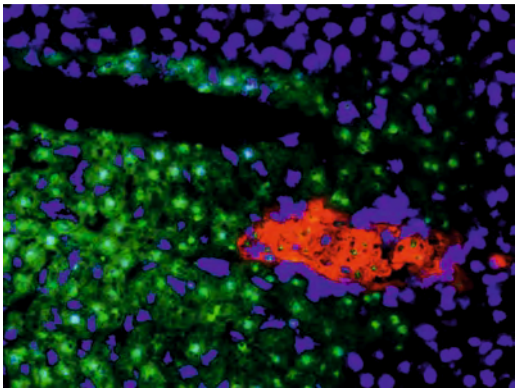
Inhibition of gelatinase activity in pancreatic islet graft reduces cellular inflammation and restores islet function

NEELAM LINGWAL



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INAUGURALDISSERTATION

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vorgelegt von

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Dedicated to My Parents….

Thesis Planning

The present thesis is divided into five different chapters, which are as follows-

Chapter 1- Introduction including a literature review and Aim
Chpetr II-Materials and Methods
Chapter III- Results
Chapter IV-Discussion
Chapter V-References

Abbreviations

BSA - Bovine serum albumin

CD11b- Cluster of differentiation molecule 11B

DMEM - Dulbecco's modified Eagle medium

ECM- Extra cellular matrix

EDTA - Ethylenediaminetetraacetic acid

FBS - Fetal bovine serum

FGF- Fibroblast growth factor

HBSS - Hank's buffered salt solution

HEPES - Hydroxyethyl piperazineethanesulfonic acid

HRP - Horseradish peroxidase

IBMIR- Instant blood mediated inflammatory reaction

IL β-Interleukin beta

IHC - Immunohistochemistry

ISZ-*In situ* zymography

kDa - kiloDalton

MMP- Matrix metalloproteases

MSCs- Mesenchymal stem cells

MT-MMP- Membrane type MMP

NF-κB- Nuclear Factor κB

PAGE - Polyacrylamide gel electrophoresis

STZ – Streptozocin

T1D - Type 1 Diabetes

TIMP-Tissue inhibitors of matrix metalloproteases

TGF- Transforming or Tumor growth factor

TNF α- Tumor necrosis factor alpha

Tx- Transplantation

VEGF- Vascular endothelial growth factor

Figure Legends

Chapter 1- Introduction including a literature review and Aim

Chapter II-Materials and Methods

Chapter III- Results

Fig. 16 MMP-9 inhibitor peptide and graft function.

Chapter IV-Discussion

Fig.1 Gelatinase activity decreased after islet after transplantation but increased in islet vicinity.

Fig. 2 Influence of gelatinase on islet transplantation.

Summary-

Islet transplantation provides an approach to compensate for loss of insulin producing cells in patients with type 1 diabetes. However, the intraportal route of transplantation is associated with instant inflammatory reactions against the graft and subsequent islet destruction as well. While matrix metalloproteases (MMP) 2 and 9 are involved both in remodelling of extracellular matrix and leukocyte migration, their influence on the outcome of islet transplantation has not been characterized.

Analysis of MMP-2 and MMP-9 in islet cells by zymography, gelatin dequenching assays, and western blot showed that in normal state, islets were expressing less MMP but treatment with inflammatory cytokines increased MMP-2 and MMP-9 secretions from them. However, islet transplantation in mice liver showed less MMP activity in the transplanted islet and more with the recipient liver. We observed comparable MMP-2 mRNA expressions in control and transplanted groups of mice, whereas MMP-9 mRNA and protein expression levels increased after islet transplantation. Immunostaining for CD11b (Mac-1) expressing leukocytes (macrophage, neutrophils) and Ly6G (neutrophils) revealed substantially reduced inflammatory cell migration into islet-transplanted liver in MMP-9-knockout (KO) recipients. Moreover, gelatinase inhibition resulted in a significant increase in the insulin content of transplanted pancreatic islets and reduced macrophage and neutrophil influx as compared to the control group.

These results indicate that increase of MMP-9 expression and activity after islet transplantation is directly related to enhanced leukocyte migration and that early islet graft survival can be improved by inhibiting MMP-9 (gelatinase B) activity.

Zusammenfasung-

Die Inselzelltransplantation bietet einen Ansatz in der Therapie von Patienten mit Typ 1 Diabetes, um den Verlust insulinproduzierender Zellen zu kompensieren. Allerdings ist die Transplantation auf dem Wege der intraportalen Applizierung mit einer sofortigen Entzündungsreaktion gegen das Transplantat und nachfolgend mit einer Zerstörung der Inselzellen assoziiert. Obwohl die Matrix-Metalloproteasen (MMP) 2 und 9 sowohl an der Umbildung von extrazellulärer Matrix, als auch an der Leukozyten-Migration beteiligt sind, wurde ihr Einfluss auf den Ausgang von Inselzelltransplantationen bisher nicht beschrieben.

Die Untersuchung der MMP-2 und MMP-9 in Inselzellen mittels Zymographie, Gelatine Dequenching Assay und Western Blot zeigte zum einen, dass Inselzellen unter Normalbedingungen wenig MMP exprimieren, zum anderen aber, dass sie unter Behandlung mit entzündungsfördernden Zytokinen mehr MMP-2 und MMP-9 sezernieren. Die gemessene MMP-Aktivität war jedoch nach der Transplantation von Inselzellen in die Leber von Mäusen in den transplantierten Inseln niedriger und in der Empfängerleber höher. Wir fanden vergleichbare Expressionen von MMP-2-mRNA in der Kontrollgruppe und in der Gruppe der transplantierten Mäuse, wohingegen die Expressionslevel von MMP-9-mRNA und -Protein in Inseltransplantaten höher lagen. Die immunhistologische Färbung von CD11b-(Mac-1-) exprimierenden Leukozyten (Makrophagen, Neutrophile) und Ly6G (Neutrophile) offenbarte eine erheblich reduzierte Migration von Entzündungszellen in die Leber von MMP-9-knockout (KO) Empfängertieren, in die die Inseln transplantiert wurden. Zudem führte die Hemmung der Gelatinase zu einer signifikanten Zunahme des Insulingehaltes in transplantierten Inseln aus dem Pankreas und zu einer Abnahme der Makrophagen- und Neutrophileninfiltration im Vergleich zur Kontrollgruppe.

Diese Ergebnisse deuten darauf hin, dass eine Zunahme der MMP-9-Expression und -Aktivität nach einer Inselzelltransplantation direkt im Zusammenhang mit einer gesteigerten

Leukozytenmigration steht und, dass das frühe Überleben der Transplantate durch die Hemmung der MMP-9- (Gelatinase B) Aktivität gefördert werden kann.

Chapter I

1.Matrix metalloproteases

2. Type 1 diabetes and islet transplantation

3.MMPs in diabetes and islet transplantation

4.Aim of the study

1. Matrix metalloproteases (MMPs)

MMP are a family of zinc-dependent endopeptidases composed of more than 20 members. They are involved in the turnover of the ECM in several conditions including embryonic development, inflammatory cell invasion or wound healing (1-4).

1.1 History

Matrix metalloproteinases are essential for any individual cell to interact properly with its immediate surroundings and for multicellular organisms to develop and function normally.

MMPs were for the first time discovered by Gross and Lapiere in 1962, when they observed that tadpole tail could degrade gels made of fibrillar collagen (5). They concluded that it is a matrix-degrading enzyme and named it Collagenase or MMP-1.

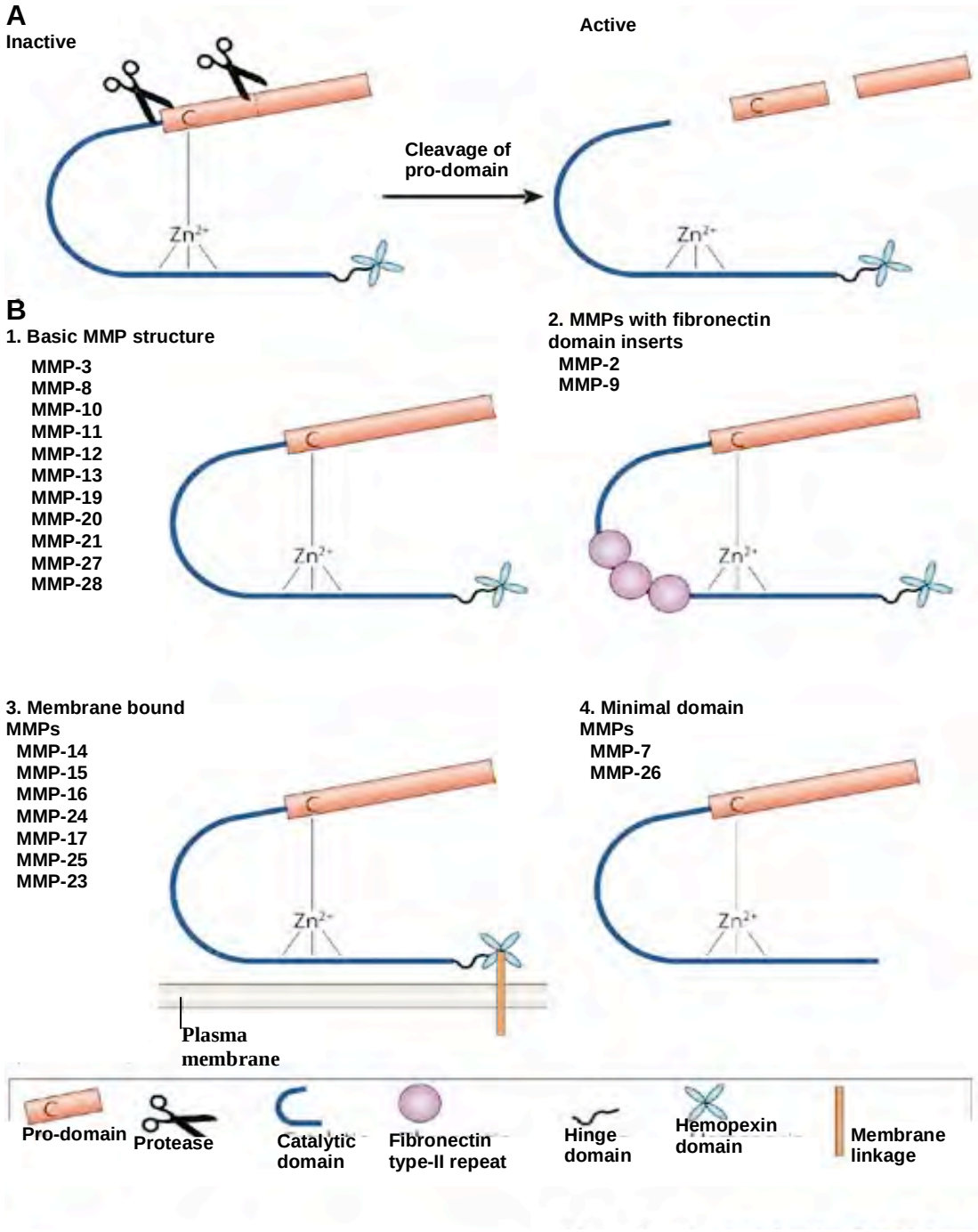
A family of related enzymes has been identified in all species and collectively called matrix metalloproteinases (MMPs) because they degrade components of ECM and they need metal ions for catalytic activity.

