Expression of laminin-332 in pancreatic islet and its effect on activation of macrophage secretory function

ARMANET, Mathieu Pierre Jean

Abstract

La laminine-332 joue un rôle important dans la biologie des îlots pancréatiques et des cellules β, in vitro. Cependant, son expression et son mode de régulation dans les îlots ainsi que son éventuel impact sur les macrophages n’ont jamais été étudiés. Dans la première partie de ce travail, nous avons montré que la laminine-332 était exprimée par les cellules endocrines des îlots humains et que l’interleukine 1 augmentait sa production, par un mécanisme intracellulaire impliquant la phospho-inositide-3-kinase. Dans la deuxième partie, nous avons démontré que la laminine-332 activait la production de plusieurs médiateurs de l’inflammation par les macrophages de rats et que les macrophages ainsi activés affectaient l’activité insulino-sécrétrice des cellules β de rat. Ce travail montre que la laminine-332 est produite de manière régulée par les îlots pancréatiques et qu’en plus de ses effets bénéfiques sur les cellules β, elle est capable de moduler le comportement des macrophages.

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Expression of laminin-332 in pancreatic islet and its effect on
activation of macrophage secretory function

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Genève, le 16 juin 2009

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“Everything will be okay in the end. If it’s not okay, it’s not the end.”

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Les îlots de Langerhans constituent la partie endocrine du pancréas et jouent un rôle majeur dans l’homéostasie énergétique et le contrôle de la glycémie. L’îlot est un réseau hétérogène de cellules endocrines comprenant quatre types cellulaires sécrétant chacun une hormone différente: les cellules β sécrètent l’insuline, les cellules α produisent le glucagon, les cellules δ sécrètent la somatostatine et les cellules PP produisent le polypeptide pancréatique. Le composant vasculaire est un autre élément important de l’îlot. Il est composé d’un réseau de capillaires sanguins bordés de cellules endothéliales. Les cellules endothéliales sont séparées des cellules endocrines par une lame basale qui constitue l’élément essentiel de la matrice extracellulaire (MEC) des îlots. Des protéines de la MEC sont également présentes dans la capsule péri-insulaire. La MEC est un réseau de macromolécules sécrétées par plusieurs types cellulaires et remplit l’espace extracellulaire. Plusieurs études fondamentales ont montré que la MEC joue un rôle actif et complexe dans la régulation du comportement des cellules qui sont à son contact, influant ainsi sur leur développement, leur migration, leur différenciation et prolifération, leur forme, leurs fonctions métaboliques et leur viabilité. L’importance de la MEC dans la biologie des îlots de Langerhans et des cellules β a été démontrée par des expériences réalisées in vitro. Certains travaux ont mis en évidence les effets bénéfiques de la laminine-332 (LN-332) sur l’activité insulinosécrétrice, la prolifération et la viabilité des cellules β primaires de rats. Cependant, aucune étude n’a cherché à déterminer l’origine cellulaire de la LN-332 dans les îlots ni à comprendre son mode de régulation.

Le diabète de type 1 (DT1) est une maladie auto-immune aboutissant à une destruction massive et irréversible des cellules β des îlots. Si l’équilibre glycémique n’est pas rétabli, de graves complications surviennent, pouvant entraîner la mort. A l’heure actuelle, le DT1 peut être traité, la plupart du temps, par la prise d’insuline exogène et, dans certains cas, par la transplantation du pancréas ou des îlots de Langerhans. Bien que la greffe pancréatique reste un traitement de choix dans la prise en charge de patients présentant une instabilité glucidique sévère, les résultats spectaculaires, rapportés par le groupe d’Edmonton, ont renouvelé l’intérêt pour la
greffe d’îlots. Cette dernière consiste à transplanter une quantité suffisante d’îlots par cathétérisation veineux portal transhépatique. Cette intervention reste encore associée à un certain nombre d’obstacles majeurs dont les origines sont multifactorielles, et peuvent entraver le succès de la greffe. Parmi ceux-ci, citons les événements inflammatoires non spécifiques impliquant les macrophages résidants du foie (cellules de Kupffer) et les monocytes circulants du sang. Les mécanismes par lesquels ces cellules peuvent agir sur les îlots sont encore très mal compris. En particulier, il reste à clarifier si la MEC des îlots joue un rôle dans l’activité cytotoxique des macrophages.

Ce travail s’articule autour de deux thématiques de recherche:

1. Caractériser l’expression de la LN-332 dans les îlots et étudier son mode de régulation.

2. Déterminer quelle est l’implication de la LN-332 dans l’activation des macrophages.

Dans la première partie du projet, nous avons réalisé l’ensemble de nos expériences sur du tissu pancréatique humain, à l’aide de techniques de biochimie, biologie moléculaire et immunohistologie. Nos analyses ont montré la présence de LN-332 dans les îlots avec une expression préférentielle dans les cellules α. En affinant nos observations microscopiques, nous avons également montré que la LN-332 ne co-localisait pas avec les granules de sécrétion des hormones. Malgré une absence de marquage extracellulaire sur les coupes histologiques de pancréas, la forme extracellulaire de la LN-332 a été identifiée dans le milieu de culture, suggérant sa sécrétion par les îlots. Parmi les différentes cytokines testées, seule IL-1β a provoqué un effet, à savoir une augmentation de la production de LN-332. Cet effet était indépendant de l’activation de la caspase 3. Par l’utilisation d’inhibiteurs pharmacologiques des voies classiques de signalisation intracellulaire nous avons mis en évidence l’importance de la phospho-inositide 3-kinase dans le contrôle de la production de LN-332. Cette observation apporte un argument supplémentaire en faveur d’un potentiel rôle de la LN-332 dans la survie cellulaire. Cependant, un
certain nombre de points reste à éclaircir. En particulier, il reste encore à démontrer si cette molécule joue un rôle biologique, *in vivo*.

Les précédentes observations nous ont encouragé à étudier les effets de la LN-332 sur la biologie des macrophages. Pour ce faire, nous avons réalisé l’ensemble des expériences décrites ci-dessous avec des cellules et des tissus prélevés chez le rat. Nous avons tout d’abord confirmé la présence de LN-332 dans l’îlot de rat ainsi que dans le milieu de culture, suggérant sa sécrétion. Les analyses histologiques de coupes de foie de rat après transplantation d’îlots ont permis d’observer une très forte accumulation de macrophages en périphérie des îlots, à la fois dans les modèles de greffes allogéniques et syngéniques. Sachant que la MEC joue un rôle fondamental dans la régulation du comportement des cellules inflammatoires, nous avons émis l’hypothèse que la LN-332 produite par les îlots pouvait moduler l’activité cytotoxique des macrophages. Les expériences réalisées *in vitro* ont démontré que les macrophages activés par la matrice 804G (source de LN-332) affectaient l’activité insulino-sécrétrice des cellules β de rat. Par la suite, nous avons testé les effets de différentes protéines matricielles, dont la LN-332, sur l’activité sécrétrice des macrophages. Les macrophages cultivés sur la matrice 804G ont montré une augmentation de la sécrétion d’IL-6 et de TNF-α ainsi qu’une augmentation de la production de NO$_2^-$ par rapport au contrôle. En présence d’un agent pharmacologique activateur des macrophages (PMA), la matrice 804G a montré sa capacité à potentialiser les effets du PMA sur la sécrétion de tous les médiateurs de l’inflammation étudiés. Des effets similaires ont été observés en utilisant de la matrice 804G dans laquelle toute trace de fibronectine avait été éliminée. En tentant de mettre en évidence les voies de signalisation impliquées dans l’activation des macrophages par la matrice 804G, nous avons montré une claire augmentation de la phosphorylation des protéines p38 et ERK. Au contraire, la phosphorylation de la protéine FAK était réduite dans les mêmes conditions. L’utilisation d’inhibiteurs pharmacologiques de p38 et ERK a permis de confirmer l’implication de ERK dans l’activation des macrophages induite par la matrice 804G. Dans cette deuxième partie, nous montrons qu’une matrice riche en LN-332 affecte l’activité sécrétrice des macrophages.
SUMMARY

The islets of Langerhans are micro-organs located in the exocrine pancreas, which are crucial for glucose homeostasis. The islet typically consists of four types of secretory endocrine cells, namely, the insulin-containing β-cells, the glucagon-containing α-cells, the somatostatin-containing δ-cells, and the pancreatic polypeptide-producing PP-cells. In addition to endocrine cells, islet has a dense capillary network, which is important to provide nutrients and disperse hormones to the systemic circulation. Islet endothelial cells are closely associated with endocrine cells and both cell types are separated by a specialized form of extracellular matrix (ECM) called the basement membrane (BM). In addition, ECM proteins are also located at the exocrine-endocrine interface. ECM is an important part of the microenvironment of cells. Indeed, signals originating from ECM proteins may influence cell behaviour and functions. Some in vitro studies have shown the beneficial effects of the ECM protein laminin-332 (LN-332) on insulin secretory activity, proliferation and viability of primary rat β-cells. However, no study tried to determine the origin of LN-332 in human islet and how its expression was regulated.

Type 1 diabetes (T1D) is an autoimmune disease that results in selective destruction of insulin-containing β-cells of the pancreas. To restore endogenous β-cell function to T1D patients, islet allotransplantation into the liver represents an attractive therapy since it can be performed as a minimally invasive procedure, when compared to whole organ transplantation. Despite encouraging results, this therapy is at a clinical experimental stage and still encounters obstacles that need to be overcome. Reasons for limited success of islet transplantation are multifactorial. One of them is related to non-specific inflammatory events occurring immediately after intra-portal infusion of islets. This inflammation involves, in part, cells of the monocyte-macrophage lineage such as Kupffer cells (liver-residing macrophages) and monocytes of the blood stream. The mechanisms by which these cells are stimulated to orchestrate inflammation are incompletely understood. More specifically, it has never been investigated whether islet ECM proteins affect cells of the monocyte-macrophage lineage.
The present thesis is structured around two themes of research. The first part is devoted to the characterization of LN-332 in human islets and to the study of its mode of regulation. The second part investigates the potential role of LN-332 on activation of rat macrophage secretory function.

By Western blotting and RT-PCR we showed that LN-332 was expressed in isolated human islets. By immunofluorescence on pancreas sections, we observed that within the islets, labelling was confined to endocrine cells. Confocal microscopy analysis on isolated islet cells revealed that labelling was granular but did not colocalize with hormone secretory granules. LN-332 was most abundant in cultured islets compared to freshly isolated islets, and was found in culture supernatant suggesting that it was secreted by islets. When islets were exposed to different cytokines, interleukin (IL)-1β, was the only one to show an effect on LN-332 expression and secretion as compared to control. LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3-K) activity, inhibited culture- and IL-1β-induced LN-332 expression in islets. In addition, IL-1β-induced LN-332 upregulation was independent of caspase 3 activation.

The first part of this thesis shows that LN-332, known to have some beneficial effect on β-cells in vitro, is expressed and secreted by human islets and conditions inducing islet stresses, such as isolation procedure and IL-1β exposure, enhance LN-332 production.

In the second part, we confirmed first the presence of LN-332 in rat islets, by immunofluorescence and Western blotting. LN-332 was also identified in islet culture media. We then investigated the effect of the LN-332-rich 804G matrix on macrophage activation. Macrophages cultured on 804G matrix showed an increased secretion of TNF-α and IL-6, and an increased production of NO₂⁻ compared to macrophages cultured on collagen, fibronectin or uncoated Petri dishes. In the presence of a macrophage-activating drug (PMA), 804G matrix potentiated the production of all inflammatory mediators studied compared to control, collagen and fibronectin. Similar effects on TNF-α and IL-6 secretion, and NO₂⁻ production were observed using fibronectin-depleted 804G matrix. After 24 hour-incubation, macrophages attached on 804G matrix showed an increased phosphorylation of p38 and ERK MAPKs when compared to control condition. In the presence of PMA,
phosphorylation of p38 and ERK was similar in both conditions. By contrast, phosphorylation of FAK was reduced under the same conditions. The use of pharmacological inhibitors of p38 and ERK MAPKs has confirmed the involvement of ERK in the activation of macrophages by the 804G matrix. Finally, 804G matrix-activated macrophages affected glucose-induced insulin secretion by rat islets. With the second part, we demonstrate that a matrix rich in LN-332 clearly affect macrophage activity.

In conclusion, from our observations on the human islet LN-332 composition (Part I) several potentially important implications can be envisioned. These are related to β-cell differentiation and proliferation, pathogenetic mechanisms of diabetes and islet transplantation. Concerning transplantation, LN-332 may improve survival of isolated islets in culture and maybe also the success of engraftment. However, once transplanted, islets are exposed to a much more complex microenvironment than in culture. The LN-332 that has some beneficial effects in vitro may represent a potential threat for islets in vivo. This adverse role of LN-332 is illustrated by our observations on the effect of LN-332 on activation of macrophage secretory function (Part II).
**ABBREVIATIONS USED IN THE TEXT**

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
</tr>
<tr>
<td>BM</td>
<td>Basement membrane</td>
</tr>
<tr>
<td>Coll</td>
<td>Collagen</td>
</tr>
<tr>
<td>EGF</td>
<td>Epithelial growth factor</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>Fn</td>
<td>Fibronectin</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>Granulocyte-monocyte colony-stimulating factor</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>Interleukin-6</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun NH2-terminal kinase</td>
</tr>
<tr>
<td>KO</td>
<td>Knock out</td>
</tr>
<tr>
<td>LN</td>
<td>Laminin</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LTA</td>
<td>Lipoteichoic acid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant protein</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MT1-MMP</td>
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</tr>
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<td>NF-κB</td>
<td>Nuclear factor-κB</td>
</tr>
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<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>Nonobese diabetic</td>
</tr>
<tr>
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</tr>
<tr>
<td>PDGF</td>
<td>Platelet-derived growth factor</td>
</tr>
<tr>
<td>PFA</td>
<td>Paraformaldehyde</td>
</tr>
<tr>
<td>PI3-K</td>
<td>Phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PNF</td>
<td>Primary non function</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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</tr>
<tr>
<td>PMA</td>
<td>Phorbol myristate acetate</td>
</tr>
<tr>
<td>T1D</td>
<td>Type 1 diabetes</td>
</tr>
<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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I. GENERAL INTRODUCTION

1. Pancreas

1.1. Pancreas anatomy

In the human being, the pancreas is a long, narrow gland that is situated transversely across the upper abdomen, behind the stomach and the spleen. The mid-portion of the pancreas lies against the dorsal spine, the abdominal aorta, and the inferior vena cava. The right side of the organ (called the head) is the widest part of the organ and lies in the curve of the duodenum (the first section of the small intestine). The left side (called the tail) ends near the spleen. The body of the pancreas is located in between (Fig. 1).

Fig. 1. Anatomy of the human pancreas. a) the human pancreas is adjacent to the duodenal part of the small intestine. b) The exocrine part of the pancreas is represented by acinar cells which produce and secrete digestive enzymes. c) Schematic representation of the endocrine part of the pancreas consisting of four hormone-producing cell types, organized in islets of Langerhans (illustration taken from Edlund, 2002, Nature Reviews Genetics).
The pancreas is derived from the foregut endoderm and comprises three main elements: exocrine tissue organized in grape-like clusters called acini, which produces and secretes a mixture of about 20 different digestive enzymes; a duct system where the enzymes are discharged upon stimulation and that transports the digestive enzymes into the duodenum; and endocrine tissue (islets of Langerhans) that secretes crucial hormones for glucose homeostasis and body energy metabolism (Meglasson and Matschinsky, 1986). The entire pancreas is covered with a thin connective tissue capsule.

1.2. Islets of Langerhans: cellular organization and function

The islets of Langerhans are small micro-organs consisting of a few thousand endocrine cells, distributed throughout the pancreas and surrounded by exocrine tissue. A normal human pancreas consists of approximately one million islets that represent 1-2% of the total pancreatic volume (Robertson, 2004). Pancreatic islets are clusters of cells that are made up of at least four different types of endocrine cells, namely the insulin-containing β-cells, the glucagon-containing α-cells, the somatostatin-containing δ-cells, and the pancreatic polypeptide-containing PP-cells. The topographical and numerical relationships of these cells are species dependent. In laboratory rodents, the cells are not randomly distributed: the predominating β-cell population forms the central core of the islet, surrounded by a peripheral mantle of non-β-cells (α-cells, δ-cells and PP-cells) arranged in a two to three layer fashion (Baetens et al., 1979; Cirulli et al., 1994; Orci and Unger, 1975). Detailed quantitative studies assessing the cell composition and organization in human islets remain sparse. When compared to rodent islets, human islets contain proportionally fewer β-cells and more α-cells. In addition, the segregation of cell types into different regions is not as clear as in rodent islets and there is a controversy about whether human islet cells really have a mantle-core organization or are randomly distributed. Indeed, some studies report that non-β-cells are intermingled with β-cells in human islets (Brissova et al., 2005; Cabrera et al., 2006) whereas other studies tried to demonstrate a distribution of islet cells in humans similar to rodents (Bonner-Weir and O'Brien, 2008; Erlandsen et al., 1976). The reality is that human islets have a
complex organization with many different profiles. Indeed, small islets exhibit a mantle-core arrangement as observed in rodent islets while larger islets have a lobulated-structure composed of several mantle-core subunits (Bonner-Weir and O'Brien, 2008; Erlandsen et al., 1976; Orci, 1976). Most of the non-β-cells are found at the islet periphery and along vasculature that penetrates larger islets, between mantle-core subunits (Bonner-Weir and O'Brien, 2008; Erlandsen et al., 1976; Grube et al., 1983). Our own experiments on the topographical relationship between insulin- and glucagon-positive cells within rodent and human islets confirmed these observations (Fig. 2, manuscript in preparation). Islets display heterogeneity not only in cell type distribution but also in size. Indeed, human islets range between 50 µm and 400 µm in diameter (Ricordi et al., 1990).

Fig. 2. Distribution of insulin- and glucagon-positive cells in human and rodents islets. Immunofluorescence staining for insulin (red) and glucagon (green) in mouse islet a), rat islet b) and human islet c) Scale bar = 40 µm.

Beside endocrine cells, the capillary network is the most conspicuous cellular component of pancreatic islets (Brissova and Powers, 2008; Orci, 1976). Indeed, islets need their own microvasculature to rapidly carry hormones and other secreted elements out of the islet, as well as to receive nutrients and regulatory factors (Bonner-Weir and Orci, 1982; Henderson, 1969). For this reason, islets are richly supplied with blood vessels. Large islets receive blood from one to three afferent arterioles, whereas the capillaries of smaller islets seem to be integrated with the exocrine capillary system (Bonner-Weir, 1993). These vessels form an intricate network, in which each endocrine cell is in contact with fenestrated endothelial cells
and from which it is separated by a specialized extracellular matrix (ECM) called the
basement membrane (BM). This latter will be discussed in chapter 2.1 below. Although
islets comprise only 1-2% of the mass of the pancreas, they receive about
10 to 15% of the pancreatic blood flow (Jansson and Carlsson, 2002; Menger et al.,
2001).

Finally, islet endocrine cells are well innervated by sympathetic,
parasympathetic, and sensory nerve fibers. The parasympathetic branch is thought to
be a regulator of the physiological islet hormone secretion (Ahren, 2000; Gilon and
Henquin, 2001).

Glucose homeostasis requires finely regulated insulin secretion by β-cells and
glucagon secretion by α-cells. The principal action of insulin is to promote the entry of
glucose into tissues and decrease hepatic gluconeogenesis, whereas glucagon
principally stimulates hepatic glycogenolysis when hypoglycaemia threatens. In
health, under fasting basal conditions insulin is secreted at a rate of ~2 pmol/kg/min
(Eaton, 1980; Polonsky et al., 1984) and after meal ingestion this rate increases by
as much as ~5–10-fold (Meier et al., 2005). Secretion of an adequate amount of
insulin is directly dependent on the total number of β-cells in the pancreas, often
referred as β-cell mass. The β-cell mass depends on a balance between growth,
neogenesis and death of β-cells. Different physiological and pathological states such
as pancreas development, pregnancy, insulin resistance, obesity and diabetes have
been reported to affect this balance and thus to influence the pancreatic β-cell mass.

1.3. Type 1 diabetes

Diabetes mellitus is a group of metabolic diseases characterized by high blood
glucose (hyperglycaemia) levels, which result from defects in insulin secretion, or
action, or both. Type 1 diabetes (T1D) is characterized by insulin deficiency
secondary to autoimmune-mediated selective destruction of islet β-cells (Atkinson
and Eisenbarth, 2001; Bach, 1997; Gepts, 1965; Soeldner et al., 1985; Tisch and
McDevitt, 1996). Experimental and clinical data accumulated over the past two
decades indicate that T lymphocytes are key elements involved in the pathogenesis
of T1D, in both human beings and rodents, such as the nonobese diabetic (NOD)
mouse (Roep, 2003). Indeed, the important contribution of CD4 and CD8 T lymphocytes toward the pathogenesis of T1D was highlighted not only by histological studies but also by the use of immunotherapies selectively targeting T lymphocytes (Goudy and Tisch, 2005; Roep, 2003). Various mechanisms for T lymphocyte-induced β-cell destruction have been proposed. These involve Fas/FasL, perforin/granzyme pathways, Rae1-NKG2D interactions, reactive oxygen species and proinflammatory cytokines (Chervonsky et al., 1997; Kagi et al., 1997; Ogasawara et al., 2004; Suarez-Pinzon et al., 2001; Thomas et al., 2004).

In addition to T lymphocytes, it has been shown more recently that B lymphocytes were also involved in T1D development (Silveira and Grey, 2006). Indeed, NOD mice deficient in B lymphocytes from birth failed to develop T1D (Serreze et al., 1996). Moreover, B lymphocytes depletion by anti-CD20 monoclonal antibody early in the course of disease development prevented T1D or delayed progression of disease in NOD mice (Xiu et al., 2008).

