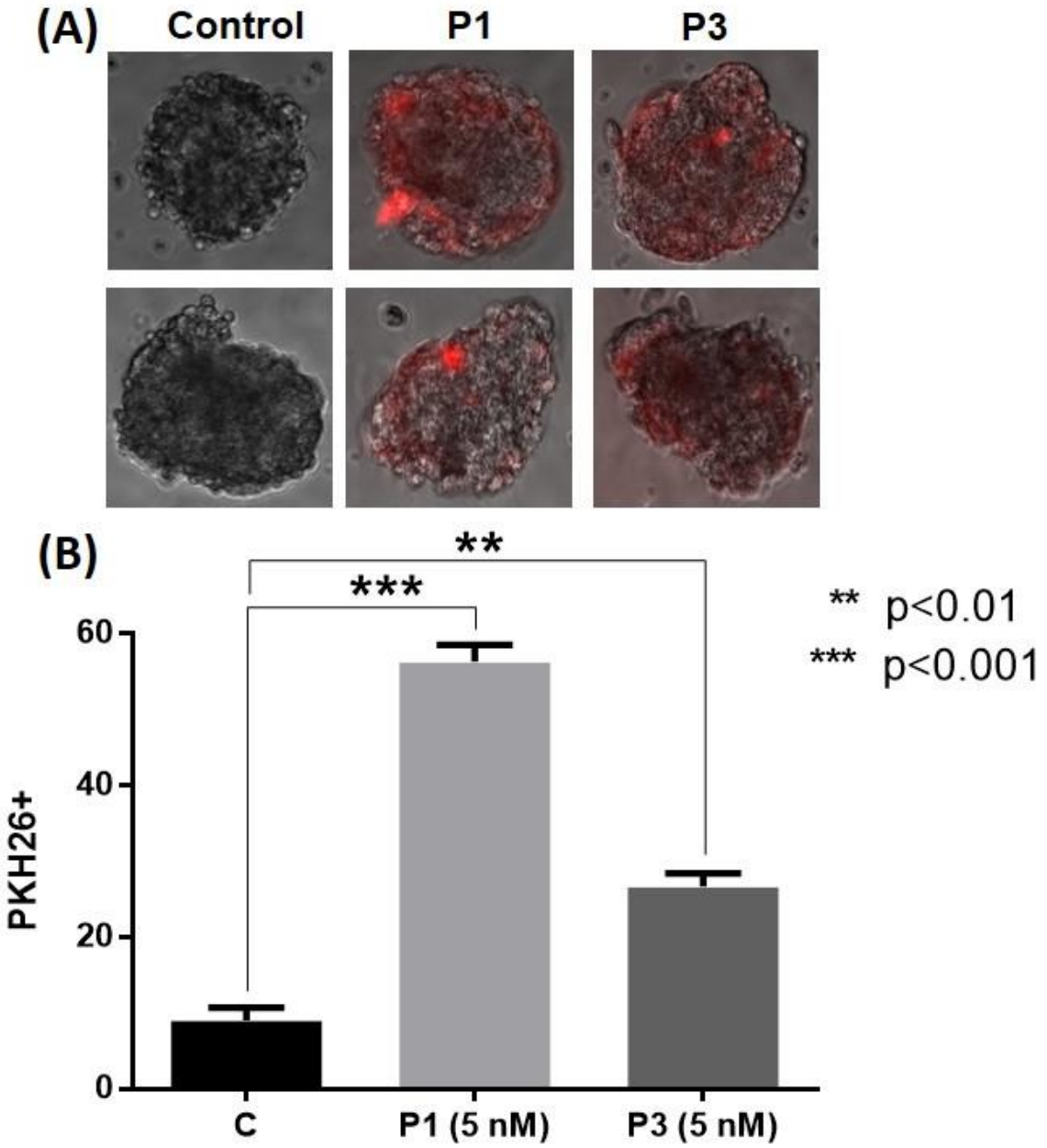

Figure 1: MPs integration into the islets

MPs integration into islet was assessed after 24 h treatment by 5 nM PhtdSer EQ of P1-, P3-MPs labeled by red PKH26 lipid probe. (A) Representative images by fluorescent microscopy (magnification 20 x). (B) Quantification of the labeled PKH26 probed pancreatic islet cells after trypsin dissociation by flow cytometry. n=3, ** $p<0.01$, *** $p<0.001$.

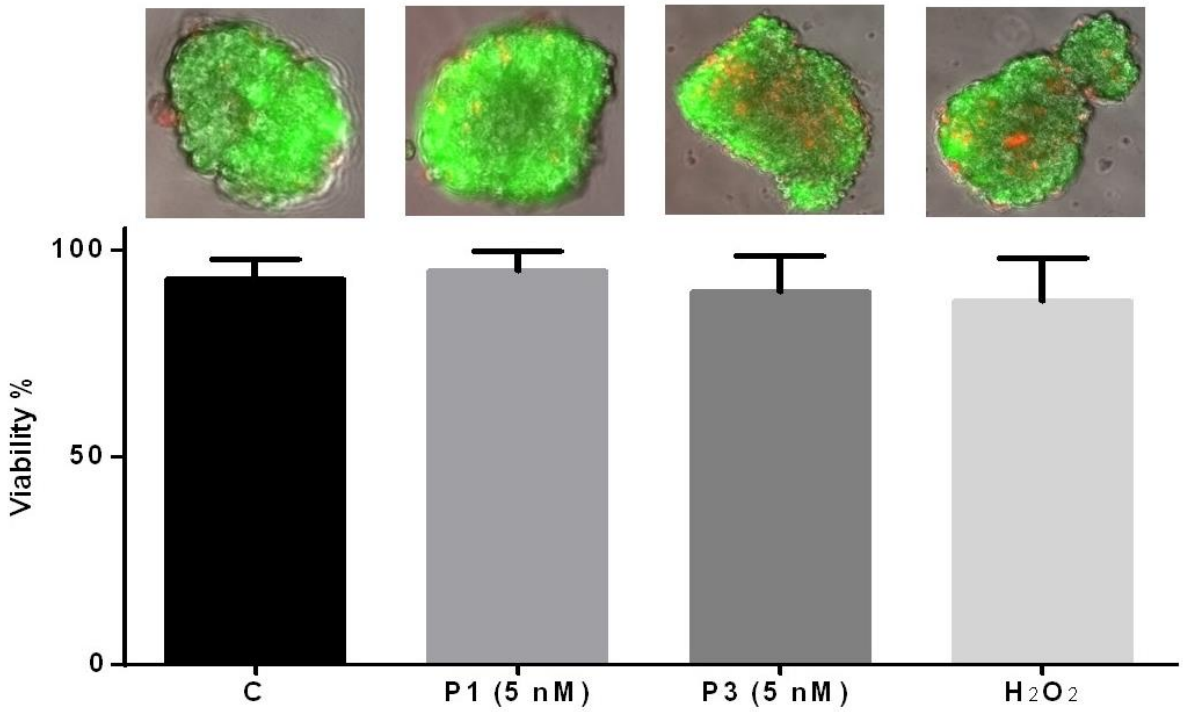

Figure 2: Islet viability

Islet viability was assessed by double propidium iodide (PI) / Fluorescein diacetate double labeling after 24 h treatment by 5 nM PhtdSer EQ of P1-, P3-MPs or 100 µM H_2O_2. Viability was calculated as the % of green and red fluorescent / total islet area (magnification 20 x), quantification was performed using Image J software, results expressed as mean ± SEM of n=5 per group.

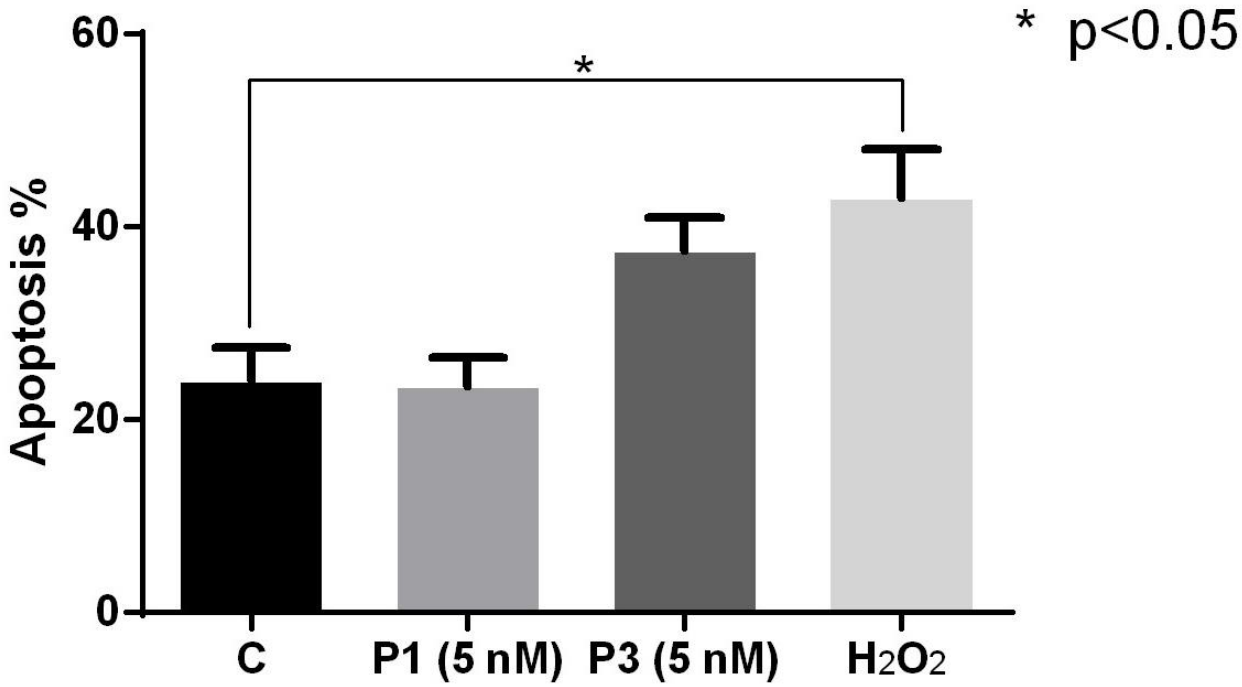

Figure 3: Apoptosis in isolated pancreatic islets

Islet apoptosis was evaluated after trypsin dissociation into cell suspension following a 24 h treatment by 5 nM PhtdSer EQ of P1-, P3-MPs or 100 µM H_2O_2. Cell suspension was analysed by flow cytometry after propidium iodide (PI) / FITC-Ann-V double labeling. Results are expressed as mean ± SEM of n=3 per group * $p<0.05$.

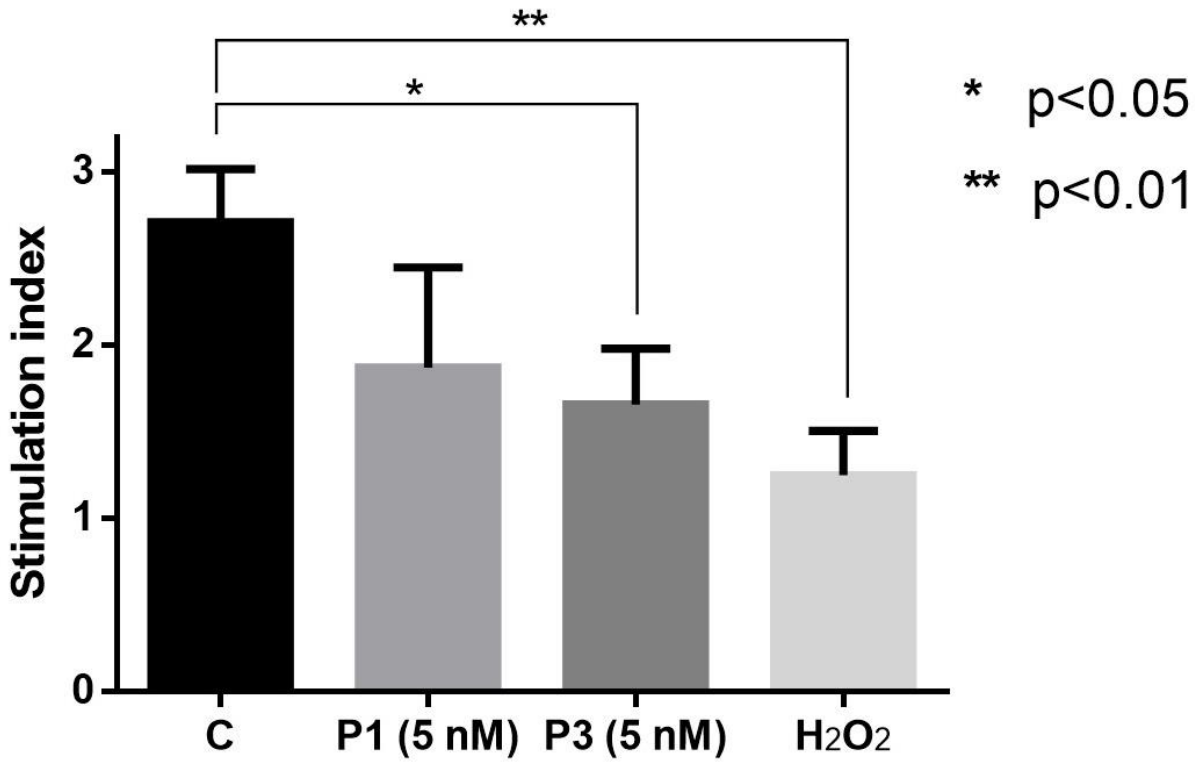

Figure 4: Islet Function test

Islet function was evaluated by the stimulation index (insulin amount secreted after incubation in KREBS solution supplemented with 25 mM glucose / amount of insulin secreted after incubation in KREBS solution supplemented with a 2.5 mM glucose) after 24 h treatment by 5 nM PhtdSer EQ of P1-, P3-MPs or 100 µM H_2O_2. Results are expressed as mean ± SEM of n=3 per group * $p<0.05$, ** $p<0.01$.

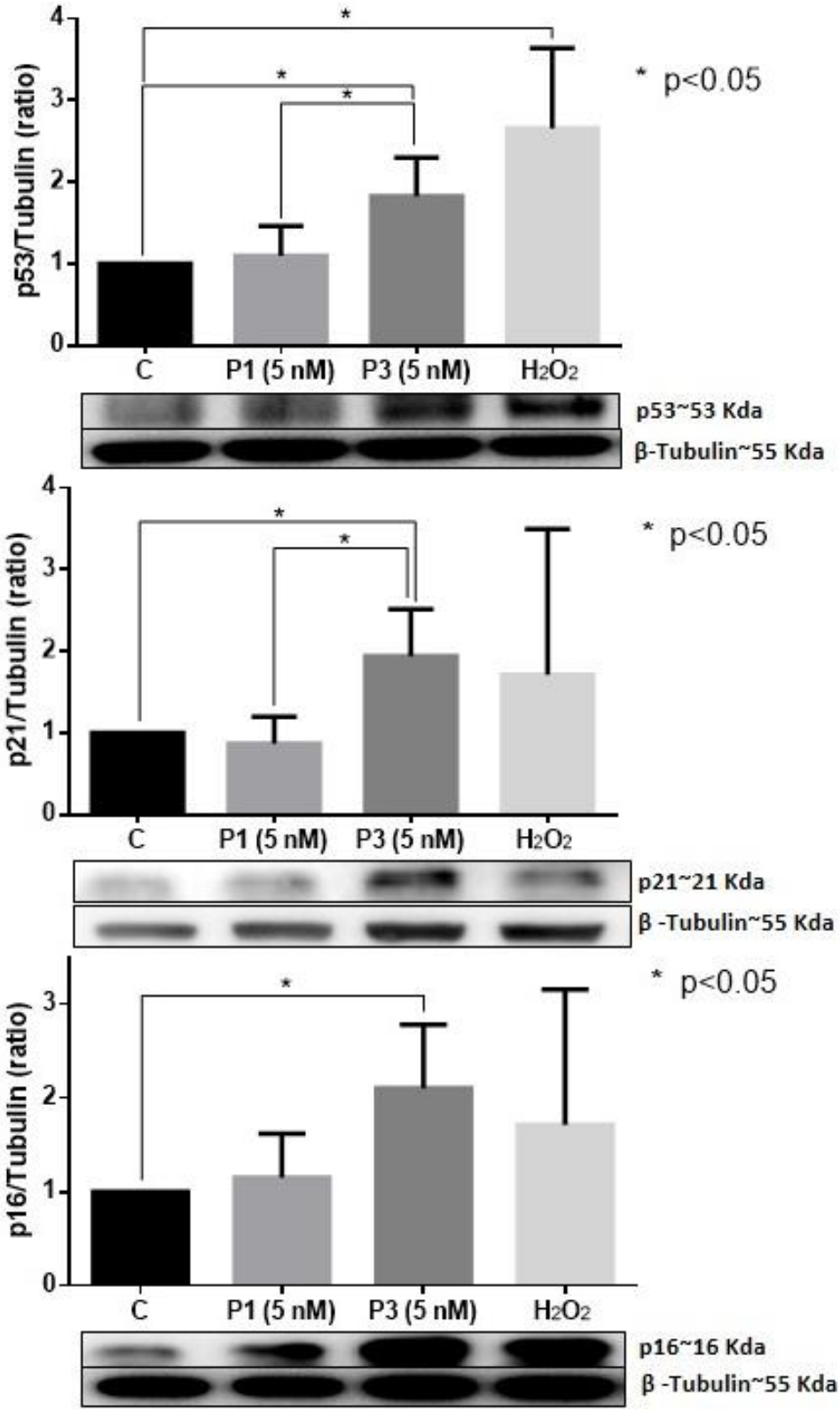

Figure 5: Expression of senescence markers in MPs treated pancreatic islets
The senescence markers p53, p21, and p16, after 24 h treatment by 5 nM PhtdSer EQ of P1-, P3-MPs or 100 µM H_2O_2, were measured by Western blot (lower panel representative blot, upper panel: cumulative data). Quantification of Chemiluminescence as a ratio of protein of interest/ housekeeping protein levels (Tubulin) performed by Image J software. Results are expressed as means ±SEM of n=3 per group, * p<0.05.

General Discussion

Islet transplantation as new treatment of T1D

The available most common treatment of T1D, which is administering several injections of insulin daily, is still not sufficient to cure such a drastic complication. The development in the diabetes domain has led to the debut in the era of pancreatic islet transplantation that consists of grafting the functional unit of insulin secretion that is directly dedicated to controlling blood sugar. This is indeed a promising noninvasive therapy in case of high glycemic instability. This technique aims to match the efficacy of the reference replacement technique namely pancreas transplantation.

However, two major issues remain obstacles in the islet transplantation path. First, the quality and functionality of the isolated islets and their preservation immediately after transplantation to favor the engraftment of sufficient amounts of viable islets. Secondly, the interaction of the islets with the recipient's portal vein components (endothelial and circulating). Indeed, the islet contact with portal blood triggers inflammation and procoagulation reactions known as IBMIR that is the leading cause of early loss of islets.

In our work, two main pieces of evidence of the impact of aging have been identified:

1- The premature aging of the pancreas with respect to that of the vasculature shown in middle-aged rats. Middle-aged rats exhibited early signs of altered pancreas morphology and function, whereas the vascular function remained unchanged compared to young rats

2- The pro-senescent role of endothelial MPs on the function of isolated islets.

Our data are indicative of distinct aging processes in the pancreatic tissue compared to the vascular compartment. Nevertheless, nothing is known of the role of MPs generated during IBMIR that were found pro-thrombotic *in vitro*. Most probably, these circulating TF^+-MPs are shed from leucocyte and endothelial cells in the vicinity of the islet embolization site. Nevertheless, little is known about the role of ischemia itself on the TF expression by islet cells, TF sorting into MPs and their eventual pro-senescent action within the islet tissue. Indeed, data from the laboratory demonstrated *in vitro* the noxious pro-thrombotic effect of endocrine TF^+-MPs in response to ischemia [426] as well as the pro-senescent and pro-thrombotic autocrine action exerted by endothelial TF^+-MPs [347]. Interestingly, data from a pilot study performed at Strasbourg University Hospital in islet-grafted patients indicate that rejection is associated with the elevation of circulating MPs of endocrine origin.

Aging of the pancreas and islet dysfunction

In organ transplantation and particularly in pancreatic islet transplantation, donor age is an important determinant for the efficacy of graft function and survival, because of the limited number of organs donors and of the extended life span. The control of age-related senescence is a prospect for organ re-conditioning and improved graft function to limit cell cycle arrest and protect cell repair potency of the graft. With aging, the immune system also undergoes a progressive modification in the number and/or function of immune cells termed immunosenescence, which may affect the transplantation success.

In our first rat model, we give evidences that donor age has an impact on organ function possibly through senescence and oxidative stress. By comparing young and middle-aged rats, we showed morphological modifications of the pancreas islets and altered response to hyperglycemia and oxidative stress (pancreatic ROS). Senescence markers like p53 and its downstream targets p21 and p16, were also up regulated in the pancreatic tissue indicating the initiation of senescence pathways. However, the circulating MPs measured in the peripheral blood as surrogates of vascular cell injury remained unchanged as well as the vascular function in mesenteric arterial rings. Therefore, we conclude that pancreatic islet alterations in middle-aged rats occur before any macro-vascular dysfunction. It has to be noted that our data are only related to whole pancreatic tissue. However, other groups could identify islet senescence in middle-aged and its progression in elderly rats by measuring p16, in line with our observations. Indeed, they identified the SA-β Gal senescence staining only in the endocrine tissue [427]. In addition, Tamura *et al.* showed that the telomeres of β-cells become shortened with normal aging process which lead to a reduction in their number and may be a major step responsible for the onset of T2DM [324]. These observations are in accordance with our findings, showing a reduced β-cell area in middle-aged rats and the reduced pancreas function.

Altogether, our data show that the pancreas is an early sensor of oxidative stress that might be the driving force to senescence appearing earlier in the pancreas compared to the vessels (see Figure 1, article 1, complementary data). In addition, the islet exposure to oxidative stress suppresses the expression of the pancreatic and duodenal homebox-1 (PDX-1) that is a key transcription factor for β-cell differentiation. Therefore, this can participate in the alteration of insulin secretion and decreasing the ability of β-cells to replicate. Indeed, the pancreas could be considered an early sensor of oxidative stress and senescence in middle-aged rats. These observations, therefore question the impact of the age of the islet donor on the success of the

pancreatic islets transplantation and the interest of a pharmacological pre-conditioning of the islet graft. Indeed, we could evidence in isolated rat islets the accelerated appearance of senescence mediated by endothelial MPs suggesting their possible early role. Other data from the laboratory showed that immunosuppressive drugs have distinct effects on islet survival and may alter their function [428]. Until now, no specific clinical study on islet pre-conditioning has been conducted. Liraglutide, a GLP1 analogue was studied as a complementary treatment to limit insulin dependence in islet transplanted patients. However, the study was terminated because of the very low recruitment rate [429]. A part from senescence, putative targets for islet pre-conditioning are numerous and include the mediators of oxidative stress as well as pro-inflammatory and thrombogenic actors of the IBMIR.

Islets revascularization and endothelial senescence

Islets revascularization and tissue homeostasis

Islet revascularization constitutes a major challenge to ensure the endocrine function of the graft. It is dependent on the reconstitution of the intra-islet vessel endothelial lining from three different sources: the transplant recipient's cells, the intraislet donor cells constituting 7-40% of the islet endothelial cells and a minor source of endothelial progenitor cells migrating from the recipient's bone marrow. Within the aged islets, senescent endothelial cells are likely the main endogenous source of endothelial cells and a contributor to the shedding of pro-senescent MPs. It is well known that aging comes with structural and functional modifications of the vasculature, which lead to the dysfunction of the endothelium and smooth muscle cells. Therefore, preserving the islet endothelium from accelerated senescence could favor the better outcome of the transplantation.