It took almost a decade after their discovery, when researchers discovered for the first time that MMPs are synthesized as inactive zymogens and they require activation (6), and demonstrated that MMPs can be inhibited by their natural endogenous metalloproteinase inhibitors called tissue inhibitors of metalloproteinase or TIMPs(7) .

1.2 Structure

Based on their structural characteristics they are classified into two types -secreted MMPs and membrane-anchored MMPs. MMPs are composed of protein domains (fig 1). Generally they have an N terminal signal sequence (which is also known as pre-domain) that directs secretion of MMP. The pre-domain is followed by pro-domain that makes the enzyme inactive. Once this pro-domain is removed, the MMP gets activated (8). The catalytic domain is adjacent to the pro-domain and possesses a zinc-binding site. Most of the MMPs have hemopexin-like domains at the C-terminal, which mediate substrate and inhibitor interaction. Catalytic domain is separated from hemopexin-like domain by a small hinge region which influences

substrate specificity (9).



A. Page-McCaw et al. Nature Reviews Molecular Cell Biology 8, 221–233 (March 2007)

Fig 1 Structure of MMPs. They are generally secreted in an inactive form by the interaction between thiol group of a pro-domain cysteine residue and the zinc molecule of the catalytic domain (A). Based on their structure we can divide them into four parts. First class of MMPs have the basic structure while the second group of MMPs have 3 fibronectin repeats in their catalytic domain. Third class of MMPs are with membrane linkage region and the fourth group are with minimal domain structure (B).

Some of the MMPs such as MT-MMPs possess membrane-anchored domain and some of the

MMPs lack hemopexin-like domain (Figure 1B,1-4). This domain influences TIMP binding,

some of the substrate binding and enzymatic activity. For example, MMP-1 needs both ends for cleaving collagens whereas hemopexin domain performs local unwinding of its triple-helical structure and sequential cleavage of each alpha-chain individually (10). MMP-2 and MMP-9 also contain a module of three fibronectin-type-2 repeats, which they need for binding with their substrate (11).

1.3 **MMPs regulation**

Generally, MMPs are not expressed in healthy tissue but their expression can be observed in fetal development, inflammatory reactions and in tissue remodelling (1-4). Regulation of MMPs is complex and tightly regulated at different levels such as, at the protein level by their endogenous activator and inhibitors, the secretion level, the cell surface localization and their own degradation after secretion. Details of regulation pathways are mentioned below.

1.3.1 Regulation at the transcription level

MMPs are mostly regulated at the transcription level except MMP-2, which expresses constitutively and co-regulates with TIMP-2 and MMP-14. There are several factors such as cytokines-interleukins, interferon, TNF and growth factors - FGF, VEGF, TGF, which regulate MMP expression in different cell types. Most of the MMP promoters contain cis-elements which can regulate MMP's gene expression by trans-activators, for e.g.- AP-1, PEA3, Sp-1,B-catenin/Tcf-4 and NF-κB (12).

There are some other factors such as phorbol esters, integrin-derived signals and cell stress that stimulate or suppress MMP's gene expression (13-15).

1.3.2 Endogenous inhibition of MMPs

MMPs can be inhibited by their natural inhibitors, which are known as tissue inhibitors of metalloproteases (TIMPs). TIMPs are 20-29 kDa-secreted proteins that inhibit MMPs in stoichiometric fashion (16). For example, TIMP-2 binds the hemopexin domain of MMP-9 less readily compared with MMP-2 (17) and TIMP-2, 3 can inhibit MT1-MMP but TIMP-1 cannot (18). TIMPs also regulate target genes and are tissue specific.

Alpha2-macroglobulin is another major endogenous inhibitor of MMPs (19). It is an abundant plasma protein that essentially inhibits MMPs in tissue fluids, whereas TIMPs act on the cellular level. Alpha2-macroglobulin/MMP complexes are cleared irreversibly by scavenger receptors while TIMPs inhibit MMPs in a reversible manner.

1.3.3 Regulation at secretion level

After translation MMPs are constitutively secreted but some of them reside within the intracellular compartment. For example MMP-9 is synthesized by neutrophilic granulocytes in the bone marrow and stored in tertiary granules. Once granulocyte are activated they secrete these MMPs (20). Plasmin and thrombin activate MMP-12 secretion from macrophages but without inducing transcription of MMP-12 (21).

1.3.4 Regulation from inactive form to active form

MMPs are generally secreted in the inactive form and get activated when cysteine-to-zinc switch is opened by normal proteolytic removal of the propeptide domain (22). Some MMPs such as MMP-11, MMP-27 and MT-MMPs contain furin-like-enzyme motif, which can become active by intracellular subtilisin type intracellular serine proteases before they reach the cell surface (23). The extracellular activation of most MMPs can be initiated by other already activated MMPs or by several serine proteinases that can cleave peptide bonds within MMP pro-domains (24)

1.3.4 Regulation of pericellular proteolytic activity

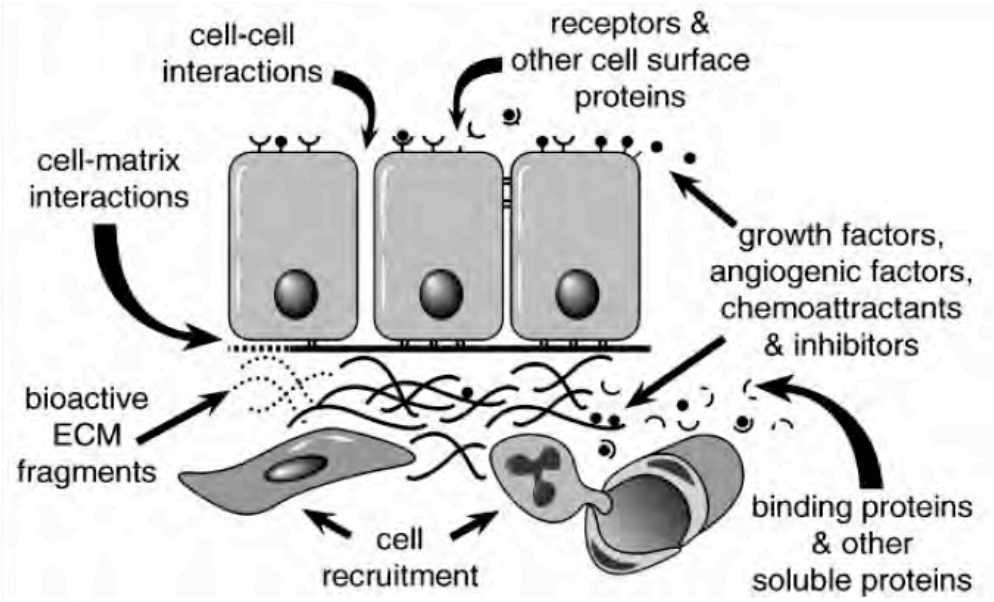
MMPs can also be regulated in pericellular space by regulating membrane bound MT-MMPs expression by binding of MMPs to cell surface receptor such as MMP-2 binds with alpha v beta 3-integrin (25) and MMP-9 to CD44 (26). Presence of cell surface receptors for enzymes such as uPA/plasmin, thrombin and elastase (27-29) also influence MMP functions as catalytic processes enhance MMP activity in the pericellular space, limit the inhibitors of MMPs and thereby increase the probability of proteolysis of MMP targets.

1.3.5 MMPs catabolism

MMP degradation or clearance is another way of inactivating them. It has been shown that alpha2-macroglobulin forms a complex with MMP and by endocytosis permanently clears this complex (19). Thrombospondin (TSP) has also been implicated in the clearance of MMPs. The low-density lipoprotein receptor-related protein (LRP) mediates the endocytic clearance of various proteinases and proteinase inhibitor complexes, including thrombospondin (TSP)-dependent endocytosis of MMP-2 (30).

1.4 Possible mode of action

Matrix metalloproteases can degrade not only extracellular matrix but they also provide structural support to the cells. In addition they cleave several cell surface receptors and cellular junctions and thereby change signalling pathways and cell-cell interactions.



M.D. Sternlicht and Z.Werb, Annu. Rev. Cell Dev. Biol. 2001. 17:463–516

Fig 2 Possible mode of action. MMPs can influence a lot of cellular functions such as- degradation of ECM, cell migration, cleavage of cell surface receptor, cleavage of cell-cell adhesion and removal of ECM binding protein or growth factors.

They release signalling molecules and growth factors from ECM and influence inflammatory reactions and cell migration (Fig 2). Some of their main modes of action (Fig 2) are shown below.

1.4.1 Remodelling of extracellular matrix (ECM)

ECM is a complex structural entity, which provides support for cells. The ECM is composed of three major insoluble structural classes of molecules: collagens, elastins, and specialized proteins such as fibrillin, fibronectin, laminin and proteoglycans. MMPs can degrade different components of the ECM, which are shown in Fig 3.