Apart from diabetogenic T and B lymphocytes, experimental data suggest that cells of the monocyte-macrophage lineage are involved in the final stage of autoimmune-mediated β-cell destruction (Calderon et al., 2006; Jun et al., 1999). Indeed, it was shown first that depletion of monocytes in vivo hampered development of T1D. Then, activated macrophages isolated from infiltrated islets exhibited in vitro cytolytic activity toward primary islet β-cells (Calderon et al., 2006). Using transgenic mouse models, Martin and colleagues demonstrated that monocytes could be recruited to islets when the chemokine CCL2 (also known as monocyte chemoattractant protein-1 (MCP-1)) was expressed transgenically in β-cells (Martin et al., 2008). These cells of the innate immune system were able to destroy β-cells, resulting in T1D even in the absence of mature B and T lymphocytes, suggesting that monocytes and macrophages play a more important role in the onset of the disease than previously suspected (Martin et al., 2008). Therefore, all these results permit us to add the cells of the monocyte-macrophage lineage to the growing list of immune cells involved in T1D development and islet β-cell destruction.

T1D is characterized by two distinct histopathological stages: peri-insulitis and diabetes. Peri-insulitis consists of an initial infiltration of leukocytes including activated cells of the monocyte-macrophage lineage, B lymphocytes, T lymphocytes and natural killer cells around the islets without apparent effects on β-cells. These
cells are attracted to the islets by chemokines which are chemoattractant proteins (Baggiolini and Loetscher, 2000; Bendall, 2005) produced by cells in response to infection or cell damages (Bendall, 2005). Leukocytes express chemokine receptors, and when a chemokine interacts with an appropriate receptor, cells migrate toward the source of chemokine production. For example, CCL2, is produced in response to inflammatory stimulus by many cells, including cells of the monocyte-macrophage lineage, lymphocytes, and endothelial cells (Oppenheim et al., 1991). Peri-insulitis is then followed by a more aggressive step wherein leukocytes actively invade the islets and kill β-cells, leading to β-cell mass reduction, and consequently diabetes.

Chronologically, T1D begins with a declining phase of insulin secretion over the years and is followed by a developing hyperglycaemia phase due to a declining β-cell mass (Lo et al., 1992; Srikanta et al., 1984). In humans, up to 40% loss of β-cells can be tolerated without significant deterioration of glucose tolerance (Butler et al., 2003), but additional reduction in β-cell mass leads to hyperglycaemia.

T1D is lethal unless a treatment with exogenous insulin via injections replaces the missing hormone, or a functional replacement of the destroyed pancreatic β-cells is provided via pancreas or pancreatic islet transplantation. The different treatments to cure T1D will be briefly discussed in the following section.

1.4. Islet transplantation as a means to cure type 1 diabetes

The major goal in treating T1D is to minimize any elevation of blood glucose without causing abnormally low levels of blood glucose. Exogenous insulin therapy has greatly improved over the last twenty years. Although major advances in insulin treatment have been achieved, hypoglycaemic unawareness remains an obstacle for optimal glycaemic control in many patients (Cryer, 2002). This complication not only impairs quality of life but is also life-threatening (Shu and He, 1993). β-Cell mass replacement therapy could provide a cure for T1D. In this context, whole-organ pancreas transplantation is a procedure that can lead to euglycaemia and insulin-independence in the vast majority of recipients, with graft survival rates as high as 78% at five years (Sollinger et al., 1998). However, despite significant progress, pancreas transplantation is still associated with peri-operative mortality and
significant morbidity (Humar et al., 2000; Manske, 1999). Allogeneic islet transplantation is an attractive alternative to pancreas transplantation because it can be performed as a minimally invasive percutaneous procedure, in which islets are infused into the liver by portal vein embolization (Oberholzer et al., 1999). In addition, islet transplantation is a procedure with low morbidity as compared to whole pancreas transplantation (Berney et al., 2001a). Permanent normoglycaemia is not always achieved by islet transplantation, but in most cases a stabilization of glycaemia is obtained and at least severe hypoglycaemia is prevented. The experimental and clinical results obtained so far are encouraging and the demonstration that allogeneic islet transplantation may lead to prolonged insulin independence is a new hope in the care of patients with T1D.

1.4.1. Obstacles to islet engraftment

Despite encouraging results, islet transplantation is at a clinical experimental stage and still encounters obstacles that need to be overcome. Indeed, grafted islets are exposed to a number of adverse conditions that lead to poor engraftment and loss of islets, early after transplantation (Berney et al., 2001b). Several possible reasons can explain this impairment in islet graft success and survival.

Chronologically, the isolation procedure itself is the first cause of β-cell mass loss. Enzymatic digestion of the pancreas by collagenase separates islets from their initial environment composed of ECM proteins and surrounding exocrine tissue. Isolated islets lose some of their characteristics as micro-organs because of loss of vascularization (which feeds islets), innervation and cell-to-ECM contacts with the surrounding tissue. As isolated islets are avascular, they are more sensitive and specifically prone to hypoxia, at least during the time elapsing before neovascularization of the graft (Menger et al., 1992). Moreover, several studies using islets from diverse species, including pig, dog, non-human primate, and human have demonstrated that islet isolation procedure is associated with increased cell death and β-cell dysfunction even several days following isolation (Paraskevas et al., 1999; Rosenberg et al., 1999; Thomas et al., 2001; Thomas et al., 1999; Wang and Rosenberg, 1999).
Other events including primary non-function (PNF), immunological rejection and the recurrence of the autoimmune process of the disease contribute to the loss of the transplanted islets. PNF refers to all non-specific inflammatory events that occur immediately after intraportal infusion of islets, including inflammatory elements of the hepatic microenvironment in which islets are implanted. These inflammatory elements involve liver endothelial cells, Kupffer cells (residing macrophages of the liver), and components of the blood stream, such as complement, monocytes (Bennet et al., 2000; Bennet et al., 1999) and T lymphocytes. Islet damages induced by activated macrophages, including Kupffer cells, are mediated by cytokines, nitric oxide (NO) and oxygen free radicals (Kaufman et al., 1990; Rabinovitch et al., 1996). The use of macrophage-depleting drugs in murine and canine models has been shown to improve early graft survival (Bottino et al., 1998; Kaufman et al., 1994; Stephanian et al., 1992). These experiments point out the crucial role played by the cells of the monocyte-macrophage lineage in PNF.

1.4.2. Cells of the monocyte-macrophage lineage

Macrophages comprise a heterogeneous population of cells that belong to the mononuclear phagocyte system. They play an essential role in development, homeostasis, repair, remodelling processes, initiation and mediation of immune and inflammatory responses observed in graft rejection as well as in the initiation of T1D (Lacy, 1994). These cells have at least three major functions: (1) antigen presentation, (2) phagocytosis of self or foreign agents, (3) and immunomodulation through production of cytokines and various growth factors (Kasahara and Matsushima, 2001).

Macrophages represent the terminally differentiated stage of cells that originate from a myeloid progenitor cell in the bone marrow (Fogg et al., 2006). The immature cells differentiate into monocytes and leave the bone marrow to enter the blood vessels, where they circulate from one to several days. Then, they cross the subendothelial BM and immigrate to various organs or tissues to differentiate into specialized organ macrophages, in response to local environment (van Furth, 1989) (Fig. 3). The precise external signals that control differentiation of monocytes into tissue macrophages are incompletely defined. However, several molecules, including
monocyte colony-stimulating factor, granulocyte-monocyte colony-stimulating factor (GM-CSF), MCP-1 and/or interleukin (IL)-3 are considered as potential candidates implicated in the differentiation process. During this step, cells grow in size and increase their lysosomal enzymes, content of mitochondria and metabolism (Valledor et al., 1998).

Usually, resident macrophages are designated by their organ of residence; for example, microglia are localized in the brain, mesangial cells in the kidney, osteoclasts in the bone, and Kupffer cells in the liver. It is important to note that liver contains the largest number of macrophages in the body (Crofton et al., 1978; Munthe-Kaas et al., 1975). The observation of macrophages in mature organs was made possible through the use of transgenic mice in which macrophages were recognized specifically by their green fluorescence (Sasmono et al., 2003).

Cells of the monocyte-macrophage lineage are dynamic and heterogeneous cells. This is due to different mechanisms governing their differentiation, tissue distribution, and responsiveness to stimuli (Gordon, 2003; Mosser, 2003) and to the microenvironment that may also constrain the functional properties of macrophages (Lambrecht, 2006; Lumeng et al., 2007a; Lumeng et al., 2007b).

The diversity of macrophage functions has led to further subclassify macrophages according to their cellular behaviour and response to cytokines. Classically activated macrophages are involved in phagocytosis and production of pro-inflammatory cytokines. Alternatively activated macrophages are a heterogeneous group of cells induced by the T lymphocyte cytokines IL-4 and IL-13, as well as glucocorticoids and transforming growth factor (TGF)-β (Mantovani et al., 2004). Alternatively activated macrophages have a role in tissue remodelling and repair and also carry out immunoregulatory functions and promote angiogenesis.
1.4.3. Inflammation and activation of cells of the monocyte-macrophage lineage

Inflammation is a complex, highly regulated sequence of events that may be provoked by a variety of stimuli including pathogens, noxious mechanical and chemical agents. Inflammation may be observed during bacterial infection as well as after organ transplantation. Cells of the monocyte-macrophage lineage contribute to the development of inflammatory response by secreting an array of cytokines and chemokines in a tissue microenvironment. Proinflammatory cytokines such as IL-1β and tumour necrosis factor (TNF)-α are activators of macrophages and upregulate the expression and biosynthesis of cytokines and chemokines.
Cells of the monocyte-macrophage lineage exist either at the resting or activated state during the inflammatory response. They can be activated to fulfil their function either at the monocyte stage in the vessels or at the macrophage stage within tissues. When activated, cells increased their size, their production of lysosomal enzymes and their active metabolism, and have a greater ability to phagocyte and kill self or non-self cells. The term “activated” must not be confused with “differentiated”, which applies to monocytes in the process of differentiation. Generally, it is accepted that classically activated cells of the monocyte-macrophage lineage develop in response to two successive signals. The first one is the cytokine interferon (IFN)-γ which primes cells for activation and the second signal is provided by microbial products such as lipopolysaccharides (LPS) or lipoteichoic acid (LTA) from the surface of Gram-negative or Gram-positive bacteria, respectively (Chen et al., 1992; Ginsburg, 2002; Tobias and Ulevitch, 1993). Although natural killer cells produce IFN-γ, the main source of this cytokine remains the T lymphocyte cells (Young, 2006). LPS and LTA activate cells of the monocyte-macrophage lineage by binding to toll-like receptors (TLR) expressed on cell membrane (Medzhitov et al., 1997). Both of these stimuli generally induce the production of TNF-α by the cells of the monocyte-macrophage lineage, which itself also activates these cells. The combination of TNF-α and IFN-γ results in optimal cell activation.

At the molecular level, binding of LPS or other activating ligands to TLR lead to the activation of signalling cascade pathways including nuclear factor (NF)-κB (Muller et al., 1993) and mitogen-activated protein kinase (MAPK) family members (Chan and Riches, 1998; Dong et al., 2002; Hambleton et al., 1996; Liu et al., 1994; Scherle et al., 1998; Vanden Berghe et al., 1998; Weinstein et al., 1992). MAPKs constitute more than a dozen proteins belonging to three families, extracellular signal-regulated kinase (ERK), p38, and c-Jun NH2 terminal kinase (JNK) (Cobb, 1999; Kyriakis and Avruch, 1996). p38 is the most studied MAPK in terms of inflammatory responses. Among the four isoforms that have been described, p38α is the predominant form expressed in cells of the monocyte-macrophage lineage and may play a major role in the inflammatory response (Hale et al., 1999). After activation, MAPKs translocate to the nucleus where they activate several targets such as transcription factors that regulate various genes encoding inflammatory mediators. These transcription factors include ELK1 (Janknecht et al., 1993; Marais
et al., 1993), ATF2 (Livingstone et al., 1995), and c-jun (Derijard et al., 1994) that induce activation of other transcription of early genes involved in biological processes such as cell activation, proliferation and transformation.

These classically activated cells of the monocyte-macrophage lineage become strongly microbicidal and act as important immune effector cells to process and present antigen through major histocompatibility complex-II, to promote lymphocyte Th1 type inflammation via the production of IL-12, and to mediate cytotoxicity via production of inducible nitric oxide synthase (iNOS) and cytokines such as TNF-α (Van Ginderachter et al., 2006). In the murine and rat system, activated cells of the monocyte-macrophage lineage are easily identified by virtue of their production of nitric oxide (NO) (Hibbs, 2002; MacMicking et al., 1997). On the opposite, in the human system, the activation of these cells is more difficult to determine, as monocyte-derived macrophages from the peripheral blood generally do not produce NO in response to the classical activating stimuli. For this reason, other criteria permitting the identification of human activated macrophages have to be considered: upregulation of surface molecules such as major histocompatibility complex-II and CD86 and an enhanced ability to present antigen and kill intracellular pathogens.

Once activated, cells of the monocyte-macrophage lineage may secrete toxic oxygen species, reactive nitrogen, such as NO, and a large number of proinflammatory cytokines, including IL-1, IL-6, TNF-α, IFN-α/β, IL-10, IL-12, and IL-18 (Le Meur et al., 2002; Okamura et al., 1998). IL-1, IL-6, TNF-α, and IFN-α/β are cytokines also involved in the regulation of haematopoiesis (Sieff C.A., 1998). NO is a highly reactive free radical, produced from the amino acid L-arginine. It is a small, uncharged molecule that diffuses across biological membranes. Nitrite (NO$_2^-$) is a stable product of NO, and determination of NO$_2^-$ in the culture medium provides a reliable indication of the amount of NO produced by cultured cells during prolonged time periods (Gross and Wolin, 1995; Moncada and Higgs, 1993).
1.4.4. Cells of the monocyte-macrophage lineage and their interaction with extracellular matrix

In addition to tissue macrophages, during an inflammatory response, circulating blood monocytes respond to chemotactic factors and subsequently exude into inflamed tissues or inflammatory lesions. In order to reach the site of inflammation, monocytes have to cross the subendothelial BM and underlying interstitial structures, which are both rich in ECM proteins. It is also in this ECM-rich environment that monocytes are differentiated into macrophages and activated to perform their functions.

Haskill and colleagues were the first to identify adherence to substrates as a powerful inducer of gene expression in human monocytes (Haskill et al., 1988). Since then, different studies demonstrated the impact of ECM on the biology of cells of the monocyte-macrophage lineage. Fibronectin promoted cell migration (Hemler, 1990) and phagocytosis (Hemler, 1990; Newman and Tucci, 1990), and has been shown to induce a rapid mRNA expression of several inflammatory genes (Juliano and Haskill, 1993). Adherence of monocytes to ECM also modulated the secretion of inflammatory mediators such as proteases (Xie et al., 1993), superoxide anion $O_2^-$, prostaglandin E2, and thromboxane (Gudewicz et al., 1994), and finally cytokines such as IL-1β, IL-8, TNF-α and GM-CSF (Eierman et al., 1989; Juliano and Haskill, 1993; Kasahara et al., 1993; Wesley et al., 1998; White et al., 2001). Adherence alone is not sufficient to allow translation and secretion of cytokines. Other stimuli are necessary to synergize ECM effects. For example, adherence of monocytes to ECM substrates markedly potentiated LPS-induced TNF-α production (Bauer et al., 2000; Haskill et al., 1988) and production of IL-8 in response to a wide variety of stimuli such as TGF-β (Wang and Fu, 2001).

Attachment of cells of the monocyte-macrophage lineage to ECM substrates is mediated predominantly by the members of the integrin family of adhesion molecules (Hemler, 1990). In the case of this cell type, the key integrin involved in adhesion to ECM substrates belong mainly to the subclass of the β1 integrin family. Interactions between cells of the monocyte-macrophage lineage and ECM through integrins generate “outside-in” signals.
Monocytes express a broad panel of different integrins including $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ (de Fougerolles and Koteliansky, 2002). Among these integrins, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ are constitutively produced and expressed at high levels on the cell surface of monocytes, while $\alpha_2\beta_1$ is expressed at lower levels, and $\alpha_1\beta_1$ is upregulated upon monocyte activation (Hemler, 1990; Rubio et al., 1995). More recently, in murine macrophages, discoidin domain receptor, a non integrin collagen receptor, has been shown to induce iNOS expression and consequently NO biosynthesis (Kim et al., 2007). Studies of intracellular signal transduced by integrins have revealed some signalling pathways involved in cell biology and activation of the cells of the monocyte-macrophage lineage. Some classical integrin-induced signalling pathway will be reviewed in section 2.4.1.

Concomitantly with the effect of ECM on the biology of cells of the monocyte-macrophage lineage, a symmetrical relationship is possible between ECM and these cells. Indeed, exposure of macrophages to inflammatory stimuli can induce the expression of several serine proteases, matrix metalloproteases (MMPs) and heparanases that modify ECM molecules via enzymatic degradation (Schor et al., 2000). This degradation may have several functions, including: (1) the facilitation of movement across the BM and tissue parenchyma, (2) the release of ECM-bound chemokines and cytokines, (3) and the release of fragments of ECM molecules, which may in turn activate cells of the monocyte-macrophage lineage. Particularly, many fragments of fibronectin have been shown to be potent stimuli for these cells (Barilla and Carsons, 2000). For instance, a 110-120-kDa fragment of fibronectin has been shown to bind to macrophages via the $\alpha_5\beta_1$ integrin receptor and transduce signal that either directly activate cells or prime cells for activation by other ligands (Trial et al., 2004). In addition, a cryptic peptide in the $\alpha$ chain of LN-511 has been shown to induce recruitment of cells, expression of cytokines such as TNF-$\alpha$, and release of proteases such as MMP-9, in a murine macrophage cell line (Adair-Kirk et al., 2005).
2. The extracellular matrix

General characteristics of the ECM with regard to organization and composition are well known. Organized fibrous polymers, such as collagen, elastin, and resilin, are embedded within an amorphous mixture of non-fibrous components known as the fundamental substance and composed predominantly by proteoglycans. The relative proportion of fibrous and non-fibrous components partially dictates the physical properties of a particular ECM. This three-dimensional organized network provides scaffolding, support, and strength to tissues and organs. The ECM is not a static structure, but is continually produced and remodelled by numerous and different cells. The ECM has long been recognized as an adhesive and support structure (Schnaper and Kleinman, 1993) and more recently has been shown to play a significant role in regulating the behaviour of cells. ECM proteins engender changes in cell shape and movement, bind growth factors, and facilitate cell-to-cell and cell-to-ECM interactions. These interactions operate through receptor-mediated signalling and directly or indirectly modulate the cell response to growth factors. Classic examples of cell-to-ECM interactions involve the cell surface receptors of integrin family. However, there are also non-integrin-based interactions between ECM and cell surface receptors. The ligand domain in ECM component may be cryptic, that is, exposed only after the ECM is modified. These bioactive ECM domains, designated “matricryptins”, have functions generally distinct from the native molecule (Davis et al., 2000; Schenk and Quaranta, 2003). In addition, the ECM serves also as a reservoir of biologically active molecules, such as cytokines and growth factors (Sternlicht and Werb, 2001). For example, basic fibroblast growth factor (bFGF), vascular endothelial growth factors (VEGF), platelet-derived growth factors (PDGF) or TGF are sequestered in the ECM and can be released after the ECM remodelling and/or degradation (Kalluri, 2003; Vlodavsky et al., 1991).

In tissues, the ECM exists as two main forms: the interstitial matrix present in connective tissues and the BM, a condensed layer adjacent to epithelial cells (Fig. 4).
Fig. 4. The basement membrane and connective tissue underlying an epithelial cell sheet.

2.1. The basement membrane

The BM is a specialized form of ECM, described as a 50- to 100-nm thin homogeneous sheet of proteins located at the interface between parenchymal cells and the surrounding stroma (Erickson and Couchman, 2000; Kalluri, 2003). The BM acts as a selective barrier and serves as a foundation on which cells are anchored and from which they receive signals for their functions, including maintenance of tissue-specific morphology, differentiation, survival, and gene activation (Watt, 2002). At the electron microscopy level, the BM is seen as a three layer component: the laminae lucida, densa (constituting together the basal lamina), and reticularis. The lamina lucida contains cell adhesion molecules and their receptors. The lamina densa is a sheet of connective tissue made up of type IV collagens, laminins (LNls), entactin (nidogen), and heparin sulfate proteoglycans. The lamina reticularis is located at the ECM side of the lamina densa, and contain anchoring fibrils. Virtually, all epithelial, endothelial, neuronal, fat and muscle cells in adult animals settle on a BM. The composition of the BM is cell-type, species and developmental stage specific (Miner and Yurchenco, 2004). The heterogeneous molecular compositions and biochemical complexity of different organ BMs are nearly as diverse as their unique specific biological functions.
2.2. The extracellular matrix of islet of Langerhans

The ECM plays a crucial role in foetal and postnatal pancreas development, and participates in maintaining tissue integrity in adult life. Early, during normal embryonic pancreas development, immature precursor endocrine cells bud from the primitive pancreatic duct epithelium, migrate through the ECM, and associate to form islets. Among the ECM proteins, LN-111 (which is expressed only in the embryonic pancreas) was shown to be required for differentiation of isolated E13.5 mouse pancreas cells into insulin-positive β-cells (Jiang et al., 1999). Moreover, Bonner-weir and colleagues reported that pancreatic ducts isolated from human pancreas expanded and differentiated into glucose responsive islet tissue in culture, when overlaid with LN-111-rich ECM (Bonner-Weir et al., 2000). Another study showed that rat pancreatic endocrine cells embedded in three-dimensional gel composed of type I collagen reorganized into islet-like structure with non-random distribution of islet cells similar to that observed in vivo (Montesano et al., 1983a). Altogether, these data demonstrated the importance of ECM proteins on islet cell differentiation and islet morphogenesis.