In addition, the recipient's vascular altered health and the occurrence of IBMIR are two other causes of accelerated endothelial senescence in the hepatic microvessels at the vicinity of the implanted islets. The therapeutic targeting of pro-senescent mediators such as MPs, PAI-1, interleukins and HMGB1, may prove beneficial through systemic and local reduction of senescence associated noxious effects [317]. In particular, diabetic patients who have altered endothelial functions and elevated circulating inflammatory mediators, preservation from accelerated senescence should improve the transplantation outcome by reducing the IBMIR response.

Noteworthy, T2DM also leads to highly elevated inflammatory status associated with the growth of adipose tissues. Senolytic drugs could therefore, be of benefit by delaying the

aggravation of β-cell dysfunction. In this respect, also bone marrow mesenchymal stem cells (BM-MSCs), known to display anti-inflammatory and cyto-protective mediators have been proposed as complementary cell therapy in islet transplantation. Depending on their niche, they can differentiate into many types of cells such as osteoblasts, adipocytes but also into cells of endodermal origin such as insulin secreting cells [430]. Although, BM-MSCs have other properties like a powerful immunosuppressive effect [431] and an ability to release pro-angiogenic factors [432]. From these two crucial properties, the co-transplantation of islets of Langerhans with BM-MSCs is highly promising. In a recent study by Cao XK *et al.* applied this new approach using tissue engineering. They found that the combination of islets with bone marrow mesenchymal stem cells (BM-MSCs) promotes angiogenesis by the migration of BM-MSCs to transplanted islet, where it will differentiate into vascular smooth muscle cells (VSMC), vascular endothelial cells (VEC) and secrete vascular endothelial growth factor (VEGF). In addition, they found that it improved allograft immune tolerance of grafted islets through the increase of donor lymphocytic chimerism [134].

Altogether, BM-MSCs improve transplantation by promoting the development of new insular vascularization and by limiting the cytotoxic effects of hypoxia. Moreover, the cytokines secreted by the BM-MSCs locally control the differentiation of the self-reactive monocytes, thus preventing the autoimmune destruction of the grafted islets [431].

To ensure rapid vascularization of the grafted islets, another approach can be the direct enrichment in endothelial cells and the improvement of their capability to form mature and functional vessels. For this purpose, Quaranta *et al.* co-transplanted pancreatic islets with endothelial progenitors in diabetic rats and obtained the restoration of the glycemic control as well as the establishment of a de novo adequate supply of vascular network up to 6 months after the transplant [433]. In this respect, little is known about the specific role of intra-islet endothelial cells, their sensibility to oxidative stress and induced senescence. Most importantly, the effect of pro-senescent endothelial MPs trapped within the islet on revascularization remains to be fully explored (see next paragraph).

Endothelial senescence

Contrastingly to our results, a recent study by Almaca *et al.* who could not evidence that β-cell function decline with age in a murine model [434]. However, one interesting demonstration of the work is that islets from aged mice can be rejuvenated after revascularization when transplanted into the interior chamber of the eye of young mice with diabetes. These

rejuvenated islets were able to enable sufficient glycaemia control. By contrast, transplantation in old diabetic rats led to limited control of the glycaemia on a shorter period of time. This observation is highly indicative of the role of the vessels in islets engraftment and function. Indeed, aged islets have inflamed and fibrotic blood vessels that highly express endothelial ICAM-1 and are infiltrated by macrophages [434].

Altogether, the above data indicate that endothelial senescence is a process associated to diabetes and that the systemic influence of islets is dependent on the vascular health and function. Circulating endothelial MPs have been claimed as a marker of endothelial dysfunction and loss of vascular competence in diabetes [350]. In our work, we could not evidence any significant variation of circulating MPs in middle-aged rats, although pancreas tissues showed early signs of dysfunction associated with markers of senescence.

However, this does not preclude to the impact of endothelial senescence in the hepatic microvessels nor on their role as pro-senescent mediators toward the grafted islets. Our second part of work have revealed that pro-senescent endothelial MPs induce the up-regulation of senescent markers in the isolated islets and the decrease in the ability to secrete insulin in response to glucose. Cellular senescence therefore becomes an active area of research that has promising pharmacological and therapeutic applications in islet transplantation.

Oxidative stress induces endothelial cell senescence and dysfunction, thereby switching the vaso-protective endothelium into a thrombogenic surface exposing TF, pro-adhesive proteins and secreting pro-inflammatory cytokines [347]. At the end of the liver microvessels, the highly fenestrated endothelium is extremely sensitive to ROS and may therefore release high concentrations of pro-senescent MPs at the vicinity of the niche of the grafted islets. Such a noxious environment may prove a driven force for IBMIR and local coagulation that will limit the islet perfusion and survival (Figure 35).

A side from C-peptide and insulin measurements, there is a need for the monitoring of islet function and viability in islet-transplanted patients. Total circulating MPs were demonstrated by our laboratory to be a promising post-transplant prognosis markers for graft dysfunction before the fall of C-peptide. However, since endothelial MPs convey pro-senescent and pro-thrombotic (AT1R, ACE, Caspase and TF) proteins, one could anticipate that pro-senescent MPs could also be of interest in the accurate detection of islet loss.

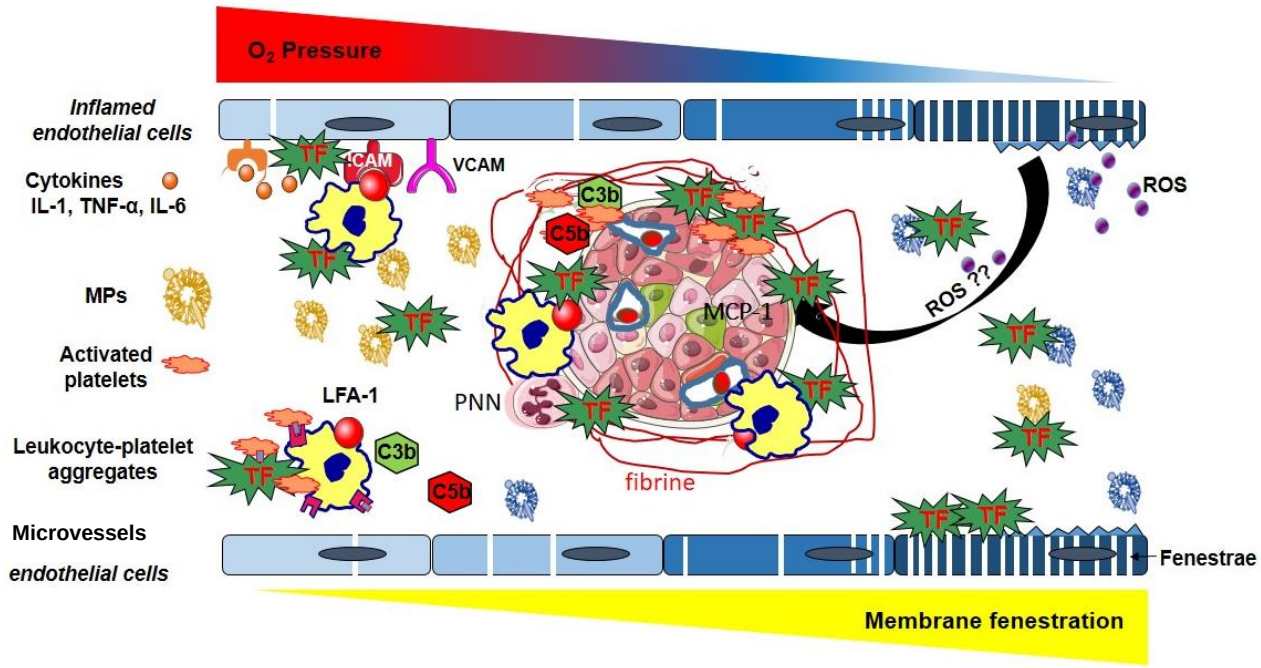

Figure 35 : Endothelium damage occurs at the end of the hepatic microvessels and within the islets

Hepatic endothelial cells are highly fenestrated at sites of islet engraftments. Owing to the low oxygen pressure, they are also highly redox sensitive, a putative cause of endothelial dysfunction contributing to ischemia-reperfusion-induced local coagulation. In addition, they would be susceptible to redox sensitive induced endothelial senescence with shedding of highly pro-senescent MPs that expose active TF (active TF is also expressed by cytokine activated monocyte (yellow cells), neutrophil (pink) and endocrine β-cells).

Microparticles, as cellular effectors in pancreatic islet transplantation

Besides their role as a biomarker constituting the signature of the cellular homeostasis, MPs act as cellular effectors by their ability to fuse with the plasma membrane of target cells, enabling the transfer of the MPs content consisting of active proteins or lipids and membrane receptors. MPs effects can be autocrine or paracrine. Studies show that MPs can also carry and transfer genetic material like DNA, RNA and miRNAs [354, 355].

A recent study by our laboratory confirmed that pro-senescent MPs are characteristic of tissue damage. Indeed MPs circulating in acute coronary syndrome (ACS) patients contain a proportion of pro-senescent endothelial MPs, compared to healthy individuals. In addition, such pro-senescent endothelial MPs induce a thrombogenic endothelial phenotype *in vitro* with the expression of tissue factor and ACE activities and with AT-1 angiotensin 2 receptor overexpression. Conversely, to the highly pro-senescent activity of MPs isolated from atherosclerotic plaques, MPs from internal mammary arteries remain ineffective, indicating that MPs properties reflect the tissue state of cellular activation [347]. In our study, concerning the middle-aged rats we could also evidence that MPs of vascular origin did not vary, whereas oxidative stress and senescence were already established within the pancreas (see article 1).

Of note, MPs derived from senescent ECs promote an early oxidative stress *in vitro*. Most likely by the up-regulation of NADPH oxidase and COX-2, the activation of mitochondrial respiratory enzymes, the down-regulation of eNOS and induced ECs senescence at concentrations that can be reached during ACS. In addition, it promotes ECs thrombogenicity through the up-regulation of TF expression and activity and procoagulant MPs shedding [435]. Since endothelial cells are pivotal in the revascularization of islets after transplantation, we sought to determine whether pro-senescent endothelial MPs induce premature senescence of isolated pancreatic islets. Furthermore, the deciphering of the underlying mechanisms could prove interesting for the development of senolytic drugs to protect islets function in aging individuals or to prevent accelerated islet loss and dysfunction pre- and post-transplantation.

Our data described in article 2 show that pro-senescent endothelial MPs are paracrine cellular effectors of senescence within the freshly isolated pancreatic islets. Indeed, other cells and not only endothelial cells constituting the functional structure of the islet were targeted by the MPs like endocrine cells. This result is in accordance and extends the data from Burger *et al.*. They found that treatment with MPs or H_2O_2 was associated with a shift from a proliferating to a non-proliferating phenotype. This indicated that MPs induce premature senescence in primary

cultured ECs [226]. Most importantly, we could show that endothelial pro-senescent MPs lead to the early alteration of islet function after 24h when viability was still not affected. It was clearly shown that MPs integrate easily after 24h in islet (P1-MPs 56 ± 2%, P3-MPs 27 ± 1.8%) and that despite a lower integration into target cells pro-senescent P3-MPs were highly noxious. However, while the transfer of information conveyed by P3-MPs could be evidence by functional assay. It yet remains to identify which molecule and pathways are responsible for their pro-senescent effect. Another pharmacological target in the preservation from accelerated cell aging is the secondary generation of pro-senescent MPs emerging from new senescent cells pointing at the modulation of PhSer translocation from the inner to the outer leaflet of the plasma membrane (see paragraph VII-2.1). For instance, MPs shedding could be inhibited through the action of antagonist to TMEM16F that supports the Ca^{2+} dependent scrambling of phospholipids [436].

Microparticles in transplantation

Because IBMIR is a form of ischemia-reperfusion leading to endothelial, platelets and leucocyte activation, one can anticipate that these cells produce a high amount of pro-coagulant MPs bearing TF and possibly pro-oxidant and pro-senescent. Interestingly, the transplanted islets can be therefore targeted by endothelial MPs of two different territories: the liver endothelial cells at the extremity of the capillary that are extremely sensitive to oxidative stress and the intra-islets endothelial cells already submitted to ischemia owing to the isolation procedure [437].

There is clear evidence that the measurement of circulating MPs in the peripheral blood could become a useful non-invasive tool for the monitoring of transplant dysfunction especially when biopsies are non-informative or dangerous (see paragraph VII-6). However, the question of the thresholds defining a risk of cellular or humoral rejection must be determined for each type of transplantation. A new approach for the determination of these thresholds in the pancreatic islets could be the constitution of a score including various MPs origin (in the case of islet transplantation: endocrine and liver derived MPs combined to CD8/CD4/CD20 bearing MPs). Another possibility would be the coupling of these MPs origins with the examination of their pro-senescent contents.

The dual faces of MPs: good or bad?

In addition to being biomarkers of vascular and graft damages, MPs are generally considered as noxious elements because of a long history of reports on their pro-coagulant, pro-

inflammatory and pro-oxidant activity within the vascular compartment, together with several data indicating their inhibiting effect on vascular repair. Indeed, MPs were demonstrated to be biomarkers of primary and secondary cardiovascular risk [438]. Furthermore, high levels of pro-coagulant MPs of endothelial and leucocyte origin were associated with disseminated intravascular coagulopathy (DIC) in septic patients [439]. Recent data coming from *in vitro* studies show the beneficial effect of certain sub-population of EMPs that could modulate cell death mechanisms, stimulation of vascular repair and cyto-protective activities supported by activated protein C (APC) [440].

Under pathological acute circumstances, EMPs were first considered as conveyers of deleterious biological information for endothelial function leading to DIC and cell death. They were proven to cargo therapeutically infused APC, owing to the presence of EPCR, the APC receptor. Furthermore, the injection of such APC, EPCR positive MPs to naïve septic rats did modify vascular reactivity in the recipient and reduced inflammation in cardiac and vascular tissues [441, 442]. Furthermore, other groups have demonstrated that the cyto-protective effects of APC is also conveyed by APC positive EMP that inhibits the release of procoagulant MPs and decreases endothelial cells apoptosis [356].

Other eventual beneficial effects of MPs could be their proliferative action that facilitates tissues repair. For example, EMPs stimulate the angiogenic process where from one side it can be harmful like in cancer dispersion and proliferative diabetic retinopathy, or can be beneficial as in the case of vascular repair in ischemic tissues [443]. Also Cantalupi *et al.* show that macrovesicles derived from endothelial progenitor cells that also contain MPs can protect the kidney from ischemic acute injury [221].

All together, these data highlight the need for new *in vivo* models to study the role of MPs in tissues and fluids in order to determine how are they contributing to organ homeostasis. Hence, there is no doubt that the future research will have a big challenge to find answers for many questions like the pathophysiological role of MPs, the mechanisms controlling endothelial vesiculation and MPs shedding, decode the mechanisms governing MP binding to target cells and the most important challenge will be to find pharmacological modulations that control MPs generation.

Conclusion & Perspectives

In conclusion, all our data suggest that the pancreas is an early sensitive organ to senescence associated with age, particularly during the transplantation of pancreatic islets. It also indicates that at least in the rat model, the pancreas is an early sensor of oxidative stress even before any sign of vascular injury and that middle-aged healthy individuals presented different islet morphology and early signs of pancreas dysfunction and senescence that is very important to take it in to consideration in pancreas or islet transplantation.

In addition, our second part of the study has shown a deleterious effect of the pro-senescent endothelial MPs (P3) on the functionality of the rat pancreatic islets. This deleterious effect is observed together with an increase of cellular senescence possibly related to a paracrine effect of endothelial MPs. These data are strongly in favor of the existence of a negative cross-talk between endothelial cells and pancreatic islet cells via endothelial pro-senescent MPs. Indeed, the deleterious effect of the pro-senescent MPs, were clearly evidenced by our *ex vivo* studies. The inhibition of such a noxious effect should prove beneficial for the outcome of islet graft transplantation. Altogether, findings from our team and others underscore the fact that age-related or premature cell senescence is pivotal in transplantation but also as a contributive process of ischemia-reperfusion induced vascular damages and dysfunction as observed during IBMIR.

Furthermore, our data also indicate that the preconditioning of the islet could ameliorate the success of the graft by limiting the age-related dysfunction in the donor organ before its implantation. Our results may also bring to light a better understanding of the impact of aging on pancreas function. The scientific statements that were evoked by the present study offer valuable information for the pathophysiology of pancreas and stand as a solid proof of concept for the further development of therapeutic strategies to improve pancreatic islet transplantation.

Further studies will relay on the establishment of reproducible *ex vivo* and *in vivo* models mimicking the situation of pancreatic islet transplantation in diabetic animals. For instance, the islet senescence model that we developed was not yet challenged with blood components. This could be realized by adapting the tubing loop reported by Akima *et al.* [444]. The pharmacological drugs initially investigated in such standardized model should however be further evaluated *in vivo*. For this final step, the intraportal graft model in the Spiegel lobe in rats is a reference technique to study the implantation of islets in a restricted area of the liver. This offers the advantage of a minimal need for MPs since the number of transplanted islets would be minimal. In addition, such model will allow the investigation of MPs from diabetic patients of different ages or isolated from a senescence cellular model. These MPs, which can be labeled by specific markers, could be administrated as modulators of islet function

intravenously or in the eye chamber as recently proposed in the study of the role of the vasculature on the aged-islets rejuvenation in young recipient [434].

The pharmacological pre-conditioning of pancreatic islets has been poorly investigated in animal models. However, the activated protein C (aPC), an anticoagulant protein recently recognized as a biased ligand initiating the PAR-2 receptors mediated anti-inflammatory and anti-apoptotic, could contribute to islet protection. Indeed, in engineered mice over-expressing the aPC receptor (EPCR), grafted islet function was preserved [445]. Furthermore, aPC itself was shown to protect isolated islets *ex vivo* in a tubing loop filled with blood, thereby indicating its usefulness to counteract the IBMIR reaction [444]. Similarly, thrombomodulin, another anti-coagulant endothelial protein that shifts the procoagulant action of thrombin towards anti-coagulant properties through the activation of protein C that initiate retro-control inhibition of thrombin generation was recently demonstrated an interesting tool to preserve islet function in diabetic transplanted mice [446]. Indeed, when administrated intravenously as a liposomic suspension, thrombomodulin preferentially accumulated within the liver thereby facilitating the inhibition of IBMIR. Another form of pre-conditioning could be the targeting of the floppases that favor MPs shedding. Putative candidate are TMEM16F, other true or occasional scramblases that could be targeted by specific inhibitors. Alternatively, Annexin V could be used to constitute protective shield around the activated cells, thereby counteracting the MP shedding process. This type of PhSer targeting could be beneficial during acute cell responses as observed during IBMIR or conversely in the progressive cell activation and MPs shedding observed during senescence. Because pro-senescent endothelial MPs are noxious to the islet function as demonstrated in our work, the senescence mediators such as proteins involved in the local Angiotensin system or in the control of ROS generation could also be targets in new protective strategies of the transplanted islets, especially in the progressive establishment of the chronic islet graft dysfunction.

In conclusion, pro-senescent MPs appear as prime effectors of pancreatic tissue damage. The deciphering of the underlying mechanisms of their noxious effects needs further studies *in vitro* and *in vivo* to ensure the development of new drugs or therapeutic strategies to limit either their generation or reprogram their properties thereby promoting a local pharmacological modulation through targeted delivery. Indeed, the mechanisms of MPs interaction and capture by cells as well as their ability to convey active molecules remain poorly known in tissues such as the liver or the pancreas.

Discussion Générale (en Français)

La transplantation d'îlots comme nouveau traitement du diabète de type 1

Le traitement quotidien du diabète de type 1 (DT1), qui consiste en plusieurs injections d'insuline, n'est pas encore suffisant pour contrôler la glycémie. La transplantation d'îlots pancréatiques est une alternative au traitement classique. Elle consiste à greffer l'unité fonctionnelle de la sécrétion d'insuline, directement asservie au contrôle de la glycémie. C'est une thérapie non invasive prometteuse en cas d'instabilité glycémique élevée. Cette technique est une alternative à la transplantation de pancréas tout en étant aussi efficace.

Deux obstacles majeurs posent problème dans la greffe d'îlots. Premièrement, pour permettre la greffe d'une quantité suffisante d'îlots viables, il est important de préserver la qualité et la fonctionnalité des îlots isolés. Deuxièmement, le contact des îlots avec les composants de la veine porte du receveur (endothéliaux et circulants) générent des interactions spécifiques. En effet, le contact des îlots avec le sang porte déclenche une inflammation et des réactions pro-coagulantes connues sous le nom d'IBMIR (Instant Blood Mediated Inflammatory Reaction), principale cause de perte précoce d'îlots.

Dans notre travail, deux éléments principaux de l'impact du vieillissement ont été identifiés :

1- Le vieillissement prématuré du pancréas par rapport à celui du système vasculaire chez les rats d'âge moyen. Les rats d'âge moyen présentaient des signes d'altérations morphologiques et fonctionnelles précoces du pancréas, alors que la fonction vasculaire restait inchangée par rapport aux rats jeunes.

2- Le rôle des MPs endothéliales pro-sénescentes sur la fonction des îlots isolés.

Nos données montrent des processus de vieillissement distincts dans le tissu pancréatique par rapport au compartiment vasculaire. Par ailleurs, les MPs générées au cours de la réaction IBMIR sont suceptibles d'induire un processus de sénéscence prématurée au niveau des cellules de l'îlot greffé. Nos travaux *in vitro* ont montré un effet pro-thrombotique et pro inflammatoire de ces MPs. Ces données suggérent que les MPs-FT^{+} (microparticules porteuses du facteur tissulaire) circulantes libérées à partir de cellules leucocytaires et endothéliales au voisinage du site d'implantation des îlots exercent un effet délétére *in vivo* au cours de la greffe.

Toutefois, le rôle de l'ischémie elle-même sur l'expression du facteur tissulaire par les cellules des îlots est peu connu, tout comme son impact sur l'expression de MPs-FT^{+} et l'action pro-sénescente de ces dernières. En effet, les données du laboratoire ont démontré *in vitro* l'effet prothrombotique nocif des MPs-FT^{+} endocrines en réponse à l'ischémie [426], ainsi que l'action autocrine pro-sénescente et pro-thrombotique exercée par les MPs-FT^{+} endothéliales [347]. Il

est intéressant de noter que les données d'une étude pilote menée aux Hôpitaux universitaires de Strasbourg chez des patients greffés d'îlots indiquent que le rejet des îlots est associé à l'élévation des MPs circulantes d'origine endocrine.

Vieillissement du pancréas et dysfonctionnement des îlots

En raison de l'allongement de la durée de vie et du nombre limité d'organes, l'âge du donneur est déterminant en ce qui concerne la survie et la qualité du greffon, notamment en greffe d'îlots pancréatiques. Le contrôle de la sénescence liée à l'âge est une perspective intéressante pour le pré-conditionnement ce qui permet d'améliorer la fonction du greffon en limitant l'arrêt du cycle cellulaire et en favorisant la réparation cellulaire du greffon. Avec le vieillissement, le système immunitaire subit également une modification progressive du nombre et / ou de la fonction de ses cellules. Ce phénomène, appelé immuno-sénescence, peut affecter le succès de la transplantation.

Dans notre première modèle de rat, nous avons montré que l'âge du donneur a un impact important sur la fonction de l'organe, notamment par le biais de la sénescence et du stress oxydant. En comparant les rats jeunes et d'âge moyen, nous avons observé des modifications morphologiques importantes des îlots pancréatiques, une augmentation de la production de ROS (espèces réactives d'oxygène) et une régulation altérée de la glycémie. Une surexpression des marqueurs de sénescence p53, p21 et p16 a également été observé dans le tissu pancréatique, indiquant une initiation des voies de sénescence. Cependant, la concentration des MPs circulantes dans le sang périphérique, mesurée en tant que substituts de la lésion des cellules vasculaires, est restée inchangé tout comme la fonction vasculaire des anneaux artériels mésentériques. Par conséquent, nous en avons conclu que les altérations du pancréas et des îlots pancréatiques chez les rats d'âge moyen se produisent avant toute dysfonction macro-vasculaire. Il faut noter que nos données ne concernent que le tissu pancréatique entier. Cependant, d'autres groupes ont pu identifier la sénescence des îlots chez les rats d'âge moyen et sa progression chez les rats âgés et ont montré en accord avec nos travaux une augmentation de p16. En effet, l'expression de sénescence par la mesure de l'activité SA-β Gal a été mise en évidence uniquement dans le tissu endocrine [427]. En outre, Tamura et al. ont montré que les télomères des cellules β sont raccourcis, avec un processus de vieillissement normal. Cela conduit à réduire leur nombre, ce qui peut constituer une étape majeure dans l'apparition du DT2 (diabète type 2) [324]. En accord avec ces observations, nous avons également montré en

histologie une diminution de la surface des cellules β chez les rats d'âge moyen, associée à une diminution de la fonction pancréatique.