ECM not only provides cell support but is also a site of embedded growth factors and cytokines. It releases cryptic sites that are mostly made by ECM degradation, for instance MMPs cleave native fibrillar collagen and expose cryptic RGD sites that can be ligated by alpha v-beta 3 integrin, and this interaction promotes the expansion of tumor cells (31). Another component of basement membranes, laminin-5, has a cryptic site, which is the γ2 chain of laminin that induces epithelial cell migration (32). MMP-2 and MMP-9 expose a cryptic epitope within collagen IV that supports endothelial proliferation (33, 34).

Enzymes	ECM substrates
Secreted-type MMP	
Collagenases	
Interstitial collagenase (MMP-1)	Collagens I, II, III, VII and X; gelatins; aggrecan; link protein; entactin; tenascin; perlecan
Neutrophil collagenase (MMP-8)	Collagens I, II and III; gelatins; aggrecan; link protein
Collagenase-3 (MMP-13)	Collagens I, II, III, IV, IX, X and XIV; aggrecan; Fn; tenascin; osteonectin; Ln; Perlecan
Gelatinases	
Gelatinase A (MMP-2)	Gelatins; collagens IV, V, VII, X and XI; Ln; Fn; elastin; aggrecan; link protein
Gelatinase B (MMP-9)	Gelatins; collagens III, IV and V; aggrecan; elastin; entactin; link protein, vitronectin; N-telopeptide of collagen I
Stromelysins	
Stromelysin-1 (MMP-3)	Aggrecan; decorin; gelatins; Fn; Ln; collagens III, IV, IX and X; tenascin; link protein; perlecan
Stromelysin-2 (MMP-10)	Aggrecan; Fn; Ln; collagens III, IV and V; link protein
Matrilysins	
Matrilysin-1 (MMP-7)	Aggrecan; gelatins; Fn; Ln; elastin; entactin; collagen IV; tenascin; decorin; link protein
Matrilysin-2 (MMP-26)	Gelatin; collagen IV; Fn; fibrinogen; vitronectin
Furin-activated MMP	
Stromelysin-3 (MMP-11)	Fn; Ln; aggrecan; gelatins
Epilysin (MMP-28)	Unknown
Other secreted-type MMP	
Metalloelastase (MMP-12)	Elastin; aggrecan; Fn; collagen IV; osteonectin; Ln; nidogen
RASI-1 (MMP-19)	Collagen IV; gelatin; Fn; tenascin; aggrecan; COMP; Ln; nidogen
Enamelysin (MMP-20)	Amelogenin; aggrecan; gelatin; COMP
MMP-21	Unknown
MMP-27	Unknown
Membrane-anchored MMP	
Type I transmembrane-type MMP	
MT1-MMP (MMP-14)	Collagens I, II and III; gelatins; aggrecan; Fn; Ln; fibrin; Ln-5
MT2-MMP (MMP-15)	Fn; tenascin; nidogen; aggrecan; perlecan; Ln
MT3-MMP (MMP-16)	Collagen III; Fn; gelatin
MT5-MMP (MMP-24)	PG
GPI-linked MMP	
MT4-MMP (MMP-17)	Gelatin; fibrinogen
MT6-MMP (MMP-25)	Gelatin; collagen IV; fibrin; Fn; Ln
Type II transmembrane-type MMP	
MMP-23	Gelatin

Fig 3 MMPs and their substrates.

1.4.2 MMPs regulate inflammatory reaction

Inflammation is always characterized by several steps such as recruitment of leukocytes, activation of leukocytes, plasma derived inflammatory mediators and increase in inflammatory cytokine secretion. Though inflammation is necessary for host defence, it is harmful if unregulated or excessive. Increase in MMPs expression has been associated with almost every inflammatory disease. MMPs regulate several inflammatory mediators such as cytokines and chemokines and establish chemokine gradients in inflamed tissues that regulate migration of leukocytes to sites of inflammation (35-37).

Leukocytes release MMPs and extravagate by cell-cell junction, by cleaving components of the basement membrane or interstitial matrix. MMPs are also known to influence leukocyte migration by increasing chemokine activation, for instance MMP-9 can activate chemokines CXCL5, CXCL8, CXCL6 (38) and cytokine TGF-beta (39). Similarly MMP-2, MMP-9 and MMP-3 activate IL1-beta precursor and MMP-3 can degrade active IL1-beta (35). During inflammation neutrophils release MMP-8 and MMP-9 which degrades type 1 collagen and generates an acetylated tripeptide mimicking the chemotactic effects of chemokine ligand 8(CXCL8) and enhances neutrophil recruitment to site of inflammation (40).

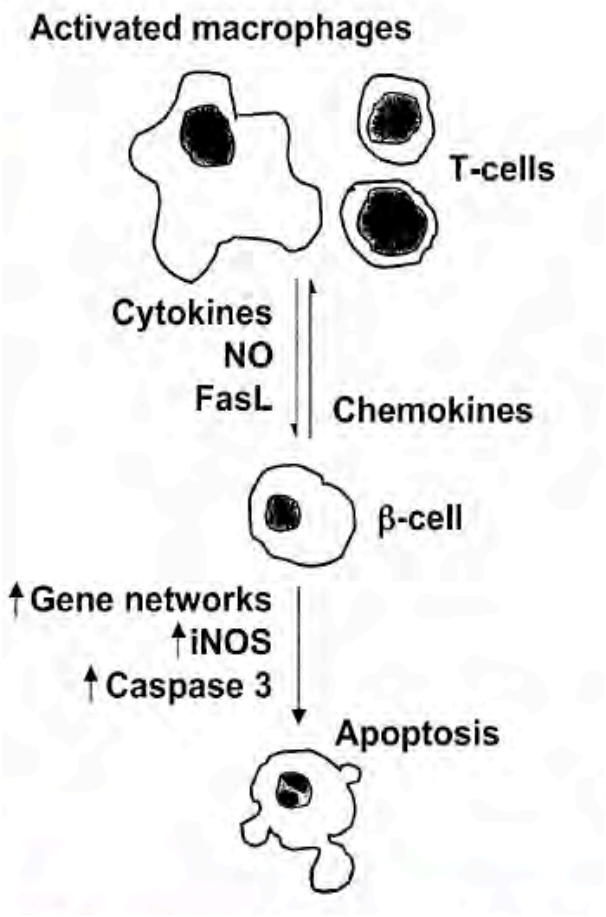
1.4.3 Cleavage of cell surface molecules

MMPs can release cell surface molecules and thereby influence a lot of signalling pathways. Matrix metalloproteinase 9 (MMP-9/gelatinase B) proteolytically cleaves ICAM-1 and participates in tumour cell resistance to natural killer cell-mediated cytotoxicity (41). MMP-3 cleaves soluble L-selectin from leukocytes and releases active heparin binding EGF-like growth factor from cell surface (42, 43). MMP-7 cleaves the NR1 subunit of the *N*-methyl-d-aspartate (NMDA) receptor, which is associated with NMDA stimulated calcium flux in neurons. (44).

2. Type 1 diabetes and islet transplantation

Type 1 diabetes is defined as an autoimmune disease that results in the permanent destruction

of insulin producing beta cells of the pancreas. In this disease the immune system self-destroys beta cells in the islets of Langerhans of the pancreas and eliminates insulin production. Even though it can occur at any age usually it starts in people younger than 30 and is therefore also known as juvenile onset diabetes (45).



Cnop M et al, *Diabetes* 54 Suppl 2:S97-107, 2005

Fig 4 Type 1 diabetes. In type I diabetes immune cells destroy beta cells of pancreas and thereby patients need to inject insulin.

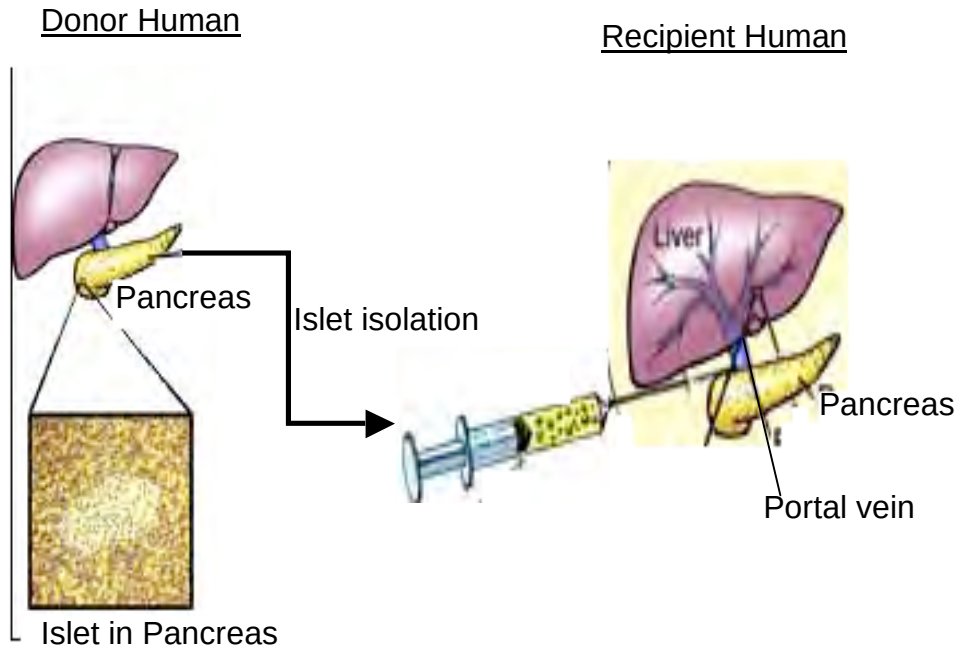
2.1 Treatment of T1D

Type 1 diabetes is associated with metabolic abnormalities, which can lead to several chronic and degenerative complications in patients, including retinopathy, nephropathy, neuropathy, atherosclerosis and lipid disorders. It can be treated by either injecting exogenous insulin or by performing whole pancreas or islet transplantation. Recent studies have shown that transplantation is a better approach than exogenous insulin therapy as transplantation can not

only restore insulin production, but also reduce diabetic complications more effectively. On the whole, organ transplantation is associated with major surgery and the risk of surgical complications, islet cell transplantation is less critical and can be obtained by minor surgery (45).

2.2 Islet cell transplantation

Islet cell transplantation provides an approach used in patients with type 1 diabetes to compensate for loss of insulin producing cells. In this therapeutic procedure, islet cells are injected into the liver via the portal vein, where they take up residence and begin to produce insulin (45, 46).