In the adult pancreas, ECM is certainly the most important component of the islet microenvironment. It serves as a cellular scaffold, and functions to regulate proliferation, survival, and differentiation. In situ, islets are surrounded by a peri-insular capsule mainly composed of collagen type IV and moderate collagen type I (van Deijnen et al., 1992; White et al., 1999), fibronectin (Hamamoto et al., 2003) and LN (Jiang et al., 2002). Within islets, a BM is separating endothelial cells from endocrine cells. The cellular origin of this BM is still a matter of debate. Some authors suggested that BM was mainly deposited by endothelial cells (Nikolova et al., 2006); and others by endocrine cells (Virtanen et al., 2008).

Evidence for a role of ECM on islet and β-cell function comes from in vitro studies. Indeed, islets cultured on matrices containing different ECM proteins exhibited improved viability (Lucas-Clerc et al., 1993), proliferation, and insulin secretion (Hulinsky et al., 1995a; Hulinsky et al., 1995b). In addition, individual ECM components also affected motility (Kaido et al., 2004a), survival (Ris et al., 2002) and proliferation (Beattie et al., 1997; Hayek et al., 1989) of β-cells. More recently, using a matrix secreted by the rat bladder carcinoma 804G cell line, it has been
demonstrated that LN-332 was able to increase insulin secretion (Bosco et al., 2000) and to induce proliferation of rat \(\beta\)-cells \textit{in vitro} (Parnaud et al., 2008). LN-332 also exhibited some protective effect against apoptosis (Hammar et al., 2004). Consequently, LNs and especially LN-332 seem to be of crucial importance in islet biology. The next sections will present general concepts of LNs.

2.3. Laminins

2.3.1. Structure of laminins

LNs are large heterotrimeric glycoproteins whose molecular masses vary between 400 kDa and 900 kDa. All members of the LN family are composed of three different polypeptide chains termed \(\alpha\)-, \(\beta\)- and \(\gamma\)-chain (Colognato and Yurchenco, 2000). The first LN, presently known as LN-111, was isolated over twenty years ago from the matrix of the mouse Engelbreth–Holm–Swarm tumour as a cross-shaped protein of disulfide-linked chains of about 200 and 400 kDa. Following molecular cloning of these three chains (\(\alpha_1\), \(\beta_1\), and \(\gamma_1\)) at the end of the 1980s, several isoforms were isolated, revealing a previously unsuspected heterogeneity in LNs. Today, 11 distinct chains of LN (five \(\alpha\)-chains, three \(\beta\)-chains and three \(\gamma\)-chains) have been identified and described so far in vertebrates (Miner and Yurchenco, 2004) (Fig. 5). These chains assemble into at least 15 different LN heterotrimer isoforms in mammals (Aumailley et al., 2005; Burgeson et al., 1994; Koch et al., 1999; Libby et al., 2000). The different LNs identified so far and their heterotrimeric compositions are listed in Table I. The number of combinations that can be assembled from the three chains exceeds the number of LNs identified. It is likely that additional heterotrimers will be discovered, though it appears that some chains, such as \(\beta_3\) and \(\gamma_2\), have only limited allowable associations.

Each chain of the LN is composed of rod-like, globular, and coiled-coil regions (Fig. 5). The largest chain is the \(\alpha\)-chain which contains a 100-kDa C-terminal globular domain termed the G domain. The G domain can be subdivided into five self-folding modules termed LN G (LG) domains (LG1 through LG5) (Timpl et al.,
These LG domains are involved in interactions with cellular receptors such as integrins and dystroglycans.

**Fig. 5. Domains of LN α, β, and γ chains.** Long arms of each chain are composed of α helical coils (LCC). Short arms of each chain comprise rodlike epithelial growth factor (EGF)-like tandems (LEa, LEb, LEc) and globular domains (LN, L4a, L4b, L4, LF). α-Chains have a distinctive globular domain, consisting of five repeats (LG1–5), at the C-terminal of the long arm. β-Chains have a characteristic interruption of the coiled-coil structure, the Lβ domain. Black arrows indicate cleavage sites (illustration adapted from Tzu and Marinkovich, 2008, The International Journal of Biochemistry and Cell Biology).
The N-terminal is more associated with LN polymerization of the molecule but in some case, it can also interact with integrin receptors (Patarroyo et al., 2002). The β- and γ-chains are also involved in interactions with molecules present in the ECM. β-Chains have a characteristic interruption of the coiled-coil structure, known as the LN Lβ domain which is one major characteristic that distinguishes them from the γ-chains. In the majority of LN isoforms, chain assembly results in a typically cross-shaped structure when viewed by rotary shadowing electron microscopy (Martin and Timpl, 1987) (Fig 6A). The separate chains are held together at the coiled-coil regions by disulfide bonds (Beck et al., 1993; Ekblom et al., 2003; Miner and Yurchenco, 2004). Figure 6B shows the structure of two typical LN heterotrimers.

<table>
<thead>
<tr>
<th>Former name</th>
<th>Chain composition</th>
<th>New nomenclature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminin-1</td>
<td>α1β1γ1</td>
<td>LN-111</td>
</tr>
<tr>
<td>Laminin-2</td>
<td>α2β1γ1</td>
<td>LN-211</td>
</tr>
<tr>
<td>Laminin-3</td>
<td>α1β2γ1</td>
<td>LN-121</td>
</tr>
<tr>
<td>Laminin-4</td>
<td>α2β2γ1</td>
<td>LN-221</td>
</tr>
<tr>
<td>Laminin-5A</td>
<td>α3Aβ3γ2</td>
<td>LN-332</td>
</tr>
<tr>
<td>Laminin-5B</td>
<td>α3Bβ3γ2</td>
<td>LN-332</td>
</tr>
<tr>
<td>Laminin-6</td>
<td>α3β1γ1</td>
<td>LN-311</td>
</tr>
<tr>
<td>Laminin-7</td>
<td>α3β2γ1</td>
<td>LN-321</td>
</tr>
<tr>
<td>Laminin-8</td>
<td>α4β1γ1</td>
<td>LN-411</td>
</tr>
<tr>
<td>Laminin-9</td>
<td>α4β2γ1</td>
<td>LN-421</td>
</tr>
<tr>
<td>Laminin-10</td>
<td>α5β1γ1</td>
<td>LN-511</td>
</tr>
<tr>
<td>Laminin-11</td>
<td>α5β2γ1</td>
<td>LN-521</td>
</tr>
<tr>
<td>Laminin-12</td>
<td>α2β1γ3</td>
<td>LN-213</td>
</tr>
<tr>
<td>Laminin-14</td>
<td>α4β2γ3</td>
<td>LN-423</td>
</tr>
<tr>
<td>Laminin-15</td>
<td>α5β2γ3</td>
<td>LN-523</td>
</tr>
</tbody>
</table>
**Fig. 6. Structure of LN.**

A) Image representing a LN variant, as viewed by rotary shadowing electron microscopy (image taken from Marinkovich, 1992, Journal of Cell Biology).

B) The classic cruciform structure is representative of most LN heterotrimers as shown in the example of LN-111 and LN-332 (illustration taken from Marinkovich, 2007, Nature Reviews Cancer).
2.3.2. Laminin expression in tissues

LNs are synthesized by numerous cell types, and expression of LN isoforms, particularly their α-chain, is cell- or tissue-specific. Expression patterns of LN are regulated both temporally and spatially during development. For example, the α1-chain, found in LN-111 and LN-121, is widely expressed during early embryogenesis. It is expressed predominantly in pre- and post-implantation stages and is important for epithelial development in many tissues including pancreas and islet morphogenesis (Burgeson et al., 1994; Jiang et al., 1999). In adult, its expression is restricted to reproductive organs, kidney, and liver (Ekblom et al., 2003). The α2-chain, present in LN-211, LN-221 and LN-213, is mostly localized to the neuromuscular system such as BM of the myofiber sarcolemma and the neuromuscular junction. The α3-chain, present in LN-332, LN-311 and LN-321, is found in skin and other epithelia. LN-332, LN-311 and LN-321 are often linked together in human skin and amnion (Hirosaki et al., 2002). LN-332 is also confined to BM underneath epithelia with secretory or protective functions, such as in salivary glands and skin (Aumailley and Rousselle, 1999). The α4-chain, found in LN-411, LN-421 and LN-423, is localized in mesenchymal and endothelial cells (LN-411). It was also demonstrated that cells derived from the bone marrow were able to secrete LN-411 (Wondimu et al., 2004). The α5-chain, found in LN-511, LN-521 and LN-523, is widely expressed throughout the body, in adult epithelial, neuromuscular and vascular tissues. As α1-, α5-chain is found in significant amount during embryogenesis (Colognato and Yurchenco, 2000). LN-511 is also a major component of the BM of vascular endothelial cells.

β3- and γ2-Chains are selectively expressed by epithelial cells, whereas β1-, β2-, and γ1-chains have wide pattern of expression.

LNs are mainly, but not exclusively, found in BMs, and BMs always contain at least one α-chain (Aumailley and Smyth, 1998; Colognato and Yurchenco, 2000; Miner et al., 1997). Commonly, two or three α-chains are found in a single BM, and its LN composition may vary during development and maturation. Interestingly, both epithelial and mesenchymal cells contribute to the synthesis of LN chains of a common BM (Hynes, 1992). Table II illustrates the main organs in which the different LN α-, β- and γ-chains have been identified.
Table II. System and tissue distribution of laminin genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>System</th>
<th>Tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>LAMA1</td>
<td>Nervous</td>
<td>Brain, spinal cord</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Liver, pancreas, prostate, salivary glands</td>
</tr>
<tr>
<td></td>
<td>Immune</td>
<td>Spleen, Thymus</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Lung, kidney, retina, placenta</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMA2</td>
<td>Muscular</td>
<td>Cardiac muscle, skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>Nervous</td>
<td>Peripheral nerve, meninges</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Pancreas, adrenal glands, salivary glands</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Lung, placenta, kidney, bladder, capillaries, testis, skin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMA3</td>
<td>Nervous</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Prostate, intestinal epithelial</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Skin, lung, epithelial of urinary tract</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMA4</td>
<td>Nervous</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Muscular</td>
<td>Cardiac muscle</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Ovary, Intestines, placenta, lung, blood vessels, dermis, muscle</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMA5</td>
<td>Muscular</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Prostate, liver</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Lung, retina, kidney, placenta</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMB1</td>
<td>Nervous</td>
<td>Brain, spinal cord</td>
</tr>
<tr>
<td></td>
<td>Muscular</td>
<td>Cardiac muscle</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Prostate, pancreas</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Lung, kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMB2</td>
<td>Nervous</td>
<td>Brain, spinal cord</td>
</tr>
<tr>
<td></td>
<td>Muscular</td>
<td>Cardiac muscle, skeletal muscle (synaptic cleft)</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Prostate, pancreas</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Lung, kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMB3</td>
<td>Nervous</td>
<td>Brain, spinal cord</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Skin, lung, kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMC1</td>
<td>Muscular</td>
<td>Cardiac and skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>Nervous</td>
<td>Brain</td>
</tr>
<tr>
<td></td>
<td>Secretory</td>
<td>Prostate, liver, pancreas</td>
</tr>
<tr>
<td></td>
<td>Others</td>
<td>Lung, kidney</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAMC2</td>
<td>Muscular</td>
<td>Skeletal muscle</td>
</tr>
<tr>
<td></td>
<td>Nervous</td>
<td>Brain, spinal cord</td>
</tr>
</tbody>
</table>
Of the many LN isoforms, LN-332 is unique in both structure and activity. Indeed, the β3- and γ2-chains differ significantly from other LN chains. β3-Chain contains LN domain but lacks the L4 domain and has substantially less LE repeats. On the opposite, γ2-chain contains L4 domain but lacks LN domains. α3-Chain was first identified with a truncated N-terminal. A recent study has characterized a new LN-332 isoform that contains a long α3-chain (Kariya et al., 2004). Thus, α3-chain is expressed as two splice variants in LN-332: α3A with a truncated N-terminal and α3B with an extended N-terminal. Although LN-332B has not been detected in cultured cells, it appears to be expressed in normal tissues at higher levels than LN-332A (Mizushima et al., 1996).

The main role of LN-332 in normal tissues is in the maintenance of epithelial-mesenchymal cohesion in tissues exposed to external disruptive forces. LN-332 is secreted by keratinocytes and expressed in the dermo-epidermal junction of the skin (Carter et al., 1991; Rousselle et al., 1991) but also in stratified squamous epithelial tissues, transitional epithelia, lung, kidney and various glands (Miosge et al., 2002). The crucial role of LN-332 in epidermal adhesion was further highlighted by the demonstration that mice deficient in one of the three genes encoding the α3- (LAMA3), β3- (LAMB3) and γ2-chains (LAMC2) died within five days of birth because of severe skin blistering that resembles that of Herlitz’s junctional epidermolysis bullosa in humans (Kuster et al., 1997; Meng et al., 2003; Ryan et al., 1999). In vitro, LN-332 promotes attachment, spreading, scattering and migration of human keratinocytes by interacting mainly with integrin α3β1, α6β1 and α6β4 integrins at far lower concentrations than other cell adhesion proteins (Delwel et al., 1994; Marchisio
et al., 1993; Rousselle and Aumailley, 1994). As discussed above, LN-332 also induces attachment and spreading of rat β-cell and affects their insulin secretion, viability and proliferation (Bosco et al., 2000; Hammar et al., 2004; Parnaud et al., 2008).

2.3.4. Laminin and laminin-332 biosynthesis, secretion and extracellular processing

Most LN chains are distinct LAMA\textsubscript{1-5}, LAMB\textsubscript{1-3}, and LAMC\textsubscript{1-3} gene products. Exceptions are the alternatively spliced α3A and α3B variants (Galliano et al., 1995; Miner et al., 1997; Ryan et al., 1994). All LN chains have N-terminal secretion signals that target them to the endoplasmic reticulum. Individual LN chains are then co-translationally glycosylated with high mannose oligosaccharide side chains at the N-terminus, within the rough endoplasmic reticulum. Glycosylation is essential to stabilize the chains and protect them from degradation (Morita et al., 1985). The assembly of the heterotrimer begins by the formation of a stable disulfide-linked βγ heterodimer. Then, it is followed by incorporation and disulfide cross-linking of the α-chain to the heterodimer, which drives secretion of the heterotrimer (Marinkovich et al., 1992; Yurchenco et al., 1997). This last stage is the rate-limiting step for secretion (Matsui et al., 1998; Matsui et al., 1995; Schneider et al., 2007). Before being secreted, the LN heterotrimer undergoes terminal glycosylation within the Golgi apparatus (Morita et al., 1985). Once in the extracellular environment LN can undergo proteolytic processing of its chain. Until now, extracellular processing has only been observed for the α2-, α3A-, α4-, α5- and the γ2-chains (Aumailley et al., 2003; Talts et al., 1998: Talts et al., 2000; Veitch et al., 2003). Extracellular processing of LN by specific enzymes has been mostly studied on LN-332 (Goldfinger et al., 1998; Hintermann and Quaranta, 2004; Koshikawa et al., 2004; Matsui et al., 1995; Mydel et al., 2008; Ogawa et al., 2004; Sugawara et al., 2008).

LN-332 is initially secreted as a heterotrimer containing a 325-kDa (α3B) or 190-kDa (α3A) α3-chain, a 140-kDa β3-chain, and a 155-kDa γ2-chain. But under appropriate conditions, in healthy cells, chains undergo further processing to smaller sizes. The α3-chain can be processed at both the C- and N-terminal ends. One
enzymatic cleavage occurs within the identical C-terminal end of the α3A and α3B variants, leading to the removal of the LG4 and LG5 domains and the conversion of the chains into 165-kDa and 280-kDa chains, respectively. The α3A chain can be converted to an even smaller chain of 145-kDa by processing of its N-terminal end. (Aumailley et al., 2003). In this case, cleavage occurs at the LEc region (Fig. 5). Plasmin, MMP-2, membrane type-1 (MT1)-MMP and bone morphogenetic protein-1 (BMP-1) are thought to be key enzymes involved in C-terminal α3 processing (Veitch et al., 2003). As LG domains are involved in binding to cell surface receptors such as integrins, syndecans and dystroglycans, processing of the α3-chain at the C-terminus is thought to regulate cellular movement. In human skin BM, the α3 processed form at the C-terminal end is present in all LN-332 (Aumailley et al., 2003) whereas the processed form at the N-terminal end is found in only half of LN-332 (Marinkovich et al., 1992). LN-332 containing the unprocessed α3A chain is expressed at the leading edge of actively migrating epithelial cells and has been shown to promote epithelial cell migration and proliferation through interaction with α3β1 integrin and stimulation of the MAPK signalling cascade (Gonzales et al., 1999). LN-332 containing the processed form of α3A stimulates keratinocyte-matrix attachment and hemidesmosome formation (Goldfinger et al., 1999; Goldfinger et al., 1998).

Only few studies focused on human LN-332 β3-chain processing. β3-Chain proteolysis carried out by MT1-MMP and MMP-7 has been reported in human prostate cancer cells (Udayakumar et al., 2003) and in human colon cancer cells, respectively (Remy et al., 2006). Cleavage occurs at the N-terminus and reduces the chain from 140-kDa to 80-kDa. β3-Chain processing seems to be species-dependent, as rat β3 chains do not undergo processing (Udayakumar et al., 2003). These studies concluded that β3 processing could facilitate tumour cell migration.

Concerning γ2-chain, numerous studies have focused on its processing which is also species-dependent. The γ2-chain is a 155-kDa chain that undergoes processing at the N-terminal. MMP-2 and MT1-MMP are two candidates involved in cleaving rat γ2-chain to 80-kDa and 100-kDa forms (Koshikawa et al., 2000). In addition, human γ2-chain may get processed by mammalian Tolloid MMP found in skin, resulting in a 105-kDa γ2-chain (Veitch et al., 2003). Upon tissue remodelling the 105-kDa γ2-chain may be further proteolysed within the N-terminus giving rise to
an 80-kDa protein (Giannelli et al., 1997; Koshikawa et al., 2005). In the process, an internal DIII fragment (25-27-kDa) is also generated. This DIII fragment has EGF-like properties and may bind to the EGF receptor (Schenk et al., 2003a). Once fixed to the erb1 domain of the receptor, it initiates the ERK signalling pathway. All these events lead to hemidesmosome disassembly, necessary for cellular movement. ErbB1 also activates expression of MMPs which contributes to further processing of γ2-chain for cell migration. It is important to note that the γ2-chain and its proteolytic fragments are found at the invasion front of tumours, where they may support cancer invasion (Koshikawa et al., 1999; Pyke et al., 1994).

Different studies attempted to clarify the factors that could regulate the synthesis and secretion of LNs. Cytokines (TGF-α, TGF-β1 and TNF-α) and lysophospholipids A and C were found to induce LN-332 expression in acute wound fluid (Amano et al., 2004). More recently, IL-1β was shown to increase expression of LN β1-chain in rat fibroblasts and immortalized alveolar type II epithelial cells (Furuyama et al., 2008). In addition, LN β2 mRNA expression was increased in rat glomerular (Richardson et al., 1995) and alveolar epithelial cells (Kumar et al., 1995) treated with IL-1β and TGF-β1, respectively. The effects of these cytokines and transcription factors on LN gene expression are not well understood because of the diversity of LN isoforms and their specific expression in various cell types. Additional studies are required to clarify the mechanisms underlying the roles of these cytokines in the enhanced secretion of BM macromolecules.

### 2.4. The cell laminin receptors

To understand the function of LNs, it is necessary to consider the receptors whereby cells recognize and respond to LNs. Numerous cell membrane molecules have been identified as receptors for LN and other BM proteins. They are usually divided into two classes: integrin and non-integrin receptors (summarized in table III).
### Table III. Laminin receptors and their notable laminin ligands

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Ligands</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Integrins</strong></td>
<td></td>
</tr>
<tr>
<td>α1β1</td>
<td>LN-111, LN-211</td>
</tr>
<tr>
<td>α2β1</td>
<td>LN-111, LN-211</td>
</tr>
<tr>
<td>α3β1</td>
<td>LN-332, LN-511, LN-521</td>
</tr>
<tr>
<td>α6β1</td>
<td>LN-332, LN-511, LN-521</td>
</tr>
<tr>
<td>α6β4</td>
<td>LN-332, LN-511, LN-521</td>
</tr>
<tr>
<td>α7β1</td>
<td>LN-211, LN-221</td>
</tr>
<tr>
<td>αVβ3</td>
<td></td>
</tr>
<tr>
<td><strong>Non-integrins</strong></td>
<td></td>
</tr>
<tr>
<td>Dystroglycan</td>
<td>LN-111, LN-211, LN-121, LN-221</td>
</tr>
<tr>
<td>Syndecans</td>
<td>LN α-chain</td>
</tr>
<tr>
<td>Lutheran blood group glycoprotein</td>
<td>LN-511, LN-521</td>
</tr>
</tbody>
</table>

Integrins are cell surface adhesion receptors composed of α- (120-180-kDa) and β-subunits (90-110 kDa) which form heterodimers. Each subunit has an extracellular domain, a single transmembrane region and a short cytoplasmic domain associated with the actin cytoskeleton and affiliated proteins (Hynes, 2002). To date, 18 α- and 8 β-subunits that assemble into at least 24 distinct integrin isoforms have been identified in vertebrates. The variations in subunit associations confer ligand binding specificities to the cell. Different integrins can recognize the same ligand and, conversely, integrins composed of a common subunit have different ligand binding specificities. Several integrin heterodimers have been found to bind to purified LNs or to recombinant or synthetic fragments of LN. These include integrin α1β1, α2β1, α3β1, α6β1, α6β4, α7β1 and αVβ3 (Colognato and Yurchenco, 2000; Delwel et al., 1996; Givant-Horwitz et al., 2005; Sasaki and Timpl, 2001). Each integrin recognizes particular sequences of LN α-chain and thus binds only to a specific set of LNs. These interactions occur mostly at the C-terminal LG domain of the LN α-chain, although in some cases, they can also take place at the N-terminal. More recently,
interactions of integrins with LN β- and γ-chains have also been reported (Patarroyo et al., 2002). For instance, a glutamic acid residue (Glu1607) at the third position from the C-terminal of the LN γ-chain has been shown to play an important role in LN-integrin interactions (Ido et al., 2007).