Au total, nos données montrent que le pancréas est un indicateur précoce de stress oxydant puissant inducteur sénescence qui apparait précocement par rapport au vaisseau (voir figure 1, article 1, données complémentaires). De plus, l'exposition de l'îlot au stress oxydatif supprime l'expression de PDX-1 (Home box pancréatique et duodénale-1) qui est un facteur de transcription clé pour la différenciation des cellules β. Par conséquent, ceci peut participer à l'altération de la sécrétion d'insuline et à la diminution de la capacité des cellules β à se répliquer. En effet, le pancréas pourrait être considéré comme un marqueur précoce du stress oxydatif et de la sénescence chez les rats d'âge moyen. Ces observations soulignent l'importance de l'impact de l'âge du donneur d'îlots précocement sur le succès de la transplantation d'îlots pancréatiques et l'intérêt d'un pré-conditionnement pharmacologique d'une greffe d'îlots. En effet, nous avons mis en évidence le rôle précoce des MPs endothéliales sur l'apparition de la sénescence dans des îlots isolés de rats. D'autres données du laboratoire ont montré que les médicaments immunosuppresseurs ont des effets distincts sur la survie des îlots et peuvent altérer leur fonction [428]. Jusqu'à présent, aucune étude clinique spécifique sur le pré-conditionnement des îlots n'a été menée. Le Liraglutide, un analogue de GLP1, a été étudié comme un traitement complémentaire pour augmenter l'insulino-indépendance chez les patients transplantés d'îlots. Cependant, l'étude s'est terminée en raison du faible nombre de patients inclus dans la cohorte [429]. A côté de la sénescence, les cibles putatives pour le pré-conditionnement des îlots sont nombreuses et incluent les médiateurs du stress oxydatif ainsi que les acteurs pro-inflammatoires et thrombogènes de l'IBMIR.

Revascularisation des îlots et sénescence endothéliale

Revascularisation des îlots et homéostasie tissulaire

La revascularisation des îlots constitue un défi majeur pour assurer la fonction endocrinienne du greffon. Elle dépend de la reconstitution des vaisseaux endothéliaux intra-îlots à partir de trois sources différentes : les cellules du receveur, les cellules du donneur (intra-îlots) qui constituent 7-40% des cellules endothéliales des îlots et une source mineure de cellules progénitrices endothéliales migrant de la moelle osseuse du receveur. Au sein des îlots âgés, les cellules endothéliales sénescentes sont probablement la principale source endogène de cellules endothéliales et contribuent à la production de MPs pro-sénescentes. Il est bien connu que le vieillissement s'accompagne de modifications structurelles et fonctionnelles de la

vascularisation, qui conduisent au dysfonctionnement de l'endothélium et des cellules musculaires lisses. Par conséquent, préserver l'endothélium des îlots de la sénescence accélérée pourrait améliorer le résultat de la transplantation.

En outre, l'altération vasculaire du receveur et l'apparition de l'IBMIR sont deux autres causes de sénescence endothéliale accélérée dans les capillaires hépatiques au voisinage de l'îlot greffé. Le ciblage thérapeutique des médiateurs pro-sénescents tels que les MPs, PAI-1, interleukine et High-mobility group box-1 (HMGB1) par exemple, peut s'avérer bénéfique en permettant une réduction systémique et locale des effets nocifs associés à la sénescence [317].

En particulier, chez les patients DT1 qui ont une fonction endothéliale altérée et des médiateurs inflammatoires circulants élevés, la protection contre la sénescence accélérée devrait améliorer le résultat de la transplantation en réduisant la réponse IBMIR.

Il est à noter que le DT2 conduit également à un état inflammatoire très élevé associé à une croissance des tissus adipeux. Les médicaments sénolytiques pourraient donc être avantageux en retardant l'aggravation du dysfonctionnement des cellules β. A cet égard, des cellules souches mésenchymateuses de moelle osseuse (BM-MSC), connues pour présenter des médiateurs anti-inflammatoires et cyto-protecteurs pourraient être intéressantes comme thérapie cellulaire complémentaire dans la transplantation d'îlots. En fonction de leur niche, ces cellules peuvent se différencier en de nombreux types de cellules tels que les ostéoblastes, les adipocytes mais aussi les cellules d'origine endodermique comme les cellules sécrétrices d'insuline [430]. De plus, les BM-MSCs ont d'autres propriétés, comme par exemple un puissant effet immunosuppresseur [431] et une capacité à libérer des profacteurs angiogéniques [432]. C'est ainsi que la co-transplantation îlots de Langerhans/BM-MSCs s'avère très prometteuse. Dans une étude récente, Cao XK et al. ont appliqué cette nouvelle approche en utilisant l'ingénierie tissulaire. Ils ont constaté que la combinaison d'îlots avec des cellules souches mésenchymateuses de moelle osseuse (BM-MSCs) favorise l'angiogenèse par migration des BM-MSCs vers l'îlot transplanté. Ces cellules se différencient par la suite en cellules vasculaires musculaires lisses (VSMC), cellules vasculaires endothéliales (VEC), et sécrètent le facteur de croissance endothélial vasculaire (VEGF). En outre, ils ont constaté qu'elles amélioraient la tolérance immunitaire des allogreffes d'îlots greffés par augmentation du chimérisme lymphocytaire du donneur [134].

En conclusion, les BM-MSCs améliorent la transplantation en favorisant le développement d'une nouvelle vascularisation insulaire et en limitant les effets cytotoxiques de l'hypoxie. De

plus, les cytokines sécrétées par les BM-MSCs contrôlent localement la différenciation des monocytes auto-réactifs, empêchant ainsi la destruction auto-immune des îlots greffés [431].

Une autre approche permettrait d'assurer une vascularisation rapide des îlots greffés. Elle consiste à enrichir les îlots en cellules endothéliales et à améliorer leur capacité à former des vaisseaux matures et fonctionnels. C'est ainsi que Quaranta et al. ont co-transplanté des îlots pancréatiques avec des progéniteurs endothéliaux chez des rats diabétiques. Ils ont observé une restauration du contrôle glycémique ainsi que l'établissement de novo d'un réseau vasculaire suffisant jusqu'à 6 mois après transplantation [433]. A l'heure actuelle, il y a encore peu de connaissances sur le rôle spécifique des cellules endothéliales intra-îlots, ainsi que sur leur sensibilité au stress oxydatif et la sénescence induite. Plus important encore, l'effet des MPs endothéliales pro-sénescentes piégées à l'intérieur de l'îlot sur la revascularisation reste à explorer complètement (voir le paragraphe suivant).

Sénescence endothéliale

Nos données sont en contradiction avec l'étude récente d'Almaca et al. qui a démontré que la fonction des cellules β ne diminue pas avec l'âge dans un modèle murin [434]. Cependant, leur étude a révélé que les îlots de souris âgées pouvaient exprimaient moins la sénescence après revascularisation dans la chambre interne de l'œil de jeunes souris atteintes de diabète. De plus, les îlots ainsi rajeunis ont permis un contrôle suffisant de la glycémie. En revanche, la transplantation chez les rats diabétiques âgés a conduit à un contrôle limité de la glycémie sur une période de temps plus courte. Cette observation est très révélatrice du rôle des vaisseaux dans la greffe et la fonction des îlots. En effet, les îlots âgés contiennent des cellules endothéliales et sont infiltrés par les macrophages [434] qui sont le siège d'une inflammation et d'une fibrose et qui expriment fortement l'ICAM-1.

L'ensemble de ces données indiquent que la sénescence endothéliale est un processus associé au diabète et que son influence sur lîlots dépend du niveau d'activation de la cellule endothéliale et de la fonction vasculaire. Les MPs endothéliales circulantes ont été revendiquées comme marqueurs du dysfonctionnement endothélial et de la perte de compétence vasculaire dans le diabète [350]. Dans notre travail, nous n'avons pas pu mettre en évidence de variation significative des concentrations des MPs circulantes chez les rats d'âge moyen, bien que les tissus du pancréas présentaient des signes précoces de dysfonctionnement associés aux marqueurs de la sénescence.

Cependant il est fortement probable que la sénescence endothéliale des capillaires hépatiques exerce un rôle sur les îlots en tant que médiateur pro-sénescent au cours de la greffe. En effet,

dans notre deuxième partie du travail, nous avons montré que les MPs endothéliales pro-sénescentes induisent une régulation positive des marqueurs sénescents dans l'îlot isolé et la diminution de la capacité à sécréter de l'insuline en réponse au glucose. La sénescence cellulaire devient donc un domaine de recherche actif qui présente des applications pharmacologiques et thérapeutiques prometteuses dans la transplantation d'îlots.

Le stress oxydatif induit la sénescence et le dysfonctionnement des cellules endothéliales, ce qui fait passer la surface de l'endothélium d'un état vaso-protecteur à un état thrombogène en exposant le FT, les protéines pro-adhésives et les cytokines pro-inflammatoires sécrétrices [347]. À la fin du capillaire hépatique, l'endothélium hautement fenêtré est extrêmement sensible aux ROS et peut donc libérer de fortes concentrations de MPs pro-sénescentes au voisinage de la niche des îlots greffés. Un tel environnement peut s'avérer propice au déclenchement de l'IBMIR et de la coagulation locale, ce qui limitera la perfusion et la survie des îlots (voir Figure 35).

A côté des mesures du peptide C et de l'insuline chez les patients transplantés d'îlots, il est nécessaire de surveiller la fonction et la viabilité des îlots. Notre laboratoire a démontré que les MPs totales circulantes étaient un marqueur prometteur après la transplantation d'îlot et avant la chute de la concentration en peptide C. Cependant, comme les MPs endothéliales véhiculent des protéines pro-sénescentes et pro-thrombotiques (AT1R, ACE, Caspase et FT), on pourrait imaginer que les MP pro-sénescentes pourraient également être intéressantes dans la détection efficace et précoce de la perte des îlots.

Microparticules comme effecteur cellulaire dans la transplantation d'îlots pancréatiques

En plus de leur rôle de biomarqueur de l'homéostasie cellulaire, les MPs agissent comme effecteurs cellulaires par leur capacité à fusionner avec la membrane plasmique des cellules cibles. Cela permet le transfert du contenu de MPs, constitué de protéines actives, de lipides et de récepteurs membranaires, vers une cellule cible. Les effets des MPs peuvent être autocrines ou paracrines. Des études ont montré que les MPs peuvent également transporter et transférer du matériel génétique comme l'ADN, l'ARN et les miARN [354, 355].

Une étude récente de notre laboratoire a confirmé que les MPs pro-sénescentes sont caractéristiques des dommages des tissus. En effet, les patients atteints du syndrome coronarien aigu (ACS) voient leur taux circulant de MPs endothéliales pro-sénescentes augmenter par rapport aux sujets sains. Ces MPs endothéliales pro-sénescentes induisent *in vitro* un phénotype endothélial thrombogénique. Elles expriment notamment le facteur tissulaire, possèdent des

activités ACE (Angiotensin Converting Enzyme) et surexpriment le récepteur AT-1 de l'angiotensine 2. Contrairement à l'activité pro-sénescente des MPs isolées de plaques athérosclérotiques, les MPs des artères mammaires internes restent sans effet. Cela indique que les propriétés des MPs reflètent l'état tissulaire de l'activation cellulaire. [347]. Dans notre étude concernant les rats d'âge moyen, nous avons pu constater que les concentrations des MPs d'origine vasculaire ne variaient pas alors que le stress oxydatif et la sénescence étaient déjà établis dans le pancréas (voir article 1).

Il est à noter que les MPs issues de cellules endothéliales sénescentes induisent un stress oxydant précoce *in vitro*, très probablement par la régulation positive de la NADPH oxydase et de la COX-2, via l'activation des enzymes respiratoires mitochondriales, la régulation négative de l'eNOS en induisant la sénescence de cellules endothéliales à des concentrations pouvant être atteintes pendant le syndrome coronarien aigu De plus, elle favorise la thrombogénicité des cellules endothéliales par augmentation de l'expression et de l'activité du FT et la libération de MPs procoagulantes [435].

Puisque les cellules endothéliales jouent un rôle essentiel dans la revascularisation de l'îlot après la transplantation, nous avons cherché à déterminer si les MPs endothéliales pro-sénescentes induisaient une sénescence prématurée des îlots pancréatiques isolés. De plus, la compréhension des mécanismes sous-jacents pourrait s'avérer intéressante pour le développement de médicaments senolytiques afin de protéger les îlots fonctionnels chez les individus vieillissants ou pour prévenir une perte et un dysfonctionnement accéléré des îlots avant et après la transplantation.

Nos données, décrites dans l'article 2, montrent que les MPs endothéliales pro-sénescentes sont des effecteurs cellulaires paracrines de la sénescence dans les îlots pancréatiques fraîchement isolés. En effet, d'autres cellules comme les cellules endocrines qui constituent la structure fonctionnelle de l'îlot ont été ciblées par les MPs et pas seulement les cellules endothéliales. Ce résultat confirme et complète les données de Burger et al. où ils ont constaté que le traitement par MPs ou H_2O_2 était associé à un passage d'un phénotype proliférant à un phénotype non proliférant, ce qui indiquait que les MPs induisaient une sénescence prématurée dans les cellules endothéliales primaires [226]. Plus important encore, nous avons pu montrer que les MPs pro-sénescentes endothéliales conduisaient à une altération précoce de la fonction des îlots après 24 h, alors que la viabilité n'était pas affectée. Nous avons clairement montré que les MPs s'intègrent facilement après 24h dans l'îlot (MPs-P1 56 ± 2%, MPs-P3 27 ± 1,8%) et que malgré

une intégration plus faible dans les cellules cibles, les MPs-P3 pro-sénescentes étaient très nocives.

Cependant, bien que le transfert d'informations véhiculées par les MPs-P3 puisse être prouvé par un test fonctionnel, il reste à identifier quelle molécule et quelles voies sont responsables de leur effet pro-sénescent. Une autre cible pharmacologique dans la protection du vieillissement cellulaire accéléré serait l'inhibition de la génération secondaire de MPs pro-sénescents faisant émerger de nouvelles thérapeutiques visant à moduler la translocation PhtdSer du feuillet intérieur vers l'extérieur de la membrane plasmique. Par exemple, la sécrétion de MPs pourrait être inhibée par l'action de l'antagoniste de TMEM16F qui supporte le scramblase des phospholipides dépendant de Ca^{2+} [436].

Microparticules en transplantation

La réaction IBMIR est une forme d'ischémie-reperfusion qui conduit à l'activation des cellules endothéliales, plaquettaires et leucocytaires. On peut ainsi émettre l'hypothèse que, suite à cette réaction, ces cellules produisent une grande quantité de MPs pro-coagulantes portant le facteur tissulaire avec d'éventuelles propriétés pro-oxydantes et pro-sénescentes. De façon intéressante, les îlots transplantés peuvent donc être ciblés par des MPs endothéliales qui proviennent de 2 territoires différents: les cellules endothéliales du foie, extrêmement sensibles au stress oxydatif, et les cellules endothéliales intra-îlots déjà soumises à l'ischémie du fait de la procédure de l'isolement [437].

Il est clair que la mesure des MPs circulantes dans le sang périphérique pourrait devenir un outil non invasif utile pour la surveillance du dysfonctionnement de la transplantation, surtout lorsque les biopsies sont non informatives ou dangereuses (voir paragraphe VII-6). Cependant, la question des seuils définissant un risque de rejet cellulaire ou humoral doit être déterminée pour chaque type de transplantation. Une nouvelle approche pour la détermination de ces seuils dans les îlots pancréatiques pourrait être la constitution d'un score incluant l'origine de diverses MPs. Dans le cas de la transplantation d'îlots pancréatiques, les MPs sont d'origine endocriniennes et dérivées de foie combinés à CD8 / CD4 / CD20. On pourrait également s'intéresser à la fois à l'origine et au contenu pro-sénescent des MPs.

Le double visage des MPs: bon ou mauvais?

En plus d'être des biomarqueurs des dommages vasculaires et des greffons, les MPs sont généralement considérées comme des éléments nocifs, en raison de leurs propriétés pro-

coagulantes, pro-inflammatoires et pro-oxydantes et de leur effet inhibiteur sur la réparation vasculaire.

En effet, les MPs ont été démontrés comme étant des biomarqueurs du risque cardiovasculaire primaire et secondaire [438]. De plus, des taux élevés de MPs pro-coagulantes d'origine endothéliale et leucocytaire ont été associés à une coagulopathie intravasculaire disséminée (DIC) chez un patient septique [439]. Des données récentes provenant d'études *in vitro* ont montré l'effet bénéfique de certaines sous-populations de MPs endothéliales qui pourraient moduler les mécanismes de mort cellulaire, stimuler la réparation vasculaire et les activités cytoprotectrices médiées par la protéine C activée (APC) [440].

En effet, dans des circonstances pathologiques aiguës, les MPs endothéliales ont d'abord été considérées comme des transporteurs d'informations biologiques délétères pour la fonction endothéliale menant à la DIC et à la mort cellulaire. Des travaux réalisés au laboratoire ont montré l'intérêt thérapeutique potentiel des MPs par le transfert de l'activité protéine C activée à des cellules cibles via la présence d'EPCR (le récepteur d'APC). De plus, l'injection des MPs porteuses d'APC à des rats septiques naïfs a modifié la réactivité vasculaire chez le receveur et réduit l'inflammation dans les tissus cardiaques et vasculaires [441, 442]. De plus, d'autres groupes ont démontré que les effets cyto-protecteurs de l'APC sont également véhiculés par les MPs endothéliales porteuses d'APC. Cela inhibe la libération de MPs procoagulantes et diminue l'apoptose des cellules endothéliales [356].

Les MPs peuvent dans certaines conditions exercaient des effets bénéfiques, comme leur action proliférative qui facilite la réparation des tissus. Les MPs endothéliales stimulent le processus angiogénique. Cette stimulation peut être nocive, comme dans le développement des cancers et la rétinopathie diabétique proliférative, mais peut aussi être bénéfique, comme c'est le cas dans la réparation vasculaire des tissus ischémiques [443]. Cantalupi et al. ont montré que les microvésicules dérivées de cellules progénitrices endothéliales qui contiennent également des MPs peuvent protéger le rein contre une lésion aiguë ischémique [221].

Ensemble, ces données soulignent la nécessité de développer de nouveaux modèles *in vivo* pour étudier le rôle des MPs dans les tissus et les fluides afin de comprendre comment elles contribuent à l'homéostasie des organes. Les perspectives de recherche devront s'intéresser aux rôles physiopathologiques des MPs, aux mécanismes de contrôle de vésiculation endothéliale et de libération des MPs. Il faudra également décoder les mécanismes de liaison des MPs aux cellules cibles et réussir à moduler de manière pharmacologique les mécanismes qui contrôlent la génération des MPs.

Conclusions et Perspectives (En Français)

En conclusion, toutes nos données suggèrent que le pancréas est un organe précocement sensible à la sénescence liée à l'âge. Il est également indiqué qu'au moins dans le modèle de rat, le pancréas est un indicateur précoce de stress oxydatif avant tout signe de lésion vasculaire. Nos données suggèrent que les individus en bonne santé d'âge moyen présentent des îlots morphologiquement altérés, des signes précoces de dysfonction pancréatique et de la sénescence. Ces éléments sont très importants à prendre en considération dans la transplantation de pancréas ou d'îlots où l'âge des donneurs est de plus en plus élevé.

De plus, la deuxième partie de notre étude a montré un effet délétère des MPs pro-sénescentes endothéliales (P3) sur la fonctionnalité de l'îlot pancréatique de rat. Parallèlement à cette observation, nous avons montré une augmentation de la sénescence cellulaire, éventuellement liée à un effet paracrine des MPs endothéliales. Ces données sont fortement en faveur de l'existence d'un rétro-contrôle négatif entre les cellules endothéliales et les cellules des îlots pancréatiques via les MPs endothéliales pro-sénescentes. En effet, l'effet délétère des MPs pro-sénescentes a été clairement mis en évidence par nos études *ex vivo*. Inhiber un tel effet pourrait s'avérer bénéfique pour la greffe d'îlots. Tous ces résultats ont montré que la sénescence cellulaire liée à l'âge est un facteur à prendre en compte dans la transplantation mais aussi comme étant un processus contributif de l'ischémie-réperfusion qui induit des dommages vasculaires et des dysfonctionnements, comme observé au cours de l'IBMIR.

De plus, nos données suggèrent que le pré-conditionnement de l'îlot pourrait améliorer le succès du greffon en limitant le dysfonctionnement lié à l'âge dans l'organe du donneur avant son implantation. Nos résultats ont également permis une meilleure compréhension de la manière dont le vieillissement affecte la fonction du pancréas. Les résultats de cette étude donnent ainsi des informations sur la physiopathologie du pancréas et constituent une base solide pour le développement de stratégies thérapeutiques permettant d'améliorer la transplantation d'îlots pancréatiques.

Par la suite, d'autres études *ex vivo* et *in vitro* seront réalisées afin de développer un modèle de transplantation d'îlots pancréatiques chez les animaux diabétiques. Par exemple, le modèle de sénescence des îlots que nous avons développé n'a pas encore été étudié en présence des composants sanguins. Ceci pourrait être réalisé en appliquant le modèle de boucle fermée « tubing loop » rapportée par Akima et al. [444]. L'effet pharmacologique des médicaments initialement étudiés *in vitro* devra être évalué *in vivo*. Pour cette dernière étape, le modèle de greffe intraportale dans le lobe de Spiegel chez le rat est une technique de référence qui permet

d'étudier l'implantation d'îlots dans une zone restreinte du foie. Cette technique a l'avantage d'utiliser une quantité de MPs moins importante que dans les modèles de greffe d'îlots dans le fois entier. Ces MPs, qui peuvent être identifiées par des marqueurs spécifiques, pourraient être administrées en tant que modulateurs de la fonction des îlots par voie intraveineuse ou dans la chambre oculaire, comme récemment proposé dans l'étude du rôle du système vasculaire sur le rajeunissement des îlots âgés chez le jeune receveur [434].

Le pré-conditionnement pharmacologique des îlots pancréatiques a été peu étudié dans des modèles animaux. Cependant, l'APC qui est une protéine anticoagulante récemment reconnue comme un ligand activant les récepteurs PAR-2 à médiation anti-inflammatoire et anti-apoptotique, pourrait contribuer à la protection des îlots. En effet, chez des souris génétiquement modifiées qui surexpriment le récepteur à l'aPC (EPCR), la fonction des îlots greffés a été préservée [445]. De plus, on a montré que l'aPC elle-même protégeait des îlots isolés *ex vivo* dans un modèle de tubing loop contenant du sang, t indiquant ainsi son utilité pour empêcher la réaction IBMIR [444].

De même, la thrombomoduline, une autre protéine endothéliale anticoagulante qui est capable de déplacer l'action procoagulante de la thrombine vers des propriétés anti-coagulantes, par l'activation de la protéine C et qui initie l'inhibition du rétro-contrôle de la production de thrombine, a récemment été identifiée comme un outil intéressant pour préserver la fonction des îlots chez les souris diabétiques transplantés [446]. En effet, lorsqu'elle est administrée par voie intraveineuse sous la forme d'une suspension liposomique, la thrombomoduline s'accumule préférentiellement dans le foie, facilitant ainsi l'inhibition de l'IBMIR.