Adapted Juan J. Blondet et al, Surg Clin N Am 87 (2007) 1477–1501

Fig 5 Islet transplantation process. It includes islet isolation from donor pancreas and then isolation of islet followed by injection of islet in recipient liver.

As in normal conditions insulin from pancreatic beta cells is secreted directly into portal vein system and degraded in the liver, it was hypothesised that the islet transplantation in liver can provide a similar physiological situation. Later several islet transplantation experiments confirmed this hypothesis. This method has another advantage of having minimal invasion procedure (46).

2.3 History of islet cell transplantation

First time in 1892 Minkowsky reported that removal of pancreas was associated with diabetes in dog and in 1893 English surgeon Watson Williams transplanted sheep pancreatic fragments into a 15 year old boy with end stage type 1 diabetes however the boy died after few days due to immune rejection of islets which at the time people were not aware of (45).

Paul E. Lacy at Washington University proposed that islet cell transplantation may be a better option than insulin treatment as it can not only cure diabetes but can also prevent chronic complications of diabetes. In 1972, he successfully reversed diabetes by transplanting pancreatic islets in a rodent model sytem (47). In 1986 an automated method of islet isolation was resolved and this yielded a sufficient number of islets. In 1990, for the first time Pittsburgh's group performed successful human islet allograft which was achieved by using a steroid free immunosuppressor with insulin independence up to 5 years (48). In 1996, by including the use of endotoxin-free reagents and improved peritransplant recipient treatment, the Giessen group headed by R.G. Bretzel and B. Hering were able to achieve a 100% initial graft function and a 40% insulin independence after 1 year following transplantation (45). In 2000 Shapiro and colleagues introduced one of the most sucessful protocols in transplantation history, which was named Edmonton protocol (49). In this protocol rapamycin was used as an immunosuppressor. From this time more then 100 transplantations have been carried out in North America and Europe by using Edmonton protocol with some small changes and insulin independence was achieved in almost 80% patients.

2.4 Factors influencing graft

Though islet cell transplantation provides a hope for type 1 diabetes patients, still the rate of success of isolated islet transplantation is limited due to inflammatory, apoptotic and coagulation processes that occur in the hepatic environment and these appear to be responsible for the failure of islet transplantation (50-53). It is estimated that almost 50% islet mass damage is experienced immediately after islet transplantation. Factors that are playing

an important role in early islet loss are as follows:

2.4.1 Hypoxia

Hypoxia is a pathological condition in which the tissue has unduly low oxygen supply. Pancreatic islets are richly vascularized in the normal state. Arterial blood flow in the pancreas is quite high, 40 mmHg in comparison to portal venous blood 10-15 mmHg, and this creates a hypoxic situation in islet vicinity after transplantation to liver (54).

It has been shown that hypoxia is an important factor, which is affecting islet cell transplantation in liver by inducing apoptosis in beta cells after transplantation (55). Hypoxia can also reduce NF-κB activation and thereby can inhibit transcription of anti-apoptotic genes (55).

2.4.2 Instant blood mediated inflammatory reaction (IBMIR) and innate immune system

Islet cell transplantation is also associated with instant blood mediated inflammatory reaction (IBMIR), which is occurring when islets are coming in direct contact with blood after transplantation (56). This blood coagulation process in the islet surrounding is starting from tissue factors (CD142, exposed in islets disrupted from endothelial cells). Tissue factor generates thrombin and promotes activation of the complement system (56; 57). Immune cells migrate from the blood stream to the liver and into the direction of the transplanted islets (50). Inhibition of thrombin activity or its interference with the tissue factor prolongs the function of intraportal islet allografts (58; 59).

It has been shown that even in syngenic islet transplantation into non-autoimmune diabetic mice there is almost 60% of islet loss within three days of transplantation due to necrosis and apoptosis (60). A key role of macrophages (CD11b positive cells) and their inflammatory cytokines was shown in islet survival and their depletion was observed, effectively delaying xenogenic islet rejection (61; 62).

Increased inflammatory cytokine (IL1, IFN-γ, IL-6) and NO level was observed within few hours of transplantation and it was concluded, that although cells from innate immune system

(including Kupffer cell) and hepatocytes are potential sources of these factors (53), neutrophil granulocytes were also playing the most important role in early islet graft loss (50). It was also reported that CD11bGR1 positive cells (neutrophil) are instrumental in early graft loss by increasing local inflammatory cytokine production (51). Not only cells from innate immune system, but from adaptive system such as T-lymphocytes are accountable for allograft malfunction (63).

2.4.3 Other Factors

There are several other factors that can induce later islet graft loss such as islet amlyoid formation and lipotoxicity. It has been described that islet amlyoid formation occurs in isolated human cultured islets (64) and a recent study indicates that amyloid forms after clinical islet transplantation. Lipogenesis is the process where acetyl-CoA is converted to fats and it has been shown that after islet transplantation insulin induces lipogenesis in nearby hepatocytes. Blocking of lipogenesis improved islet graft survival (65).

3. MMPs in diabetes and islet transplantation

Cell extracellular matrix (ECM) interactions have been shown to influence islet integrity, proliferation, differentiation and insulin secretion (66-68). MMPs were for the first time discovered in islet by Barro et al in 1997 when he found that islets are capable of secreting gelatinase.

Cells from innate immune system such as neutrophils and macrophages express and secrete gelatinases (69). Previous studies have shown that such cells contribute to the first line of rejection following islet transplantation (50; 51). Moreover, elevation of MMP-9 plasma levels was observed in diabetic patients (70; 71), while in mice with acute pancreatitis, trypsin induced the activation of MMP-2 and 9. When activated by endogeneous trypsin, MMP-9 was diabetogenic, as it cleaved secreted insulin (71; 72). Tissue inhibitor of MMP-1 (TIMP1) protects against apoptosis and restores glucose-stimulated insulin release of islets in the presence of cytokines (73). Also, inhibition of pancreatic MMP-9 activity suppressed leukocyte migration and inflammation in type 1 diabetes (74). Basement membrane-degrading gelatinases, such as MMP-9, play an important role in immune-mediated tissue destruction (2,3). Thus, MMP-9 may serve as a candidate target for therapeutic intervention, as gelatinase activity could change the ECM composition of transplanted islets and, at the same time, promote leukocyte migration into the graft.

The present study was therefore undertaken to investigate the role of gelatinases in islet transplantation.

4. Aims of the study

Intraportal islet transplantation is characterized by an instant inflammatory reaction against the graft with subsequent islet destruction. It is known that gelatinases (matrix metalloproteases 2 and 9) are involved in both inflammation and leukocyte migration. Therefore, we investigated the influence of gelatinase on the outcome of islet transplantation.

The general aim of the present work was to investigate the role of gelatinases in islet cell transplantation into liver tissue and to provide measures for the protection of transplanted islets against the infiltration of immune cells.

Following specific aims were addressed to achieve our goal-

1- Gelatinase secretion from islets after isolation with and without cytokines treatment.

2- Association of gelatinase activity to islet transplanted liver.

3- Immune cell migration in islet transplanted liver

4- Effect of gelatinase inhibition on islet graft survival and immune cell migration.

Chapter II-

1 Material

2 Methods

1 Materials

1.1 Chemicals

Product	Manufacturer
Acetic acid	Roth
Agarose(LM-MP)	Sigma
Agarose	Invitrogen
Ammonium persulfate (APS)	Bio-Rad
Bovine serum albumin (BSA)	Sigma
Calcium chloride (CaCl2)	Sigma
Calcein-AM solution	Sigma
Collagenase D	Roche
DNAse I	Roche
DQ-Gelatin	Invitrogen
DMEM 41966 Medium	Invitrogen Gibro
Dimethyl sulfoxide (DMSO)	Fluka
ECL Western Blotting Substrate	Thermo Scientific Pierce
EDTA	Fluka
Ethanol	Merck
Ethylendiaminetetraacetic acid (EDTA)	Fluka
Fetal calf serum (FCS)	Bio West
Formaldehyde	Roth
Glucose	Sigma
Glycine	Roth
Gelatine	Sigma
HEPES	Sigma
Hoechst 33342	Sigma

Hydrochloric acid (HCl)	Merck
Isopropanol	Merck
Magnesium chloride (MgCl2)	Merck
Magnesium sulfate (MgSO4)	Merck
β-Mercaptoethanol	Fluka
Methanol	Merck
Percoll	GE-Healthcare
Potassium chloride (KCl)	Fluka
RPMI-1640	Gibco
Tissue-Tek O.C.T. Compound	Sakura
Skim milk powder	Merck
Sodium azide (NaN3)	Fluka
Sodium chloride (NaCl)	Roth
Sodium dodecyl sulfate (SDS)	Bio-Rad
Sodium dihydrogen phophate (NaH2PO4)	Acros organics
di-Sodium hydrogen phosphate (Na2HPO4)	Merck
Sodium hydroxide (NaOH)	Fluka
Sodium orthovanadate (Na3VO4)	Sigma
N,N,N',N'-Tetra-methyl-ethylenediamine (TEMED)	Bio-Rad
Thymidine	Sigma
Tris-base	Acros organics
Triton X-100	Sigma
Trypsin/EDTA	Invitrogen Gibro
Tween 20	Merck
Zink Chloride	Sigma

1.2 List of antibodies-

Primary Antibody	Dilution	Company
Guinea Pig Anti-insulin, polyclonal	1:500 (IF)	DAKO
Rat Anti-Mouse Ly-6G , monoclonal	1:150 (IF,FACS)	eBioscience
Rat Anti-Mouse CD11b	1:100 (IF)	Immunotool
Rat anti Mouse CD11b (PE)	1:100(FACS)	BD-Pharmingen
Rat anti Mouse GpIIb/IIIa (FITC)	1:100(IF)	BD-Pharmingen
Rat anti-Mouse F4/80	1:100(IF)	AbD Serotec
MMP-2	1:1000(WB)	Calbiochem
MMP-9	1:1000(WB)	Millipore
Rat anti Mouse CD147	1:150 (IF)	Abcam

Secondary antibody	Dilution	Company
Rhod Red-X-APure Donkey Anti-Guinea Pig	1:400 (IF)	Jackson ImmunoResearch
Rhod Red-X-APure Donkey Anti Rat	1:400 (IF)	Jackson ImmunoResearch
FITC-APure Donkey Anti-Guinea Pig	1:400 (IF)	Jackson ImmunoResearch
FITC-APure Donkey Anti Rat	1:400 (IF)	Jackson ImmunoResearch

1.3 Kits-

Kits	**Company**
BCA Protein Assay Kit	Thermo Scientific Pierce
Porcine insulin ELISA kit	DRG Instruments
SuperScript® III Reverse	Transcriptase Invitrogen
RNeasy Mini	Qiagen

1.4 Primers

Primers for Murine	Product Size
MMP-2	
Forward Primer 5´-AGATCTTCTTCTTCAAGGACCGGTT-3´	
Reverse Primer 5´-GGCTGGTCAGTGGCTTGGGGTA-3´	225 bp.
MMP-9	
Forward Primer 5´-GTTTTTGATGCTATTGCTGAGATCCA-3´	
Reverse Primer 5´-CCCACATTTGACGTCC AGAGAAGAA-3´	208 bp.
GAPDH	
Forward Primer–5´-GGAGCGAGACCCCACT AACAT-3´	
Reverse Primer- 5´-GC GGAGATGATGACC CTTTT-3´	135 bp.