The importance of LN-receptor interactions and particularly the C-terminal LG domains of the LN α-chain have been highlighted in human and mouse models in which LN-receptors or LNs are functionally absent. Junctional epidermolysis bullosa, a skin blistering disease characterized by separation at the lamina lucida of the epidermal BM zone, is caused by recessive mutations in the genes encoding integrin α6β4 (ITGA6 and ITGB4) or LN-332 (LAMA3, LAMB3, and LAMC2) (Varki et al., 2006).

Several studies explored the expression of integrin heterodimers within the pancreas and pancreatic islets. The existence of α3β1 and α6β1 integrin isoforms was demonstrated in primary rat islet cells. The integrin β1-subunit was shown to have an important role in rat β-cell attachment and spreading in vitro (Bosco et al., 2000; Kantengwa et al., 1997). In the porcine, canine, hamster and human pancreas, integrin α3-subunit was only expressed on islet cells. Integrin αV-subunit was only detected in human and canine pancreas. Finally, integrin β1-subunit was found to be expressed in the human pancreas (Wang et al., 1999).

Concerning the non-integrin cell surface receptors, three main proteins with high affinity for LNs have been identified: two of these classes are syndecans and dystroglycans which belong to the heparan sulphate proteoglycans (Okamoto et al., 2003; Wizemann et al., 2003). The third class of non-integrin receptor is the Lutheran blood group glycoprotein. This protein belongs to the immunoglobulin superfamily and has recently been recognized as a LN-binding receptor with specific affinity for the LN α5-chain (Eyler and Telen, 2006; Kikkawa and Miner, 2005; Kikkawa et al., 2002). However, the physiological functions of this molecule remain obscure. As the Lutheran gene is alternatively spliced, the protein exists either as a short or long protein. By RT-PCR, it has been demonstrated that human islets express the longer form of Lutheran protein (Otonkoski et al., 2008).
2.4.1. Cell signalling initiated by integrins

Integrins initiate not only adhesion and cytoskeletal organization through direct physical interactions with other proteins, but also may activate a large variety of signal transduction events that modulate many aspects of cell behaviour including proliferation, survival/apoptosis, shape, polarity, motility, gene expression, and differentiation (Belkin and Stepp, 2000; Giancotti and Ruoslahti, 1999; Miranti and Brugge, 2002; Schwartz and Ginsberg, 2002). Integrins do not have any enzymatic activity themselves, but are associated with a broad variety of signalling and/or adaptor proteins involved in different signal transduction pathways, as depicted in figure 7. An early insight into a possible signalling role played by integrins was provided by the observation that integrin-mediated adhesion and/or clustering of integrins lead to enhanced activation (resulting in tyrosine residue phosphorylation) of a cytoplasmic integrin-associated protein kinase, now known as focal adhesion kinase (FAK) (Schaller et al., 1992). In addition to FAK, activity of a multitude of signalling molecules, including phosphatidylinositol 3-kinase (PI3-K), protein kinase B (PKB/Akt) and the MAPK ERK may be regulated by integrin-mediated adhesion (Howe et al., 2002; Parsons, 2003; Schwartz, 2001). These proteins have all been implicated in the integrin-mediated protection of rat β-cells against various apoptotic stimuli and in the control of gene expression (Hammar et al., 2004).

Fig. 7. Integrin signalling (Figure taken from Hynes, 2002, Cell)
II. PROJECT AIMS

Previous studies of ECM composition in pancreas, including islets, have generated confusion resulting from a limited range of available tools and thus incomplete analysis. The exact composition and localization of ECM components, and their cellular origin is still a subject of controversy. Among all the different ECM proteins identified in the pancreas and within and/or around pancreatic islets, LNs and particularly LN-332 seem to be of particular interest. Indeed, several \textit{in vitro} models of cell-to-ECM contacts have shown how signals originating from LN-332 were important for rat \(\beta\)-cell function. However, no study has investigated whether LN-332 was expressed in pancreatic islets, and information concerning the cellular origin of LN-332 in islets and its secretion is missing.

Islets are particularly vulnerable in the initial days after transplantation (Davalli et al., 1996). Islet cell dysfunction and damage take place before immunological rejection of the graft and is known as PNF. Although PNF is more common in xenogeneic and allogeneic transplants, it has also been described in syngeneic islet transplants (Biarnes et al., 2002; Kaufman et al., 1990), confirming the involvement of non-immunological processes. The reasons of PNF are multifactorial, and cells of the monocyte-macrophage lineage appear as a key element in orchestrating inflammation by increasing expression of inflammatory mediators that participate in the destruction of transplanted islets (Berney et al., 2001a; Gysenmans et al., 2003; Rabinovitch and Suarez-Pinzon, 1998). The mechanisms by which cells of the monocyte-macrophage lineage are stimulated to secrete inflammatory mediators are incompletely understood. More specifically, it has never been investigated whether islet ECM proteins affect cells of the monocyte-macrophage lineage.

This work was divided in two sections. In the first part, we were interested in studying LN-332 in pancreatic islet and addressed the following questions:

- Is LN-332 expressed and secreted by islet endocrine cells?
- Can stress conditions, such as isolation procedure or cytokine exposure, regulate LN-332 expression?
To answer these questions, we decided to use human islets. The results of this study are presented in the PART I of this thesis.

In the second part, we focused on the interaction between cells of the monocyte-macrophage lineage and ECM proteins, and addressed the following questions:

- Does LN-332 induce pro-inflammatory mediator secretion by the cells of the monocyte-macrophage lineage?
- What are the cell signalling pathways involved in LN-332-induced pro-inflammatory mediator secretion by these cells?

To answer these questions, we used an in vitro system in which freshly isolated rat peritoneal macrophages were seeded on different ECM proteins. As source of LN-332 we used the well characterized rat bladder carcinoma 804G cell line. The results of this study are presented in the PART II of this thesis.
III. PART 1

Regulated laminin-332 expression in human islets of Langerhans.

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1. Abstract

Laminin-332 (LN-332) is a basement membrane component known to exert a beneficial effect on rat pancreatic \(\beta\)-cells, *in vitro*. In this work we analysed expression of LN-332 in human islets, its expression after inflammatory insults by cytokines, and the molecular mechanisms responsible for this effect.

By Western blotting and RT-PCR we showed that LN-332 was expressed in isolated human islets. By immunofluorescence on pancreas sections, we observed that within the islet, labelling was confined to endocrine cells. Confocal microscopy analysis on isolated islet cells revealed that labelling was granular but did not colocalize with hormone secretory granules. LN-332 was most abundant in cultured islets compared to freshly isolated islets, and was found in culture supernatant suggesting that it was secreted by islets. When islets were exposed to interleukin (IL)-1\(\beta\), expression and secretion of LN-332 increased as compared to control. No effect was observed with tumour necrosis factor (TNF)-\(\alpha\) and interferon (IFN)-\(\gamma\). LY294002, an inhibitor of phosphatidylinositol 3-kinase (PI3-K) activity, inhibited culture- and IL-1\(\beta\)-induced LN-332 expression in islets.

These results show that LN-332, known to have some beneficial effect on \(\beta\)-cells *in vitro*, is produced and secreted by endocrine islet cells and is upregulated by stressing conditions such as culture and IL-1\(\beta\)-exposure.

Key words: extracellular matrix, cytokines, PI3-K
2. Introduction

Laminins (LN) are a family of large heterotrimeric glycoproteins synthesized and secreted by a wide variety of cells and are present in basement membranes of various epithelia. The LN molecules consist of three subunits (or chains) linked by disulfide bonds to form the well-known cross-shape structure. To date, more than 15 LN isoforms with different combinations of $\alpha$1-5, $\beta$1-3, and $\gamma$1-3 chains have been identified in human and mouse (Kariya and Miyazaki, 2004; Marinkovich, 2007; Miner and Yurchenco, 2004; Miyazaki, 2006). LNs play important roles in both tissue construction and regulation of cellular functions such as adhesion, migration, proliferation, differentiation and programmed cell death (Colognato and Yurchenco, 2000). These properties are mediated by integrins that are expressed at the cell membrane and function as membrane receptors for extracellular matrix proteins, including LNs (Ekblom, 1996).

Of all LN isoforms, LN-332 is unique in both structure and activity. It consists of three polypeptide chains, $\alpha$3, $\beta$3, and $\gamma$2, encoded by the LAMA3, LAMB3, and LAMC2 genes, respectively (Burgeson et al., 1994). The short arms of the three LN-332 chains are truncated and lack some domains present in other LNs, and the $\beta$3 and $\gamma$2 chains are specific of LN-332. LN-332 is produced as a precursor consisting of a 200-190-kDa $\alpha$3 chain, a 145-kDa $\beta$3 chain and a 155-kDa $\gamma$2 chain. After its secretion, the precursor undergoes specific extracellular proteolytic cleavages resulting in the mature form of LN-332 containing 165-kDa $\alpha$3 and 105-kDa $\gamma$2 ($\gamma$2') chains (Marinkovich et al., 1992). LN-332 effects are mediated by integrins $\alpha$3$\beta$1, $\alpha$6$\beta$1, and $\alpha$6$\beta$4 (Rousselle and Aumailley, 1994). LN-332 was originally found as an anchoring filament component of keratinocytes (Rousselle et al., 1991) and successively showed to promote cellular adhesion, motility, and cell scattering in culture (Bachy et al., 2008; Ogawa et al., 2007). Defects in genes encoding LN-332 and auto-antibodies against this protein cause diseases, such as epidermolysis bullosa and cicatricial pemphigoid, characterized by skin adhesion disorder (Christiano et al., 1996). In addition, inflammatory cytokines involved in wound healing enhance LN-332 expression in keratinocytes (Amano et al., 2004; Korang et al., 1995).
Some studies have revealed the essential role of cell-to-ECM interactions mediated by LN-332 in the function of isolated pancreatic islet cells. Integrins α3β1 and α6β1 were identified in rat pancreatic β-cells (Bosco et al., 2000; Kantengwa et al., 1997) and rat β-cells cultured on the LN-332-rich matrix produced by 804G cells spread out and secreted twice more insulin in response to glucose compared to cells cultured on plastic or poly-L-lysine (Bosco et al., 2000). It has been demonstrated that these effects were mediated by β1 integrin-LN-332 interactions (Parnaud et al., 2006). Furthermore, 804G matrix and purified LN-332 promoted proliferation of rat β-cells (Parnaud et al., 2008) and protected these cells from apoptosis induced by pro-inflammatory cytokines such as TNF-α, IL-1β and IFN-γ (Hammar et al., 2004).

Even though the role of LN-332 on the function of rat β-cells has been extensively documented, the expression of LN-332 by islet cells has been poorly characterized. In one previous study, we have shown expression of LN γ2 chain in human and rat islets (Parnaud et al., 2006). Here we show that LN-332 is expressed, secreted and regulated in human islets, suggesting that LN-322 might be physiologically relevant in human islets.
3. Materials and Methods

3.1. Reagents and antibodies

PD98059 (MEK1 inhibitor), SB203580 (P38 MAPK inhibitor), SP600125 (JNK inhibitor II) and LY294002 (phosphatidylinositol 3-kinase (PI3-K) inhibitor) were purchased from Calbiochem (Darmstadt, Germany). Recombinant human interferon (IFN)-γ was purchased from Invitrogen (Carlsbad, USA). Recombinant interleukin (IL)-1β, IL-1α, tumor necrosis factor (TNF)-α, and oncostatine-M were purchased from Biosource (Nivelles, Belgium). IL-8 and TGF-β1 were purchased from Peprotech (London, UK). Anakinra, an interleukin-1 receptor antagonist (IL-1Ra), was from Amgen (Thousands Oaks, USA). LN-332, purified from the culture medium of human SCC25 cells (hLN-332), the rabbit polyclonal antibody L132, raised against the purified native human LN-332 (Remy et al., 2006) and the mouse monoclonal antibody BM165 raised against LN α3 chain (Rousselle et al., 1991) were provided by Patricia Rousselle. Mouse monoclonal anti LN α3 chain was purchased from RnD Systems (Abingdon, UK). Goat polyclonal anti-LN β3 and -LN γ2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Mouse monoclonal anti-actin antibody was purchased from Chemicon International (Temecula, USA), horseradish peroxidase-conjugated goat-anti-mouse, goat-anti-rabbit and donkey-anti-goat antibodies from Amersham Biosciences, mouse monoclonal anti-glucagon antibody from Sigma-Aldrich (Buchs, Switzerland), and guinea-pig anti-insulin antibody from Dako (Baar, Switzerland). Guinea-pig anti-pancreatic polypeptide antibody was from Linco Research (Missouri, USA) and rat anti-somatostatin antibody was from Chemicon International (Temecula, USA). Rabbit polyclonal anti-caspase 3 and -cleaved caspase 3 antibodies were from Cell Signalling Technology-Bioconcept (Allschwil, Switzerland). Mouse anti-pan-ductal membrane carbohydrate antigen 19-9 (CA19-9) antibody was from NovoCastra (Newcastle upon Tyne, UK). Alexa-Fluor 633 anti-mouse IgG was from Santa Cruz Biotechnology (Santa Cruz, USA). Newport green PDX acetoxyethyl ether (NG-PDX-Ac), pluronic F-127 and 7-aminoactinomycin D (7-AAD) were purchased from Molecular Probes (Leiden, The Netherlands). Cisplatin was from Sigma-Aldrich (Buchs, Switzerland).
3.2. Islet isolation and culture

Human islets were isolated at our institution from human pancreata harvested from brain dead heart-beating multiorgan donors and provided by the islet for research distribution programme through the European Consortium for Islet Transplantation, under the supervision of the Juvenile Diabetes Research Foundation. The use of human islets for research was approved by our local institutional ethical committee. Islets were isolated after enzymatic ductal perfusion as previously described (Bucher et al., 2005) then washed and finally cultured in non-adherent culture flasks (Gibco, Paisley, Scotland) with CMRL 1066 medium (Sigma-Aldrich, Buchs, Switzerland) containing 5.6 mmol/l glucose and, 25 mmol/l N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 2 mmol/l glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (hereafter, referred to as complete CMRL) supplemented with 10% fetal calf serum (FCS) at a density of 20000 islet equivalents (IEQ) in 30 ml. After 12 to 24 hours at 37°C, culture medium was renewed and islets were further cultured at 24°C for 1 to 3 additional days before to be used for experiments.

3.3. Islet treatment with cytokines

To study the effects of cytokines on LN-332 expression, 4000-5000 IEQ, freshly isolated or cultured as described above, were incubated for 24 hours at 37°C in 2 ml complete CMRL supplemented with 1% human albumin (ZLB, Bern, Switzerland) and completed with the following cytokines: IL-1α (10 ng/ml), IL-1β (10 ng/ml), IFN-γ (1000 U/ml) and TNF-α (5 ng/ml), IL-8 (10 ng/ml), TGF-β1 (25 ng/ml) and oncostatine-M (20 ng/ml), a cytokine of the IL-6 family. The following combination of cytokines was also tested: IL-1β (10 ng/ml), IFN-γ (1000 U/ml) and TNF-α (5 ng/ml). When required, islets were incubated 1 hour prior IL-1β treatment with 10 µg/ml anakinra, an IL-1 receptor antagonist (IL-1Ra). To investigate the signalling pathways involved, 4000-5000 IEQ were pretreated for 1 hour with PD98059 (50 µmol/l), SB203580 (10 µmol/l), SP600125 (50 µmol/l) or LY294002 (50 µmol/l), followed by a 6-hour incubation with IL-1β (10 ng/ml) in a final volume of 2 ml of complete CMRL supplemented with 1% human albumin. To study caspase 3 activation, islets were
incubated in presence of IL-1β (10 ng/ml) or cisplatin (500 µmol/l) in the same conditions of culture. Islets were then processed for quantitative PCR or Western blotting.

3.4. LN-332 secretion

In order to determine whether LN-332 is secreted, 4000-5000 IEQ were incubated in 2 ml of complete CMRL without human albumin or FCS, in the absence or presence of 10 ng/ml of IL-1β. The serum-free conditioned medium was harvested after 48 hours and cleared from floating cells and debris by centrifugation. It was then subjected to protein precipitation with 10% trichloroacetic acid (Applichem, Cheshire, USA). The resulting pellet was resuspended in 66 µl lysis buffer (100 mmol/l Tris, 5% SDS, 5 mmol/l EDTA) in order to obtain a 30-fold concentration. Aliquots were assayed for LN-332 by Western blotting.

3.5. Semi-quantitative PCR

Total RNA was extracted from cultured islets using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) according to the manufacturer protocol. For quantitative analysis, RNA levels were assessed by measuring the optical density (A260 = 40 µg/ml). 2100 Bioanalyzer (Agilent Technologies, Morges, Switzerland) was used for quality control of the RNA samples before any application. First-strand cDNA synthesis was performed using SuperScript II reverse transcriptase (Invitrogen, Lucerne, Switzerland). As DNase was not added to the RNA extracts, negative reverse transcription was also performed to check for DNA contamination. Real-time RT-PCR primers were designed with Primer express 2.0 (Applied Biosystems, Rotkreuz, Switzerland). The specific human primers used were forward 5'-CACCTGCCAGCACTCAAGAG-3' and reverse 5'-AGGGATCCTCAGTGTGCAA-3' (LAMA3), forward 5'-CAGCAGCTTGCAGAGG-3' and reverse 5'-TGTTTTATTCTCTCAATCCCTCTTG-3' (LAMB3), forward 5'-CGGATTCGTTCGTCAGG-3' and reverse 5'-CGTTTTTTGTTTGATCCTGTTT-
Three housekeeping genes were used as controls: *Eef1a1*, forward 5'-AGCAAAAATGACCACCAATG-3' and reverse 5'-GGCCTGGATGTTGAGGATA-3'; *18S*, forward 5'-AGTCCCTGCTTTGTACACA-3' and reverse 5'-GATCCGAGGGCCTCCTAAAC-3'; and *GusB*, forward 5'-CCACCAGGACCACAT-3' and reverse 5'-AGTCAAATATGTGTTGACACAAAGTA-3'. PCRs were done in triplicate, with 4 specimens per condition, and were labelled with SYBR green master mix (Applied Biosystems). Fluorescence was quantified with the Prism 7900 HT sequence detection system (Applied Biosystems). Raw Ct (threshold cycle) values obtained with SDS 2.0 (Applied Biosystems) were used to calculate the normalization factor and the fold change with the geNorm script, as published (Vandesompele et al., 2002). No change was scored when p<0.05. All experiments were performed at the Genomics Platform of our institution.

3.6. Western blotting

Islets, sorted β-cell and non β-cell fractions were resuspended in lysis buffer (100 mmol/l Tris, 5% SDS, 5 mmol/l EDTA) supplemented with protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) diluted 7x from the stock solution and 1 mmol/l phosphatase inhibitor sodium orthovanadate (Sigma-Aldrich, Buchs, Switzerland). Protein concentration was determined by the dye method using a detergent compatible protein assay kit (Bio-Rad laboratories, Hercules, USA). Equivalent amounts (10 to 50 µg) of protein from each sample were fractioned by electrophoresis in either 7.5% or 12% polyacrylamide gels. Proteins were electrically transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA) using a constant current of 450 mA for 75 minutes. PVDF membranes were saturated with 5% non fat dried milk and 0.1% Tween-20 for 1 hour and then probed with primary antibodies, overnight at 4°C. Antibody against LN-332 (L132 antibody) was diluted 1:1000, antibody against LN α3 chain (BM165) was diluted 1:800, antibodies against LN β3 and LN γ2 chains were diluted 1:50, antibodies against caspase 3 and cleaved caspase 3 were diluted 1:1000. The antibody against β-actin diluted 1:4000 was used as control for loading. After 60-minute incubation at room temperature with
appropriate horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories, Hercules, USA), diluted 1:6000, membranes were developed and visualised by enhanced chemiluminescence according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Piscataway, USA). In some experiments, proteins were quantified by densitometry using Quantity One software (Bio-Rad laboratories, Hercules, USA).

3.7. Islet cell preparation

Islets were washed three times with phosphate-buffered saline without Mg$^{2+}$ and Ca$^{2+}$ (PBS). Aliquots of $10^4$ islets were digested with 1 ml ready to use Accutase (PAA Laboratories GmbH, Pasching, Austria), and incubated at 37°C for 9-10 minutes, with occasional pipetting through a 1 ml-plastic tip. Digestion was stopped by adding 10 ml cold complete CMRL supplemented with 1% human albumin, and cells were washed twice with the same medium. Cells were then counted and aliquots of either $3 \times 10^5$ or $1 \times 10^6$ cells were incubated for 24 hours in 10 cm diameter, non-tissue culture-treated Petri dishes containing 10 ml complete CMRL supplemented with 1% human albumin.