Une autre forme de pré-conditionnement pourrait être le ciblage des floppases qui favorisent la libération des MPs. Plusieurs candidats potentiels ont déjà été identifiés comme cibles d'inhibiteurs spécifiques. Il s'agit notamment du TMEM16F, ainsi que d'autres scramblases vraies ou occasionnelles. D'autre part, l'annexine V pourrait être utilisée pour constituer un bouclier protecteur autour des cellules activées, ce qui contrebalançait le processus de libération de MPs. Ce type de ciblage de PhtdSer pourrait être bénéfique pendant les réponses cellulaires aiguës comme observé pendant la réaction IBMIR, ou inversement dans l'activation cellulaire progressive et la libération de MPs observées pendant la sénescence.

Notre travail a montré un effet néfaste des MPs endothéliales pro-sénescentes sur la fonction des îlots. Ainsi, les médiateurs de la sénescence, tels que les protéines du système local de l'angiotensine ou encore les protéines impliquées dans la génération de ROS, pourraient être

des cibles potentielles de nouvelles stratégies de protection des îlots transplantés.

En conclusion, les MPs pro-sénescentes apparaissent comme les principaux effecteurs des lésions des tissus pancréatiques. Les mécanismes d'interaction et de capture des MPs par les cellules, ainsi que leur capacité à véhiculer des molécules actives restent mal connus dans des tissus tels que le foie ou le pancréas. La compréhension des mécanismes sous-jacents et de leurs effets nocifs nécessite de nouvelles études *in vitro* et *in vivo* pour assurer le développement de nouveaux médicaments ou stratégies thérapeutiques. Les objectifs de ces traitements seraient de limiter leur génération, reprogrammer leurs propriétés, favorisant ainsi une modulation pharmacologique locale par une administration ciblée.

Communications and Publications

➢ *Communications*

❖ Oral Communications

- **KASSEM M**, NIAZI Z, ABBAS M, SAMAMA B, AUGER C, CONSTANTINESCU A, VAUCHELLES R, LESSINGER JM, BOEHM N, SCHINI-KERTH V, TOTI F, KESSLER L. Influence de la sénescence endothéliale sur le devenir de l'îlot pancréatique chez le rat : rôle de l'âge du donneur et impact sur la fonction du greffon, XXIème Journée Annuelle Association EST-TRANSPLANT, Mardi 24 novembre 2015, **Strasbourg-France**

- **KASSEM M**, ABBAS M, KREUTTER G, El HABHAB A, AUGER C, BOEHM N, SCHINI-KERTH V, TOTI F, KESSLER L, Early signs of endocrine pancreas abnormalities and senescence in middle age rats with normal vascular function: impact for pancreatic islet transplantation. SFT (Société Francophone de Transplantation), 1-4 Décembre 2015**, Lille-France**. Transplant. Int. February 2016 Volume 29, Issue Supplement S1 Pages 1–24.

❖ Commentary posters

- **KASSEM M**, NIAZI Z, ABBAS M, SAMAMA B, AUGER C, CONSTANTINESCU A, VAUCHELLES R, LESSINGER JM, BOEHM N, SCHINI-KERTH V, TOTI F, KESSLER L. Morphological and functional abnormalities of the endocrine pancreas in middle age rat: Impact for transplantation, CPBI (Congrès de la Société de Physiologie et de Biologie Intégrative) 4-6 May 2015, **Strasbourg-France**. Acta Physiologica Volume 214, Issue Supplement S700, Pages 1-92, May 2015.

- **KASSEM M**, NIAZI Z, ABBAS M, VAUCHELLES R, AUGER C, ANTAL MC, SCHINI-KERTH V, TOTI F, KESSLER L, The Endocrine Pancreas, an Early Sensor of Senescence in Middle-Aged Rats: Impact for Transplantation. ADA (American Diabetes Association), 10-14 Juin 2016, **New Orleans, LA-USA.**

❖ Posters

- **KASSEM M**, NIAZI Z, ABBAS M, SAMAMA B, AUGER C, CONSTANTINESCU A, VAUCHELLES R, LESSINGER JM, BOEHM N, SCHINI-KERTH V, TOTI F, KESSLER L. Morphological and functional abnormalities of the Endocrine pancreas in middle age rat: Impact for transplantation, JCI (Campus Illkirch day) 13-14 April 2015, **Illkirch-France**.

- **KASSEM M**, NIAZI Z, ABBAS M, SAMAMA B, AUGER C, CONSTANTINESCU A, VAUCHELLES R, LESSINGER JM, BOEHM N, SCHINI-KERTH V, TOTI F, KESSLER L. Morphological and functional abnormalities of the Endocrine pancreas in middle age rat: Impact for transplantation, FMTS (Fédération de Médecine Translationnelle de Strasbourg) 16-17 April 2015, **Strasbourg-France**.

- **KASSEM M**, El HABHAB A, KREUTTER G, ABBAS M, AUGER C, BOEHM N, SCHINI-KERTH V, TOTI F, KESSLER L, Anomalie morphologique et senescence précoces du pancréas endocrine des rats d'âge moyen avec fonction vasculaire normale. SFD (Société Francophone du Diabète), 22-25 Mars 2016, **Lyon-France**. Diabetes et metabolism 2016 Vol 42 - Supplément 1 - mars 2016.

➢ ***Publications***

❖ Papers associated with the subject

- **KASSEM M,** NIAZI Z, ABBAS M, EL-HABHAB A, KREUTTER G, BENKHIAT S, ANTAL MC, AUGER C, SCHINI- KERTH V, TOTI F, KESSLER L. Senescence of Pancreas in Middle-aged Rats with Normal Vascular Function (Accepted 20 December 2016 in the journal Annals of Transplantation).

- **KASSEM M,** KREUTTER G, BLATZINGER P, EL HABHAB A, ABBAS M, SCHINI-KERTH V, KESSLER L, TOTI F. Impact of pro-senescent endothelial Microparticles on rat pancreatic islet (in preparation)

❖ Contributions to the research team's work

- GLEIZES C, KREUTTER G, ABBAS M, **KASSEM M**, CONSTANTINESCU A, BOISRAME-HELMS J, YVER B , TOTI F, KESSLER L. β cell membrane remodeling and procoagulant events occur in inflammation-driven insulin impairment: A GLP-1 receptor dependent and independent control. J Cell Mol Med. 2016 Feb; 20(2):231-42.

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Annexes

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β cell membrane remodelling and procoagulant events occur in inflammation-driven insulin impairment: a GLP-1 receptor dependent and independent control

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Abstract

Inflammation and hyperglycaemia are associated with a prothrombotic state. Cell-derived microparticles (MPs) are the conveyors of active procoagulant tissue factor (TF) and circulate at high concentration in diabetic patients. Liraglutide, a glucagon-like peptide (GLP)-1 analogue, is known to promote insulin secretion and β-cell preservation. In this *in vitro* study, we examined the link between insulin impairment, procoagulant activity and plasma membrane remodelling, under inflammatory conditions. Rin-m5f β-cell function, TF activity mediated by MPs and their modulation by 1 μM liraglutide were examined in a cell cross-talk model. Methyl-β-cyclodextrine (MCD), a cholesterol depletor, was used to evaluate the involvement of raft on TF activity, MP shedding and insulin secretion as well as Soluble N-éthylmaleimide-sensitive-factor Attachment protein Receptor (SNARE)-dependent exocytosis. Cytokines induced a two-fold increase in TF activity at MP surface that was counteracted by liraglutide. Microparticles prompted TF activity on the target cells and a two-fold decrease in insulin secretion *via* protein kinase A (PKA) and p38 signalling, that was also abolished by liraglutide. Large lipid raft clusters were formed in response to cytokines and liraglutide or MCD-treated cells showed similar patterns. Cells pre-treated by saturating concentration of the GLP-1r antagonist exendin (9-39), showed a partial abolishment of the liraglutide-driven insulin secretion and liraglutide-decreased TF activity. Measurement of caspase 3 cleavage and MP shedding confirmed the contribution of GLP-1r-dependent and -independent pathways. Our results confirm an integrative β-cell response to GLP-1 that targets receptor-mediated signalling and membrane remodelling pointing at the coupling of insulin secretion and inflammation-driven procoagulant events.

Keywords: insulin • β cell • microparticles • tissue factor • lipid raft • exocytosis • ion channels • GLP-1 receptor

Introduction

In diabetes patients, MPs, that are surrogates of cell activation, were reported to circulate at high concentration, even in well-controlled type 2 diabetes (T2DM) patients [1–4]. Microparticles are submicron fragments of the plasma membrane released in biological fluids and in the peri-cellular environment under conditions of metabolic or apoptotic stress [5, 6]. The release of MPs is prompted by a drastic plasma membrane remodelling and the translocation of anionic phospholipids from the inner to the outer leaflet. Microparticles contain a broad array

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of imbedded active proteins and therefore act as cellular effectors through the delivery of biological signals to target cells. In the vessel, MPs support coagulation owing to the exposure of the anionic phospholipid phosphatidylserine (PhSer) and to the presence of active TF [7, 8]. Tissue factor is the membrane initiator of coagulation and controlled by an early responsive gene, the expression of which is induced under pro-inflammatory conditions, mostly in endothelial and monocyte cells [9]. Highly procoagulant MPs of endothelial origin and conveying active TF are detected in patients with diabetes [10], and were associated with prothrombotic state [11–13]. In stimulated cells, TF activity at cell surface is potentiated by the exposed PhSer. Lipid rafts are dynamic cholesterol-enriched microdomains that contribute to TF activity and its regulation by ensuring the spatial clustering of TF and exposed PhSer [14–16]. Relationships between lipid rafts and insulin secretion have been reported in studies [17–20], describing the regulation of ion channels and exocytosis, particularly *via* raft-embedded SNARE proteins [21, 22].

Liraglutide is a GLP-1 analogue that belongs to the incretinomimetics class of drugs. In the treatment of T2DM, the beneficial effects of liraglutide rely on their ability to improve glycemic control, insulin secretion and promote β-cell survival [23–25]. In a previous work, we have shown that Liraglutide decreases TF activity measured at β-cell surface and reduces MPs shedding under oxidative and cytokine stress conditions [26].

In the present work, we investigated the role of TF-bearing MPs on the impairment of insulin secretion by Rin-m5f β cells, submitted to prolonged hyperglycaemic conditions and pro-inflammatory stress. Because MP shedding is the consequence of membrane remodelling and TF activity is potentiated by PhSer translocation across the membrane as well as raft concentration, we investigated the effect of liraglutide and raft disruption on TF activity and insulin secretion. The incidence of the GLP-1 receptor (GLP-1r) signalling was investigated using exendin (9-39), a GLP-1r antagonist.

Materials and methods

Cell culture

Rat β cells, Rin-m5f (CRL-11605™; ATCC, Manassas, VA, USA), were seeded at 125,000 cells/cm^2 in RPMI 1640 medium (PAN™ Biotech GmbH, Aidenbach, Germany) containing 4.5% glucose, 10 mM HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) 2 mM glutamine, 1 mM sodium pyruvate and supplemented with 10% foetal bovine serum (Gibco, Saint Aubin, France) and 20 μg/ml gentamycine (Lonza, Basel, Switzerland). Cells were cultured at 37°C and 5% CO_2 in a humidified atmosphere.

Cellular models of stress and pharmacological modulation

Rin-m5f were chosen as an adequate model for the study of the β-cell response to prolonged inflammation and hyperglycaemia, submitted to 24–48 hrs cytokine and oxidative stress. Indeed Rin-m5f are not responsive to a short metabolic raise by glucose stimulation, but develop apoptosis after prolonged exposure to H_2O_2 [26]. Stress was applied when cells reached 70% of confluence as reported elsewhere [27]. Inflammatory stress was induced by a 24 hrs treatment with the combination of 50 U/ml of IL-1β (Sigma-Aldrich, St. Louis, MO, USA) and 1000 U/ml of TNF-α (Sigma-Aldrich), further referred to as 'cytokines' throughout the manuscript. Cytokine effects were compared to those prompted by H_2O_2 application, a well-established treatment leading to Rin-m5f dysfunction. Oxidative stress was induced by 100 μM H_2O_2 in fresh medium during 6 hrs. Cell supernatants were collected at the end of each stress procedure and kept at 4°C until measurement.

Pharmacological inhibition of PKA was achieved by pre-treatment with 10 μM H89 during 30 min. before 24 hrs incubation with MPs. Inhibition of K^+-ATP channels and Ca^{2+} channels was performed by continuous exposure to 10 μM Amlodipine and 0.25 mM Diazoxide, for the cytokine or H_2O_2 respective incubation times. In all experiments, liraglutide (Novo Nodisk, Bagsvaerd, Denmark) was added at the concentration of 1 μM as proposed by other investigators [28–31].

Insulin measurement

Insulin released in the supernatant after 24 hrs, was assessed by ELISA assay with the matrix solution, according to supplier recommendations (ELISA Kit Rat/Mouse Insulin; Millipore, Molsheim, France).

MP generation, harvest, and quantification

Microparticles were harvested from the supernatants of stimulated cells under sterile conditions 24 hrs after the initiation of the cytokine or H_2O_2 treatment (see above and as described elsewhere [26]). Detached cells and debris were discarded by differential centrifugation steps and MPs washed in HBSS and concentrated by two-centrifugation steps (13,000 × g, 1 hr) and kept at 4°C for not more than 3 weeks.

Total MP concentration was determined by prothrombinase assay as previously described [26]. Briefly, MP captured onto insolubilized Annexin-5 were incubated with blood clotting factors (FXa, FVa, FII) and $CaCl_2$ [32]. Conversion of prothromobin to thrombin was revealed by chromogenic substrate, using a spectrophotometric reader at 405 nm. Results were expressed as nanomolar PhtdSer equivalent (nM PhtdSer eq.) by reference to a standard curve constructed using liposomes of known concentration and PhtdSer eq. proportion [33].

MP-mediated cell cross-talk

Microparticles generated by oxidative stress (MPox) and by cytokine stress (MPcyt) were applied to naïve Rin-m5f cells (70% confluence) at a final concentration of 10 nM PhtdSer eq. during 24 hrs. In some experiments, 1 μM liraglutide was added to the cell medium and isolated MPs could be pre-incubated with an antibody to tissue factor (HTF-1, kind gift of Prof. N. Mackmann, Chapel Hill, USA).

Measurement of TF activity

After 6 hrs stimulation, TF activity was measured in supernatants and at the surface of washed target cells through its ability to promote the

activation of factor X (150 nM; Hyphen Biomed, Neuville-sur-Oise, France) by factor VII(a) (5 nM; Novoseven, Hillerød, Denmark). The reaction was allowed to proceed for 15 min. at 37°C, 0.1 mM CS11, a chromogenic substrate for factor Xa (Hyphen Biomed, Neuville-sur-Oise, France), were added and absorbance recorded at 405 nm (65). Results were expressed as fM TF activity per 50,000 living cells by reference to a standard curve established with known amounts of highly purified, lipidated recombinant human tissue factor (ADF Biomedical, Neuville-sur-Oise, France).

Western blot analysis

After treatment, cells were washed twice with PBS and then lysed in TRIS (trishydroxyméthylaminométhane) buffer containing protease inhibitors (5 μg/ml leupeptin, 5 mM benzamidine) and 2% Triton® X-100 on ice. Total proteins (30 μg) were separated by electrophoresis on 10% SDS-polyacrylamide (Sigma-Aldrich) gels as previously described [34]. Blotting membranes were incubated with the different primary antibodies directed against rat-phosphorylated p38 (1:1000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat-cleaved caspase 3 (1:1000 dilution; Cell Signaling Technology, Danvers, MA, USA) and rat GLP-1 receptor (1:1000 dilution; Alomone Labs, Jerusalem, Israel), overnight at 4°C. Detection of β-tubulin was used for normalization. After washing, membranes were incubated with the secondary antimouse IgG antibody (1:10,000 dilution; Cell Signaling Technology) at room temperature for 60 min. Pre-stained markers (Invitrogen™, Carlsbad, CA, USA) were used for molecular mass determinations. Immunoreactive bands were detected by enhanced chemiluminescence (GE Healthcare, Amersham, UK). Density analysis was performed with ImageQuant LAS 4000 imager (GE Healthcare).

TF labelling

Cells were submitted to both stress for1 hr up to 8 hrs, washed, fixed with Fix and Perm® (Sigma-Aldrich), and kept at 4°C before incubation with FITC-conjugated (Fluorescein isothiocyanate) rabbit anti-rat TF (dilution: 1:50; Life Science), Saint Louis, MO, USA during 30 min. in darkness. Tissue factor expression-associated green fluorescence was quantified by flow cytometry (FACS-scan cytometer; Becton Dickinson, San José, CA, USA) set at logarithmic gain. Around 10,000 events were recorded for each sample.

Raft labelling

Cells were cultured in eight-well culture chambers (Sarstedt, Numbrecht, Germany) and pre-treated for 1 hr with 10 mg/ml of MCD before application of H_2O_2 (1 hr) or cytokines (4 hrs) and with continuous treatment by MCD or by Liraglutide. After treatment, cells were washed, fixed and kept at 4°C before labelling with 2 μg/ml of biotinylated subunit B of toxin cholera (Sigma-Aldrich) for 30 min., washing and labelling with streptavidine-phycoerythrine (Sigma-Aldrich) for 30 min. After washing and strip mounting, cells were observed by fluorescent confocal microscopy. Insulin secretion and MP shedding were assessed in harvested supernatant. In some experiments TF activity was measured at the surface of unfixed cells.

Insulin exocytosis blockage and labelling

Rin-m5f were cultured on eight-well culture plates. Tetanus toxin (20 nM) was added during 30 min. in a depolarization medium prepared from RPMI medium, to enable toxin internalization [35]. Supernatant was withdrawn, fresh medium added and oxidative or cytokine stress applied during 6 hrs. Cells were washed, fixed and permeabilized using Fix and Perm® and kept at 4°C. After 3 washes, guinea pig anti-rat insulin antibody (dilution: 1:100; Abcam, Cambridge, UK, 30 min., RT) was applied. Washed cells were incubated with FITC-goat anti-Guinea pig IgG (dilution: 1/130; Abcam, 30 min., RT). Control conditions consisted of the labelled unstimulated or stimulated cells incubated with the secondary antibody (data not show). After washing and strip mounting, cells were observed by fluorescent confocal microscopy. The proportion of cells exhibiting normal pattern of exocytosis was counted and expressed as per cents of total cells.

Blockade of the GLP-1r

Cells were pre-treated for 1 hr with 200 nM of the GLP-1r antagonist, exendin fragment (9-39) (Sigma-Aldrich) [36]. It was previously verified that 200 nM exendin led to maximal inhibition of insulin secretion by Rin-m5f after 24 hrs and 48 hrs incubation. The supernatant was withdrawn and exendin (9-39) continuously applied with either cytokines or H_2O_2 in fresh medium during 6 hrs.

Statistical analysis

Data are expressed as mean ± S.E.M. and analysed using GraphPad Prism5 (GraphPad Software, La Jolla, California, USA)®. Statistical analysis between two groups was carried out using unpaired Student's *t*-test. A $P < 0.05$ was considered significant. Experiments were performed in triplicate.

Results

Microparticles released in response to oxidative and cytokine stress carry active TF and liraglutide reduces MP-prompted TF activity

Compared to MPs shed from untreated cells, MPs released after oxidative and cytokine treatment bore highly active TF (MPox: 247.7 ± 1.6 fM/50,000 cells and MPcyt: 63.3 ± 5.9 fM/50,000 cells *versus* 37.6 ± 2.4, $P < 0.0001$ and $P = 0.003$ respectively). Incubation of the cells with liraglutide prevented the generation of TF activity in the supernatant with a significant two-fold decrease in TF activity, regardless of the nature of the stress (MPox: 175.0 ± 15.4 fM/50,000 cells, $P = 0.01$ and MPcyt: 38.0 ± 3.9 fM/50,000 cells, $P = 0.008$, Fig. 1A). Microparticles generated by oxidative stress collected in the supernatant from cells treated by H_2O_2 were also able to prompt TF activity at the surface of naïve target cells (from 149.6 ± 13.3 fM/50,000 cells to 285.0 ± 14.3 fM/50,000 cells; $P < 0.0001$) that was reduced in the presence of Liraglutide, TF activ-

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ity decreasing to 185.1 ± 10.8 fM/50,000 cells ($P = 0.0001$, Fig. 1B).

Liraglutide prevents the impairment of insulin secretion induced by TF$^+$-MPs

Microparticles also behaved as cellular modulators of the insulin production, concentrations of insulin being significantly reduced in the supernatants of cells treated by MPox or MPcyt (MPox: 28.8 ± 2.1 ng/ml/50,000 cells, MPcyt: 39.1 ± 0.3 ng/ml/50,000 cells *versus* 52.1 ± 1.8 ng/ml/50,000 cells, $P < 0.0001$ and $P = 0.02$ respectively). Liraglutide counteracted the MP-driven impairment of insulin secretion and prompted a high yield of insulin secretion similar to that observed in control cells (MPox: 88.3 ± 6.0 ng/ml/50,000 cells, MPcyt: 58.2 ± 1.4 ng/ml/50,000 cells, Fig. 2A). Nevertheless, liraglutide was more efficient in MPox-treated cells (fourfold yield for MPox, 30% yield for MPcyt).

Pre-treatment of MPs by an anti-TF antibody before incubation with target cells prevented the MP-driven drop in insulin secretion, concentrations in supernatant being significantly increased from 26.8 ± 3.6 ng/ml/50,000 cells to 89.6 ± 4.8 ng/ml/50,000 cells in MPox-treated cells ($P < 0.0001$) and from 38.6 ± 2.2 ng/ml/50,000 cells to 49.7 ± 3.7 ng/ml/50,000 cells in MPcyt-treated cells ($P = 0.02$; Fig. 2B). These data indicate a contribution of the active TF borne by MPs to the target cell response. Addition of H89, a PKA inhibitor, to MP-treated cells led to an approximate 30% decrease in insulin secretion that was completely reversed by liraglutide, regardless of the stress condition (Fig. 2C) suggesting a MP-driven alteration of the PKA-dependent response of β cell. Western blots of MP-targeted cell lysates also indicated an elevated phosphorylation of p38, a MAP Kinase involved in the regulation of insulin secretion and inflammatory MP release, that was also limited by liraglutide (Fig. 2D).

Liraglutide does not modify the expression of TF at cell membrane

Because the enhanced TF activity at cell and MP surface could be the result of an up- regulation of TF expression, the kinetics of TF exposure was examined under both stress conditions. After 1 hr oxidative stress, TF expression at cell surface was dramatically elevated (from 11.5 ± 0.2 MFI a.u. in untreated cells to 26.1 ± 0.2 MFI a.u. in 1 hr-treated cells $P < 0.0001$) and remained significantly higher than baseline thereafter (Fig. 3A). Optimal expression of TF was observed after 4 hr exposure to cytokines (up to 24.5 ± 0.9 MFI a.u. in 4 hr-treated cells *versus* 11.5 ± 0.2 MFI a.u. in untreated; $P = 0.001$) and TF membrane expression returned to baseline after 8 hrs (Fig. 3B). No effect of liraglutide could be observed whatever the stress (Fig. 3A and B). These data indicate that liraglutide does not modify the expression and exposure of TF, but only alters its activity.