1.5 Recombinant Cytokines

Cytokines	Company
IL1-beta	Calbiochem
TNF-alpha	R&D System

1.6 Instruments and Software

Instruments	Company
FACSCalibur	Becton Dickinson
Cell Quest software	Becton Dickinson
Fluorescent Microscope	Leica DMLB
Gel Doc	Vilber Lourmat
Bio 1D	Vilber Lourmat
Elite Glucometer	Bayer
Fluorescence Plate Reader	Berthold Technologies-Mithras LB940

ELISA Reader	DRG Instruments
Statistic Analysis	GraphPad prism

2. Methods

2.1 Islets isolation and culture

2.1.1 Mouse islets isolation

Pancreatic islets were isolated after giving anaesthesia to mice. Pancreas was distended with Hanks balanced salt solution (HBSS) containing collagenase (2mg/ml) via the main bile duct. Pancreas was surgically removed and the donor was sacrificed. Digestion was performed at 37°C for 10 minutes in water bath with gentle shaking and terminated by ice cold HBSS. The resultant cell suspension of cells was centrifuged at 1000 rpm for 1 min followed by two times washing with HBSS. Islets were handpicked under stereomicroscope. Islets were cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10%fetal calf serum, penicillin (100 U/ml), and streptomycin (100µg/ml).

2.1.2 Pig islets isolation

Pig islets were isolated using previously described techniques of collagenase digestion and Ficoll purification (75). Briefly, islets from a single pig pancreas were isolated after vascular flush with University of Wisconsin solution (Du Pont Critical Care, Waukegan, USA). The quality of islet isolation was evaluated by trypan blue exclusion, dithizone staining and glucose-stimulated insulin secretion to check viability, purity and function. Animal research was approved by Regional Commission Giessen (Germany) under the code number GI20/11-Nr.15/2006. Animal husbandry was performed according to the German Animal Welfare Law as published in the latest version under http://bundesrecht.juris.de/tierschg.

Pig islets were cultured at non-CO_2 air in CMRL 1066 (PAA, Pasching, Austria) supplemented with 25 mM HEPES, 20% heat-inactivated pig serum, 100 U/ml penicillin (Biochrom, Berlin, Germany), 100 µg/ml streptomycin and 2 mg/ml glucose.

2.2 Gelatin Zymography

2.2.1 Measurement of protein concentration

Cells were cultured in serum free medium. Culture conditioned medium was collected and

protein concentration was measured by Bicinchoninic acid assay (BCA) kits (Pierce). Whenever islet-transplanted liver tissue was used then it were homogenised by using lysis buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.1% SDS, and 0.1% Na-deoxycholate) and were centrifuged for 20 min at 4°C at 9000 *g*. Protein concentration was measured by BCA protein assay (Pierce) for all samples. BCA assay was first time discovered by Paul K Smith at the Pierce Chemical Company. In this assay peptide bonds in protein reduce Cu2+ ions form cupric sulfate to Cu1+ and the amount of Cu2+ reduced is proportional to the amount of protein. Later two molecule of Bicinchoninic acid chelate with each Cu1+ ion and form a purple colourd product that absorbs light at 562 nm. The amount of protein present in solution can be quantified by comparing with known concentration of protein solution such as BSA.

2.2.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS – PAGE) and zymograpy

Equivalent amount of protein volume was adjusted to equal amount with 1x SDS-sample buffer without EDTA and B-mercaptoethanol. Samples were then electrophoresed on SDS-PAGE containing substrate gelatine (0.2%) that can be readily cleaved by MMPs. The following solutions were used-

Seperation gel (8%)		Stacking gel(5%)	
Lower Buffer	3.75 ml	Upper Buffer	2.5ml
AA(30%)	4ml	AA	1.65
H2O	5.8 ml	H2O	5.7ml
APS(10%)	100µl	APS	100µl
TEMED	10µl	TEMED	10µl
Gelatine	0.2%		
Lower Buffer-	1.5 M Tris/HCL pH-8.8		
	0.4% SDS		
Upper Buffer-	1 M Tris/HCL pH-6.8		

0.4% SDS

5x SDS running buffer- 1.250M Glycine

0.125 M Tris

1% SDS

After electrophoretic separation of proteins, gels were washed thrice, 15 min each, in 2.5%triton X-100. Gels were then treated with incubation buffer for 2days at 37°C and then stained with Coomassie Brilliant Blue R-250.

Incubation Buffer- 50mM Tris-HCL, pH 7.6,

0.1% NaN3,

1% Triton X-100,

10 mM $CaCl_2$

1 M $ZnCl_2$

After destining the gel clear bands indicated gelatinase activity. Gels incubated with incubation buffer containing 5 mM EDTA were used as negative control.

2.3 Western blot

For detection of MMP-2, serum-free conditioned medium was collected from islets with and without treatment. Equivalent amount of protein was separated by SDS –PAGE (as described above but without gelatin) and transferred to PVDF membrane at 4° C. Membranes were blocked by incubating with 5% skim milk powder (Fluka). Membranes were washed with TBST (0.1% Tween 20) and incubated with primary antibody anti-MMP-2 (Calbiochem) overnight at 4°C. Subsequently, membranes were washed thrice with TBST (0.1% Tween) at room temperature and incubated with polyclonal rabbit anti-mouse HRP conjugated antibody for one hour. Protein expression was detected by enhanced chemiluminescence system (Pierce).

Transfer buffer 50 mM Tris

	40 mM Glycine 0.04% (w/v) SDS 20% Methanol
10 x TBS	250 mM Tris pH 7.4 1.5 M NaCl 50 mM KCl

2.4 Islet transplantation

Diabetes was induced in C57Bl/6 recipients (12 weeks old, male) by a single injection of 200 mg/kg streptozotocin (Sigma, Munich, Germany) intraperitoneally and blood glucose levels were monitored by glucometer Elite (Bayer, Leverkusen, Germany). Mice with a non-fasting blood glucose concentration of more than 16.7 mmol/L for two consecutive days were selected for transplantation. Recipients were anesthesized with avertine and maintained with isoflurane. Two thousand islet equivalent (IEQ) porcine islets washed with PBS were transplanted into the liver via the portal vein of mice with a 27-gauge needle using a previously described method with modifications (75). MMP-9 KO and respective control mice (6-7 weeks old) were a gift from Eva Korpos, (Westfälische Wilhelms-Universität, Münster, Germany). They were originally generated at the University of Leuven, Belgium (76) and backcrossed with C57Bl/6 mice for more then 10 generations. Mice were characterized for absence or presence of MMP-9 by using both RT-PCR and gel gelatin zymography. Similar transplantation method was used for transplanting pig islet in MMP-9 KO mice.

2.5 RT-PCR

Following islet transplantation animals were killed after 4 h and total RNA was prepared from liver by using TRIzol reagent (Invitrogen, Darmstadt, Germany) according to the manufacturer's instructions to analyze m-RNA expression of MMP-2 and MMP-9. Reverse transcription was performed for 15 min at 70°C followed by 2 min incubation at 95°C for denaturation. Amplification started by 15s at 94°C, 20 s at 60°C, and 10s at 72°C for 39

cycles. RT-PCR products were loaded on 2% agarose gels and analyzed after staining with syber green (Invitrogen) using imaging system BIO 1D (Vilber Lourmat, Eberhardzell, Germany).

2.6 *In situ* gelatin zymography with immunohistochemistry

Cryostat sections (7 µm) of pancreas and islet-transplanted liver were fixed in precooled acetone for 10 min. The sections were washed thrice with PBS. Following blocking with 3% donkey serum, sections were incubated with polyclonal guinea pig anti-swine insulin primary antibody (Dako, Glostrup, Denmark) for 2 h. After using a donkey anti-guinea pig secondary antibody (RhodRedx, Jackson ImmunoResearch, Hamburg, Germany), *in situ* zymography was performed. DQ gelatin (10%, Invitrogen) that was dissolved in 1% agarose LM-MP (Roche, Grenzach, Germany) containing 10 µM $ZnCl_2$, 1 mM $CaCl_2$, 1 µl DAPI was put on top of the cover slip to cover each tissue. Slides were kept in a moist chamber at 37°C and at time 0 (immediately after placing sections on the slides) as well as after 1 h, sections were viewed in a fluorescent microscope (Leica DMLB, Germany) and processed in Leica Application Suite. Gelatin gel with captopril (10 mM, Sigma) was taken as negative control. The presence of autofluorescence was excluded by incubating sections in agarose-containing medium without DQ-gelatin.The presence of autofluorescence was tested by incubating sections in agarose-containing medium without DQ-gelatin

2.7 Immunohistochemistry

Pig islets were transplanted into mice liver and after 4 h; islet-transplanted livers were embedded in TissueTek OCT compound and snap-frozen in liquid nitrogen. Frozen sections (7 µm) were prepared, using a cryostat and were stored at -20°C until used for staining. Sections were fixed in 10% acetone at -20°C and blocked with 10% donkey serum. After washing with PBS the sections were incubated overnight at 4°C with rat anti-mouse Ly-6G (Gr1, Clone RB6-8C5, eBioscience, Frankfurt, Germany) and rat anti-mouse CD11b (Mac-1, ImmunoTools, Friesoythe, Germany) in a humidifier. The sections were washed in PBS and

incubated with secondary antibodies FITC-donkey anti-rat for CD11b and Ly-6G (all from Jackson ImmunoResearch) for 1 h at 22°C. Sections were washed again with PBS and counterstained with DAPI being inspected under the fluorescent microscope. The same method was used for MMP-9 staining in control and transplanted liver whereby rabbit anti-MMP-9 polyclonal antibody (Millipore) was used as primary and RhodRedx-donkey anti-rabbit as secondary antibody. For insulin staining guinea pig anti-insulin primary antibody and FITC-donkey anti-guinea pig (Jackson ImmunoResearch) secondary antibody was used. In addition, some sections were incubated with rat anti-mouse F4/80 (AbD Serotec, Dusseldorf, Germany) primary antibody and RhodRedx -donkey anti-rat secondary antibody. F4/80 positive cells were counted (200x magnification) in fields of liver sections without islet or in the proximity of transplanted islets at different time points.