3.8. Histochemical analysis

Expression of LN-332 and islet hormones was analysed by immunofluorescence, either in isolated islet cells or in pancreas sections. Aliquots of $10^4$ islet cells were attached for 60 minutes at 37°C into Cunningham chambers. These chambers were rinsed with PBS solution, fixed in 4% paraformaldehyde (PFA) for 20 minutes, rinsed again with PBS solution and treated for 20 minutes with 0.1% Triton X-100 and exposed to a 0.1% bovine serum albumin (BSA) containing-PBS solution for 45 minutes. Samples of human pancreata were harvested in PBS solution and fixed 24 hours in 4% PFA at room temperature. Tissues were embedded in paraffin and sectioned for histology. Five-micrometer sections were deparaffinized and soaked in 10 mmol/l citrate buffer (pH 6) in a microwave oven at 600 W for 3 x 5 minutes for
antigen retrieval. The slides were then cooled at room temperature, rinsed with PBS and used immediately for immunofluorescence labelling. In both pancreas sections and islet cells, non specific binding sites were blocked with 0.1% BSA solution for 45 minutes. The whole immunofluorescence procedure was carried out at room temperature and all reagents were diluted in PBS. Sections and cells were then incubated for 2 hours with primary antibodies in blocking solution. The dilutions were 1:100 for L132 antibody, 1:1500 for the guinea-pig anti-insulin antibody, 1:4000 for the mouse anti-glucagon antibody, 1:1000 for guinea-pig anti-pancreatic polypeptide and 1:500 for rat anti-somatostatin antibody. Sections and cells were washed and incubated with specific secondary antibodies coupled either to fluorescein or to rhodamine. Specificity of the different immunostainings was confirmed with sections and cells in which primary antibodies were omitted. Sections and cells were examined with an Axioskop microscope (Zeiss, Feldbach, Germany) equipped with an AxioCam color CCD camera (Zeiss). Cells were also examined with a LSM510 Meta confocal microscopy (Zeiss).

3.9. Human β-cell purification

Islet cells were prepared as described above. The dispersed cells were then resuspended in modified Krebs–Ringer bicarbonate HEPES buffer (KRBH) (125 mmol/l NaCl, 4.74 mmol/l KCl, 1 mmol/l CaCl₂, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 5 mmol/l NaHCO₃, 25 mmol/l HEPES, pH 7.4, 0.5% BSA) containing 2.8 mmol/l glucose. Cells were incubated 1 hour at 4°C with CA19-9 antibody (1 µg/ml), rinsed twice with KRBH and reincubated for 1 hour with Alexa-Fluor 633 anti-mouse IgG. After washing steps, cells were resuspended in KRBH containing Pluronic F-127 (1.5 µl/ml) and incubated for 30 minutes at 37°C with NG-PDX-Ac (1 µmol/l). After washing, cells were incubated for 10 minutes with 7-AAD (10 µg/ml). Analysis and cell sorting were performed using a FACSvantage (BD-Bioscience, Allschwil, Switzerland). β-Cells were enriched in the 7-AAD-negative, CA19-9-negative and NG-bright populations. An aliquot of each sorted population was analysed by classical immunofluorescence for insulin and glucagon, as described above. Sorted
β-cell fractions and non β-cell fractions were cultured for 24 hours in complete CMRL supplemented with 1% human albumin, before protein extraction.

### 3.10. Detection of secreted cytokines

To measure the release of cytokines IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α by human islets, multiplex cytometric bead array (CBA) kit (BD-Bioscience, Allschwil, Switzerland) was used. The assays were performed at the core facility of the Geneva School of medicine. Briefly, 50 µl of supernatant were stained with the mixture of human cytokine capture bead suspension and the PE-conjugated detection antibodies. Standards provided with the kit were appropriately diluted and used in parallel to samples for preparation of the standard curves. After 3 hours of incubation, samples were washed and then analysed by FACSarray Bioanalyzer system, using the BD CBA software (BD-Bioscience, Allschwil, Switzerland).

### 3.11. Statistical analysis

Data were presented as means ± s.e.m. for three to five independent experiments. Differences between means were assessed either by one-way ANOVA or Student’s t-test. When ANOVA was applied, Scheffé’s least-significant difference post-hoc analysis was used to identify significant differences (p<0.05).
4. Results

4.1. LN-332 is expressed in human islet cells

Expression of LN-332 was studied first by immunofluorescence on paraffin sections of human pancreata and isolated islet cells. In pancreatic sections, staining was confined to islets. Specific labelling was neither observed in surrounding exocrine and connective tissues nor in vessels. Labelling was exclusively intracellular. All islets analysed in five different pancreata were stained. Within every islet, staining was heterogeneous with some cells strongly and others moderately stained for LN-332. To determine which types of islet cells expressed LN-332, double staining was performed for LN-332 and insulin or glucagon. Most cells moderately stained for LN-332 were insulin-expressing cells, while cells highly stained for LN-332 were predominantly glucagon-expressing cells (Fig. 1A). Furthermore, mRNA expression of LN-332 genes (LAMA3, LAMB3 and LAMC2) encoding the three peptide chains α3, β3 and γ2 was demonstrated by RT-PCR in isolated human islets. Presence of LN-332 subunits in islets was also confirmed at the protein level by Western blotting. Indeed, under reducing conditions, islet protein extract and LN-332 purified from human keratinocytes used as positive control exhibited three bands corresponding to 145-kDa β3, 155-kDa γ2, and 105-kDa processed γ2’ (Fig. 1B). This latter is considered as a processed form of LN γ2 chain found only in the secreted and mature form of LN-332. In addition, using the monoclonal BM165 antibody, the LN α3 chain (165-kDa) was also detected in islet protein extracts (Fig. 1B). We confirmed its localisation in islets by immunofluorescence on pancreatic sections (not shown). No LN-332 subunits were identified in protein extracts from exocrine tissue. In addition, the expression of the 155-kDa γ2 chain of LN-332 was studied by Western blotting in sorted β-cell and non β-cell fractions. Results indicated that non β-cell fraction contained 75.8 ± 5.6% α-cells and (n=3) (Fig. 2A) and that γ2 chain was mainly produced by cells of this fraction (Fig. 2B). As LN β3 and LN γ2 chains are specific to LN-332, we decided to use specific monoclonal antibodies directed against each of these chains, for the following Western blotting experiments.
**Fig.1.** LN-332 is expressed by endocrine cells in human pancreas. (A) Pancreatic sections were analysed by fluorescence microscopy after double labelling for insulin and LN-332 and for glucagon and LN-332. LN-332 (in green) and hormones (in red) colocalized in most cells, as confirmed by the yellow colour obtained by merging the two images. Cells expressing only LN-332 (green) are also present. Scale bar, 50 µm. (B) LN-332 purified from human SCC25 cells (hLN-332) and protein extracted from human islets were analysed by Western blotting using a polyclonal anti LN-332 antibody. Purified hLN-332 and islet proteins showed three major bands corresponding to LN β3 (145-kDa) and two forms of LN γ2 (155-kDa and 105-kDa) chains. The band corresponding to LN α3 chain (165-kDa) was also detected in islet protein extracts, using the monoclonal BM165 antibody. These results are representative of at least three independent experiments.
Fig. 2. LN γ2 chain is predominantly expressed in human non β-cells. After cell sorting the two fractions of β- and non β-cells were maintained in culture for 24 hours. (A) An aliquot of each fraction was analysed by immunofluorescence (insulin and glucagon) and scored for α- and β-cell composition. Results are expressed as means ± s.e.m. n=3. (B) Extracted proteins were analysed by Western blotting for LN γ2 chain. Immunoblots are representative of three independent experiments.

In order to investigate whether LN-332 colocalized with secretory granules, islet cells were submitted to a double immunofluorescence staining for pancreatic hormones and LN-332, and analysed by confocal microscopy. As expected, a granular staining was observed for insulin, glucagon, somatostatin and pancreatic polypeptide. Interestingly, labelling for LN-332 had a granular pattern in most cells, but did not colocalize with any of the hormones (Fig. 3).
**Fig. 3.** LN-332 and hormones do not localize in the same granules. Cells were labelled for insulin and LN-332 (A), glucagon and LN-332 (B), somatostatin and LN-332 (C) and pancreatic polypeptide and LN-332 (D) and then analysed by confocal microscopy. Merged images show that hormones (in red) and LN-332 (in green) are expressed by the same cell but do not colocalize in the same secretory granules. Scale bar 10 µm. These results are representative of at least three independent experiments.
4.2. LN-332 is secreted by human islets

In light of the known function of LN-332 as an extracellular matrix molecule and encouraged by the results showing a granular staining for LN-332, we investigated whether human islets were able to secrete LN-332. To this end, islets were cultured 48 hours in complete CMRL without serum. Concentrated conditioned medium was collected and analysed by Western blotting for LN-332 chains. Under these conditions, LN β3 chain was detected as a single band at 145-kDa and two bands were clearly detected for LN γ2 chain: a 155-kDa band and a 105-kDa band (Fig. 4). LN α3 chain was also detected as a double band around 160-170-kDa (not shown). The fact that the 105-kDa γ2’ chain is normally found in the secreted and mature form of LN-332, further supports the hypothesis of LN-332 secretion by islet cells. In these experiments, only traces of actin were detected in the conditioned medium, excluding the possibility that LN-332 chain detection in the medium was attributable to cell death.

![Image](image_url)

**Fig.4. LN-332 is secreted by human islets.** Islets were incubated for 48 hours in complete CMRL in presence or absence of IL-1β. The collected conditioned medium was concentrated by TCA precipitation. Proteins were then separated on polyacrylamide gels under reducing conditions and blotted for LN β3 and LN γ2 chains. Under both control and IL-1β conditions, LN β3 chain was detected as a single band at 145-kDa and LN γ2 chain as two bands at 155-kDa and 105-kDa. IL-1β increased the secretion of LN β3 and LN γ2 chain when compared to the control. 804G matrix was used as a positive control for LN γ2 and γ2’ chains expression. Results are representative of three independent experiments.
4.3. Expression of LN-332 in human islet is upregulated by cytokines and culture

Our aim here was to assess whether human islets were able to upregulate their LN-332 expression in response to a stress known to induce apoptosis. For this reason, expression of LN-332 chains in isolated human islets was assessed after 24-hour exposition to a pro-apoptotic cytokine cocktail containing IL-1\(\beta\), IFN-\(\gamma\) and TNF-\(\alpha\). By quantitative RT-PCR, we showed that the cytokine cocktail increased mRNA levels of the 3 genes encoding LN-332 as compared to the control condition (Fig. 5A). By Western blotting, using specific antibodies, we showed that LN\(\beta\)\(_3\) and LN\(\gamma\)\(_2\) chains were consistently increased in islets treated with the cytokines cocktail (Fig. 5B). To determine whether this effect was due to the combination of all three cytokines or to one of them in particular, islets were exposed to IL-1\(\beta\), TNF-\(\alpha\) or IFN-\(\gamma\) alone. Autoradiogram shown in figure 5B indicated that IL-1\(\beta\) had the same effect on LN\(\beta\)\(_3\) and \(\gamma\)\(_2\) chain expression as the whole cytokine cocktail, while IFN-\(\gamma\) and TNF-\(\alpha\) had limited or no effect. Expression of LN\(\alpha\)\(_3\) chain was also increased in islets exposed to IL-1\(\alpha\) compared to control (not shown). Figure 4 demonstrated that LN\(\beta\)\(_3\) and LN\(\gamma\)\(_2\) chains found in the supernatant of 24-hour-cultured islets were also increased upon exposition to IL-1\(\beta\). Since IL-1\(\alpha\) and IL-1\(\beta\) bind to the same IL-1 receptor 1 and may elicit indistinguishable cellular responses, at least \textit{in vitro} (Dinarello, 1996), we assessed the effect of IL-1\(\alpha\) on the expression of LN-332 chains. As expected, we observed that IL-1\(\alpha\) induced a similar effect on the expression of LN\(\alpha\)\(_3\), LN\(\beta\)\(_3\) and LN\(\gamma\)\(_2\) chains in human islets, as compared to IL-1\(\beta\) (not shown).

Many studies have shown pro-apoptotic effects of IL-1\(\beta\). We therefore asked whether IL-1\(\beta\)-induced production of LN\(\beta\)\(_3\) and LN\(\gamma\)\(_2\) chains observed in our experimental conditions was a consequence of apoptosis. To this end, we analysed by Western blotting the activity of caspase 3, the major effector caspase involved in apoptotic pathways. Cleaved caspase 3 expression was very low and similar in IL-1\(\beta\) treated and control conditions. In the contrary, the pro-apoptotic cisplatin drug increased the cleaved form of caspase 3 (Fig. 6A). Whereas LN\(\beta\)\(_3\) and LN\(\gamma\)\(_2\) chains were consistently increased in IL-1\(\beta\)-treated islets, their expression was slightly decreased after cisplatin treatment. (Fig. 6B).
Fig. 5. LN-332 expression in human islets is increased by IL-1β. Human islets were incubated for 24 hours in complete CMRL supplemented with 1% human albumin, in the absence or presence of 10 ng/ml IL-1β, 1000 U/ml IFN-γ and 5 ng/ml TNF-α, alone or in combination (cytokine cocktail). (A) mRNA levels of LAMA3, LAMB3 and LAMC2 were analysed by quantitative PCR in total RNA extracts from control islets (white bars) and cytokine cocktail-treated islets (black bars). Treatment, with the cytokine cocktail, significantly increased mRNA levels of the three genes. Results are expressed as means ± s.e.m. n=4; *, p<0.01. (B) Extracted islet proteins were analysed by Western blotting under reducing conditions using anti-LN β3, anti-LN γ2, and anti-actin antibodies. Cytokine cocktail and IL-1β alone increased LN β3 and LN γ2 chains expression when compared to control. Actin expression was unchanged in all conditions. Results are representative of four independent experiments.
**Fig. 6. IL-1β-induced LN β3 and LN γ2 chains expression is independent of caspase 3 activation.** Human islets were incubated for 24 hours in complete CMRL supplemented with 1% human albumin, in the absence or presence of 10 ng/ml IL-1β or 500 µmol/l cisplatin. Extracted islet proteins were analysed by Western blotting under reducing conditions using anti-caspase 3, anti-cleaved caspase 3, and actin (A), and anti-LN β3, anti-LN γ2, and anti-actin antibodies (B). Immunoblots are representative of three independent experiments.

Islet isolation procedure may result not only in destruction of islet cells themselves but also in disruption of components of the extracellular matrix that play a role in supporting islet cell viability. We hypothesized that the level of LN-332 expression should be low in islets soon after isolation and that its expression should increase after islet culture. Therefore, we studied whether LN-332 expression in islets could be restored by a culture period following the isolation procedure. The expression of LN β3 and LN γ2 chains was determined by Western blotting in freshly isolated islets and in islets cultured 24 hours at 37°C. When compared to freshly isolated islets, 24 hour-cultured islets expressed higher amount of LN β3 and LN γ2 chains (Fig. 7A). We then tested the hypothesis that IL-1β produced by 24-hour-cultured islets could be responsible for the increased expression of LN β3 and LN γ2 chains in cultured islets. To this end, islets were incubated for 24 hours with IL-1Ra immediately following the isolation procedure. IL-1Ra used at different concentrations (0.1-10 µg/ml) was unable to affect expression of LN β3 and LN γ2 chains (Fig. 7B).
By contrast, as expected, IL-1Ra inhibited IL-1β-induced expression of LN β3 and LN γ2 chains (Fig. 7C). This indicates that an IL-1-independent mechanism is involved in culture-induced LN-332 expression in islets. We hypothesized that other factors secreted by 24-hour-cultured islets could affect LN β3 and LN γ2 chains expression. Among the different proteins analysed, two cytokines, IL-8 and IL-6 were detected in the culture supernatant (Fig.8). We therefore questioned whether these two cytokines released by islets had similar effects as IL-1β. Meanwhile, we tested TGF-β1, another cytokine known to induce LN-332 production in human keratinocytes (Amano et al., 2004). As shown in the autoradiogram (Fig. 9), none of these cytokines tested had an effect on the production of LN β3 and LN γ2 chains.
Fig. 7. LN-332 expression in human islets is increased when they are cultured 24 hours following their isolation. LN β3 and LN γ2 chains were analysed in islets by Western blotting immediately following their isolation and after 24 hours in culture. (A) Protein levels for LN β3 and LN γ2 were significantly increased in islets cultured 24 hours (D₁) as compared to freshly isolated islets (D₀). Representative immunoblots for LN β3 and γ2 chains are shown below each respective histogram. Results are expressed as means ± s.e.m. n=6; *, p<0.05. (B) IL-1Ra (anakinra) used at different concentrations (0.1-10 µg/ml) was unable to prevent effect of culture on LN β3 and LN γ2 chains expression. (C) By contrast, IL-1Ra inhibited IL-1β-induced expression of LN β3 and LN γ2 subunits. Results are representative of three different experiments.
**Fig. 8. Cytokines secreted by human islets.** Following isolation procedure, human islets were incubated 24 hours under control conditions and supernatant was recovered for cytokine analysis (n=9).

**Fig. 9. Effect of different cytokines on LN ββ and LN γγ chain expression.** Human islets were incubated for 24 hours in complete CMRL supplemented with 1% human albumin, in the presence of 10 ng/ml IL-8, 20 ng/ml oncostatine-M and 25 ng/ml TGF-β1. Extracted islet proteins were analysed by Western blotting under reducing conditions using and anti-LN β3- and anti-LN γ2 chain and anti-actin antibodies. Results are representative of three different experiments.
4.4. PI3-K signalling pathway controls β3 and γ2 chains expression

Different studies indicate that cellular responses to IL-1β are mediated by intracellular cascades involving activation of c-jun NH2-terminal kinase (JNK), P38 kinase or extracellular signal-regulated kinase (ERK) (Larsen et al., 1998). Even if less attention has focused on PI3-K and its target Akt as a downstream effector of IL-1, evidence has provided that IL-1 activates PI3-K/Akt pathway in different systems (Cahill and Rogers, 2008); (Diem et al., 2003; Pousset et al., 2000; Reddy et al., 1997; Sizemore et al., 1999). Moreover, it has been suggested that activation of PI3-K/Akt pathway may be a mechanism for protecting cells against adverse stimuli (Alessi and Cohen, 1998) and may play a role in the regulation of β-cell survival (Tuttle et al., 2001). In order to understand the relative contribution of ERK, P38, JNK and PI3-K signalling pathways in the control of LN-332 chain expression and secretion upon IL-1β stimulation, islets were pre-incubated 1 hour prior IL-1β exposure, in the presence or absence of PD98059, SB203580, SP600125 or LY294002, inhibitors of ERK, P38, JNK, and PI3-K, respectively. After 6-hour incubation with IL-1β, total proteins were extracted from islets and Western blotting was performed for LN β3 and LN γ2 chains. LN β3 and γ2 chain expression was significantly increased by IL-1β compared to control. As shown in autoradiograms and histograms (Fig. 10A), ERK, P38 and JNK inhibitors did not affect IL-1β-induced expression of LN β3 and LN γ2 chains. By contrast, PI3-K inhibitor abolished the effect of IL-1β on LN β3 and γ2 chain expression (Fig. 10A).

To study whether PI3-K was also able to affect culture-induced LN-332 expression, islets were incubated for 24 hours in the presence or absence of LY294002 immediately following the isolation procedure. As depicted in autoradiograms and histograms (Fig. 10B), LY294002 completely prevented culture-induced expression of LN β3 and LN γ2 chains.
Fig. 10. Expression of LN β3 and LN γ2 chains is regulated by PI3-K. Prior IL-1β stimulation, islets were pre-treated 1 hour with indicated inhibitors. After 6-hour incubation with IL-1β, proteins were extracted and analysed by Western blotting for expression of LN β3 and γ2 chains. Among all inhibitors tested, LY294002 is the only one that inhibits the effects of IL-1β. Representative immunoblots and densitometric analysis are shown (A). Results are expressed as means ± s.e.m. n=3-6; *, p<0.05 vs control; **, p<0.05 vs IL-1β-treated islets. To determine whether PI3-K was also able to affect culture-induced LN-332 expression, islets were incubated for 24 hours in presence or absence of LY294002 immediately following isolation. Representative immunoblots for LN β3 and LN γ2 chains are shown beside each respective histogram (B). Results are expressed as means ± s.e.m. n=3; *, p<0.05 vs freshly isolated islets; **, p<0.05 vs 24 hour-cultured islets.
5. Discussion

In this study, we described the expression of LN-332 in human pancreatic islets. Using an anti-LN-332 polyclonal antibody that recognizes the three chains of LN-332, we clearly showed that this LN isoform was expressed within islets. In one previous study (Parnaud et al., 2006), we reported expression of LN γ2 chain by non-β-cells. Here, we have shown that in addition to glucagon, pancreatic polypeptide and somatostatin-producing cells, insulin-producing cells also express LN-332.

Expression of extracellular matrix proteins by islet cells is still a subject of debate. Lammert group argued that in mouse islets, basement membrane was present exclusively around capillaries and that β-cells were only required to induce basement membrane protein secretion by endothelial cells (Nikolova et al., 2006). This model is supported by the observation that LNs and collagen IV mRNA are expressed by isolated islet endothelial cells but not by isolated β-cells (Nikolova et al., 2006). This view has been challenged by Virtanen and coll. (Virtanen et al., 2008) who presented multiple lines of evidence for a double-layered basement membrane in human islets: one layer is facing endocrine islet cells and another layer is facing endothelial cells. The authors cannot determine which cells formerly produce proteins for endocrine basement membrane in human islets. However, their immunological and ultrastructural studies strongly suggest that endocrine islet cells are directly involved in basement membrane formation. Our observations showing LN-332 in endocrine islet cells are consistent with this hypothesis and further permit us to underline the complexity of extracellular matrix formation in islets.