Raft integrity is targeted by liraglutide in stimulated cells and is critical to MP release, TF activity and insulin secretion

Treatment of Rin-m5f with MCD completely abolished cellular TF activity (Fig. 4A) and MP shedding under both stress conditions (Fig. 4B). In addition, raft disruption restored insulin secretion (7.2 ± 0.2 ng/ml/50,000 cells in H_2O_2-treated cells *versus* 12.9 ± 0.06 ng/ml/50,000 cells in MCD-H_2O_2-treated cells, $P = 0.002$; 10.1 ± 0.5 ng/ml/50,000 cells in cytokine-treated cells

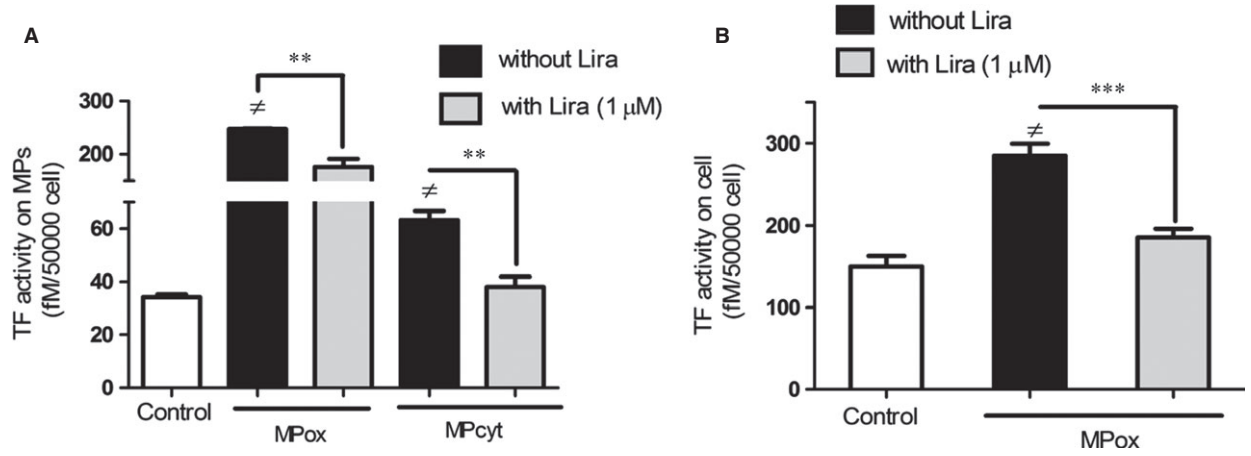

Fig. 1 Liraglutide decreases TF activity borne by MPs (**A**) and on target cells (**B**). (**A**) MPs were harvested from supernatants of H_2O_2 or cytokine-treated cells incubated in the presence (grey bars) or not (black bars) of Liraglutide (Lira). The TF activity was assessed by Tenase assay. (**B**) 10 nM MPox were applied to naïve Rin-m5f cells in the presence or absence of Lira. Empty bars: unstimulated cells. Data normalized as fM TF per 50,000 cells and expressed as mean ± S.E.M. (MPox, MPcyt: MPs produced by H_2O_2 or cytokine stimulation; $n = 6$ ≠: *versus* unstimulated cells; $^{**}P < 0.01$, $P < 0.0001$,).

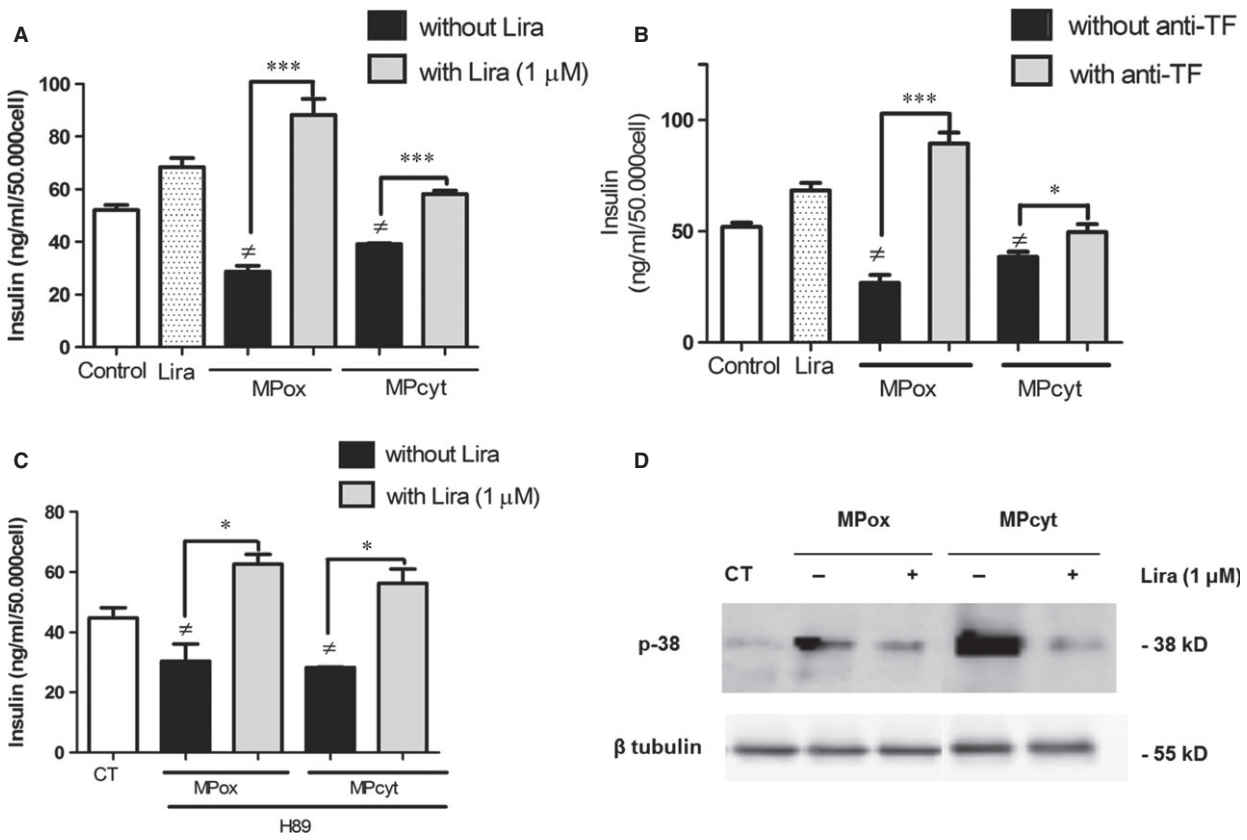

Fig. 2 Liraglutide prevents the TF^+-MP-mediated impairment of insulin secretion (**A** and **B**) through PKA (**C**) and MAP Kinase p38 signalling (**D**). (**A**) Rin-m5f were incubated with 10 nM MPox or MPcyt in the presence (grey bars) or absence (black bars) of liraglutide (lira) during 24 hrs. (**B**) 10 nM MPs were pre-treated with anti-TF (grey bars) or irrelevant antibody (black bars). Secreted insulin was measured in the supernatant. (**C**) Cells were pre-treated with H89, washed and submitted to 10 nM MPox or MPcyt in the presence or absence of Lira. (**D**) Western blot of MPox- and MPcyt-treated cells lysates. Empty bars: unstimulated cells. Data expressed as mean ± S.E.M. ($n = 4$; ≠: *versus* unstimulated cells; MPox, MPcyt: MP produced by H_2O_2 or cytokine stimulation $*P < 0.05$, $***P < 0.0001$).

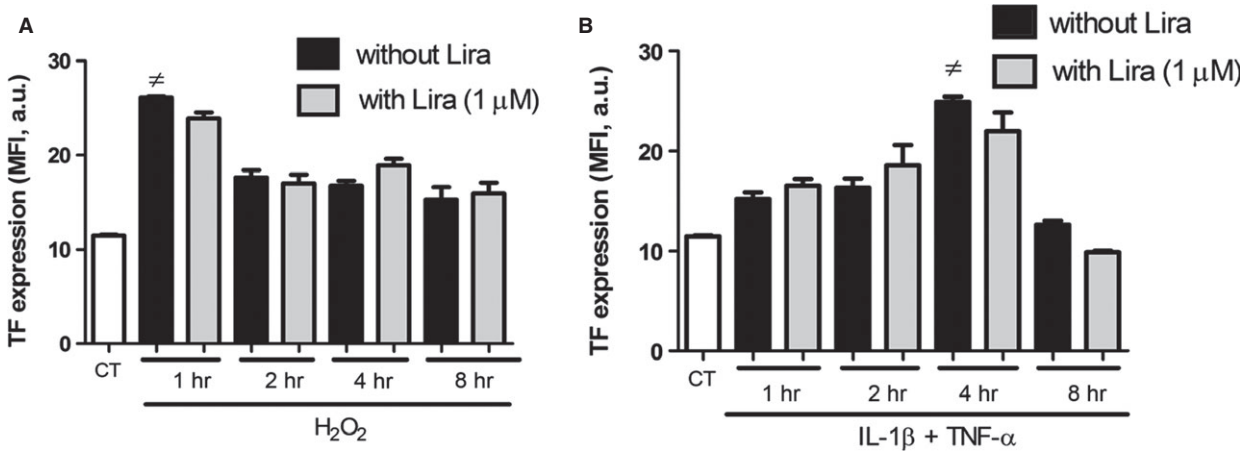

Fig. 3 Expression of TF after oxidative (**A**) and cytokine stress (**B**). Oxidative (**A**) and cytokine (**B**) stress, were applied to Rin-m5f (1–8 hrs). Fluorescence intensity was quantified by flow cytometry after TF-immunostaining. Data expressed as mean ± S.E.M. ($n = 3$; Lira: Liraglutide; MFI: Mean Fluorescence Intensity; ≠: *versus* unstimulated cells).

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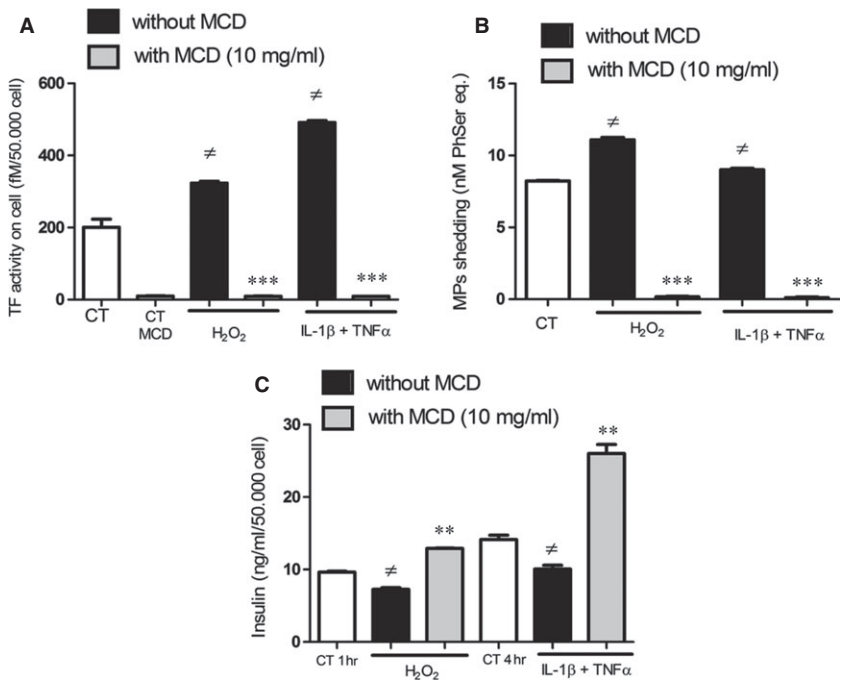

Fig. 4 Raft disruption by MCD inhibits TF activity (**A**), MP shedding (**B**) and restores insulin secretion (**C**). Cells were treated by H_2O_2 during 1 hr, or cytokines during 4 hrs, in the presence (grey bars) or absence (black bars) of MCD. Empty bars: unstimulated cells, dotted bars: cells treated by MCD alone. Data expressed as mean ± S.E.M. ($n = 5$; MCD: methyl-β-cyclodextrin; Lira: Liraglutide; ≠: *versus* unstimulated cells; $^{**}P < 0.01$, $^{***}P < 0.0001$).

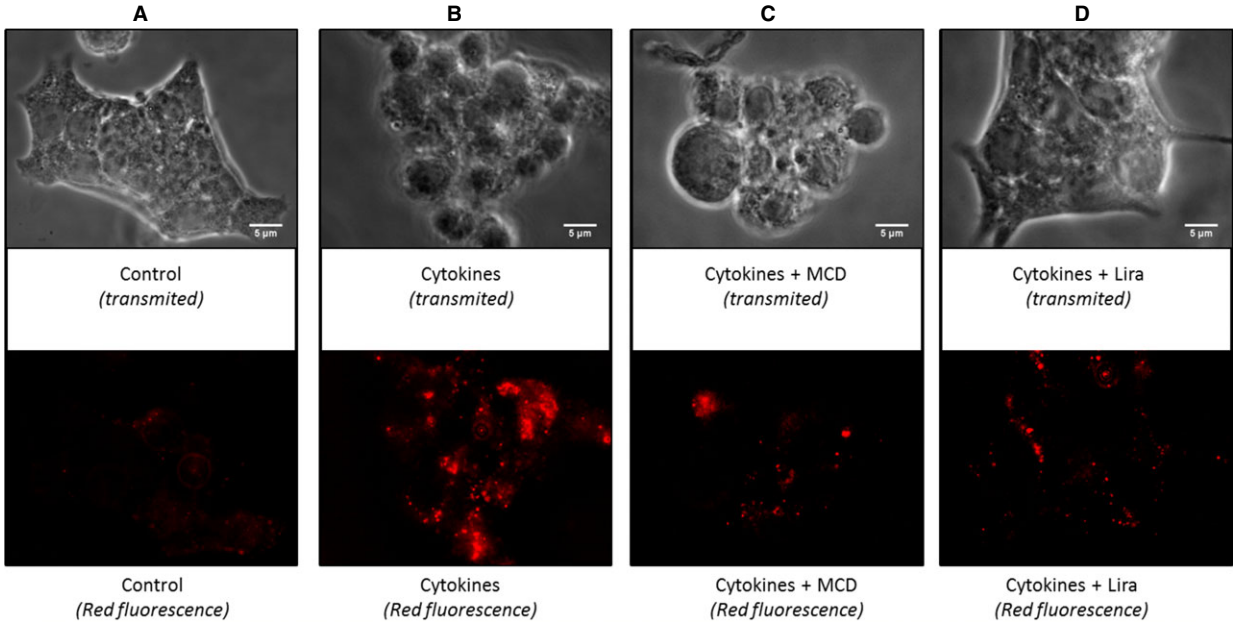

Fig. 5 Lipid rafts cluster under inflammatory conditions and are disrupted by liraglutide. After stimulation by cytokines, in the presence (**C**) or absence of MCD (**B**) or in the presence of liraglutide (lira) (**D**), cells were fixed and raft labelled by PE-cholera toxin. Stimulated and unstimulated (**A**) cells were observed by fluorescent confocal microscopy (×100).

versus 26.0 ± 1.3 ng/ml/50,000 cells in MCD-cytokine-treated cells, $P = 0.007$; Fig. 4C). Staining of the GM1 ganglioside, a marker of lipid rafts, using the fluorescent cholera toxin demonstrated the formation of large lipid raft clusters in response to cytokines by confocal microscopy (Fig. 5B). This major membrane remodelling could not be detected in MCD-treated stimulated cells in which the raft disruption led to a pattern of small rafts spread over the whole cell surface (Fig. 5C). Raft staining of liraglutide-treated cells revealed patterns close to those of MCD-treated stimulated cells (Fig. 5C and D).

Liraglutide modulates insulin exocytosis and K_{ATP} and Ca^{2+} channels activity

To further investigate the role of raft clustering on insulin secretion and its modulation by liraglutide, ionic channels activity and exocytosis were assessed through pharmacological inhibition and direct staining.

Addition of the K^+ channel inhibitor, Diazoxide, to H_2O_2 or cytokine-treated cells led to an approximate 85% decrease in insulin secretion that was counteracted by liraglutide. Similar results were obtained with the Ca^{2+} channel inhibitor, Amlodipine (Fig. 6A and B). The alteration of insulin secretion prompted by MPs was also mediated although K_{ATP} and Ca^{2+} channels (data not shown).

Insulin staining revealed a typical pattern of abnormal exocytosis, the protein accumulating close to the inner leaflet of the membrane and the insulin cytosol content appearing low by comparison. Conversely, treatment by Liraglutide led to a homogenous distribution of the β-cell insulin content with a decreased proportion of cells that expressed an abnormal exocytosis pattern (Fig. 7A–C). Moreover, 1 μM Liraglutide allowed a higher insulin release in cell supernatant (10.2 ± 0.1 ng/ml/50,000 cells in H_2O_2-treated cells *versus* 13.5 ± 0.6 ng/ml/50,000 cells in liraglutide-treated counterparts, $P = 0.04$; 10.5 ± 1.3 ng/ml/50,000 cells in cytokine-treated cells *versus* 16.4 ± 0.9 ng/ml/50,000 cells in liraglutide-treated counterparts, $P = 0.03$, Fig. 7D).

The beneficial effects of liraglutide rely on GLP-1r-dependent and -independent pathways

Because the effects of liraglutide seemed dependent on membrane remodelling, we suggested that a part of them are independent of GLP-1r. Cell pre-treatment by saturating concentration of the GLP-1r antagonist, Exendin (9-39), led to a partial abolishment of the liraglutide-driven insulin secretion, by approximately 50% in H_2O_2-treated cells and 23% in cytokine-treated cells (Fig. 8A). Interestingly, exendin (9-39) abolished the liraglutide-driven reduction of TF activity under oxidative stress, but not under inflammatory conditions with values remaining significantly lower (cytokines: 95.6 ± 6.0 fM/50,000 cells *versus* cytokines-exendin–liraglutide: 71.8 ± 3.3 fM/50,000 cells, $P = 0.007$; Fig. 8B). Western blots also showed that liraglutide reduced caspase-3 cleavage by about 30% in H_2O_2 or cytokine-challenged cells, with no significant variation between exendin (9-39) pre-treated and untreated cells (Fig. 9A and B). Similarly, liraglutide reduced MP shedding by about 25% in H_2O_2 or cytokine-treated cells, with no significant alteration by exendin (9-39) pre-treatment (Fig. 10A and B). No effect on GLP-1r expression by the exendin-treated or untreated cells could be observed by Western blot (Fig. 11A and B).

Discussion

In the present work, we demonstrated that TF borne by MPs modulate insulin secretion in targeted β cells. Because TF activity is highly

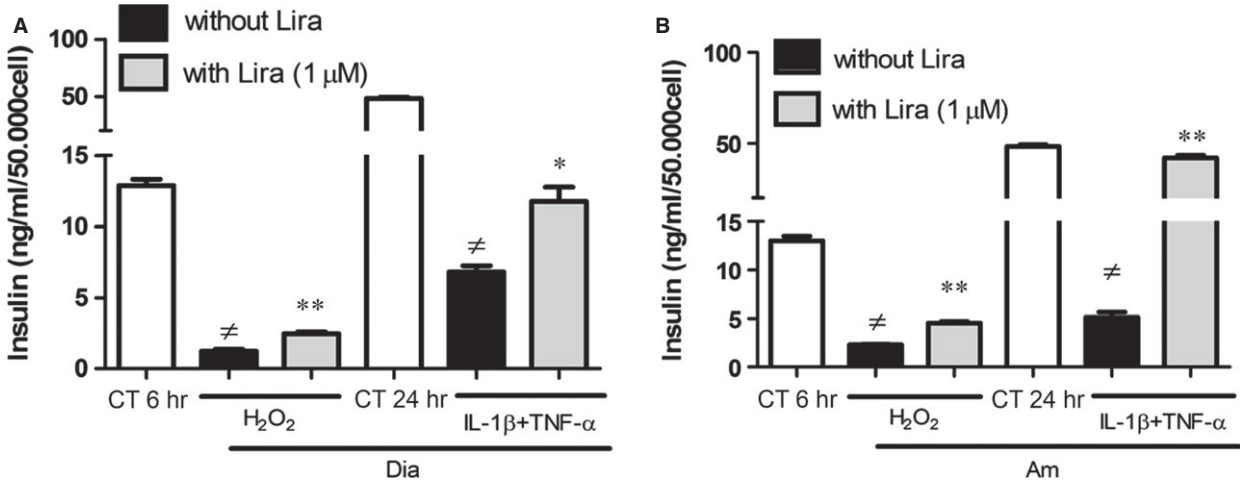

Fig. 6 Liraglutide maintains insulin secretion after pharmacological inhibition of K^+ (**A**) and Ca^{2+} (**B**) channels. Cells were treated with Diazoxide (Dia) (**A**) or Amlodipine (Am) (**B**) and H_2O_2 during 6 hrs or cytokines during 24 hrs, in the presence (grey bars) or absence (black bars) of liraglutide (lira). Insulin secretion was assessed by ELISA. Empty bars: unstimulated cells. Data represent the mean ± S.E.M. ($n = 3$; ≠: *versus* unstimulated cells; $*P < 0.05$, $**P < 0.01$).

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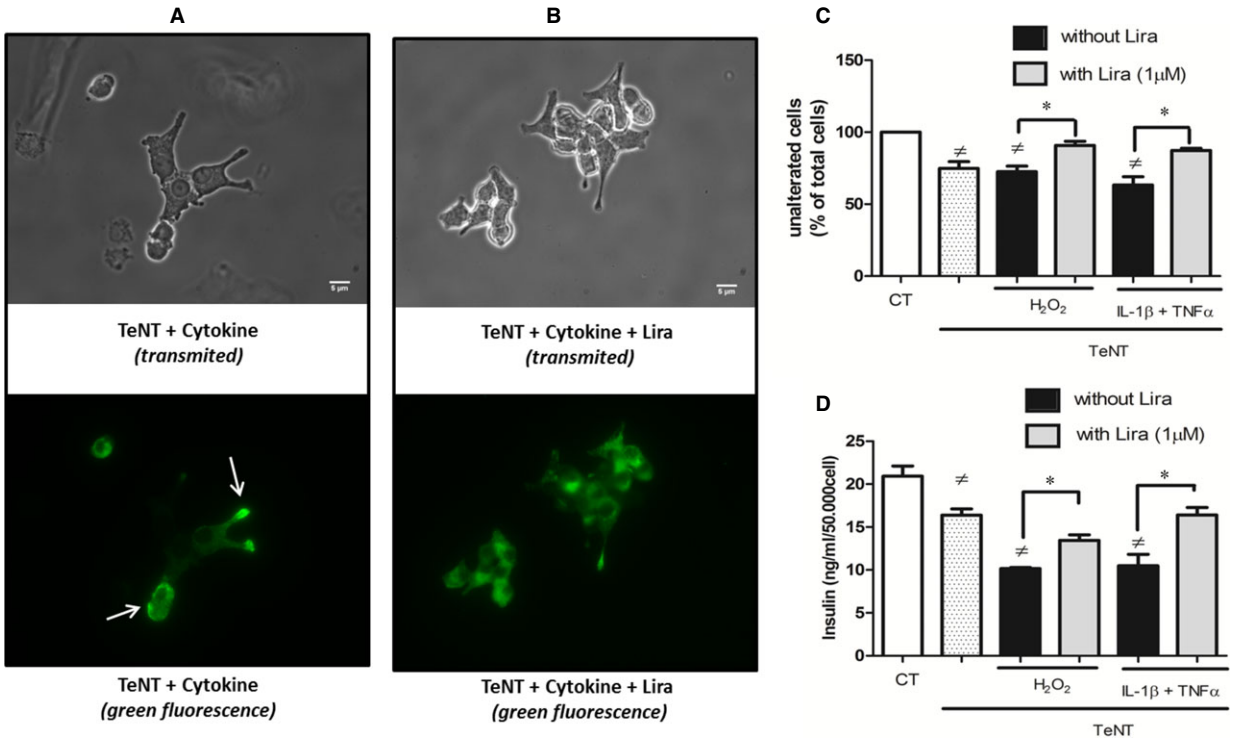

Fig. 7 Liraglutide restores insulin release after SNARE blockade by tetanus toxin. Pre-treated cells with tetanus toxin (TeNT) were incubated with cytokines in the presence (**B**) or absence (**A**) of liraglutide (lira) during 6 hrs. Fixed cells were observed by fluorescent confocal microscopy (×100) after insulin immunostaining. (**C**) Percentage of unaltered cells. (**D**) Insulin released after 6 hrs stress in supernatant. Empty bars: unstimulated cells, dotted bars: cells treated by TeNT alone. Data expressed the mean ± S.E.M. ($n = 3$; ≠: *versus* unstimulated cells; $*P < 0.05$).