2.8 Liver leukocyte preparation

Islet-transplanted livers were perfused through the vena cava with 20 ml of PBS, followed by the removal of the gall bladder and careful excision of the liver from the abdomen. Isolation of single cells by mechanical disruption in RPMI (RPMI without phenol red containing 10% FCS, 25 mM HEPES) with surgical scissor was performed. This preparation was dissolved in RPMI containing collagenase and DNase (RPMI without phenol red containing 10% FCS, 25 mM HEPES, 0.5 mg/ml collagenase D, 0.025 mg/ml DNaseI) and kept for 45 min at 37°C with shaking (1 cycle/s). The reaction was stopped by addition of 20 mM EDTA. After passing the content through 100 μm cell strainer, RPMI containing 5% FCS was added, followed by centrifugation at 1250 rpm for 15 min at 4°C. The cell pellet was mixed with 40% Percoll and then poured on top of 70% Percoll. After centrifugation at 2000 rpm for 20 min at 20°C leukocyte were removed from the interphase of the gradient, washed with RPMI and used for analysis by FACSCalibur (Becton Dickinson, Heidelberg, Germany).

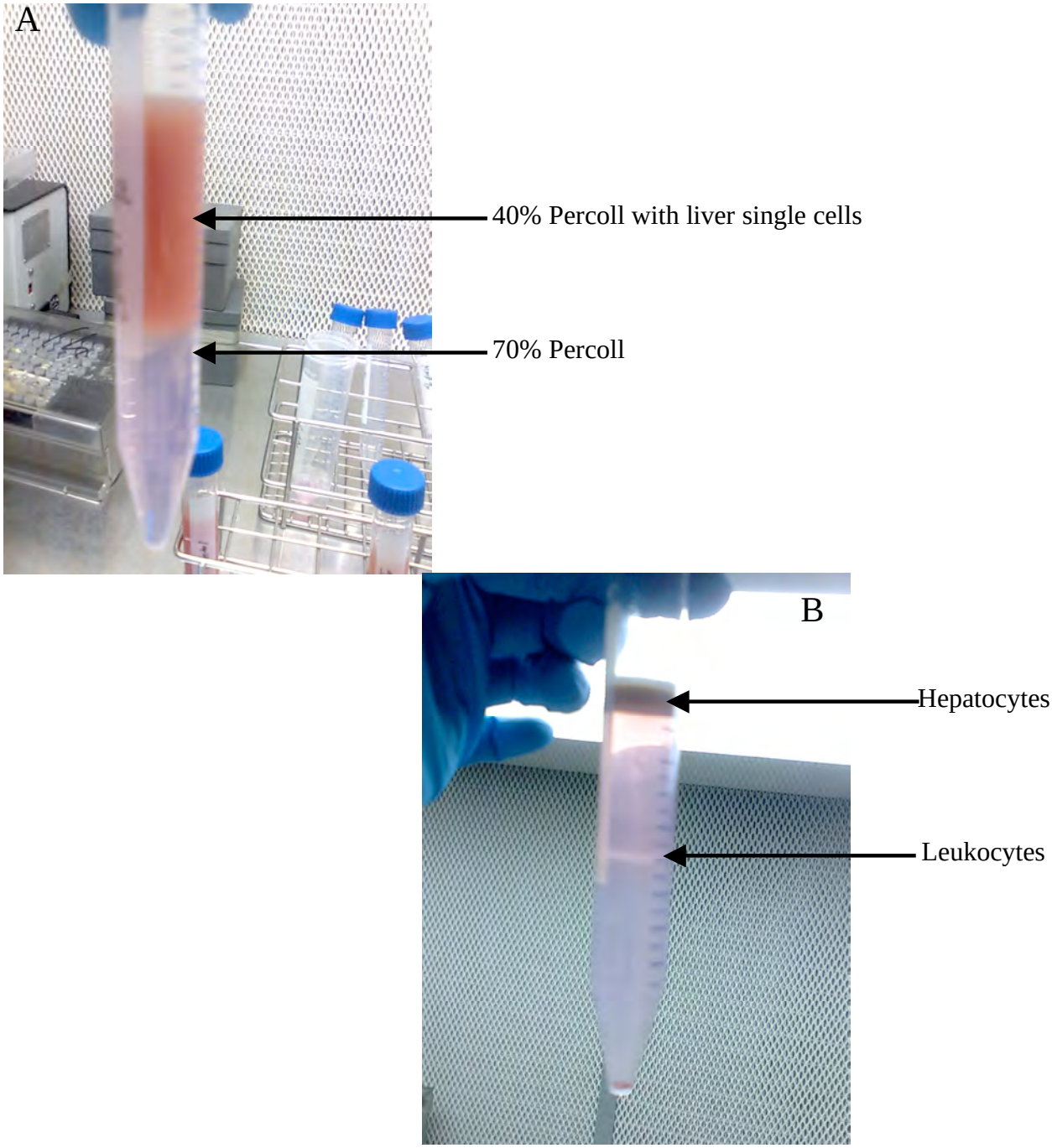


Fig 1 Liver leukocyte preparation. Liver single cells was mixed with 40% percoll and poured on top of 70% Percoll (A). After centrifugation without breaks leukocyte cells were removed from interphase of gradient (B).

2.9 Antibodies and flow cytometric analysis

one million cells were suspended in PBS containing 0.3% BSA and 0.1% sodium azide. For inhibiting non-specific binding of antibodies, Fc-receptors were blocked by pre-incubating cells with monoclonal antibody directed against the FcR III/II CD16/CD32 (BD Biosciences). Cells were incubated with 1 μg/ 10^6 cells of the CD11b antibody (PE-conjugated anti-CD11b, BD-bioscience) and Gr1 (FITC- conjugated anti Gr1, BD-bioscience) for 45 min at 4°C and washed twice. They were analyzed by using Cell Quest software for FACSCalibur (Becton Dickinson).

3.0 Peritoneal macrophage isolation

Four percent brewer's thioglycollate medium (Becton Dickinson, Heidelberg, Germany) was injected into the peritoneal cavity of each mouse. Peritoneal fluid was obtained 72h after injection by flushing the peritoneal cavity with ice-cold serum free RPMI 1640 medium using a 22G needle. Fluid was centrifuged in a 15 mL conical centrifuge tube at 1200 rpm for 5 min at 4^oC, and the pellet was resuspended in RPMI 1640 medium supplemented with 10% heat-inactivated FCS, 15 mM HEPES buffer and 1% penicillin/streptomycin. Cell lysis buffer was used for red blood cell lysis. Remaining cells were counted and checked for viability by the trypan blue dye exclusion method. Six million cells were cultured in a 70 mm petridish and incubated at 3h at 37^oC for adherence. Non-adherent cells were removed by washing with serum-free RPMI 1640 medium.

3.1 Cell migration/invasion assay

Under sterile conditions 12-well transwell plates (Greiner bio one, Frickenhausen,Germany) with 8 μm pore size matrigel coated filters (BD-Matrigel) were used. Lower wells contained 600 μl of islet supernatant, whereby the upper wells contained a final volume of 250 μl after the addition of cells. To begin the assay, 0.5×10^6 cells in medium were added to the upper wells in the absence and presence of MMP inhibitors (5 mM captopril or 10 μM GM6001), and were allowed to migrate for 48 h at 37 °C in a 5% CO_2 humidified atmosphere. After completion of migration cells were labeled with 450 μl of calcein AM (Sigma) that was added

to the lower chamber at 8 µM final concentration. Cells migrating to the underside of the filter were removed by using trypsin EDTA and detected using a fluorescence plate reader (Berthold Technologies-Mithras LB940, Bad Herrenalb, Germany).

3.2 Transplantation experiment with captopril

Captopril was dissolved in normal saline and injected subcutaneously for 7 days to mice (0.5 mg/kg, n=10, C57 Bl/6) starting from the day of transplantation. Two thousand islet equivalents were transplanted into the portal vein of diabetic mice. Recipients were anesthesized with 2.5% avertine (Sigma). Fourteen days after transplantation grafted livers were retrieved for insulin extraction as described before (75). The transplants were homogenized mechanically and dissolved in acid ethanol and the supernatants were collected for quantification of insulin by ELISA (DRG Instruments, Marburg, Germany).

3.3 Islet transplantation with MMP-2/9 inhibitor

A hydrophobic cyclic peptide that acts as an inhibitor of MMP-2 and 9 (IC_{50} 10 mM) was used to confirm results obtained from captopril treatment (MMP-2/9 inhibitor III, Calbiochem-Merck,NottinghamUK). Pig islets were transplanted to mice liver and MMP-2/9 inhibitor was injected intravenously for 7 days into mice (15 mg/kg, n=4, C57 Bl/6) as described previously (75). Two thousand islet equivalents were transplanted into the portal vein of diabetic mice. Recipients were anesthesized with 2.5% avertine (Sigma).

3.4 Statistics

Student's t-test and one-way Anova was applied for analysis of data. A value of $p < 0.05$ was considered significant (*$p<0.05$, **$p< 0.001$, ***$p<0.0001$).