LN staining was heterogeneous according to islet cell types, with non-β-cells presenting a stronger labelling than β-cells. The reason for this heterogeneity remains to be clarified. It is unlikely that LN-332 plays a more important role in non-β-cells expressing higher amounts of LN-332. Indeed, at least in human islets, β-cells are intermingled with non-β-cells (Brissova et al., 2005) and both endocrine cell types are certainly layered on a basement membrane with a unique molecular composition. Even if LN-332 was secreted exclusively by non-β-cells, it should anyway exert an effect on β-cells. Staining was neither observed in endothelial cells, that have been described as a major source of the LN-411 and LN-511 isoforms (Hallmann et al., 2005), nor in the basement membrane that separates endothelial cells from
endocrine cells. Absence of staining in the basement membrane does not preclude that LN-332 is present. It is possible that its expression is too low to be detectable by immunofluorescence or that tight interaction between LN-332 and other proteins of the basement membrane or cell membrane receptors may render LN-332 epitopes inaccessible to antibodies. Another possibility is that LN-332 chains produced by islet cells are not integrated into the basement membrane. Indeed, many works have shown that LN-332 is a multifunctional protein with cell adhesion, migration, proliferation and scattering functions (Parnaud et al., 2008; Giannelli et al., 2000). Many of these functions do not necessarily require that LN-332 is a part of extracellular matrix. For instance, cell scattering activity of LN-332 in carcinoma cells has been shown to be mediated by a soluble form of LN-332 and is independent of specific LN-332 integrin receptors (Grassi et al., 1999). In addition, it cannot be excluded that the LN-332 chains may be secreted and have a biological function as monomer. For instance, LN γ2 chain has been found to be expressed as a monomer in several types of cancer cells where it can play a role as modulator of tumour cell behaviour (Koshikawa et al., 1999; Koshikawa et al., 2008). It is well documented that a domain of LN γ2 chain (domain DIII) containing an EGF-like repeat can be released by the action of MMPs. This released DIII fragment can act as a ligand for the EGF receptor eliciting intracellular signals (Schenk et al., 2003a).

Several studies already showed an intracellular labelling for LN γ2 chain in both healthy cells, such as human foetal teeth, (Salo et al., 1999) and pathological cells, such as gastric carcinomas (Koshikawa et al., 1999). Here, by confocal microscopy, we demonstrated that cytoplasmic LN-332 staining has a granular pattern that does not colocalize with the granular staining for pancreatic hormones. LN biosynthetic/secretory pathways have been poorly investigated. Few studies reported LN chain assembly within the endoplasmic reticulum and the Golgi apparatus (Matsui et al., 1995; Morita et al., 1985; Peters et al., 1985). However, there is no information concerning LN-332 translocation from the Golgi apparatus to the cell membrane leading to its secretion. The granular staining observed may represent LN-332 in the process of its synthesis and/or transport to the cell membrane. More studies are required to confirm this hypothesis.
Our study failed to show LN-332 expression in the extracellular environment in pancreatic sections. However, we have data suggesting that LN-332 is secreted by islets, at least in vitro. First, LN β3 and LN γ2 chains were detected in the medium of 48-hour-cultured islets. Second, the 105-kDa γ2' chain, a constituent of the secreted and mature form of LN-332 (Ghosh and Stack, 2000), was observed in the medium of cultured islets. When submitted to IL-1β treatment, expressions of LN β3, LN γ2 and LN γ2' chains were markedly increased in the islet culture medium, further supporting the hypothesis that LN-332 secretion is regulated. In this conditioned medium, only traces of actin were detected excluding the possibility that LN-332 detection in the medium was attributable to cell death.

Another striking result is that IL-1 increased both LN-332 gene expression and protein synthesis. IL-1 is produced by islet cells and has been shown to impair glucose-stimulated insulin production in islets (Eizirik, 1991; Giannoukakis et al., 2000) and to increase β-cell death (Hoorens et al., 2001; Steer et al., 2006). In some pathological conditions, such as diabetes and pancreatitis, islets are submitted to cytokine-mediated inflammatory insults (Mandrup-Poulsen, 2001), and similar inflammatory mechanisms have been shown to occur after islet transplantation (Montolio et al., 2007). It was demonstrated that in reaction to cytokine attack, epithelial cells such as keratinocytes and intestinal cells upregulated LN expression (Amano et al., 2004; Francoeur et al., 2004; Korang et al., 1995). The increased LN-332 production elicited by cytokines in the wound area presumably enhances wound repair by stimulating adhesion and migration of keratinocytes. Previous studies also suggested that LN-332 may have some cellular protective effects in islets (Bosco et al., 2000; Hammar et al., 2004; Parnaud et al., 2006) and that secretion of cytokines, including IL-1, was increased by rat β-cells under conditions that improve their function and survival (Ribaux et al., 2007). These observations combined with our result showing that IL-1β increased LN-332 expression suggest that this cytokine may have some beneficial effects on β-cells, at least indirectly.

In this study, we reported that expression of LN β3 and LN γ2 chains was lower in freshly isolated islets as compared to 24-hours-cultured islets. We hypothesized that stresses attributable to isolation could affect the expression of LN-332 in islets. Our results clearly showed that the effect of culture on LN-332 expression was independent of IL-1β. First, IL-1β was undetectable in the islet
conditioned medium, and second, IL-1Ra added to the medium of freshly isolated islets did not inhibit the effect of culture on LN-332 expression. Other factors secreted by islets themselves or contaminating cells, such as ductal and endothelial cells and macrophages, may affect expression of LN-332. For instance, IL-6 and IL-8 that we have identified in large amounts in islet conditioned medium could be two potential candidates. In addition, growth factors, such as HGF, TGF, PDGF and EGF, that have been shown to improve islet graft survival and function (Yamaoka and Itakura, 1999) and to induce production of LN-332 in other cell types, such as smooth muscle cells (Kingsley et al., 2002a; Kingsley et al., 2002b) and keratinocytes (Amano et al., 2004) could be also involved. IL-8, TGF-β1 and oncostatine-M, a cytokine of the IL-6 family showed no effect on the production of LN β3 and LN γ2 chains. However, we cannot exclude that other secreted factors are involved. Particular attention could be focused on HGF, shown to be produced by pancreatic endothelial cells and its predominant expression during pregnancy coincides with a peak of proliferation of β-cells (Johansson et al., 2006). We can speculate that HGF produced by islet endothelial cells may stimulate endocrine cells to produce LN-332, which in turn may induce proliferation of β-cells.

In an attempt to understand which signalling pathway was involved in IL-1β-induced-LN-332 expression, we used several specific inhibitors of ERK, P38, JNK and PI3-K. Our results showed that PD98059, SB203580, and SP600125 do not inhibit expression of LN-332. By contrast, LY294002, an inhibitor of PI3-K, inhibited IL-1β-induced expression of LN-332. Consequently, we propose that the PI3-K pathway is involved in the regulation of LN-332 production by islet cells. This hypothesis is supported by the observation that deactivation of the PI3-K pathway in a carcinoma cell line using wortmannin decreases the secretion of LN-332 (Baba et al., 2008). Interestingly, PI3-K inhibitor also inhibited culture-induced LN-332 production. Factors such as HGF, TGF, PDGF and EGF have been shown to activate PI3-K (Gentilini et al., 2007; Henson and Gibson, 2006; Roggia et al., 2007; Yao et al., 2008) and are therefore good candidates for culture-induced LN-332 production in islets.

In summary, our results indicated that LN-332 was expressed and secreted by human islets and that condition, inducing islet stresses such as isolation procedure and exposure to IL-1β enhanced LN-332 production. Altogether, these observations
can be considered as prerequisites necessary to hypothesize that the \textit{in vitro} effect of LN-332 on $\beta$-cell secretion, viability and replication are of physiological relevance as well.

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IV. PART 2

Effects of LN-332 on activation of rat peritoneal macrophage secretory functions

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1. Abstract

**Background and aims:** Macrophages play a critical role in the inflammatory responses occurring after islet transplantation as well as in the onset of type I diabetes. Whether extracellular matrix (ECM) in islets affects inflammatory processes mediated by macrophages is still unknown. The characteristic γ2 chain of laminin-332 (LN-332) has been detected by Western blotting and immunochemistry in islets. The aims of this study are i) to investigate whether LN-332 is able to activate macrophages and ii) to elucidate the signalling pathways involved in this process.

**Material and methods:** As source of LN-332, we used a matrix produced by a rat bladder carcinoma cell line that also contains fibronectin (804G matrix). Rat peritoneal macrophages were harvested after intraperitoneal injection of soluble starch and seeded either on uncoated (control), collagen-, fibronectin-, 804G matrix- or fibronectin-depleted 804G matrix coated Petri dishes. After a 24h-incubation in the presence or absence of 1 µg/ml phorbol myristate acetate (PMA), we measured tumour necrosis factor (TNF)-α and interleukin (IL)-6 secreted and nitrite (NO\textsubscript{2}\textsuperscript{-}) produced by peritoneal macrophages, by ELISA and the Griess reaction, respectively. Activation of p38 mitogen-activated protein kinase (MAPK), Extracellular signal-regulated kinase (ERK) MAPK and focal adhesion kinase (FAK) were analysed by Western blotting.

**Results:** We confirmed by immunofluorescence and Western blotting that LN γ2 chain was mainly expressed in rat islets and was localized in α-cells. In absence of PMA, secretion of TNF-α and IL-6 and production of NO\textsubscript{2}\textsuperscript{-} were increased in macrophages cultured on 804G matrix compared to macrophages cultured on collagen, fibronectin or uncoated Petri dishes. In presence of PMA, 804G matrix potentiated the secretion and production of all inflammatory mediators studied compared to control, collagen and fibronectin. Similar effects on TNF-α and IL-6 secretion and as well as NO\textsubscript{2}\textsuperscript{-} production were observed using fibronectin-depleted 804G matrix. After 24 hour-incubation, macrophages attached on 804G matrix showed an increased phosphorylation of p38 and ERK MAPKs when compared to control. In the presence of PMA, phosphorylation of p38 and ERK was similar in both conditions. By contrast, phosphorylation of FAK was reduced under the same conditions. **Conclusion:** LN-332-rich 804G matrix potentiated the secretion
of TNF-α and IL-6, and the production of NO₂⁻ by peritoneal macrophages. These effects are likely to be mediated by activation of ERK MAPK.
2. Introduction

Pancreatic islet transplantation into the liver is an attractive strategy to replace pancreatic endocrine function in patients with type 1 diabetes (Berney et al., 2001a). Indeed, clinical trials have demonstrated that pancreatic islet transplantation can result in remarkable improvement in the quality of life of patients (Shapiro et al., 2000; Shapiro et al., 2006). However, a substantial part of the graft is functionally impaired or lost in the following days after transplantation. In addition to uncontrolled cell damage caused by the isolation process (Kaufman et al., 1988), pancreatic islets face non-specific inflammatory events that develop in and around the graft, and may further cause irreversible cell damage and death (Berney et al., 2001a). These phenomena imply cells of the monocyte-macrophage lineage since the use of macrophage-depleting drugs in murine and canine models of islet transplantation has been shown to improve early graft survival (Bottino et al., 1998; Kaufman et al., 1994).

Macrophages comprise a heterogeneous population of versatile cells that play an essential role in host development, normal homeostasis, repair and remodelling processes and in the initiation and mediation of immune and inflammatory responses (Mosser and Edwards, 2008). Upon classical stimulation with lipopolysaccharide (LPS) and/or cytokines or chemical agents such as phorbol-myristate-acetate (PMA), macrophages produce a broad array of powerful chemical substances including pro-inflammatory cytokines and reactive oxygen species (Chen et al., 1992; Kurosaka et al., 1998; Smith et al., 1998; Tobias and Ulevitch, 1993). LPS activates mitogen-activated protein kinases (MAPKs) including c-Jun NH2-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (Guha and Mackman, 2001). Other investigators reported the involvement of MAPKs in LPS-induced cytokine production (Novogrodsky et al., 1994; Swantek et al., 1997).

In the course of an inflammatory response, cells of the monocyte-macrophage lineage respond to chemotactic factors and subsequently migrate through the subendothelial basement membrane (BM) and underlying interstitial structures, which are both rich in extracellular matrix (ECM) proteins (Worthylake and Burridge, 2001). Haskill and colleagues were the first to identify adherence to ECM as a powerful inducer of gene expression in monocytes (Haskill et al., 1988). Furthermore,
adherence of monocytes to ECM also modulate the secretion of inflammatory mediators such as proteases (Xie et al., 1993), superoxide anion $O_2^-$, prostaglandin E2, thromboxane (Gudewicz et al., 1994), and cytokines (Eierman et al., 1989; Juliano and Haskill, 1993; Kasahara et al., 1993; Wesley et al., 1998; White et al., 2001). In addition to native ECM, several in vitro studies reported that proteolytic ECM fragments (cryptic domains) or synthetic peptides corresponding to ECM sequences affected multiple functions and properties of inflammatory cells (Adair-Kirk et al., 2005; Adair-Kirk and Senior, 2008).

Pancreatic islets are surrounded by an ECM that contains fibronectin (Fn), collagen (Coll) types IV, V, and laminins (LNs), (Van Deijnen et al., 1994; Wang and Rosenberg, 1999). LNs are major ECM components present in all BM and play essential roles in both BM construction and regulation of cellular functions (Miner and Yurchenco, 2004). Within the pancreatic islet, BM was observed only around capillaries (Wang and Rosenberg, 1999). In humans, Coll type IV was detected in association with the pancreatic islet microvasculature (Kaido et al., 2004b). More recently, LN $\gamma_2$ chain has been identified in rat and human pancreatic islets, suggesting the existence of LN-332 isoform in pancreas and more specifically in pancreatic islets (Parnaud et al., 2006). Following islet isolation, the native ECM organization may be disrupted and consequently reveal cryptic domains. After transplantation into the liver, these ECM proteins and/or cryptic domains, may initiate the inflammatory response leading to early graft failure. The mechanisms by which cells of the monocyte-macrophage lineage are activated in the early graft failure are incompletely understood. More specifically, the potential involvement of LN-332 in stimulating macrophage secretory activity has never been investigated. A better understanding of these mechanisms, could be useful to improve the survival of transplanted $\beta$-cell mass.

The aims of this work were i) to investigate whether LN-332 was able to activate macrophages and ii) to elucidate the signalling pathways involved in this process, using a rat cellular model.
3. Materials and Methods

3.1. Reagents and antibodies

Antibodies against phospho-ERK (Thr202/Tyr204), ERK, phospho-p38 (Thr180/Tyr182) and p38 MAPKs were purchased from Cell Signalling Technology-Bioconcept (Allschwil, Switzerland), goat anti-LN γ2 and rabbit anti-ED2 antibodies from Santa Cruz Biotechnology (Santa Cruz, USA), rabbit anti-glucagon antibody from Dako (Switzerland, Baar), rabbit anti-fibronectin antibody from Sigma-Aldrich (Buchs, Switzerland) and mouse anti-ED1 antibody from Serotec (Oxford, UK). SB203580 (p38 MAPK inhibitor) and PD98059 (ERK MAPK inhibitor) were from Calbiochem (Darmstadt, Germany), and phorbol 12-myristate 13-acetate (PMA) and dimethyl sulfoxide (DMSO) were from Sigma-Aldrich (Buchs, Switzerland). Propidium iodide (PI) and fluoresceine diacetate (FDA) were from Sigma-Aldrich (Buchs, Switzerland). All culture media, reagents and materials were certified endotoxin free.

3.2. Animals

Male Sprague Dawley and Lewis rats were obtained from Janvier Laboratory (Le Genest-Saint-Isle, France) at 8 weeks of age and were used as source of macrophages and pancreatic islets. The experiments were carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of the University of Geneva School of Medicine and the veterinary Authorities of the state of Geneva.

3.3. Preparation of primary rat peritoneal macrophages

Adherent cells isolated from the peritoneal cavity of rats Sprague Dawley were used as a source of macrophages. To this end, a solution of 5% soluble starch (Sigma-Aldrich, Buchs, Switzerland) was injected intraperitoneally into rats, at a dose of 5 ml/100 g of body weight. Seventy-two hours later, rats were anesthetized with
isoflurane (Abbott AG, Baar, Switzerland) and ascites was harvested. Then, ascites cells were washed with phosphate-buffered saline (PBS). Contaminating red blood cells were removed by exposing the ascites cells to 0.2% NaCl for 30 seconds, after which the isotonicity (0.9%) was adjusted with 1.6% NaCl. Before culture, cell viability was assessed by PI/FDA staining and trypan blue exclusion tests. To determine the purity of the preparations, aliquots of isolated cells were injected into Cunningham chambers, fixed with paraformaldehyde (PFA) 4%, specifically labelled using anti-ED1 antibody and finally analysed by fluorescence microscopy.

3.4. Peritoneal macrophage adhesion test

Starch-elicited peritoneal macrophages were resuspended in RPMI-1640 medium (Sigma-Aldrich, Buchs, Switzerland) containing 2 mmol/l glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (hereafter, referred to as complete RPMI). To study the effects of ECM on peritoneal macrophages, 50 µl-aliquots of cells (2.5 x 10^5 cells) were plated on Petri-dishes coated or not with collagen (Coll), fibronectin (Fn), 804G matrix (804G) or Fn-depleted 804G matrix (804G-Fn⁻).

The cells were then immersed in 1.5 ml of complete RPMI supplemented or not with PMA (1 µg/ml) and incubated at 37°C. After 24 hours, cell adhesion was analysed by phase-contrast microscopy, proteins were extracted for Western blotting analysis and conditioned media were collected for subsequent analysis. To investigate the signalling pathways induced by 804G matrix, cells in suspension were treated for 1 hour with 10 µmol/l SB203580 or 50 µmol/l PD98059, then plated and incubated 6-hour at 37°C in 1.5 ml of complete RPMI.

3.5. ECM preparation

804G cells were grown in DMEM (Invitrogen Basel, Switzerland) supplemented with fetal calf serum (FCS) and glucose (5.6 mmol/l). At confluence, cells were washed and maintained for three days in DMEM without FCS. Conditioned medium (hereafter referred to as 804G matrix) was centrifuged at 1200 rpm for 5 minutes, filtered (0.22
µm), and frozen at -20°C. Absence of endotoxin (< 0.1 EU/ml) contamination in the 804G matrix was demonstrated using a limulus amebocyte lysate test kit (Lonza, Basel, Switzerland). An 804G matrix depleted in Fn (804G-Fn⁻) was prepared by affinity chromatography over a gelatine-sepharose column (Pharmacia biotechnology, St.-Quentin Yvelines, France) following manufacturer’s instructions.

3.6. Coating of plastic Petri dishes with ECM proteins

Fifty µl-aliquots (50) of crude Coll (extracted from rat tail tendons (Montesano et al., 1983b)), Fn (100 µg/ml) (Biomedical Technologies Inc., Stoughton, USA), 804G matrix, or Fn-depleted 804G matrix were layered at the center of adherent 35-mm culture Petri dishes. Dishes were kept in a damp box at 37° C for at least two hours before being rinsed three times with sterile distilled water and air dried. Uncoated Petri dishes were used as negative controls.

3.7. Nitrite determination

Nitric oxide (NO) is a gaseous free radical with a shorter half-life of seconds. The more stable NO metabolite nitrite (NO₂⁻), provides a surrogate marker and quantitative indicator of NO production. NO₂⁻ concentration was assessed by the Griess reaction. To this end, peritoneal macrophage culture supernatants were transferred onto a 96 well plate (BD-Biosciences, Allschwill, Switzerland) and 100 µl of Griess reagent consisting of 50 µl of 1% sulfanilamide (Sigma-Aldrich, Buchs, Switzerland) and 50 µl of 0.1% naphthylethylenediamine dihydrochloride (Sigma-Aldrich, Buchs, Switzerland) was added. The absorbance was measured at 540 nm, using a microplate reader (Bioconcept, Allschwil, Switzerland), and NO₂⁻ concentrations were calculated from a sodium nitrite standard curve.
3.8. Cytokine analysis

IL-1β, IL-6, and TNF-α secreted by peritoneal macrophages following exposure to individual ECM protein components were measured by ELISA according to the manufacturer’s instructions (R&D Systems, Abingdon, UK).

3.9. Western blotting

Western blotting of proteins extracted from peritoneal macrophages was performed as described in detail above (part I). Briefly, attached cells were washed with ice-cold PBS to remove any non adherent cells. The remaining cells were immersed in lysis buffer (100 mmol/l Tris, 5% SDS, 5 mmol/l EDTA) supplemented with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), diluted 1:7, and 1 mmol/l sodium orthovanadate (Sigma-Aldrich, Buchs, Switzerland), and detached using a cell scraper. The lysate was then homogenized by sonication (2x5 seconds). Protein concentration was determined by a dye method using a detergent compatible protein assay kit (Bio-Rad laboratories, Hercules, USA). Equal amounts of total proteins were suspended in sample buffer 5x (625 mmol/l Tris, pH 6, 10% SDS, 50% (v/v) glycerol, 0.25% bromophenol blue, 25% 2-mercaptoethanol) and boiled for 5 minutes prior to loading. Proteins from each sample were fractioned on 10% or 12% polyacrylamide gels. After separation on SDS-PAGE gel, all samples were electroblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, USA). PVDF membranes were saturated for 1 hour with 5% non fat dried milk and 0.1% Tween-20. When anti-phospho protein antibodies were used, membranes were saturated for 1 hour with 5% BSA 0.1% Tween-20. An enhanced chemiluminescence protein detection kit (Amersham Pharmacia Biotech, Piscataway, USA) and a Kodak image station were used for visualization of the bands.
3.10. Rat islet isolation

Pancreatic islets were isolated from Sprague Dawley rats by a modification of the method of Sutton et al. (Sutton et al., 1986). Rats were anesthetized with a saline solution (NaCl 0.9%) of rompun (Bayer AG, Switzerland) and ketasol (Dr. E. Graub AG, Bern, Switzerland). After a median laparotomy, pancreas was exposed and cannulated downstream into the bile duct. The pancreatic duct was clamped at its duodenal outlet, and 20 mg of collagenase type XI (Sigma-Aldrich, Buchs, Switzerland) diluted in 10 ml ice-cold Hank’s balanced salt solution (HBSS) containing 2.35 mmol/l CaCl$_2$ and 25 mmol/l N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES) was injected through the cannula. The distended pancreas was immediately dissected out, washed quickly with PBS and stored in a plastic tube in ice. The enzymatic digestion was performed in a water-bath at 37°C during 19 minutes. The reaction was stopped by adding 50 ml ice-cold HBSS, containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich, Buchs, Switzerland). Undigested pancreatic tissue was removed by filtering it through a 400 µm mesh tea-strainer. The filtered tissue was transferred into a plastic tube and centrifuged for 1 minute at 1000 rpm. Pancreatic islets were then separated from exocrine tissue by centrifugation over a discontinuous ficoll gradient (Sigma-Aldrich, Buchs, Switzerland) constituted of the following density: $D_1 = 1.108$, $D_2 = 1.096$, $D_3 = 1.069$. The pancreatic islets were collected from interface between densities $D_2$ and $D_3$ after a 20-minutes centrifugation without brake (4°C, 200 0 rpm), and further purified by handpicking when necessary.