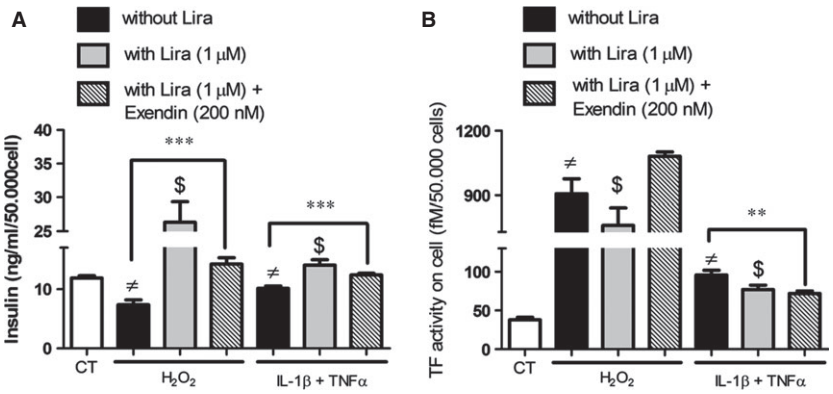

Fig. 8 The effects of liraglutide on insulin secretion (**A**) and TF activity (**B**) rely on GLP-1r -dependent and -independent pathways. Cells were submitted to 1 hr treatment with the GLP-1r antagonist exendin (9-39), before application of H_2O_2 or cytokines and exendin during 6 hrs, in the presence (grey bars) or absence (black bars) of liraglutide (lira). Insulin secretion (**A**) and TF activity (**B**) were assessed. Empty bars: unstimulated cells, striped bars: exendin (9-39). Data expressed as mean ± S.E.M. ($n = 3$; ≠: *versus* unstimulated cells; $: *versus* H_2O_2- or cytokine-treated cells; $**P < 0.01$; $***P < 0.0001$).

dependent on membrane remodelling, we questioned the significance of membrane alteration in the cytoprotection exerted by liraglutide [26]. Under cytokine and oxidative stress conditions, our data indicate that raft disruption abolishes the raise in TF activity and MP shedding, and restores insulin secretion. Liraglutide treatment led to a disrupted raft-pattern similar to that observed after MCD treatment. Pharmaco-

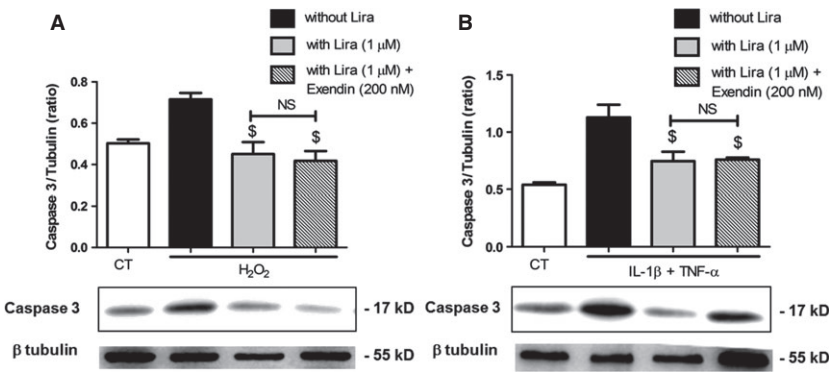

Fig. 9 The effects of liraglutide on caspase 3 activation after oxidative (**A**) and cytokine (**B**) stress rely on GLP-1r -dependent and -independent pathways. Cells were submitted to 1 hr treatment with the GLP-1r antagonist exendin (9-39), before application of H_2O_2 or cytokines and exendin (9-39) during 6 hrs, in the presence (grey bars) or absence (black bars) of liraglutide (lira). Cleaved caspase 3 was demonstrated by Western blot on cell lysates. Empty bars: unstimulated cells, striped bars: exendin (9-39). Data expressed as mean ± S.E.M. ($n = 3$; \$: *versus* H_2O_2- or cytokine-treated cells; NS: non-significant).

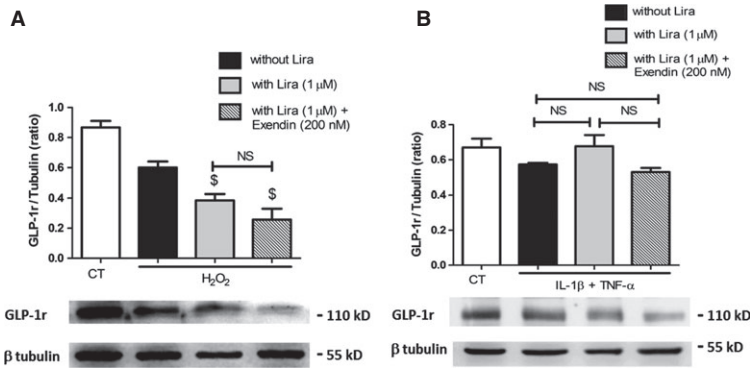

Fig. 10 Pharmacological inhibition of GLP-1r expression in liraglutide-treated cells after oxidative (**A**) and cytokine (**B**) stress. Cells were submitted to 1 hr treatment with the GLP-1r antagonist exendin (9-39), before application of H_2O_2 or cytokines and exendin (9-39) during 6 hrs, in the presence (grey bars) or absence (black bars) of liraglutide (lira). Expression of GLP-1r was assessed by Western blot in cell lysates. Empty bars: unstimulated cells, striped bars: exendin (9-39). Data expressed as mean ± S.E.M. ($n = 3$; \$: *versus* H_2O_2- or cytokine-treated cells; NS: non-significant).

logical inhibition of raft-embedded SNARE proteins and Ca^{2+} and K_{ATP} channels showed that liraglutide treatment could maintain insulin secretion. Nevertheless, pre-treatment at the saturating concentration of exendin (9-39) before application of liraglutide, did not completely abolish liraglutide effects on TF activity and insulin secretion, whereas caspase-3 cleavage or MP shedding remained unchanged.

TF is an early actor in β-cell dysfunction, whereas MPs maintain durable stress

Because MPs are pathogenic markers of cellular stress that are elevated in T2DM patients, we suggested that β cells are constantly submitted to their deleterious effects. We therefore evaluated MP effects on target cells over one cell cycle duration (24 hrs, see Fig. 2). We indeed identified TF activity and expression as early key players in insulin impairment, time course studies revealing an early cell response (1–8 hrs, see Fig. 3). Therefore, mechanisms of TF-mediated insulin secretion impairment were assessed after a short time stimulation (from 1 to 6 hrs). Distinct liraglutide modes of action could be observed, thanks to our dual functional and labelling approaches. Indeed, Liraglutide only reduced TF activity, but was ineffective on TF expression at cell membrane, at least during the first 8 hrs of treatment (see Figs 1 and 3 and previous report [26]).

MAP Kinase p38 phosphorylation as a key step in MP-mediated insulin impairment

Our data are suggestive of multiple pathways targeted by liraglutide in MP-mediated or direct stress. First, we showed that liraglutide counteracted the p38 MAP Kinase phosphorylation by

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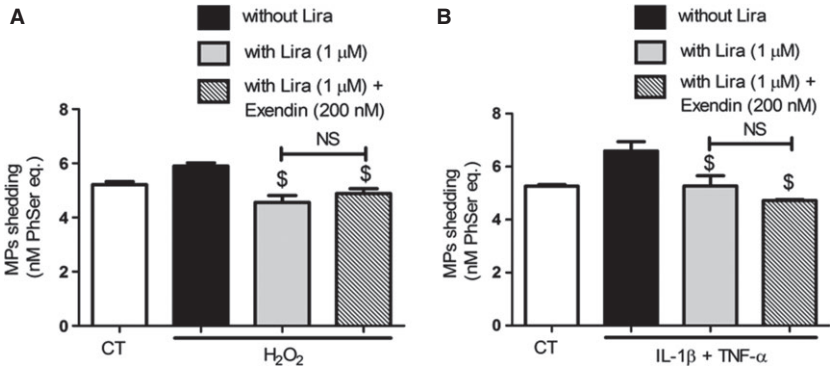

Fig. 11 The effects of liraglutide on MP shedding after oxidative (**A**) and cytokine (**B**) stress rely on GLP-1r -dependent and -independent pathways. Cells were submitted to 24 hr treatment with H_2O_2 or 48 hr treatment with cytokines in the presence (grey bars) or absence (black bars) of liraglutide (lira) and exendin (9-39). Microparticle concentration was assessed by prothrombinase assay. Empty bars: unstimulated cells, striped bars: exendin (9-39). Data expressed as mean ± S.E.M. ($n = 3$; \$: *versus* H_2O_2- or cytokine-treated cells; NS: non-significant).

MPox and MPcyt (see Fig. 2D). On line with our data, p38 was reported to favour endothelial MP shedding in a pro-inflammatory context [37] and insulin impairment by oxidative stress was associated with increased p38 phosphorylation [38]. Moreover, MPs seem to amplify the signal in target cells as MPox and MPcyt mirrored the cell response to the initial stress. Furthermore, the MP deleterious effects could be counteracted by liraglutide, making them a pharmacological target as reported by our team [26]. Furthermore, pharmacological inhibition of PKA counteracted the MP-driven fall of insulin secretion indicating that MP trigger AMP-dependent pathway (see Fig. 2C).

Are raft critical for the membrane protein control of insulin secretion?

Apart from signalling pathways, the β-cell membrane remodelling is another possible target of liraglutide. Indeed, we could evidence that liraglutide altered raft clustering prompted by inflammatory stress (see Fig. 5). These data point at an eventual modulation of the functions of raft-embedded proteins, among which are the SNARE proteins (syntaxin 1A, SNAP-25, and VAMP-2) and voltage-dependent K^+ channels (K(V)) [22, 39]. Furthermore, insulin secretion was restored by liraglutide when SNARE-dependent mechanisms were abolished by the tetanus toxin treatment. In view of previous reports showing an enhanced insulin exocytosis after raft disruption [22], it is tempting to conclude that liraglutide maintains exocytosis by acting on raft integrity. This hypothesis is strengthened by our measurement of enhanced insulin secretion after raft disruption by MCD (see Fig. 4C), as reported in INS-1 and MIN-6 rat β-cell lines [22].

Independently of raft distribution, one could consider that Ca^{2+} and K_{ATP} channels, involved in insulin secretion signalling, could constitute a target for liraglutide. We indeed demonstrated that pharmacological inhibition of these two channels led to insulin secretion impairment that was partially counteracted by liraglutide (see Fig. 6). The moderate effect, however, questions an eventual physiological role of rafts. Indeed, other authors have demonstrated that K_{ATP} channels are not embedded in rafts and that Ca^{2+} channels function is not altered by raft disruption [22].

GLP-1 analogues exert cytoprotection in ß cells through GLP-1r -dependent and -independent pathways

Our data bring new clues to the mode of action of liraglutide as a cytoprotective agent in insulin-secreting cells challenged by cytokine and oxidative stress. We show that part of the protection exerted by liraglutide is independent of the GLP-1r, in line with observations in murine endothelium, cardiac and vascular myocytes [40]. Indeed, treatment by exendin (9-39), a GLP-1r antagonist, counteracted the restoration of insulin secretion and the reduction of TF activity prompted by Liraglutide, but did not significantly alter the other cytoprotective effects triggered by the GLP-1 analogue, namely MP shedding or caspase-3 activation (see Figs 9–11). Because exendin (9-39) was used at maximal inhibitory concentration and did not significantly modify the expression of the GLP-1r, it is tempting to consider that liraglutide partly exerts cytoprotection independently of GLP-1r or through a yet unknown member of the receptor family, as suggested by recent data reported in GLP-1r knock-out mice [41]. Another possibility would be that the plasma membrane remodelling itself would modulate the potency of the GLP-1r to activate down-stream events, as recently reported by Chen *et al.* who demonstrated that specific types of endocannabinoid-like lipids regulate GLP-1r signalling [42].

Taken together, our data indicate an integrated β-cell response to GLP-1 that combines receptor-mediated signalling and membrane remodelling. One illustration of such integrated response was given by another team, showing that GLP-1r activation and raft disruption both lead to an inhibition of raft-embedded-K(V) channels, the maintenance of β-cell excitability and consecutive of insulin secretion [22, 43].

In conclusion, our work demonstrates that TF activity borne by MP released from β cells constitute an amplification loop in the insulin impairment mediated by inflammation. In addition, the ability of liraglutide to limit the raft clustering that promote MP shedding, insulin impairment and TF activity, points to membrane remodelling as a new target in insulin management.

Study limitation

This study was designed to focus on the β-cell response to inflammation-driven membrane response assessed by the shedding of TF^+-MPs and raft remodelling. No conclusion can be driven on the integrative response of β cell within the islets architecture, neither on chronic hyperglycaemia-associated stress and inflammation in the state of diabetes that would require further studies on cultured islets and in animal models of diabetes.

The Rin-m5f cells were chosen as a model to study the molecular link between prolonged pro-inflammatory signals and hyperglycaemia on β-cell functions. These cells are particularly appropriated to the study of β-cell membrane remodelling in such conditions, but do not respond to short metabolic glucose stimulation. Therefore, confirmation of our data requires studies on primary β cells isolated from islets.

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CG performed the main part of the cellular experiments, raft studies and immunofluorescence cytometry and wrote the article. GK and MA performed most Western Blots and contributed to TF activity measurement. MK contributed to cell-cultured experiments. AC and JB contributed to pharmacological modulation studies and fluorescence cytometry assessment and statistical analysis. BY performed MPs measurement and contributed to TF activity measurements. FT and LK designed the research and corrected the article.

Conflicts of interest

The authors confirm that there are no conflicts of interest.

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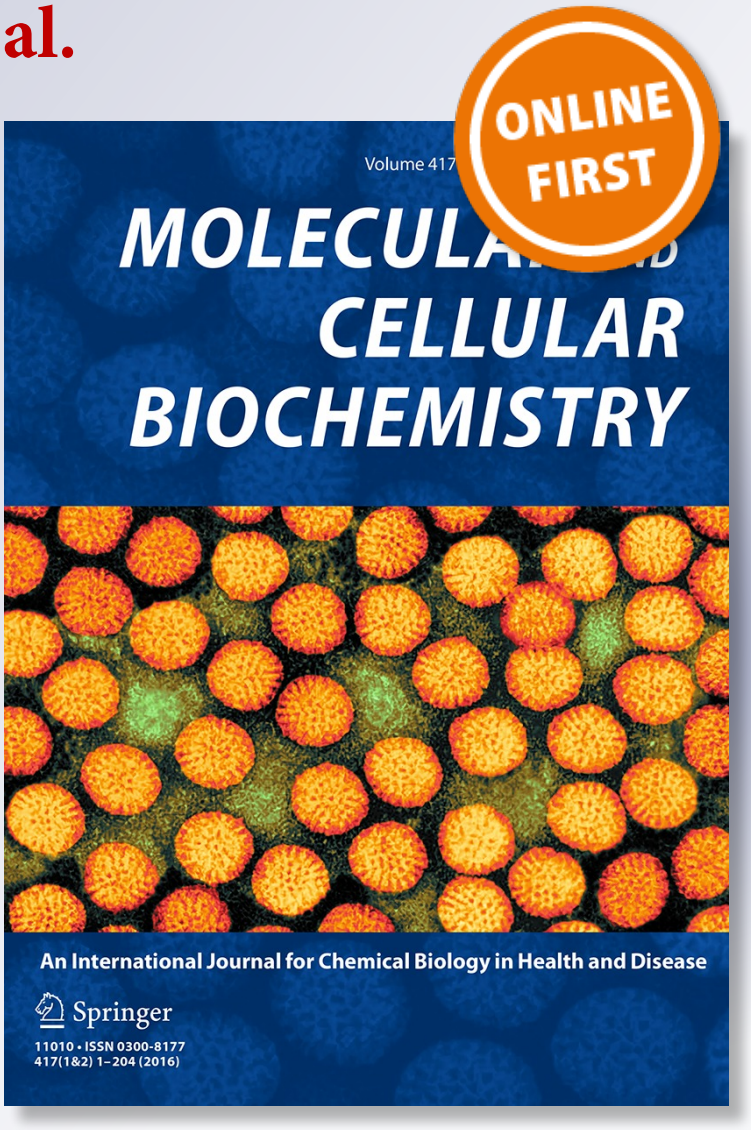

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Differential influence of tacrolimus and sirolimus on mitochondrial-dependent signaling for apoptosis in pancreatic cells

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Abstract To examine and compare the mitochondria-related cellular mechanisms by which tacrolimus (TAC) or sirolimus (SIR) immunosuppressive drugs alter the pancreatic exocrine and endocrine β-cell fate. Human exocrine PANC-1 and rat endocrine insulin-secreting RIN-m5F cells and isolated rat islets were submitted to 1–100 nM TAC or SIR. In cultures, insulin secretion was measured as endocrine cell function marker. Apoptosis was quantified by annexin 5 and propidium iodide staining. Cleaved caspase-3, Bax apoptosis indicators, and p53, p21 cell cycle regulators were detected by Western blot. Cell cycle and mitochondrial membrane potential (ΔΨm) were analyzed by flow cytometry and SA-beta-galactosidase (SA-β-gal) activity by fluorescence microscopy. Only TAC reduced insulin secretion by RIN-m5F after 24 h. TAC and SIR promoted moderate apoptosis in both PANC-1 and RIN-m5F after 24 h. Apoptosis was associated with up-regulated Bax (threefold) and cleaved caspase-3 (fivefold) but only in PANC-1, while p53 and p21 were up-regulated (twofold) in both cell lines. ΔΨm was impaired only in PANC-1 by TAC and SIR. Only SIR prompted cell cycle arrest in both cell lines. The induction of a premature senescence-like phenotype was confirmed in isolated islets by SA-β-gal activity. TAC and SIR are early inducers of pancreatic cell dysfunction and apoptosis but differentially alter endocrine and exocrine cells via mitochondrial-driven pathways. In rat islets, TAC and SIR prompt a senescence-like phenotype.

Keywords Endocrine β-cells · Exocrine cells · Tacrolimus · Sirolimus · Mitochondria-related apoptosis · Cellular premature senescence

Abbreviations

ΔΨm	Mitochondrial membrane potential
a-5	Annexin 5
a.u.	Arbitrary units
C_{12}FDG	5-Dodecanoylaminofluorescein Di-β-D-galactopyranoside
DiOC6	3,3′-Dihexyloxacarbocyanine iodide
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Fluorescein diacetate

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FITC	Fluorescein isothiocyanate
HBSS	Hank's balanced salt solution
mTOR	Mammalian target of rapamycin
mTORc1	mTOR complex 1
PBS	Phosphate-buffered saline
PI	Propidium iodide
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
SA-β-gal	Senescence-associated beta-galactosidase
SDS	Sodium dodecyl sulfate
SIR	Sirolimus
TAC	Tacrolimus

Introduction

Tacrolimus (TAC) and sirolimus (SIR) are the respective inhibitors of calcineurin and mammalian target of rapamycin (mTOR) used in the arboretum of immunosuppressive drugs to prevent the loss of graft. Although their immunosuppressive efficacy is undeniable, important side effects like nephrotoxicity, hyperlipidemia, hypertension and endocrine pancreas dysfunction have been associated with those long-term treatments [1]. Post-transplantation diabetes, as result of chronic endocrine dysfunction, is also one of the complications increasing the risk of morbidity and mortality [2].

Despite emerging data depicting the impact of TAC and SIR in endocrine cell function and fate, a significant hiatus still remains in the in-depth understanding of the underlying mechanisms. It is known, however, that targeting calcineurin by TAC impairs survival and insulin secretion in β-cells [2–4] and may alter the morphology and mass of islets [5]. SIR impairs insulin secretion, reduces mitochondrial ATP production and alters islet regeneration and proliferation [6]. It can also interfere with the survival of insulin-producing β-cells and induces apoptosis through the down-regulation of phosphorylated mTOR and inactivation of the protein kinase Akt [7].

In pancreatic exocrine cells, the effects of TAC and SIR are a matter of debate with conflicting reports on deleterious and protective outcomes of TAC usage. A worsening of pancreatitis condition in vivo [8], the association with acute pancreatitis in pancreas- and allogenic bone marrow-transplanted patients [9, 10] and impairment of pancreatic acinar cell function were reported [11]. Conversely, preventive activity against pancreatitis conditions in vivo [12, 13] or anti-inflammatory properties have been evidenced [14, 15]. Similarly, SIR was confirmed to promote ductal cell loss in vitro [16], but also to protect from acute pancreatitis-inducing conditions in vivo and in vitro [17–19].

Withal, one major aspect fairly neglected is the role of mitochondria in TAC-and SIR-mediated effects on pancreatic cells. In β-cells and isolated rat islets, however, recent data suggested that TAC may impact mitochondria by limiting its function and respiration by modulation of transcription and translation of a variety of effectors. TAC also reduces the mitochondrial mass. TAC-driven mitochondrial dysfunction was also correlated to impaired insulin secretion but not with cell death [20]. Mitochondrial downstream events explaining such cell survival remain though elusive.

Data from non-pancreatic models show that by inhibition of mTOR complex 1 (mTORc1), SIR impairs cell proliferation and survival. Mitochondrial metabolism is also regulated by mTORc1; thus, SIR is competent to interfere with the mitochondrial function [21]. In T lymphocytes, SIR has been shown to prompt the disruption of mitochondrial membrane integrity and to lower oxygen consumption and ATP levels [22]. TAC also was reported to target mitochondria and its ATP production, and to disrupt mitochondrial ion channels [23, 24] in the brain.

To the best of our knowledge, only one study describes SIR involvement in pancreatic exocrine cell proliferation and apoptosis in correlation with the up-regulation of p53 [25]. Despite the effort devoted for the understanding of the interaction between immunosuppressive drugs and mitochondria, critical questions on mitochondrial downstream signaling events involved in pancreatic cell death remain thus unanswered.

The purpose of this study was, therefore, to comparatively ascertain the interference of TAC and SIR in the fate of both exocrine and endocrine β-cells, tackling the downstream mitochondrial cellular events. In particular, we addressed the impact of TAC and SIR on cell apoptosis and function, mitochondrial viability and cell cycle progression.

Materials and methods

Cell culture, islet isolation and pharmacological treatment

Human pancreatic exocrine cell lines PANC-1 (CRL-1469^{TM}) and rat insulin-secreting β-cell line RIN-m5F (CRL-11605^{TM}) were purchased from ATCC® (Manassas, VA, USA) and grown in 10 % FBS-supplemented DMEM or RPMI 1640 media, containing 4.5 % glucose, 1 % penicillin, 1 % streptomycin and 2 mM glutamine. Cell media were purchased from PAN^{TM} Biotech (Aidenbach, Germany). Passages never exceeded the maximum number of twenty-seven. Cells were grown at 37 °C, in 5 % CO_2 humidified atmosphere.

Adherent exocrine PANC-1 and endocrine RIN-m5F cells were seeded at 30 % confluence (PANC-1: 33,000 cells/cm^2;

RIN-m5F: 120,000 cells/cm^2). After two passages from seeding (5 days), cells were washed with HBSS and treated by 1–100 nM TAC or 100 nM SIR (InvivoGen, San Diego, CA, USA), for 2–48 h, at 60–70 % confluence.