Chapter III - Results

1. Gelatinase activity with normal islet and isolated islet

2. Gelatinase activity increased in islet transplanted liver

3. Immune cell migration in islet transplanted liver

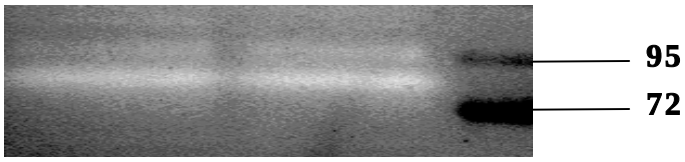
4. MMP-9 inhibition - Impact on leukocyte migration and islet function after transplantation

1. Gelatinase activity with normal islet and isolated islet

1.1 Gelatinases are secreted by pancreatic beta cells

Analysis of MMP-2 and MMP-9 secretion was performed in pig islet (Fig 1 A) and mouse islet (Fig. 1B) conditioned medium. MMP-2 (~62 kDa) and MMP-9 (pro form ~92 and active ~86 kDa) were present in cell-conditioned medium of islets. Secretion of MMP-9 was confirmed in islet-conditioned medium by zymography (I), an assay that is based on gelatinase degradation of the substrate gelatin. The presence of MMP-2 was confirmed using western blot (II). Western blot was performed most of the time for MMP-2 detection due to very faint band on Gelatine zymography. We also performed *in situ* zymography for analyzing gelatinase activity within islet in normal mouse pancreas and observed that islets are expressing gelatinase even in normal pancreas Fig. 2.

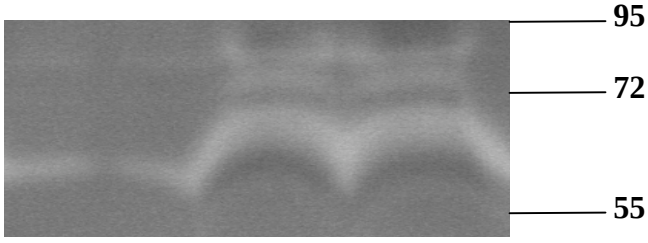
A



I

II

B



I

II

Fig.1 Expression of MMP-2 and MMP-9 in pig and mouse islets. Gelatin zymography (I) and western blot (II) revealed that MMP-2 (~62 kDa) and MMP-9 (~92 kDa, ~ 86) was expressed by pig (A) and mouse islets (B).

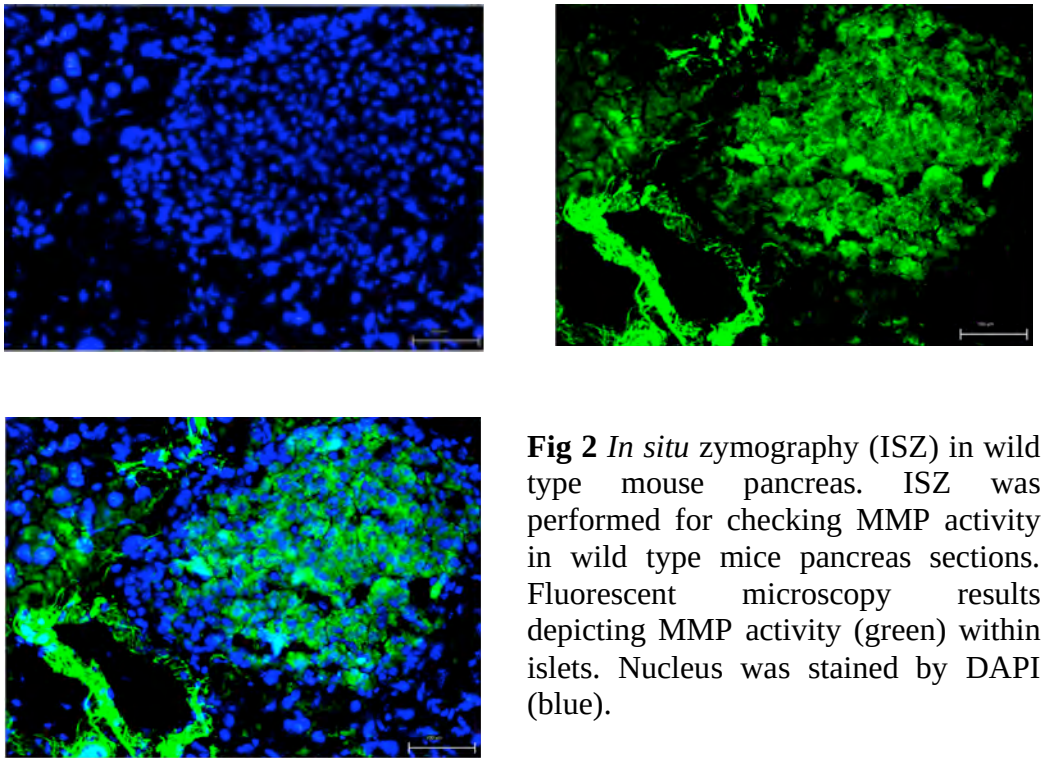


Fig 2 *In situ* zymography (ISZ) in wild type mouse pancreas. ISZ was performed for checking MMP activity in wild type mice pancreas sections. Fluorescent microscopy results depicting MMP activity (green) within islets. Nucleus was stained by DAPI (blue).

1.2 IL-1 beta and TNF alpha increased gelatinase secretion from isolated islet

Islets were isolated from pig and checked for secretion of MMP-2 and MMP-9. Since pancreatic beta cells constitutively released limited amounts of MMP-2 and MMP-9 under quiescent conditions the influence of inflammatory cytokines in the context of transplantation on MMP-2 and MMMP-9 expression was carried out by western blotting and gel gelatin zymography. A dose dependent increase in MMP-9 activation was observed with IL1-beta

(Fig 3 A) and TNF- alpha (Fig 4A), whereas only TNF- alpha induced a dose dependent MMP-2 activation (Fig 8B).

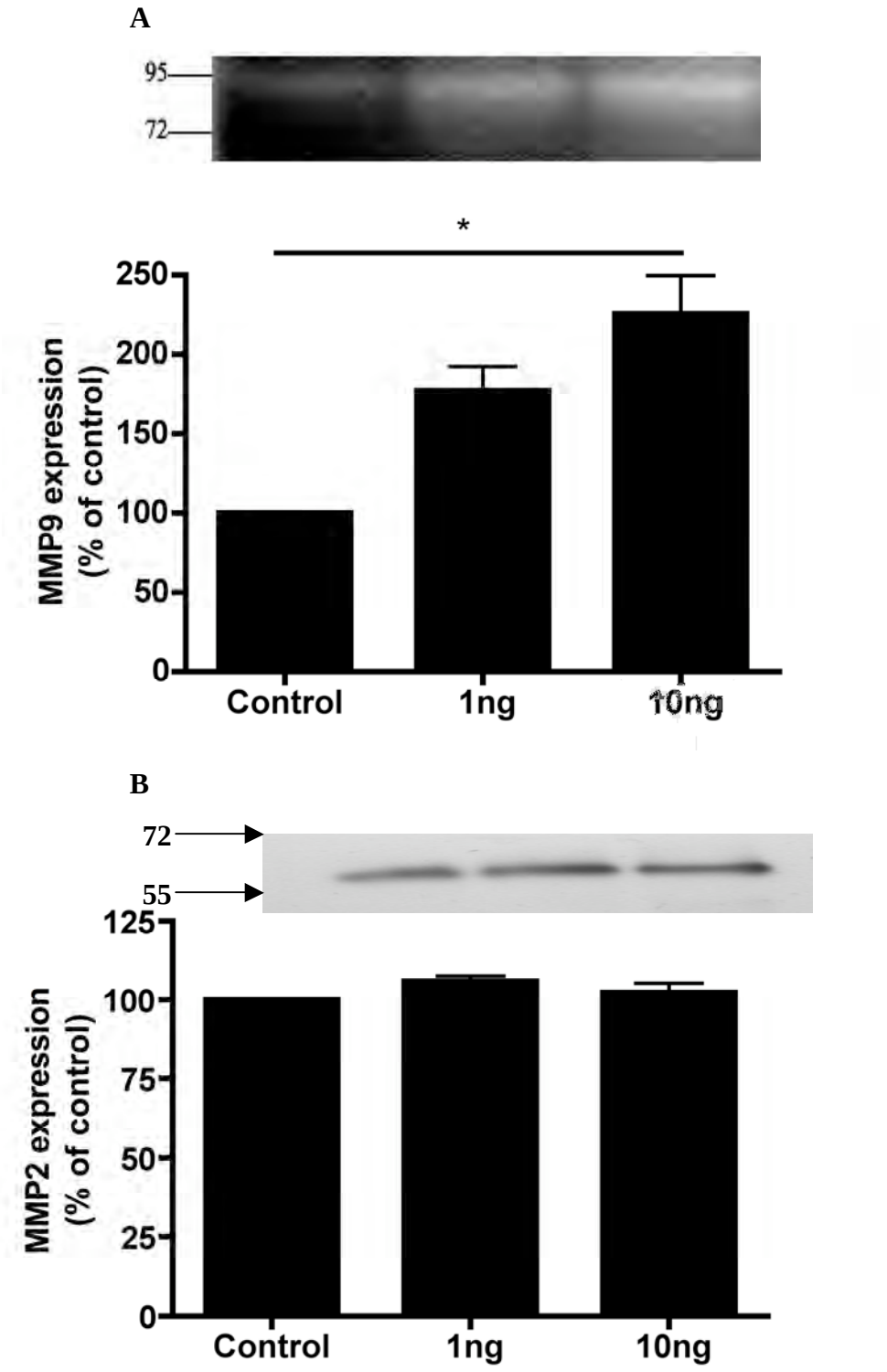


Fig. 3 MMP9 secretion from IL1-beta treated islets. Primary islets were isolated from pig and were treated with IL1- beta (1, 10ng) for 24 hrs. Gelatinase secretion in the culture medium was detected by gelatin gel zymography. Dose dependent MMP-9 activation was observed with IL1- beta treatment after 24hrs but MMP-2 activity on gel gelatin zymography after IL1-

beta treatment was not clear (A) but MMP-2 expression was not significantly deferent (B). The blots shown are representative of at least three independent experiments performed.