3.11. Preparation of islet cells

Freshly isolated pancreatic islets were washed several times with PBS. They were then resuspended into 1 ml PBS containing 0.025% trypsin (Sigma-Aldrich, Buchs, Switzerland) and 0.01% ethylene diamine tetraacetic acid EDTA. Digestion was carried out with occasional pipetting through a 1 ml-plastic tip, until only few aggregates cells remained (7-9 minutes, 37°C). The digestion was stopped by adding 10 ml ice-cold HBSS supplemented with 0.1% BSA. The cell preparation was then
centrifuged 5 minutes at 1200 rpm and cell yield was calculated using a haemocytometer. For some experiments, β-cells were purified, using a fluorescence-activated cell sorter (FACStar-Plus) (Becton Dickinson, Sunnyvale, USA), as previously described (Rouiller et al., 1990; Van De Winkel et al., 1982).

3.12. Islet and islet cell culture

Pancreatic islets or islet cells were resuspended in DMEM, supplemented with 10% FCS, 100 mmol/l sodium pyruvate, 11 mmol/l glucose, 2 mmol/l glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin (hereafter, referred to as complete DMEM). Pancreatic islets were cultured in 10-mm non adherent Petri-dishes at a density of 400 islets/10 ml DMEM whereas islet cells were seeded in 60-mm non adherent Petri-dishes at a density of 10^5 cells/4 ml DMEM to avoid any reaggregation of cells. Pancreatic islets and cells were kept for at least 20 hours at 37°C, to allow cell recovery.

3.13. Intraportal islet transplantation

Under general anaesthesia induced by inhalation of isoflurane (Abott AG, Baar, Switzerland), the peritoneal cavity of the rat was accessed through a median laparotomy. After visualization of the portal vein by extra-abdominal repositioning of the bowel, 4000 pancreatic islets were infused into the portal vein via a 22G catheter (Optiva 2, Johnson and Johnson, Spreitenbach, Switzerland). After removal of the catheter, haemostasis was performed using surgical fibrinogen and thrombin sponges (TachoSil, Nycomed, Switzerland). Sprague Dawley rats were used as pancreatic islet donors. Recipients were either Sprague Dawley rats (syngeneic group) or Lewis rats (allogeneic group). Forty-eight hours after transplantation, rats were sacrificed and the livers were removed and processed for histology.
3.14. Static incubation to assess insulin secretion

Fifty µl droplets containing islet cells and peritoneal macrophages were seeded separately into the same 35 mm-Petri dishes coated with 804G matrix. As control, 50 µl droplets containing islet cells were seeded alone. After 24-hour incubation to allow cell attachment, Petri dishes were filled with 2 ml of complete DMEM medium and further incubated for 24 hours. Cells were washed 3 times with Krebs–Ringer bicarbonate HEPES buffer (KRBH: 125 mmol/l NaCl, 4.74 mmol/l KCl, 1 mmol/l CaCl₂, 1.2 mmol/l KH₂PO₄, 1.2 mmol/l MgSO₄, 5 mmol/l NaHCO₃, 25 mmol/l HEPES, pH 7.4, 0.5% BSA) and preincubated 1 hour at 37°C. Then, cells were incubated 2 x 1 hour at 37°C, first in KRBH containing 2.8 mmol/l glucose and then in KRBH containing 16.7 mmol/l glucose. Following the last incubation, insulin was extracted from cells with acid-ethanol (hereafter referred as to insulin content). Insulin content and insulin secreted in culture for 24 hours and during incubation at 2.8 and 16.7 mmol/l glucose for 1 hour were measured by enzyme-linked immunosorbent assay (ELISA) with rat insulin as the standard (Mercodia, Uppsala, Sweden). Results were expressed as percent of insulin content.

3.15. Immunohistochemical analysis

Samples of rat pancreata and livers transplanted with islets were harvested in PBS solution and fixed 24 hours in PFA 4% at room temperature. Tissues were embedded in paraffin and sectioned for histology. Five µm tissue sections were deparaffinized. Pancreas sections were treated with trypsin 0.1% for 10 minutes and liver sections were soaked in 10 mmol/l citrate buffer (pH 6) in a microwave oven at 600 W for 3 x 5 minutes for antigen retrieval. The slides were then rinsed with PBS and used immediately for immunofluorescence labelling. The whole immunofluorescence procedure was carried out at room temperature and all reagents were diluted in PBS. Non specific binding sites were blocked with 0.1% BSA solution for 45 minutes. Pancreas and liver sections were incubated for 2 hours with primary antibodies in blocking solution. For pancreas sections, the dilutions were 1:50 for anti-LN γ2 chain antibody and 1:100 for anti-glucagon antibody. For liver sections, the dilutions were
1:600 for anti-insulin antibody and 1:50 for anti-ED1 antibody. Sections were washed and incubated with specific secondary antibodies coupled either to fluorescein, rhodamine or coumarin. Specificity of the different immunostainings was confirmed with sections in which primary antibodies were omitted. Sections and cells were examined with an Axioskop microscope (Zeiss, Feldbach, Germany) equipped with an Axiocam color CCD camera (Zeiss).

3.16. Statistical analysis

Data were presented as means ± s.e.m. for three to five independent experiments. Differences between means were assessed either by one-way ANOVA or Student’s *t*-test. When Anova was applied, Scheffé’s least-significant difference post-hoc analysis was used to identify significant differences (*p*<0.05).
4. Results

4.1. Assessment of LN-332 molecule expression in rat pancreatic islets

Our working hypothesis is that LN-332 may affect macrophage behaviour. In a previous work we demonstrated that LN-332 was present in human pancreatic islets (Parnaud et al., 2006) (part I) and here we assessed the expression of this LN isoform in rat pancreatic islets. Expression of the characteristic γ2 chain of LN-332 was studied by immunofluorescence and Western blotting (Fig.1). As in human pancreatic tissue, specific labelling was neither observed in surrounding exocrine and connective tissues nor in vessels (Fig. 1A). The restricted expression of LN γ2 chain to pancreatic islets was confirmed by Western blotting analysis of protein extracted from isolated pancreatic islets and exocrine tissue (Fig. 1B). By immunofluorescence, we observed that labelling for LN γ2 chain was restricted to endocrine cells located at the periphery of the islet core. These cells were shown to express glucagon, as determined by double immunostaining (Fig. 1A). Expression of LN γ2 chain was also analysed in FACS-purified β- and non β-cell fractions by Western-blotting. Results indicated a predominant expression of LN γ2 chain in non β-cell fraction when compared to β-cell fraction (Fig. 1B). Furthermore, the 155-kDa LN γ2 chain was also detected in pancreatic islet conditioned medium suggesting that LN-332 was secreted by insular cells (Fig. 1B).
Fig. 1. **LN γ2 chain is expressed in rat pancreatic islets.** (A) Rat pancreas sections were analysed by fluorescence microscopy after double labelling for glucagon (red) and LN γ2 chain (green). Glucagon and LN γ2 chain colocalized (yellow colour obtained by merging the two images) in cells at the periphery of islets. Scale bar, 80 µm. (B) Proteins extracted from rat islets, exocrine tissue, purified β- and non β-cells and rat islet conditioned medium were analysed by Western blotting using a monoclonal anti LN γ2 antibody. These results are representative of at least three independent experiments.
4.2. Purity, viability and function of peritoneal macrophages

In addition to macrophages, ascites contains other cell types, including lymphocytes. Thus it was necessary to ensure the quality of the ascites macrophage preparations in terms of purity, viability and function. The purity was assessed by immunofluorescence using an antibody against ED1 (Fig. 2A), a protein expressed predominantly on the lysosomal membrane of macrophages and monocytes. We found that 87 ± 3% (n=3) of the peritoneal cells were ED1-positive. PI/FDA double staining test revealed 90% of living cells in macrophage preparations (Fig. 2B-C). Similar results were obtained with trypan blue staining (not shown). Macrophages exist either at a resting steady state or at an activated state which is associated with morphological changes and increased cytokine and reactive oxygen and nitrogen species secretion. When isolated macrophages were incubated in the presence of PMA (an activator of macrophages), cell spreading occurred (Fig. 3A). At the metabolic level, cytokines (TNF-α and IL-6) released and NO₂⁻ produced in the culture medium increased (Fig 3B), as compared to macrophages incubated under control conditions. IL-1β was undetectable, neither in the control condition nor in the treated condition (not shown). Altogether, these observations confirmed the reliability of the ascites macrophage preparations for the subsequent experiments.
Fig. 2. Purity and viability of peritoneal macrophages. A solution of soluble starch 5% was injected intraperitoneally into male rats Sprague-Dawley. The peritoneal cells were harvested 72 hours later. (A) The cytoplasm of most cells was labelled in green, after immunofluorescence for ED1. Scale bar, 20 μm. (B) When submitted to a FDA/PI staining test, most cells displayed a green cytoplasm staining and red nuclei were only occasionally observed; scale bar, 30 μm. (C) Quantitative data of FDA/PI staining test showed that about 90% were FDA positive and 10% were PI positive. Results are expressed as mean ± s.e.m. n=4.
Fig. 3. Function of peritoneal macrophages. (A) Phase contrast micrographs of non activated and PMA-activated macrophages plated on uncoated Petri dishes. (B), NO₂ production and TNF-α and IL-6 secretion from macrophages attached to uncoated Petri dishes and activated or not with PMA, after 24 hours at 37°C. Results are expressed as mean ± s.e.m. of three to four independent experiments; *, p<0.05.
4.3. Adhesion to 804G matrix stimulates macrophage TNF-α and IL-6 secretion and NO₂⁻ production

Freshly isolated macrophages were layered on different substrates. The 804G cell conditioned medium (hereafter referred as 804G matrix) was used as source of LN-332. Adhesion of non activated macrophages to 804G matrix induced an increase in the secretion of TNF-α and IL-6 and production of NO₂⁻, as compared to control. By contrast, Coll and Fn had no effect. When experiments were repeated in presence of PMA, that by itself increased cytokine secretion and NO₂⁻ production, similar effect of 804G matrix was observed, suggesting that 804G matrix potentiated the effect of PMA (Fig. 4A). Again, IL-1β was undetectable in all conditions tested (not shown).

![Graphs showing cytokine and NO₂⁻ production](image)

**Fig. 4. Effect of 804G matrix on the function of peritoneal macrophages.** Macrophages were plated on uncoated Petri dishes (control), 804G matrix (804G), collagen (Coll), or fibronectin (Fn), in the absence (non activated) or presence of 1µg/ml PMA (PMA-activated). Supernatants were recovered after 24 hours and analysed by the Griess reaction for NO₂⁻ production and by ELISA for TNF-α and IL-6 secretion. Results are expressed as mean ± s.e.m. of duplicate/triplicate samples from three to seven independent experiments; *, p<0.05 vs corresponding non activated macrophages ; **; p<0.05 vs non activated control, Coll and Fn.
Since crude 804G matrix was shown to contain high molecular weight molecules such as Fn, we prepared a 804G matrix depleted in high molecular weight molecules, called hereafter Fn-depleted 804G matrix. By Western blotting, absence of Fn and presence of LN γ2 chain in Fn-depleted 804G matrix was confirmed (Fig. 5). As shown in figure 6, we observed that this Fn-depleted 804G matrix was able to induce cytokine secretion and NO$_2^-$ production from macrophages as did the crude 804G matrix. Having demonstrated that 804G matrix increased the secretion of cytokines and the production of NO$_2^-$ by peritoneal macrophages, we wanted to determine whether these effects were time-dependent. To this end, we analysed cytokines released and NO$_2^-$ produced by macrophages incubated for various intervals of time on uncoated Petri dishes or 804G matrix-coated Petri dishes, in absence or presence of PMA. The results, presented as cumulative secretion (Fig. 7), showed rapid effect of 804G matrix on secretion of cytokines and NO$_2^-$ production, in both absence and presence of PMA. When compared to the control condition, the peak of secretion was around 4-5 hours (not shown).

**Fig. 5. Analysis of 804G purified by Western blotting.** Aliquots of 804G matrix (804G) and Fn-depleted 804G matrix (804G-Fn) were separated on polyacrylamide gels under reducing conditions and blotted for LN γ2 chain and fibronectin (Fn). Fn is detected as a band at 220-kDa in 804G matrix (804G) whereas it is absent in FN-depleted 804G matrix (804G-Fn).
Fig. 6. **Effect of Fn-depleted 804G matrix on the function of peritoneal macrophages.** $2.5 \times 10^5$ macrophages were layered on uncoated Petri dishes (control) and Petri dishes coated with 804G matrix (804G) and Fn-depleted 804G matrix (804G-Fn). Supernatants were recovered after 24 hours and analysed by the Griess reaction for NO$_2$ production and by ELISA for TNF-α and IL-6 secretion. Results are expressed as mean ± s.e.m. of duplicate/triplicate samples from three independent experiments; *, $p<0.05$ vs corresponding non activated macrophages.
Fig. 7. Cumulative production of NO\textsubscript{2}\textsuperscript{-} and secretion of TNF-\textalpha{} and IL-6 in culture media by peritoneal macrophages. Rat peritoneal macrophages (3×10\textsuperscript{5} cells) were layered on uncoated Petri dishes (control) and Petri dishes coated with 804G matrix (804G), in the absence (non activated) or presence of 1\mu g/ml PMA (PMA-activated). Conditioned media were collected at the indicated period of times and analysed by the Griess reaction for NO\textsubscript{2}\textsuperscript{-} production and by ELISA for TNF-\textalpha{} and IL-6 secretion. Graphs show the cumulative production over the time. Results are the mean of two independent experiments.
4.4. Adhesion of macrophages to 804G matrix activates p38 and ERK MAPKs and deactivates FAK

Phosphorylation of p38, ERK MAPKs and FAK was analysed in macrophages cultured for 24 hours into Petri dishes coated or not with 804G matrix and in the presence or absence of PMA. Results (Fig. 8) showed that 804G matrix and PMA induced phosphorylation of p38 and ERK MAPKs and decreased phosphorylation of FAK, when compared to the corresponding control. Concerning p38 MAPK, level of phosphorylation was similar between 804G matrix and PMA, and no potentiation was observed when incubation was performed in the presence of both 804G and PMA, suggesting that similar mechanisms accounted for these effects. Concerning ERK MAPK, level of phosphorylation was also similar between 804G matrix and PMA, but a potentiation was observed when incubation was performed in the presence of both 804G and PMA, suggesting that dissimilar mechanisms accounted for these effects. The non phosphorylated forms of p38, ERK MAPKs and FAK were not affected either by 804G matrix or PMA.

4.5. ERK phosphorylation is involved in 804G matrix-induced activation of macrophages

To investigate whether p38 and ERK MAPKs pathways were involved in 804G matrix-induced activation of macrophage, we examined the effects of SB203580 and PD98059, inhibitors of p38 MAPK and ERK MAPK, respectively. To this end, macrophage secretion of TNF-α and production of NO₂⁻ were analysed after 6 hours of incubation, in the absence or presence of the specific inhibitors SB203580 and PD98059. Results (Fig. 9) showed that SB203580 as well as PD98059 inhibited PMA-induced secretion of TNF-α and production of NO₂⁻. While SB203580 did not affect their secretion and production induced by 804G matrix, inhibition of ERK MAPK activity by PD98059 inhibitor reduced the secretion of TNF-α and production of NO₂⁻ induced by 804G matrix. As a control, macrophages were incubated in the presence of DMSO (used for the dilution of inhibitors) alone and had no effect on macrophages at the concentration used (not shown).
**Fig. 8. Effect of 804G on p38, ERK MAPKs and FAK phosphorylation.** p38, ERK MAPKs and FAK phosphorylation was determined by Western blotting. 804G matrix and PMA increased phosphorylation of p38 and ERK whereas they decreased phosphorylation of FAK. Immunoblots are representative of four independent experiments.
Fig. 9. Effect of p38 and ERK MAPK inhibitors on TNF-α secretion and NO$_2^-$ production. Prior incubation on 804G matrix, macrophages were pre-treated 1 hour with SB203580 (10 µmol/l) and PD98059 (50 µmol/l). After 6-hour incubation, culture media were collected and analysed by ELISA for TNF-α secretion and by the Griess reaction for NO$_2^-$ production. SB203580 and PD98059 inhibited PMA-induced secretion of TNF-α and production of NO$_2^-$ whereas PD98059 inhibited 804G matrix-induced production of TNF-α and NO$_2^-$ and SB203580 had no effects. Results are the mean of two independent experiments.

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4.6. Macrophages are mobilized around islets transplanted into the liver

When rat pancreatic islets were transplanted into the liver (allogeneic and syngeneic graft model), a clear infiltration of ED1 positive cells all around the pancreatic islets was observed by immunofluorescence (Fig.10A). These ED1 positive cells are either Kupffer cells (liver residing macrophages) or circulating monocytes. Some of them were clearly in close contact with insulin-positive cells (Fig. 10B).

**Fig. 10.** ED1 positive cells are detected all around the transplanted islets. Forty-eight hours after transplantation, rats were sacrificed and livers were removed and processed for histology. Liver sections were analysed by fluorescence microscopy after double labelling for insulin (blue) and ED1 (green). A large number of ED1 positive cells accumulated around the islet (A) and some of them were in close contact with insulin-positive cells (B). Left scale bar, 50 µm; right scale bar, 20 µm.
4.7. 804G matrix-activated macrophages affect β-cell insulin secretion

We questioned whether macrophages and more specifically 804G matrix-activated macrophages could affect β-cell function. To answer this question, islet cells were attached on 804G matrix and further cocultured or not in presence of peritoneal macrophages also attached on 804G matrix, as shown in the figure 11.

![Fig. 11. Schematic of the experimental design.](image)

After 24 hours of culture, insulin released in the medium and insulin secreted acutely after 1 hour stimulation with glucose (16.7 mM) were analysed. As shown in figure 12A, macrophages affected insulin secreted during 24 hours and acutely during 1 hour stimulation with 16.7 mM glucose. Morphology of islet cells was also affected by macrophages. Indeed, islet cells incubated alone showed a spread out pattern, while islet cells cocultured with macrophages displayed anastomosed rodlike pattern suggesting a withdraw from the 804G matrix (Fig. 12B).
Fig. 12. Insulin secretion of islet cells is impaired by 804G matrix-activated macrophages. Islet cells were attached on 804G matrix and cultured alone (control) or in presence of peritoneal macrophages attached on 804G matrix (no contacts). After 24 hours of culture, insulin secretion was assessed by static incubation (A). Micrographs of islet cells cocultured or not with 804G matrix-activated macrophages; scale bar, 100 μm (B).
5. DISCUSSION

In a previous work (part I), we demonstrated the presence of LN-332 in human pancreatic islets. To confirm its presence in rat pancreatic islets, we used an antibody directed against the characteristic γ2 chain of LN-332 as a specific marker to detect LN-332. Histological and Western blotting analyses clearly showed that γ2 chain was predominantly expressed in α-cells, as in human. As γ2 staining was not observed in BM of pancreas sections, the only argument suggesting LN-332 secretion was the detection of γ2 chain in pancreatic islet conditioned medium.

When pancreatic islets are transplanted into the liver, they are exposed to a complex microenvironment constituted by liver endothelial cells and inflammatory elements, including cells of the monocyte-macrophage lineage (Kupffer cells). The islet graft generates an inflammatory reaction and it is likely that pancreatic islets themselves activate cells of the monocyte-macrophage lineage. This inflammation is characterized by an increased production of pro-inflammatory mediators including cytokines, chemokines, reactive oxygen and nitrogen species. It is well documented that these soluble factors have the potential to inflict damage and induce apoptosis/necrosis in different cell types, including pancreatic islets and β-cells (Berney et al., 2001a). Among the various factors affecting the behaviour of the cells of the monocyte-macrophage lineage, the proteins of the native ECM have been shown to play an important role in differentiation of monocytes into macrophages as well as activation of macrophage secretory function. Moreover, degradation products or exposure of cryptic domains of ECM components can elicit biological responses distinct from the parent molecules (Adair-Kirk et al., 2005; Adair-Kirk and Senior, 2008). Results suggested that LN-332 produced and secreted by pancreatic islets may affect macrophage behaviour. Indeed, 804G matrix, used as a source of LN-332, clearly increased IL-6 and TNF-α secretion, as well as NO2− production by macrophages, when compared to control. Although 804G matrix is rich in LN-332, it also contains other ECM components such as Fn that has been shown to induce inflammatory genes in cells of the monocyte-macrophage lineage (Juliano and Haskill, 1993). Using a Fn-depleted 804G matrix, we demonstrated that NO2− production was independent on the presence of Fn in 804G matrix. Furthermore we showed that purified Fn did not affect cytokine secretion and NO2− production by
macrophages. Similar results were observed with Coll. Overall, these results suggest that LN-332 present in 804G matrix is alone responsible for this effect. Since endotoxins are well known inducer of macrophage activation, it was necessary to confirm that 804G matrix used in our experiments was not contaminated by endotoxins. To this end we used a kit that allowed us to confirm that the level of endotoxin was inferior to 0.1 EU/ml. According to the U.S. Food and Drug Administration guideline, the upper limit of the pyrogen level is 5 EU/ml.