Pancreatic islets were isolated from young 3-month-old adult male Wistar rats (mean weight 400 g). Rats were kept under controlled conditions (12-h light/dark cycle, 22 ± 2 °C, and standard food and water ad libitum). Pancreatic islets were isolated as described elsewhere [26]. Briefly, a 10 mL of 1 mg/mL Type-XI collagenase (Sigma, St. Louis, MO, USA) suspension in HBSS was injected into the common bile duct of the euthanized animals. Pancreas was excised within 30 min, digested at 37 °C for 10 min and submitted to strong mixing. Digestion was stopped by addition of cold HBSS supplemented by 1 % bovine serum albumin. The digest was centrifuged at 300*g* for 1 min and fat removed. The pellet was submitted to three successive cold washings (4 °C) in FBS-supplemented HBSS. Isolation of islets was achieved using a three-gradient Ficoll® PM 400 (Sigma) solution (densities: 1108, 1096, 1069 kg/m^3) and centrifugation at 300*g* for 16 min at 4 °C. Islets were recovered from the interface between second and third density, and their purity evaluated by bydithizone labeling and optical microscopy. Islets were resuspended in DMEM (Gibco®, Saint Aubin, France) containing 11.2 mM glucose and supplemented with 1 % penicillin, 1 % streptomycin, glutamine and 10 % FBS. Islets were cultured in non-adherent Petri dishes and incubated at 37 °C, in 5 % CO_2 for 24–48 h before pharmacological treatment. Treatment by 100 nM TAC or SIR (InvivoGen) was applied on a number of approximately 750 islets/Petri dish, with islet size of 70–250 μM in diameter and approx. 80 % purity, during 24 h.

Measurement of insulin secretion and detection of apoptosis

Accumulation of insulin secreted in the supernatant of endocrine RIN-m5F cells was measured by ELISA using the matrix protocol recommended by the manufacturer (EMD Millipore, Billerica, MA, USA). Insulin concentration was expressed as ng/mL per number of living cells after the stimulation period.

Apoptosis was detected by flow cytometry using annexin 5 (a-5) and propidium iodide (PI) double labeling. Cells were collected, washed in cold PBS (phosphate buffered saline) and incubated with 5 μM a-5-FITC (ImmunoTools, Friesoythe, Germany) and 1 μg/mL PI (Miltenyi Biotec SAS, Paris, France) for 15 min at room temperature in the dark. Fluorescence acquisition was performed using Guava EasyCyte Plus FlowCytometry System (Millipore).

Western blot

Cells were washed with cold PBS and lysed on ice in Tris buffer containing 30 mM Tris, 150 mM NaCl, protease inhibitors (2 mM Leupeptin, 5 mM Benzamidine, 1.5 mM Pepstatin A) and 1 % Triton™ X-100 (Sigma, l'Isle d'Abeau Chesnes, France). The pH was adjusted at 7.4. Proteins from lysates (30 μg) were separated on 10 % SDS polyacrylamide (Sigma) gels and transferred onto nitrocellulose membranes (Amersham, GE Healthcare). Membranes were incubated with primary mouse or rabbit antibodies for phosphorylated mTOR (p-mTOR), cleaved caspase-3 (Cell Signaling Technology Danvers, MA, USA), Bax, p53 (GeneTex, Irvine, CA, USA) and p21 (Santa Cruz Biotechnology, Dallas, TX, USA), at 1:1000 dilution, overnight at 4 °C. After washing, membranes were incubated with the anti-mouse or anti-rabbit antibodies (Cell Signaling Technology), at 1:10,000 dilution, at room temperature for 60 min. Prestained markers were used for molecular mass determination (Euromedex, Souffelweyersheim, France). Immunoreactive bands were detected by enhanced chemiluminescence using the ImageQuant LAS 4000 imager (GE Healthcare). Numerical values used for statistical analysis were obtained using automatic algorithms by Image Studio™ Lite (LI-COR®, Lincoln, NE, USA) software v.5.2 for Windows™. Beta tubulin (Cell Signaling Technology) was used for normalization after immunological staining using the respective antibody. For each specific condition, the expression of one protein was calculated using the formula: density of specific protein/density of tubulin. All values were then reported to the control.

Mitochondrial dysfunction and oxidative stress

Mitochondrial viability was measured through the collapse of the membrane potential ($\Delta\Psi$m). Cells were stained using the specific 3,3′-dihexyloxacarbocyanine iodide (DiOC6; Sigma) dye that allows the detection by flow cytometry (Guava) of a characteristic loss of mean green fluorescence intensity owing to the damaged integrity of the mitochondrial membrane. The positively charged dye will accumulate in highly polarized mitochondria (healthy status). When the inner side of the mitochondria is less negative (depolarized, injured status), less dye will accumulate [27].

Mitochondrial reactive oxygen species (ROS) were assessed by flow cytometry using the MitoSOX™ (Life Technologies SAS) fluorogenic dye. Oxidation of the MitoSOX™ probe was evidenced by recording the mean fluorescence intensity and was confirmed by fluorescence microscopy (20× objective, 1.5 augmentation factor, Leica

FW4000). Cells were observed with identical exposure time for each set of experiments.

Assessment of cell cycle progress

For cell cycle analysis, cells were fixed by 70 % cold ethanol, washed extensively in HBSS and incubated 15 min with 10 µg/mL RNAase I (Sigma) at 37 °C. After three washes, cells were labeled with 100 µg/mL PI and fluorescence acquired by flow cytometry (FACS, Becton–Dickinson). Analysis was performed for a minimum of 5000 events.

Islet viability and labeling of SA-beta-galactosidase activity

Islet viability was measured using Fluorescein Diacetate (FDA; Sigma) and PI (Invitrogen) staining. Rat islets (100–150) were recovered in PBS and 50 µg/mL PI was added firstly, followed by 50 µg/mL FDA. After 30 s incubation, islets were passed into 35-mm Petri dish containing 2 mL PBS. Fluorescence acquisition was achieved by microscopy (100× objective) at 530 nm (green) and 600 nm (red). Viable cells were green-labeled, while dead cells were red-labeled. The ration of viable/dead cells per islet was then estimated, and a mean reported to the number of counted islets was considered.

Senescence-associated beta-galactosidase (SA-β-gal) activity was evidenced using the fluorogenic cell-permeable substrate C_{12}FDG (Life Technologies SAS, Saint Aubin, France) as previously described [28]. After immunosuppressive drug treatment, islets were washed with PBS and incubated with 300 µM chloroquine during 1 h for lysosomal alkalinization, followed by washing and incubation with 33 µM C_{12}FDG for 2 h. Cells and islets were then washed with PBS and fixed using Fixation Medium (Life Technologies SAS). The green fluorescence of the cleaved product was acquired using the Leica FW4000 platform (excitation at 480 nm and emission at 530 nm, Leica GmbH, Wetzlar, Germany), 20x objective and identical exposure time for each set of experiments (30 ms).

Statistics

All experiments were performed at least three distinct times and results expressed as mean ± SEM. Multiple *t* tests were performed for the comparisons of groups using GraphPad Prism version 6.01 for Windows™ (GraphPad Software, San Diego, CA, USA). Statistical significance was considered for $p < 0.05$.

Results

Experimental model testing and optimization

Insulin secretion

Insulin secretion was measured as an indicator of β-cell function. Concentration and time curve responses were established with increasing amounts of TAC at extended time scale up to 48 h. Only TAC effects were assessed owing to its potent activity on insulin secretion as compared to SIR [2]. TAC prompted the early decrease in insulin secretion after 4 h treatment in response to 5–100 nM (3.83 ± 0.68 vs. 0.46 ± 0.068 ng/mL; $p < 0.01$) (Fig. 1a), while 1 nM TAC had no effect. After 24 h all concentrations of TAC significantly decreased insulin secretion [from 14.2 ± 0.45 ng/mL to 12.85 ± 0.5 (1 nM); 11.88 ± 0.45 (5 nM); 11.28 ± 0.65 (10 nM)]. Higher doses (20–100 nM) showed a more pronounced effect [from 14.2 ± 0.45 ng/mL to 8.12 ± 1.5 (20 nM); 5.76 ± 1.11 (100 nM)]. The trend was kept at 48 h, when the accumulation of insulin from non-stimulated cells also slightly decreased, suggesting a saturation in the feedback of insulin signaling. Values of insulin secretion in response to all TAC concentrations reached in a plateau, probably indicating the upper limit of the endocrine function assessment in our system.

Because 24 h treatment enabled comparison between TAC concentrations outside of the stationary phase and 100 nM TAC was the peak of its inhibitory action on insulin secretion, SIR was investigated under identical conditions for comparison purposes (Fig. 1b). TAC treatment decreased the concentration of insulin detected in supernatants after 24 h (18.21 ± 0.72 vs. 11.35 ± 0.3 ng/mL per 2×10^5 cells), but no significant alteration was noticed after SIR treatment.

Production of p53, p21 and cleavage of caspase-3

To evaluate the activation of mitochondrial-dependent pro-apoptotic signaling, production of p53, p21 and the cleavage of caspase-3 were detected by Western blot at 2–48 h after treatment with the different concentrations of TAC (1–100 nM). Protein p53 and its downstream partner p21 contribute to the control of cell survival and death. Also, these two proteins contribute to the regulation of cell cycle distinctly of caspase-3 cleavage. When caspase-3 is activated due to overwhelming damage, p53 and p21 prompt apoptosis through mitochondria dysfunction [29]. After 2 h treatment, TAC increased the production of p53 and p21 when used in the range of 5–100 nM, while at 1 nM no effect was noticed (Fig. 1c). Significant increase

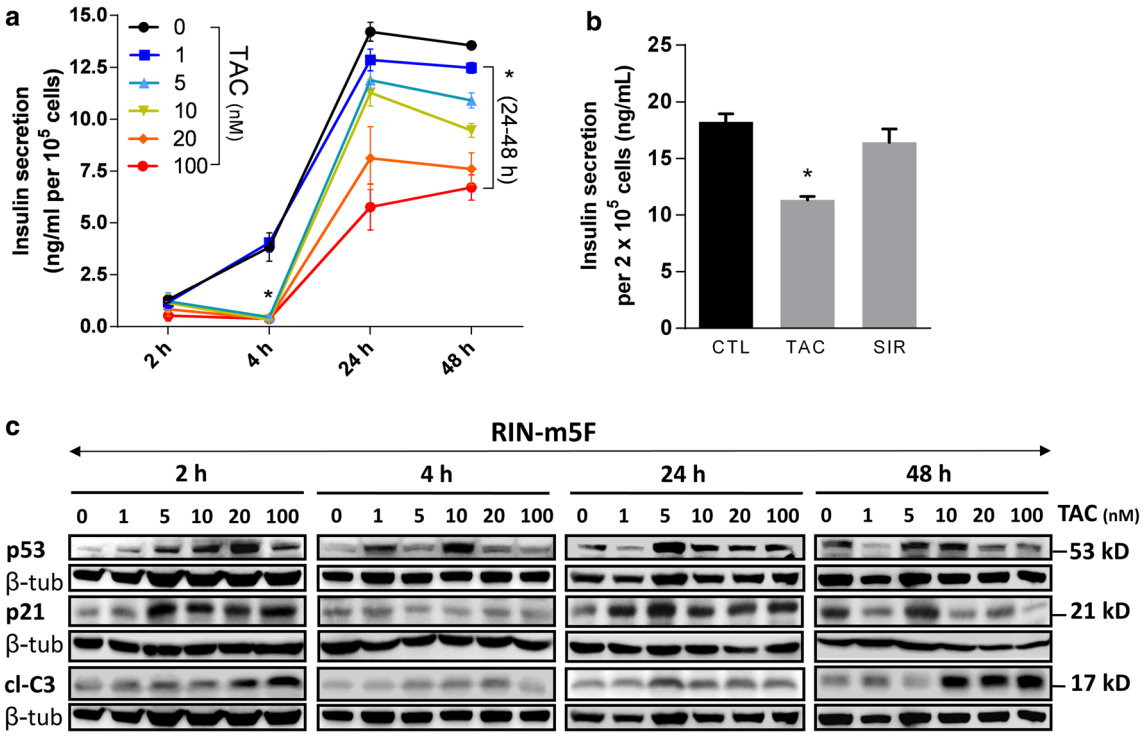

Fig. 1 Insulin secretion is impaired by TAC in a time-dependent manner. Activation of p53-related signaling. **a** The concentration of insulin secreted in the supernatant of RIN-m5F cells was measured by ELISA after incubation with 0–100 nM TAC at given time points (2–48 h) or with 100 nM SIR (24 h). **b** Values were reported to the specified number of living cells. $*p < 0.05$ versus untreated cells (CTL) (**a**, **b**), $n = 3$. **c** Endocrine RIN-m5F were submitted to increasing concentrations of TAC (0–100 nM) and production of p53, p21 and cleavage of caspase-3 were observed by Western blot (2–48 h). The images are representative for three distinct experiments

of caspase-3 cleavage was observed only at highest concentrations (20–100 nM). After 4 h treatment, no difference in caspase-3 cleavage or p21 expression was noticed between control and treated cells, and p53 was up-regulated only in the presence of 1 nM and 10 nM TAC. After 24 h treatment, significant up-regulation of p53 (5–100 nM TAC) and p21 (1–100 nM TAC) was detected, while no significant variation in the cleavage of caspase-3 was observed. At 48 h, caspase-3 cleavage dramatically increased in response to 10–100 nM TAC. These results suggested a correlation between the increased production of both p53 and p21, and the impairment of insulin secretion in early (2–4 h) and late (24–48) cell response.

Hence, 24 h and 100 nM were considered appropriate conditions for further assays, as mentioned above (see Fig. 1b). Shorter time points were irrelevant for the study of cell cycle progression [30, 31], and longer incubation led to a plateau of insulin secretion. Furthermore, longer incubation times could promote unspecific apoptosis due to cell accumulation and overexpansion. Of note, the 100 nM TAC concentration seemed a potent modulator of p53 with an initial increase in protein expression, followed by accelerated consumption and subsequent up-regulation.

Cell survival

In exocrine PANC-1 cells, TAC and SIR induced apoptosis as evidenced by the significant enhancement in the proportion of cells showing positive a-5 labeling (5.8 ± 0.6 vs. 13.5 ± 0.4 and 10 ± 1 %, respectively) (Fig. 2a, b). Similarly, TAC and SIR induced apoptosis (5.5 ± 0.6 vs. 13.6 ± 0.7 and 16.6 ± 0.9 %, respectively) in endocrine RIN-m5F cells. Western blot indicated a ~threefold increase in mTOR phosphorylation induced by TAC in exocrine cells, whereas SIR prompted a drastic fall below the control level (Fig. 2c, e). Conversely, p-mTOR level was not significantly altered by TAC in endocrine cells, but SIR induced a significant decrease below the expression level of untreated cells. TAC and SIR promoted a ~sixfold increase in caspase-3 cleavage in PANC-1 (Fig. 2d, e). In contrast, TAC and SIR both significantly decreased the production of cleaved caspase-3 in endocrine cells by half.

Mitochondrial dysfunction

As a key indicator for cell injury, mitochondrial membrane potential was measured in our experimental model (Fig. 3a, b). Treatment of PANC-1 cells by TAC

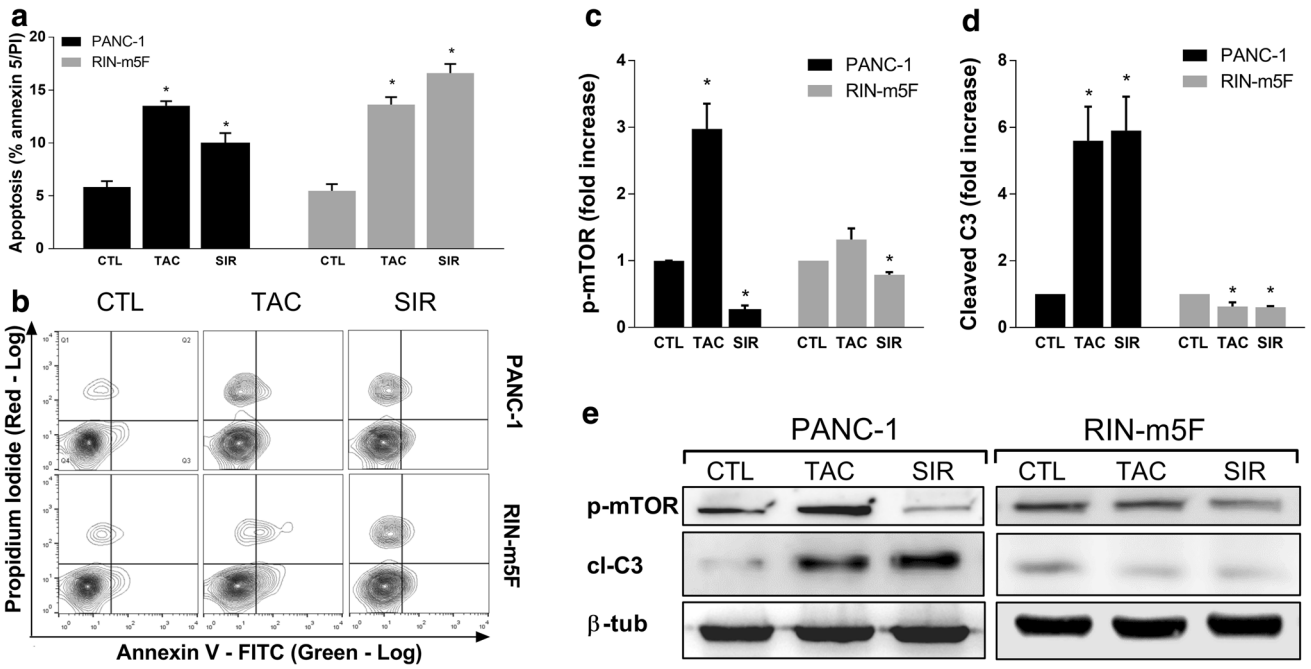

Fig. 2 TAC and SIR prompt cell death distinctly. **a** Apoptosis was evidenced by a-5-PI double staining after 24 h treatment with either 100 nM TAC or SIR. Acquisition was made by flow cytometry ($n = 3$). **b** Representative contour plots (contour level 2 %) for (**a**); gates Q1, Q2, Q3 and Q4 represent necrosis (PI+/a-5-), late apoptosis (PI+/a-5+), early apoptosis (PI-/a-5+) and viable cells (PI-/a-5-), respectively. Detection of the phosphorylated form of mTOR (**c**) and of the activated caspase-3 (**d**) by Western blot in treated cell lysates (30 µg of protein loaded in each well) was compared to untreated cells. The density of each protein blot was reported to β-tubulin, and the control was constantly considered as 1 ($n = 3$). **e** Representative image for (**c**, **d**). *$p < 0.01$ versus untreated cells (CTL)

moderately increased the population of cells with impaired $\Delta\Psi m$ (Fig. 3b). Cells with unaltered mitochondria presented a peak (P1) of superior mean fluorescence intensity (MFI) due to their negative charge. In damaged cells, a peak of low MFI was detected in TAC (P2: 18.2 ± 1.3 % of cells) as a result of mitochondria depolarization. Also, SIR induced a major loss of mitochondrial membrane integrity (P2: up to 56.5 ± 2.1 %). Unexpectedly, no effect of TAC or SIR was detected in endocrine RIN-m5F.

Detection of Bax, an inducer of mitochondrial dysfunction, also evidenced a differential effect of both drugs in exocrine and endocrine cells (Fig. 3c, f). In PANC-1 cells, TAC and SIR induced a ~3.5-fold increase of expression. In RIN-m5F cells, Bax was significantly decreased even below the control level by TAC, while SIR had no significant effect. The expression of modulators of mitochondria-driven cell survival, p53 and p21, was strongly enhanced by both TAC and SIR in exocrine and endocrine cells to a similar extent, suggesting a possible role in cell cycle regulation (Fig. 3d–f).

Oxidative stress and cell cycle arrest

The level of ROS was further examined by flow cytometry using the fluorescent MitoSOX^{TM}-specific probe to confirm an eventual cause for mitochondrial impairment (Fig. 4a, b). Treatment by TAC or SIR enhanced the proportion of exocrine cells exhibiting high MFI (median: 37 ± 1.1 a.u. in control vs. 49.6 ± 2.4 a.u. and 75.3 ± 4.3 a.u.). In endocrine cells, no significant modification was observed whatever the treatment. Data were confirmed by fluorescence microscopy (Fig. 4c).

The cell cycle (Fig. 4d, e) imbalance eventually prompted by up-regulated p53, p21 and the accumulation of ROS in the mitochondria was assessed by flow cytometry. In exocrine and endocrine cells, TAC had no significant effect on the population of cells with diploid DNA in G0/1 phase (2 N). Conversely, SIR significantly increased the percentage of cells with 2 N (PANC-1: from 30.35 ± 1.25 to 59.35 ± 3.95 %; RIN-m5F: from 67.85 ± 3.15 to 80.83 ± 2.04 %).

The cell cycle arrest induced by SIR led us to the assessment of SA-β-gal, a specific marker of cellular senescence. Because RIN-m5F are insulinoma cells with continuous replication, SA-β-gal was assessed in freshly isolated pancreatic rat islets that harbor primary cells mainly of endocrine and exocrine types. Islet purity before treatment was 80–95 %, and viability was greater than 95 % (data not shown). TAC and SIR were incubated into the islets suspension using similar settings as above, but no significant change in cell viability was noticed, whatever

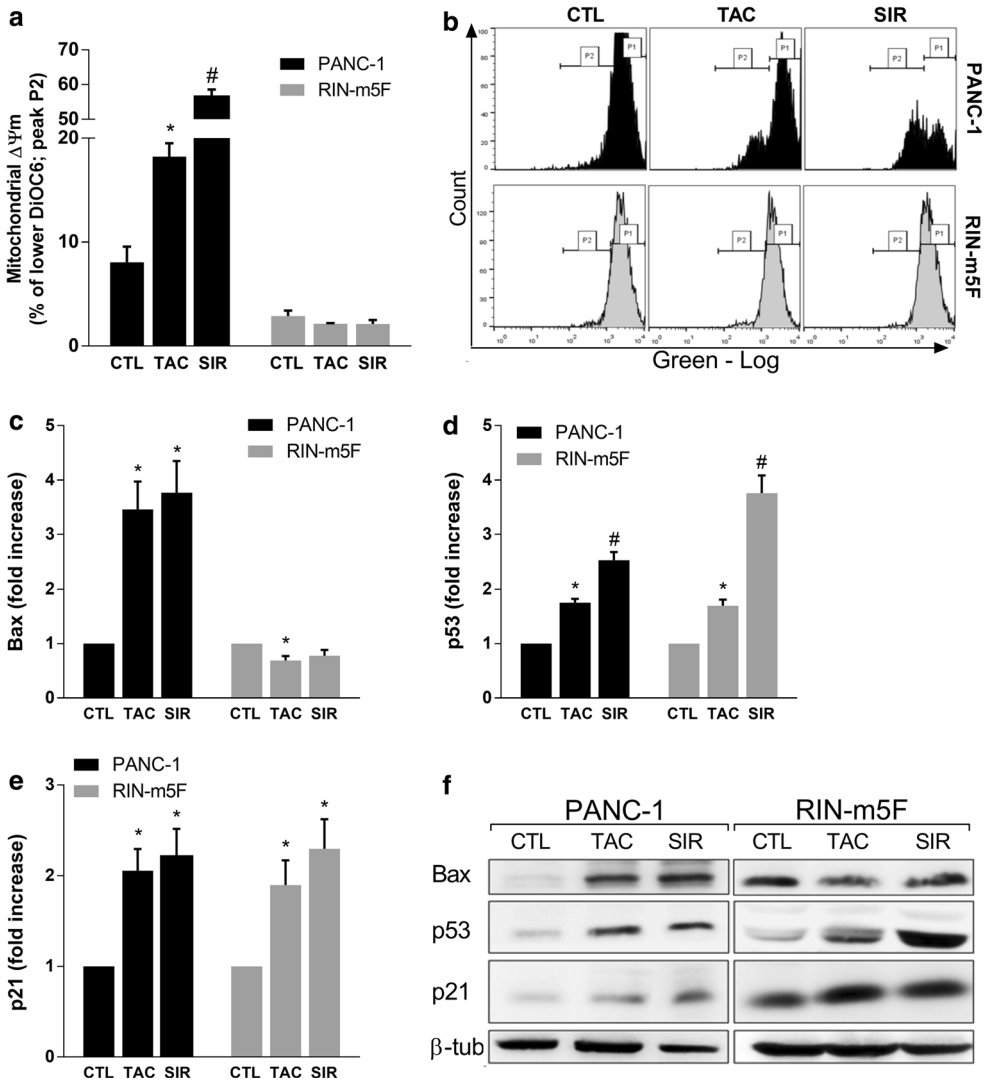

Fig. 3 TAC and SIR promote mitochondrial dysfunction by distinct pathways. **a** Proportion of cells (%) with impaired $\Delta\Psi m$ showing lower DiOC6 labeling related to the loss of mitochondrial membrane potential (MFI of P2). **b** Representative histograms for (**a**) evidencing the P1 (higher MFI) and P2 (lower MFI) populations associated to normal and damaged mitochondria. 5000 events were considered for statistical analysis; MFI (mean fluorescence intensity) in P1: ± a.u., MFI in P2 = ± a.u. Total Bax (**c**), p53 (**d**) and p21 (**e**) were quantified by Western blot in PANC-1 and RIN-m5F lysates, and 30 µg of protein were loaded in each well. Cells were treated 24 h by 100 nM TAC or SIR. The density of each protein blot was reported to β-tubulin, and the control was constantly considered as 1. **f** Representative image for (**c**–**e**). $^{*}p < 0.05$ versus untreated cells (CTL), $^{\#}p < 0.05$ versus TAC, $n = 3$

the condition (data not shown). Activity of SA-β-gal was, however, detected in islets submitted to SIR (Fig. 4f). Interestingly, TAC had a similar effect, suggesting a possible induction of a senescence-like phenotype in pancreatic endocrine cells by both immunosuppressors.

Discussion

This work aimed at a better understanding of the mitochondria-driven response induced by TAC and SIR in naive pancreatic exocrine and endocrine β-cell lineages. Measurement of apoptosis and mitochondrial dysfunction markers in those lineages points at a differential impact of TAC and SIR in the exocrine and endocrine pancreatic cell loss and dysfunction, in the absence of any other deleterious environment.

A limitation of our cellular model was the impossibility to study glucose-induced insulin secretion by endocrine cell lineage since RIN-m5F, which are grown at high glucose concentration, are generally studied as good responders to apoptotic stress and do not sense glucose elevation. In fact, this insulinoma cell line was chosen because of multiple advantages over other glucose-sensitive cells. For instance, insulinoma cells (INS-1) produce minimal insulin and require mercaptoethanol in culture, which is a reducing agent that is highly toxic and might blunt redox-sensitive apoptosis and senescence pathways. Furthermore, the mouse insulinoma cell line (MIN) exhibits stability issues, while hamster pancreatic beta cells (HIT) produce low insulin concentration [32].

Increased production of p53/p21 and endocrine function

Insulin secretion is the primary task of pancreatic endocrine β-cells. In our hands, TAC induced endocrine dysfunction in a time- and concentration-dependent manner.

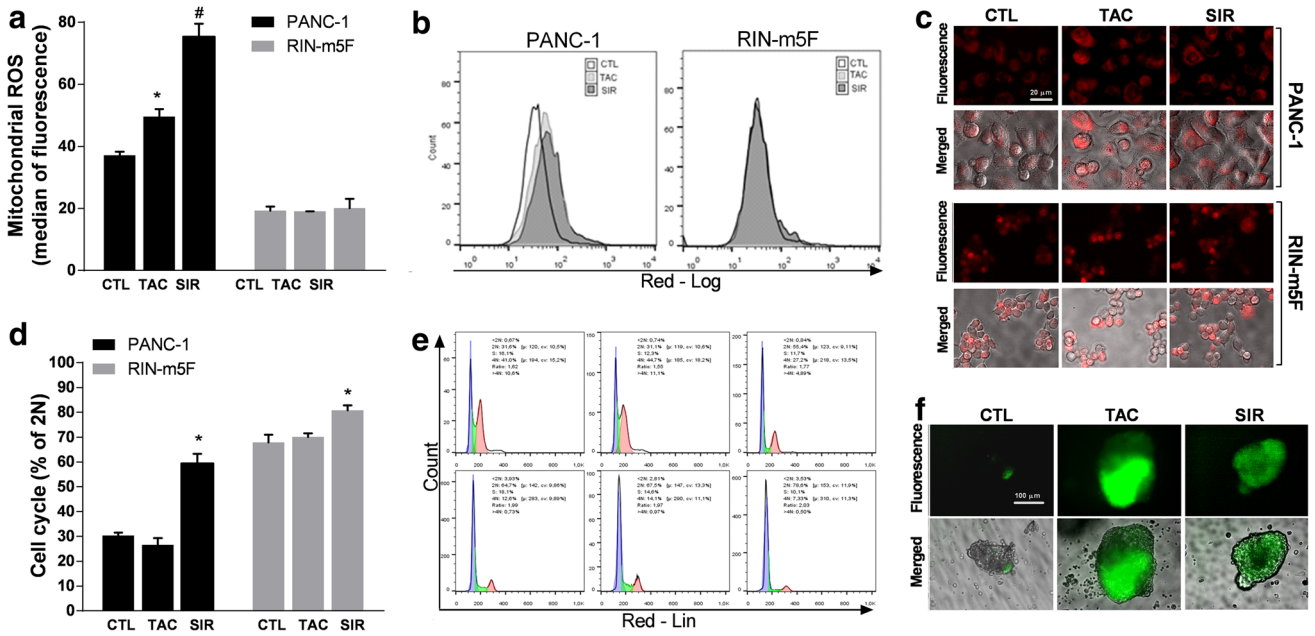

Fig. 4 TAC and SIR impact cell cycle and promote a senescence-like phenotype. **a** MFI of unfixed cells labeled with MitoSOX^{TM} fluorescent dye assessed by flow cytometry, and **b** the related histograms. **c** ROS accumulation after treatment with 100 nM TAC or SIR during 24 h was confirmed by fluorescence microscopy using the MitoSOX^{TM} probe. $^{*}p < 0.01$ versus untreated cells (CTL), $^{\#}p < 0.01$ versus TAC, $n = 3$. **d** Proportion of cells in G0/1 phase (2 N) after labeling by propidium iodide and red fluorescence acquisition by flow cytometry, following TAC and SIR treatment as above; $^{*}p < 0.01$ versus untreated (CTL), $n = 3$. **e** Representative histograms of cell populations with diploid DNA in G0/1 (2 N, *blue*), S (*green*) and G2 (4 N, *red*) phases. Univariate cell cycle model was used (FlowJo 10.0.7) indicating the following statistic data: percentage of cells in each gate, coefficients of variation (CVs). **f** 100 µM TAC- or SIR-treated pancreatic islets isolated from rats were labeled using the fluorescent C_{12}FDG substrate of SA-β-gal. Green fluorescence of the cleavage product was evidenced by epifluorescence microscopy using a 20× objective and represents cells entering in premature senescence. The *upper series* of images: fluorescence acquisition; *lower series*: merged fluorescence and light transmission images. The images are representative for a minimum of three distinct experiments performed likewise. (Color figure online)

Its impact on β-cells was seen at short time, and insulin secretion was dramatically reduced. Continuous exposure irreversibly altered insulin secretion. It is tempting to correlate these observations with the early increase in p53 and/or p21 production. But the absence of concomitant insulin impairment and up-regulation of p53 and p21 in the presence of SIR failed to provide solid evidence for a coordinated p53- and p21-mediated control of insulin secretion. We cannot exclude a possible indirect involvement of p53/p21 signaling in the modulation of insulin metabolism either. In a recent report, the role of p53 in insulin secretion has been evidenced in engineered mice [33]. Hoshino et al. demonstrated that p53 depletion can preserve insulin secretion in diabetic mice, in contrast to a previous study that found no relation between p53 and insulin secretion under distinct diabetogenic settings [34]. It is essential to mention, however, that the role of p53 in insulin secretion in the above-mentioned models was performed after the induction of diabetes. In our study, the aim was to determine whether immunosuppressors alter insulin secretion and β-cells survival through p53-related mechanisms in naïve healthy cells. Our data evidence a TAC- and SIR-driven p53 accumulation into the cytosol of endocrine cells. The up-regulation of p53 did not follow the kinetics of insulin impairment under SIR treatment. Therefore, our data are in accordance with the study by Nam et al. [34]. Although the involvement of p53 in the impairment of insulin secretion induced by TAC was not assessed by p53 activity inhibition in our study, hence we cannot exclude the conclusion of Hoshino et al. [33].

In early cell response, TAC increased the production of both p21 and p53. Conversely to p53 which remained unaltered at the lowest concentration, high p21 expression levels were detected after 24 h. Still, insulin secretion was slightly but significantly reduced, suggesting the decoupling of p53 and insulin secretion pathways (see Fig. 1a, c). However, p21 could be suspected for interference with insulin metabolism. These data are in accordance with the recent report of the modulating role of p21 on insulin production [35]. Also, Mihailidou et al. [36] showed that the gene disruption of p21 caused the decrease of insulin secretion in a murine diabetes model. These findings, along with those of Hoshino et al. point out at a regulating role of p53/p21 for insulin metabolism in conditions of hyperglycemia. However, in the literature there is no indication on a regulatory loop p21/p53 on insulin secretion under

healthy conditions. Questions remain on the role of p53/p21 on insulin secretion in the absence of any other deleterious conditions. Our investigation tackled this issue and showed the association of both proteins overexpression with insulin secretion alteration in response to TAC, while SIR had no impact on insulin release despite an increased expression of p21 and p53.

Apart from p53/p21 assessment, the effects of TAC and SIR on pancreatic endocrine cells have been recently studied on human islets [37]. TAC and SIR had no impact on basal insulin secretion, but glucose-stimulated insulin was impaired by the first only. Although the experimental model was differently designed from ours, we endorse a worsened alteration of insulin secretion induced by TAC comparing to SIR, in accordance with its deleterious properties reported clinically and experimentally.

Mitochondrial dysfunction in endocrine β-cells

Mitochondria are a central player for β-cell function and survival. Under continuous stress, p53 can activate Bax, a pro-apoptotic member of the Bcl-2 family. Following conformational shift, Bax associates the mitochondrial membrane inducing its collapse [38]. Unexpectedly, in our hands the TAC- and SIR-induced up-regulation of p53 had no impact on the β-cell membrane integrity, online with lack of Bax production increase (see Fig. 3). Absence of effect on mitochondrial membrane and on ROS accumulation was observed whatever the treatment, suggesting that mitochondria would not be key in endocrine RIN-m5F insulin secretion nor death.

In contrast, previous observation of Rostambeigi et al. [20] who evaluated the impact of TAC on mitochondrial oxygen consumption and mass in INS-1 endocrine β-cells highlighted a reduction of both indicators. However, those data are more likely pointing toward mitochondrial functional decline rather than functional damage or collapse. Moreover, the MTG (MitoTracker Green, from Thermo) dye used for mitochondrial mass assessment acts independently of membrane potential, according to the vendor. In return, the MTR (MitoTracker Red) dye that binds mitochondrial membrane in report to its potential was used solely on isolated rat islets, and no exaggerated differences were observed. Per se, no hard evidence on mitochondrial membrane integrity, thus viability, on solitary β-cells undergoing TAC treatment is available until present, and we incline to consider a lack of such effect in the given conditions. In this light, our data partially endorse those findings but address at a deeper extent the issue of immunosuppressive treatment on mitochondrial viability. Impairment of mitochondrial membrane potential promotes excessive permeabilization, ultimately leading to its collapse [38]. Therefore, it is critical to consider this parameter for mitochondrial viability assessment. In regard to SIR interference in β-cell mitochondrial function, by our knowledge it hasn't been assessed until now, our study possessing a great advantage in the head-to-head approach for TAC and SIR investigation.

Altogether, our observations displayed no argument for incriminating TAC or SIR for mitochondrial dysfunction in solitary β-cells.

Cell survival and senescence-like phenotype

Significant increase of p53 and p21 production in the absence of mitochondrial dysfunction and of caspase-3 activation, two apoptosis precursors, questioned the possibility of another metabolic phenotype as immunosuppressive treatment outcome. Depending on stress damage, p53 can initiate mitochondrial-dependent apoptosis, but also can act conjunctively with p21 as an upstream drifter for cell cycle arrest and senescence. Here was tackled the potential of immunosuppressive drugs to induce pancreatic impairment by a senescence-induced fashion, which has not been studied so far. Cellular premature senescence is characterized by an irreversible cell cycle arrest, associated beta-galactosidase activity, metabolic impairment and cell dysfunction. Various agents can trigger p53/p21 pathway, which in return regulates cell cycle progress by different mechanisms that ultimately hinder replication [39].

In addition to p53, p21, caspase-3 and mitochondrial viability regulation in our model of naive β-cell, a slight elevation in the proportion of cells undergoing G1-phase cycle arrest was induced by SIR. Critical aspects are thus met to consider that premature senescence might be a common feature in endocrine cells treated by the respective drug, in the absence of any other deleterious environment. Nevertheless, detection of SA-β-gal activity in isolated rat islets under both treatments endorses a possible immunosuppression-induced premature senescence, uncoupled from cell cycle control in case of TAC. However, we remain prudent in tying premature senescence-like phenotype to altered insulin secretion. A major impediment for such layout is the harmless impact of SIR on insulin despite its senescence triggering effect. Although recent observations shown that β-cells undergoing senescence in absence of external stress have an enhanced insulin production compared to their regular counterparts [40], the issue of stress-induced premature senescence remains challenging.

In our model, TAC and SIR induced apoptosis of endocrine cells, in defiance of mitochondria balance and caspase-3 idleness. These data suggest that the intrinsic signaling was not responsible for the observed apoptosis in RIN-m5F cells. The moderate cell death detected precludes to the conclusion that accelerated senescence is indeed a possible consequence in naive endocrine cells treated by

immunosuppressive drugs. The cells entering apoptosis might represent a small and distinct population being in an advanced replicative cycle. In this state, the mechanisms counterbalancing cell stress are decreased, and cells are deprived of their ability to block cycle progression and synthesis in support for DNA repairing. Thus, the induced stress was beyond their balancing potential, driving these cells toward death.

Modulation of exocrine cell fate by TAC and SIR

At the opposite to β-cells, we observed the activation of mitochondrial-dependent apoptosis in PANC-1 cells in response to both immunosuppressive drugs. In TAC-treated exocrine cells, one possible mechanism explaining p53 and pro-apoptotic Bax up-regulation, together with the activation of caspase-3 would be the TAC-induced overactivation of mTOR, known to partially regulate apoptosis, hence engaging the intrinsic pathway for cell death [41].

Mitochondria are a central player for both, apoptosis and senescence. In contrast to β-cells, we could evidence the disruption of mitochondrial membrane induced by both immunosuppressive drugs in exocrine cells, and our findings also indicate ROS as possible source for mitochondrial impairment. In these cells, decreased mTOR phosphorylation by SIR may have led to limited cell metabolism and a delayed cell cycle progress during the 24 h treatment. Conversely, in TAC-treated cells, the drug may have hastened the cell metabolism through major phosphorylation of mTOR, leading to an accelerated loss of cellular functions. Whatever the regulation, TAC and SIR had a higher impact on mTOR activation in exocrine cells than in β-cells.

The contribution of both immunosuppressive drugs to senescence and apoptosis pathways is suggested by the strong up-regulation of p53 and p21 and by the collapse of the mitochondrial membrane integrity [42]. Cell cycle arrest was markedly shown in SIR-treated exocrine cells, while TAC had a slightly but nonsignificant opposite trend. In TAC-treated cells, one possible explanation would be the uncoupling between the pathways prompting p53 and p21, and those prompting cell cycle arrest [29]. This hypothesis points at mTOR activation in TAC-treated PANC-1 as a possible contributor to accelerated senescence and confirms that SIR targets cell cycle, through the modulation of mTOR, a well-known sensor of the metabolic cell state. TAC and SIR may prompt an interplay between pro-senescent and pro-apoptotic pathways, tuning senescence pathways through the up-regulation of ROS, p53 and p21, with consecutive induction of Bax and caspase-3-dependent apoptotic pathways. Our observations give insights on the cellular mechanisms engaged in exocrine pancreatic dysfunction prompted by TAC and SIR that are of clinical relevance, since almost twenty percent of pancreas-grafted patients treated by these drugs are prone to pancreatic cell loss [9] by yet undefined cellular mechanism.

Altogether, our data point at an interplay between apoptosis and senescence pathways in exocrine and endocrine pancreatic β-cells, and indicate that mitochondrial membrane integrity and ROS accumulation are crucial for the exocrine cell fate in the presence of immunosuppressive drugs, even when cells are healthy. Endocrine cell alteration seems less dependent on mitochondrial viability, yet immunosuppression could lead to reduced insulin secretion. Whether p21 and p53 overexpression is a causative event in reduced insulin secretion is eventually suggested by their early up-regulation after 2 h TAC treatment, before the initiation of counterbalancing pathways. To our best knowledge, this is the first comparative study of exocrine and endocrine pancreatic cell responses to SIR and TAC, and on the impact of these drugs on pancreatic cell function and fate under resting conditions. These findings shed light on senescence as a new mechanism of pancreatic cell impairment and its possible long-term consequences in transplanted patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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EPA:DHA 6:1 prevents angiotensin II-induced hypertension and endothelial dysfunction in rats: role of NADPH oxidase and COX-derived oxidative stress

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Abstract

EPA:DHA 6:1 has been shown to be a superior omega-3 formulation inducing a sustained endothelial NO synthase-derived formation of nitric oxide (NO). This study examined whether chronic intake of EPA:DHA 6:1 prevents hypertension and endothelial dysfunction induced by angiotensin II (Ang II) in rats.

Wister rats received orally corn oil or EPA:DHA 6:1 (500 mg/kg/day) before chronic infusion of Ang II (0.4 mg/kg/day). Systolic blood pressure was determined by tail cuff sphingomanometry, vascular reactivity using a myograph, oxidative stress using dihydroethidium and protein expression by immunofluorescence and Western blot analysis.

Ang II-induced hypertension was associated with blunted acetylcholine-induced relaxations of secondary branch mesenteric artery rings affecting the endothelium-dependent hyperpolarization (EDH) and the NO components, both of which were improved by the NADPH oxidase inhibitor VAS-2870. The Ang II treatment induced also endothelium-dependent contractile responses (EDCFs), which were abolished by the cyclooxygenase inhibitor indomethacin. An increased level of vascular oxidative stress, and expression of NADPH oxidase subunits ($p47^{phox}$ and $p22^{phox}$), COX-1 and COX-2, eNOS, and Ang II type 1 receptors were observed in the Ang II group whereas SK_{Ca} and connexin 37 were down-regulated. Intake of EPA:DHA 6:1 prevented the Ang II-induced hypertension and endothelial dysfunction by improving both the NO and EDH components, and by reducing EDCFs and the expression of target proteins.

The present findings indicate that chronic intake of EPA:DHA 6:1 prevented the Ang II-induced hypertension and endothelial dysfunction in rats, most likely by preventing NADPH oxidase- and cyclooxygenase-derived oxidative stress.

Keywords

Hypertension, Vascular Endothelium, Nitric Oxide, Omega-3 Fatty Acids, Oxidative Stress

Endothelial Microparticles release after activated Protein C protect Beta cells through EPCR/PAR1 and Annexin A1/FPR2 pathways

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Keywords: Islets transplantation, Microparticles, activated protein C, annexin A1, endothelium, beta cells

Abbreviations:

ANXA1 : Annexin A1

aPC : Activated Protein C

eMP_{aPC} : Endothelial Microparticles released in response to activated Protein C

βMP : Beta cells derived Microparticles

EPCR : Endothelial Protein C Receptor

Eq : equivalent

FPR2 : Formyl Peptide Receptor 2

MP : Microparticles

PAR-1 : Protease Activated Receptor 1 , a Thrombin Receptor

PhSer : PhosphatidylSerine

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Disclosure

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Abstract

Islet transplantation is associated with early ischemia/reperfusion leading to localized coagulation and endothelial damage. Activated Protein C (aPC) limits thrombin generation and exerts endothelial cytoprotection via EPCR/PAR1 pathways. In animal models, islet cytoprotection by aPC restores islet vascularization and protects graft function. Microparticles(MP) are plasma membrane procoagulant vesicles, surrogates markers of stress, and cellular effectors. We measured the cytoprotective effects of aPC on endothelial and β-cells, and its role in the autocrine and paracrine MP-mediated cell crosstalk in the presence of oxidative stress. MP from aPC-treated endothelial(EC) or β-cells were applied to H_2O_2 treated β-cells. aPC activity was measured by enzymatic assay and ROS species by DihydroEthidium. The capture of PKH26-stained MP by β-cells and EPCR expression were probed by fluorescence microscopy, apoptosis by flow cytometry. aPC treatment enhanced both AnnexinA1 and PAR1 expression in EC and to a lesser extent β-cells. MP from aPC-treated EC protected β-cells and restored insulin secretion whereas MP from β-cells had no effect. After 24h, aPC-generated endothelial MP were integrated by 80% of target β-cells and induced a 4-fold increase in EPCR expression. Endothelial MP delivery to β-cells was concomitant with the activation of EPCR/PAR-1 cytoprotective and AnnexinA1/FPR2 pathways. These observations were confirmed in rat islets submitted to H_2O_2, that had increased viability in response to endothelial MP (62% vs 48% H_2O_2), reduced apoptosis, and preserved insulin secretion by glucose assay (High glucose : insulin 16 vs 5 ng/ml/10 islets H_2O_2). MP may prove a promising therapeutic tool in the protection of transplanted islets.

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Etude in vivo et in vitro du vieillissement des îlots pancréatiques : impact de la senescence endothéliale et des Micropaticules sur la fonction des îlots

Ce travail scientifique a abordé la problématique du vieillissement des îlots pancréatiques et l'effet de la senescence endothéliale et des microparticules (MPs) sur la fonction des îlots. Nous avons exploré l'impact du vieillissement du pancréas sur la morphologie, le devenir et la fonction de l'îlot pancréatique par analyse comparative entre pancréas de rats jeunes et d'âge moyen et le rôle des MPs endothéliales pro-sénescentes sur la fonction des îlots et leur sénescence prématurée. Nos résultats *in vivo* montrent que le pancréas est un organe précocement sensible au stress oxydant s'accumulant avec l'âge. Il conduit à la surexpression des marqueurs procoagulants et de senescence sans apparition d'apoptose. *In vitro,* les MPs de cellules endothéliales sénescentes ont un effet pro-sénescent sur les îlots pancréatiques isolés de rats jeunes avec une activité SA-β-galactosidase caractéristique, la surexpression des marqueurs p53, p21 et p16 et la réduction de la capacité de la sécrétion d'insuline en réponse au glucose. L'ensemble de nos résultats *in vivo* et *in vitro* désigne la contribution de la sénescence endothéliale comme une cause probable à la dysfonction de greffon.

Mots clés : Pancréas, greffe d'îlots, Cellule-β, sénescence, cellules endothéliales, microparticules.

This scientific work has tackled the question of the pancreatic islets aging and the effect of endothelial senescence and microparticles (MPs) on islet function. We investigated the impact of aging on pancreas morphology, fate and on the function of the pancreatic islet by comparative analysis between pancreas in young and middle-aged rats, as well as the role of pro-senescent endothelial MPs on islet function and their premature senescence. Our *in vivo* data show that the pancreas is an early sensitive organ to oxidative stress accumulating with age and leading to overexpression of the procoagulant and senescence markers without appearance of apoptosis. *In vitro*, MPs of senescent endothelial cells have a pro-senescent effect on pancreatic islets isolated from young rats with characteristic SA-β-galactosidase activity, overexpression of p53, p21 and p16 markers and reducing the ability of insulin secretion in response to glucose. Altogether, our *in vivo* and *in vitro* data indicate the contribution of endothelial senescence as a possible contributor to graft dysfunction.

Key words: Pancreas, islet graft, β-cell, senescence, endothelial cells, microparticles