We showed that the 804G matrix potentiated the effect of PMA on macrophage activation. PMA is a strong activator of protein kinase C (PKC) and has been shown previously to activate macrophages (Smith et al., 1998). The fact that 804G matrix potentiated the effect of PMA suggests that the 804G matrix acts on macrophage activation by a mechanism different of PKC. Using specific antibodies against phosphorylated forms of signalling molecules, it was clearly shown that 804G matrix triggered phosphorylation of p38 and ERK MAPKs. Phosphorylation of both of these MAPKs is a common event in the activation of inflammatory cells and many experiments revealed that activation of these signalling pathways might be induced by ECM via interaction with integrins expressed at the surface of cells. In addition, in other cell type, LN-332 has been shown to induce gene expression through an ERK MAPK-dependent pathway (Klees et al., 2005).

It is well documented that macrophages express many different integrins, including α6β1 (de Fougerolles and Koteliansky, 2002), a classical receptor for LN-332. It remains to be shown that α6β1 and LN-332 interact together.

PMA induced a similar p38 and ERK MAPK activation as compared to 804G matrix. Interestingly, when macrophages were exposed to both 804G matrix and PMA, p38 MAPK phosphorylation was similar to that observed with macrophages exposed to 804G matrix or PMA alone. These results suggest that mechanisms of p38 MAPK activation by 804G matrix and PMA are similar and could involve PKC. Different hypothesis was made for ERK MAPK, since macrophages exposed to both 804G matrix and PMA increased its phosphorylation above to that observed for macrophages exposed to 804G matrix or PMA alone. It is possible that 804G matrix may activate ERK in a PKC-independent pathway. In this context, there is accumulating evidence that ECM degradation products can stimulate the expression of inflammatory genes by a variety of immune cells at the injury site (Jiang et al.,
In this context secreted LN-332 can be proteolysed at different site in its γ2 chain and generate small fragments with biological activity. MMP-14 and -2 proteolyse γ2 chain and release a small fragment of approximately 25-27-kDa. This fragment has EGF-like properties and may bind to the EGF receptor (Schenk et al., 2003a) to initiate ERK MAPK activation. We therefore hypothesize that LN-332 present in 804G matrix may be processed by macrophages that have been shown to produce MMP-14 (Rajavashisth et al., 1999). The released 25-27-kDa LN fragment may in turn activate ERK MAPK pathway in macrophages. Supplementary experiments are required to confirm this hypothesis.

As a consequence of the effect of 804G matrix on macrophage activation and phosphorylation of signalling molecules, we assessed whether blocking any of these signalling pathways inhibited 804G matrix-induced cytokine secretion and NO\textsubscript{2}- production. Results clearly demonstrated that the p38 MAPK SB203580 inhibitor did not affect 804G matrix-induced TNF-α secretion and NO\textsubscript{2}- production but did decrease PMA-induced TNF-α secretion and NO\textsubscript{2}- production, indicating that 804G matrix activated macrophages by a p38 MAPK-independent mechanism and PMA, by a p38 MAPK-dependent mechanism. Concerning ERK MAPK involvement, preliminary results suggested that PD98059 (ERK MAPK inhibitor) decreased 804G matrix-induced production of both TNF-α and NO\textsubscript{2}- However, further experiments are needed to confirm it. Another protein of the MAPK family, JNK, has been shown to be activated during inflammation. Further studies are required to determine whether this protein is activated by 804G matrix, and whether it is involved in 804G matrix-induced activation of macrophage secretory function.

A surprising result was the finding that both 804G matrix and PMA lowered FAK phosphorylation in macrophages when compared to control. If macrophages actually interact with LN-332 present in 804G matrix through integrin receptors, one should expect an increase of phosphorylation of FAK as it does in rat β-cells. Therefore, we can hypothesize either that interactions between 804G matrix and macrophages are mediated by non-integrin receptors or that an uncommon still unknown mechanism is accountable for the 804G matrix-induced FAK dephosphorylation. This last possibility is strengthened by the observation that PMA also decreased phosphorylation of FAK in macrophages. Further investigations are
necessary to better understand why and how 804G matrix and PMA decreased phosphorylation of FAK in macrophages.

804G matrix derived from the conditioned medium of the tumoural rat bladder carcinoma 804G cell line. Therefore, we can not exclude that these cells secrete other molecules that could affect macrophage behaviour. Further studies are needed to definitively show that the effects of 804G matrix on macrophages are actually owed to LN-332. For instance, antibodies directed against LN-332 could be used. However, this approach could have the disadvantage of activating macrophages. As an alternative, purified LN-332 could be tested.

Although LN-332 was found in islet conditioned medium, it is still unknown if LN-332 produced and secreted by islets has any impact on macrophage secretory functions. One way to answer this question is to establish a monolayer of lysed pancreatic tissues on which macrophages would be seeded.

In vivo, grafted islets may be exposed to different subpopulations of macrophages including circulating monocytes and Kupffer cells. In graft experiments, an accumulation of ED1 positive cells was observed around the islet, in both allogeneic and syngeneic graft model. ED1 antibody recognizes most macrophage populations, as well as monocytes in the peripheral blood and precursor cells in the bone marrow (Damoiseaux et al., 1994). For this reason, another antibody (ED2) that recognizes antigens exclusively expressed on resident tissue macrophages was tested. Unfortunately, it failed to show which subset of cell was predominantly recruited to the site of inflammation (not shown). Cells belonging to the monocyte-macrophage lineage are characterized by high heterogeneity and plasticity (Gordon and Taylor, 2005). Once in tissues, macrophages acquire distinct morphological and functional properties dependent of the tissue and the immunological microenvironment. It is likely that, peritoneal-derived macrophages and Kupffer cells do not have the same functional properties. Therefore, it would be important to assess whether LN-332 has an impact on the function of Kupffer cells.

We tried to determine whether 804G matrix-activated macrophages did affect insulin secretion from islet cells. The results indicated that insulin secretion from islet cells was decreased by macrophages attached on 804G matrix. As in the experimental design direct cell-to-cell contacts between islet cells and macrophages were improbable, the observed effects were certainly mediated by macrophage-secreted mediators, such as TNF-α, IL-6 and NO. It remains to be shown whether
other mediators secreted by macrophages could affect insulin secretion. Also it would be interesting to assess whether mediators secreted by macrophages could affect viability of islet cells.

In conclusion, our results indicated that 804G matrix induced TNF-α and IL-6 secretion and NO₂⁻ production by peritoneal macrophages. These effects were independent on the phosphorylation of p38 MAPK. By contrast, 804G matrix effects were mediated by ERK MAPK. These effects are probably due to LN-332 present in this matrix. Moreover, 804G-activated macrophages were able to impair glucose-induced insulin secretion from β-cells.
V. GENERAL DISCUSSION AND CONCLUSION

Islets of Langerhans and LN-332
Allogeneic islet transplantation has recently emerged as one of the most promising therapeutic approaches to improve glycometabolic control in diabetic patients and, in many cases, achieving insulin independence. Unfortunately, many persistent flaws still prevent islet transplantation from becoming the gold standard treatment for T1D patients. Indeed, although approximately 80% of patients treated with islet transplantation achieve insulin independence within the first year post-transplantation (Shapiro et al., 2003), this rate of success decreases dramatically in the following years (Ryan et al., 2005) (http://www.citregistry.org/ Website of the Collaborative Islet Transplant Registry. Last accessed: March 30, 2009).

Reasons for limited success of islet transplantation are multifactorial. One of these factors should be related to the disruption of cell-to-ECM contacts that occur during the isolation procedure. In situ, islets are surrounded by a continuous peri-insular ECM that supports connection between the endocrine and exocrine tissue. In addition, a BM, primarily constituted by Coll IV, LN, and Fn, is located between endothelial and endocrine islet cells. The ECM is continually produced and remodelled by different types of cells and has been shown to play a significant role in regulating the behaviour of cells that are in its close contact, through the engagement of the integrins on the cell surface. Thus, integrin-ECM interactions mediate adhesion, provide structural support, and activate intracellular chemical signalling pathways (Jiang and Harrison, 2002; Kaido et al., 2004a; Kaido et al., 2004b). During islet isolation, enzymatic digestion of the exocrine pancreas is poorly controlled. As a consequence, the peri-insular ECM is destroyed and the interstitial BM may be digested as well. The disruption of the cell-to-ECM relationship and the loss of BM both induce apoptosis (Meredith and Schwartz, 1997).

The characterization and the role of proteins involved in islet cell-to-ECM interactions as well as reestablishment of these interactions would be instrumental to improve the survival and function of isolated islet in culture and after transplantation into T1D patients. Precedent works have studied the impact of different ECM proteins on β-cell biology. Amongst them, LN-332 has particularly attracted our attention,
since cell-to-ECM relationship mediated by LN-332 and β1 integrins has been shown to modulate the function and survival of isolated rat primary β-cells. However, the experiments were performed in an artificial system in which purified rat β-cells were layered on LN-332 produced by the rat bladder carcinoma 804G cell line. In this condition, we did not know whether LN-332 was expressed in islets and more specifically in human islets. At both morphological and physiological levels, consequent differences have been observed between rodent and human islets. For instance, rodent islets are organized in a core of β-cells surrounded by a mantle of non β-cells. This typical architecture is less evident in human where islet cells are mixed together and β-cells are arranged alternately with non β-cells all around the vessels (our unpublished data). So far, expression of LN-332 in whole islets, and more specifically in human islets, was not characterized and its study remained largely incomplete. Therefore, we aimed to better understand how LN-332 was expressed and regulated in human islets.

In the first part of the present work, we have described the heterogeneous expression of LN-332 in human islet cells with predominance in α-cells. We confirmed the presence of the characteristic γ2 chain of LN-332 in rat endocrine cells and found that it was also predominantly expressed in α-cells. In rat islet, expression of LN-332 in β-cells was less evident than in human islets. The reason for this heterogeneity in both species remains to be clarified. As most ECM proteins, LN-332 is normally deposited in BM or connective tissue. In pancreas sections, LN-332 was intracellular. Absence of staining in the BM does not preclude that LN-332 is present. It is possible that its expression is too low to be detectable by immunofluorescence or that tight interaction between LN-332 and other proteins of the BM or cell membrane receptors may render LN-332 epitopes inaccessible to antibodies. Indeed, we identified LN γ2 chain in conditioned medium of both human and rat islets, suggesting its secretion and deposition in BM. As LN-332 was undetectable in the BM of pancreas sections by classical immunofluorescence, this is the only clue that may suggest its secretion. However, the cleaved form (105-kDa γ2') was identifiable only in human and not in rat islet conditioned medium. The reason for this discrepancy remains to be elucidated.

Concerning the potential localization of LN-332 in the BM, a recent study performed by Virtanen and colleagues clearly demonstrated that the interspace
between endothelial cells and endocrine cells within islets was constituted by two BM (Virtanen et al., 2008). Their immunological and ultrastructural analyses strongly suggested that endocrine cells were directly involved in BM formation. Our observations showing LN expression in endocrine islet cells are consistent with this hypothesis and further permit us to underline the complexity of ECM formation in islets. A systematic analysis of pancreas sections by electronic microscopy could help us to determine the exact localization of LN-332.

We also showed that LN-332 expression, biosynthesis and secretion could be up-regulated in human pancreatic islets by stress conditions. Despite these results, we have no evidence about the biological activity of LN-332 and we can only speculate on the physiological role played by this protein in vitro and in vivo. In particular, we do not know if LN-332 is an essential protein for the survival and proper functioning of the islets. In addition, we do not know if it plays a similar role in stimulating insulin as it does in vitro with rat β-cells. To study its role in vitro, we should layer human islet cells on human purified LN-332 and assess insulin secretion and cell viability. One classical way to study the role of a protein in vivo is to use genetically engineered mouse in which the protein has been turned off through a gene knock-out (KO). Unfortunately, mouse deficient in one of the three genes encoding the α3-, β3- and γ2-chains of LN-332 die within five days of birth because of severe skin blistering that resembles to that of Herlitz’s junctional epidermolysis bullosa in human being (Kuster et al., 1997; Meng et al., 2003; Ryan et al., 1999). As conventional LN-332 KO mouse is lethal, the alternative to this problem should be the generation of a mouse with an islet-specific inactivation of LN-332 using the Cre (recombinase)-loxP system. This step would require the selection of the right gene to be inactivated since LN-332 is encoded by three different genes and the generation of a triple KO mouse would be fastidious. The information obtained from the literature shows that the ideal candidate to be inactivated is the LAMA3 gene. Indeed, α3-chain is essential to drive LN-332 secretion (Marinkovich et al., 1992; Yurchenco et al., 1997), and once secreted, LN-332 may interact with β1 and β4 integrins, through its α3-chain (Delwel et al., 1994; Marchisio et al., 1993; Rousselle and Aumailley, 1994). As our results have shown a predominant expression of LN-332 within α-cells, the gene encoding the recombinase should be therefore under the control of a glucagon specific promoter.
In human islets, α-cells are very often intermingled with β-cells and are in close contact with islet endothelial cells. In such an organization, it is likely that α-cells-secreted products including ECM proteins could affect β-cell behaviour. We demonstrated in a recent study in vitro that aggregates comprising a β-cell coupled to an α-cell secreted significantly more insulin than β-cells alone (Wojtusciszyn et al., 2008). This effect could be explained by a paracrine action of glucagon. Beside this paracrine hypothesis, it is conceivable that adhesion through ECM-receptor interaction also play a role in the effect of heterologous intercellular contacts on insulin secretion. Whether ECM-receptor interactions involving LN-332 are involved in insulin secretion of human β-cells remains to be investigated.

One striking result was the finding that among several cytokines tested IL-1β was the only one to induce a change in LN-332 synthesis in human islets. In the literature, we can find a plethora of information describing IL-1β as a pro-inflammatory cytokine involved in β-cell dysfunction/stunning, death and apoptosis. One can legitimately suppose that IL-1β-induced up-regulation of LN-332 is a consequence of apoptotic events. Therefore, we examined whether IL-1β induced activation of caspase-3, a protein currently described as a key element activated in islet apoptosis (Emamaullee and Shapiro, 2006; Emamaullee et al., 2007; Nakano et al., 2004). The result showed that while IL-1β stimulation increased LN-332 production, it did not induce caspase-3 activation. Moreover, cisplatin, a common apoptosis inducing drug slightly decreased LN-332 production. However, we have to consider this result cautiously and can not exclude that IL-1β activates other signalling pathways that induce apoptosis (caspase-6 or -7) or necrosis. Even if IL-1β is cytotoxic for islet β-cells, we demonstrated that its effect on LN-332 synthesis was PI3-K-dependent, a classical kinase that promotes cell survival (Datta et al., 1999). In addition, following islet isolation procedure, LN-332 was up-regulated also in a PI3-K dependent-manner. In our system, we can only speculate on the putative cytotoxic or beneficial action of IL-1β on islets. Whatever, we suggest that, beyond its recognized cytotoxic role, IL-1β could also generate a compensatory mechanism that promotes cell survival by increasing ECM protein synthesis, necessary for cell anchorage. Cell anchorage not only provides structural stability but also mediates pivotal survival signals for the cells. Integrins α3β1 and α6β1 (receptors for LN-332) have been...
shown to be expressed in islets (Bosco et al., 1989; Kantengwa et al., 1997). Therefore, it would be interesting to assess the effect of IL-1β on expression of these integrins. As cell-to-cell adhesion and cell communication are known to affect cell function in islets (Bosco et al., 1989; Meda, 2003), it would be also interesting to study the effect of IL-1β on expression of molecules involved in cell-to-cell adhesion and communication, such as E-cadherin and connexin 36, respectively.

We observed that LN-332 was lower in freshly isolated islets when compared to 24 hour-cultured islets. As IL-1β was undetectable in islet conditioned medium, it is suggested that another mechanism than IL-1β-induced LN-332 synthesis is involved. We suppose that other factors secreted by islets themselves or contaminating cells such as ductal and endothelial cells and macrophages, may affect LN-332 synthesis. In this regard, we analysed cytokines released by cultured islets, 24 hours after isolation. IL-8 and IL-6 were detected in large amounts. Moreover, when stimulated with IL-1β, cultured islets secreted at least 50 times more IL-6 and 2 times more IL-8 than non stimulated islets (not shown). We demonstrated that IL-8 and oncostatine-M, a cytokine of the IL-6 family, had no effect on LN-332 synthesis. A recent study showed that α-cells were a primary target of IL-6 actions. IL-6 promoted α-cell specific effects, including increasing glucagon expression and secretion, increasing proliferation, and preventing against metabolic stress-induced apoptosis, in vitro (Ellingsgaard et al., 2008). As α-cells are the predominant source of LN-332 and IL-6 induces metabolic changes in these cells, it would be interesting to assess whether IL-6 could affect its LN-332 expression.

**Macrophages and LN-332**

Cell-to-ECM disruption is not the only event contributing to the loss of transplanted islets. Indeed, once implanted, islets face non-specific inflammatory events that develop in and around the graft (as observed in our graft experiments), and may further cause irreversible cell damage and death (Berney et al., 2001a). These phenomena imply at least cells of the monocyte-macrophage lineage. In the course of an inflammatory response, cells of the monocyte-macrophage lineage may interact with elements of the ECM. It is well documented that native ECM or ECM fragments affect multiple functions and properties of inflammatory cells (Adair-Kirk et al., 2005; Adair-Kirk and Senior, 2008). In the first part of this thesis, we suggested that LN-332
was secreted by islets. We therefore hypothesized that LN-332 could be an element playing an important role in activating macrophages during the inflammatory responses occurring after islet transplantation.

In our experiments, we demonstrated that the 804G matrix, used as a source of LN-332, clearly increased IL-6 and TNF-α secretion, and NO\textsubscript{2} production by rat peritoneal macrophages. Cells belonging to the monocyte-macrophage lineage are characterized by high heterogeneity and plasticity (Gordon and Taylor, 2005). Once in tissues, macrophages acquire distinct morphological and functional properties dependent of the tissue and the immunological microenvironment. It is likely that, peritoneal-derived macrophages and Kupffer cells do not have the same functional properties. For this reason, it would be interesting to compare the effects of LN-332 on activation of Kupffer cells and peritoneal-derived macrophages.

Concerning the use of 804G matrix as a source of LN-332 we have to keep in mind that this matrix is secreted by a rat bladder carcinoma cell line. Therefore, we can not exclude that these cells secrete other molecules that could affect macrophage behaviour, independently on the presence of LN-332. Cells of the monocyte-macrophage lineage exhibit complex interactions with tumoural cells. In the tumour microenvironment, neoplastic cells shape the differentiation and functional orientation of tumour-associated macrophages (Sica et al., 2008). Moreover, the ability of macrophages to produce reactive nitrogen species, particularly NO is correlated with an enhanced tumouricidal activity during tumoural invasion (Mitra and Khar, 2002). For this reason it would be interesting to use a purified LN-332 instead of a carcinoma-derived matrix to confirm the implication of LN-332 in activating macrophages.

Trying to elucidate the signalling pathways involved in the effect of 804G matrix on macrophage activation, we highlighted two proteins of the MAPK family: p38 and ERK. Phosphorylation of both proteins was increased when peritoneal macrophage were in contact with 804G matrix. We then assessed whether blocking any of these signalling pathways would inhibit 804G-induced production of cytokines and NO\textsubscript{2}. Our first results indicated that 804G matrix-induced activation of macrophages was mediated by ERK MAPK. The most surprising result was that both 804G matrix and PMA lowered FAK phosphorylation in macrophages. FAK has been shown to be tyrosine-phosphorylated in response to integrin-mediated cell adhesion to the ECM (Richardson and Parsons, 1996) and LN-332 (Hammar et al., 2004). In
addition, FAK is critically involved in the regulation of macrophage motility (Owen et al., 2007) and become activated in macrophage during infection (Hudson et al., 2005). If macrophages actually interact with LN-332 present in the 804G matrix through integrin receptors, one should expect an increase of FAK phosphorylation. Therefore, we can hypothesize that either interactions between the 804G matrix and macrophages are mediated by non-integrin receptors or an uncommon and yet unidentified mechanism is accountable for the 804G matrix-induced FAK dephosphorylation. This last possibility is strengthened by the observation that PMA also decreased phosphorylation of FAK in macrophages. Further investigations are necessary to better understand why and how 804G matrix and PMA decreased phosphorylation of FAK in macrophages.

Even though we have identified the characteristic $\gamma_2$ chain of LN-332 in islet conditioned medium, suggesting LN-332 secretion, it is still unknown if LN-332 produced and secreted by islets has any impact on macrophage activation. To answer this question, the use of islet conditioned medium as a matrix should be a solution. However, one has to consider that islets secrete other factors known to activate macrophages. Another approach to determine whether islet ECM may affect macrophage activity would be to establish a monolayer of lysed pancreatic tissues on which macrophages would be seeded.

Finally, we investigated whether 804G matrix-activated macrophages did affect insulin secretion from islet cells. We observed that insulin secretion from $\beta$-cells was impaired. However we still do not know whether TNF-$\alpha$, IL-6 and or NO$_2^-$ secreted by macrophages are really responsible for the observed effect. Once activated, cells of the monocyte-macrophage lineage secrete a broad panel of inflammatory mediators. Whether other mediators secreted by macrophages could affect insulin secretion has not been yet investigated. Also, it would be important to determine how secreted mediators affect viability of islet cells.

**Conclusion**

In this work, we show for the first time that LN-332 is expressed in a regulated way in human islets. In addition, this protein that has been shown to have beneficial effects on $\beta$-cells, *in vitro*, is also secreted by islets. Several potentially important implications can be envisioned from our observations on the human islet LN-332
composition. These range from β-cell differentiation and proliferation to pathogenetic mechanisms of diabetes and finally islet transplantation. Concerning islet transplantation, LN-332 may have a major impact on islets in culture. Indeed, the provision of a matrix to support islet attachment may be an important requirement for maintaining the function and viability of islets before transplantation. However, once transplanted, islets are exposed to a much more complex microenvironment than in culture. The LN-332 that could be beneficial \textit{in vitro} represents a potential threat for islets \textit{in vivo}. Our observations on the effect of a LN-332-rich matrix on macrophage secretory function suggest it. This last issue needs to be explored more carefully to ensure a maximally physiological and safe microenvironment for the transplanted islets.
VI. REFERENCES


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