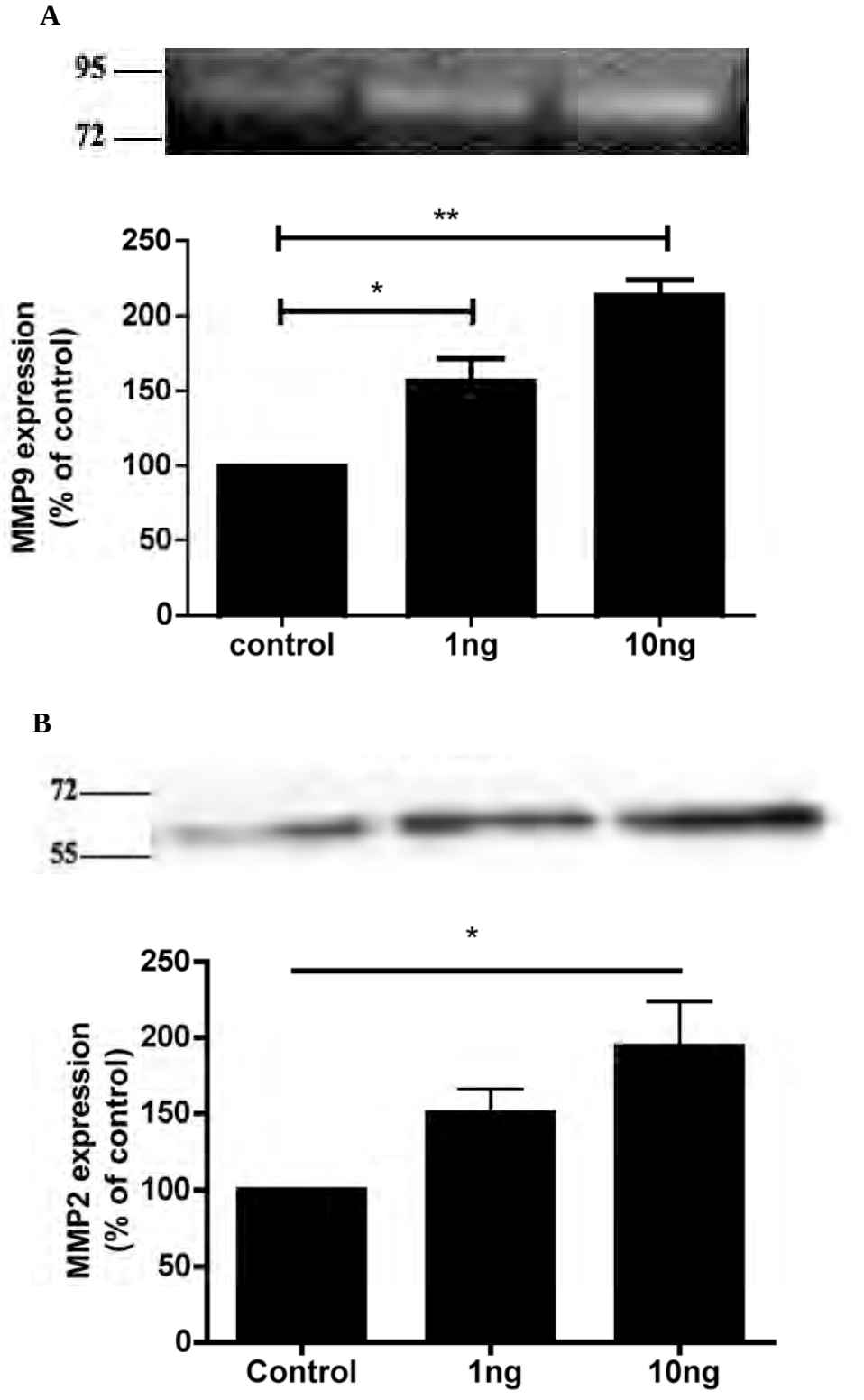


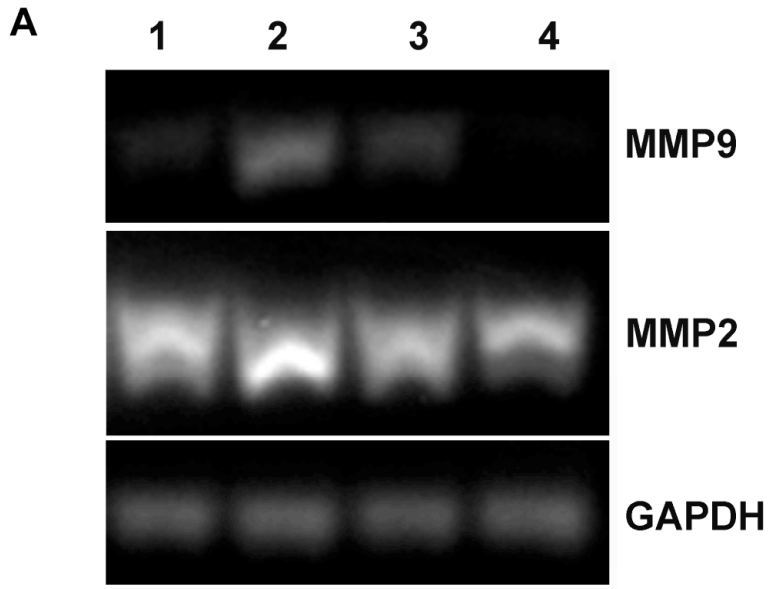
Fig. 4 TNF alpha stimulates MMP-9 and MMP-2 from pig islets. Conditioned medium were taken after 24 hrs treatments of different doses of TNF- alpha (1 ng, 10 ng) and analyzed by

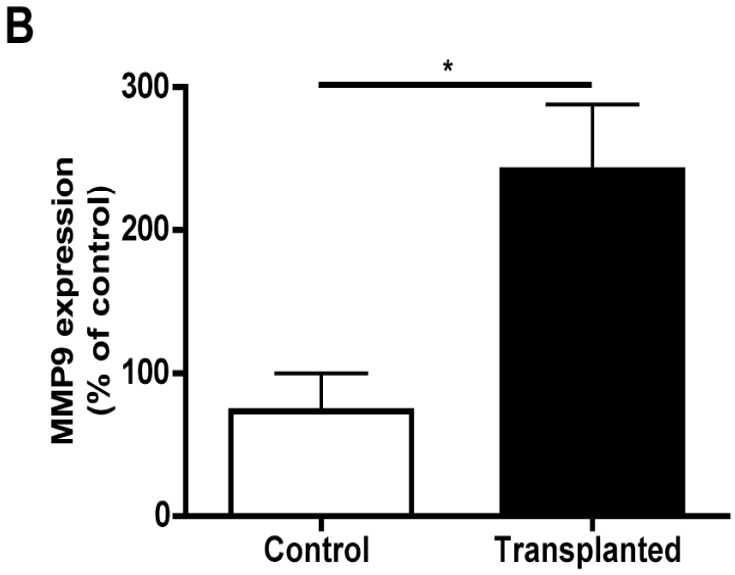
gel zymography (A) and western blotting (B). Does dependence of TNF- alpha stimulated MMP-9 (A) and MMP-2 (B) was observed.

2 Gelatinase activity increased in islet transplanted liver

To verify gelatinase expression in normal and islet-transplanted liver, RT-PCR, *in situ* gelatine zymography and immunostaining were performed. RT-PCR showed higher expression of MMP-9 in transplanted as compared to control mice (Fig. 5A). Quantification of band intensity indicated significant increases in MMP-9 mRNA (Fig. 5B) in the transplanted group as compared to control, while MMP-2 mRNA levels did not change after transplantation (Fig. 5C).

Analysis of gelatinase activity by *in situ* zymography provided information about the precise localization of MMP-2 and MMP-9 activity in liver sections by dye-quenched DQ-gelatin labeling using fluorescein isothiocyanate. The basal MMP activity was low in untreated animals (Fig. 6A), whereas in islet-transplanted liver it was increased (Fig. 6B). Next, we examined MMP-9 immunostaining within control (Fig. 6C) and islet-transplanted liver (Fig. 6D) and found the same pattern. Hepatic MMP-9 activity increased in the vicinity of the transplanted islets (Fig. 6B,D), and was also observed within them (Fig. 7A,B) but as a rare event. We did not observe gelatinase activity in islet after transplantation as it was in normal pancreas (Fig. 6 B, D).


A
1
2
3
4
MMP9
MMP2
GAPDH


B
*
300
200
100
0
MMP9 expression
(% of control)
Control
Transplanted

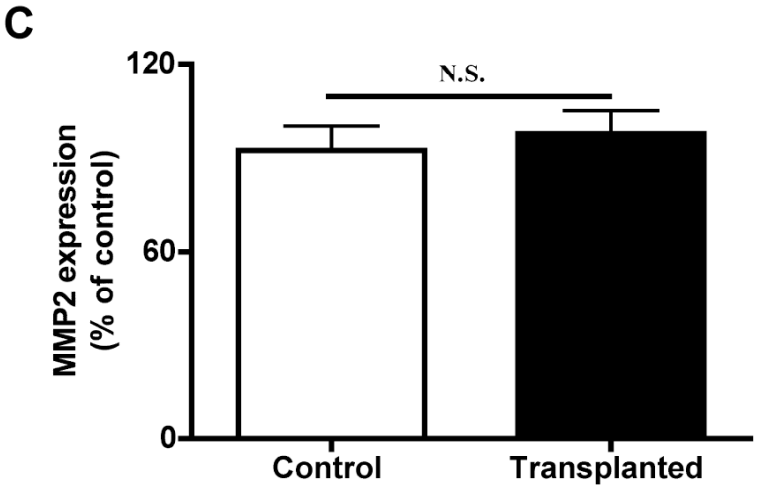

C
N.S.
120
60
0
MMP2 expression
(% of control)
Control
Transplanted

Fig. 5 MMP9 expression in transplanted liver.
mRNA levels of MMP-2 and 9 were analyzed by RT-PCR in total RNA extracts from control liver (A, lanes 1,4) and islet-transplanted liver (A, lanes 2,3). The difference between MMP-9 expressions in transplanted mice was significantly higher in comparison to control mice (B,*P < 0.05, n=3). MMP-2 mRNA level was not significantly different in control and islet-transplanted liver (C, P >0.05, n=3).

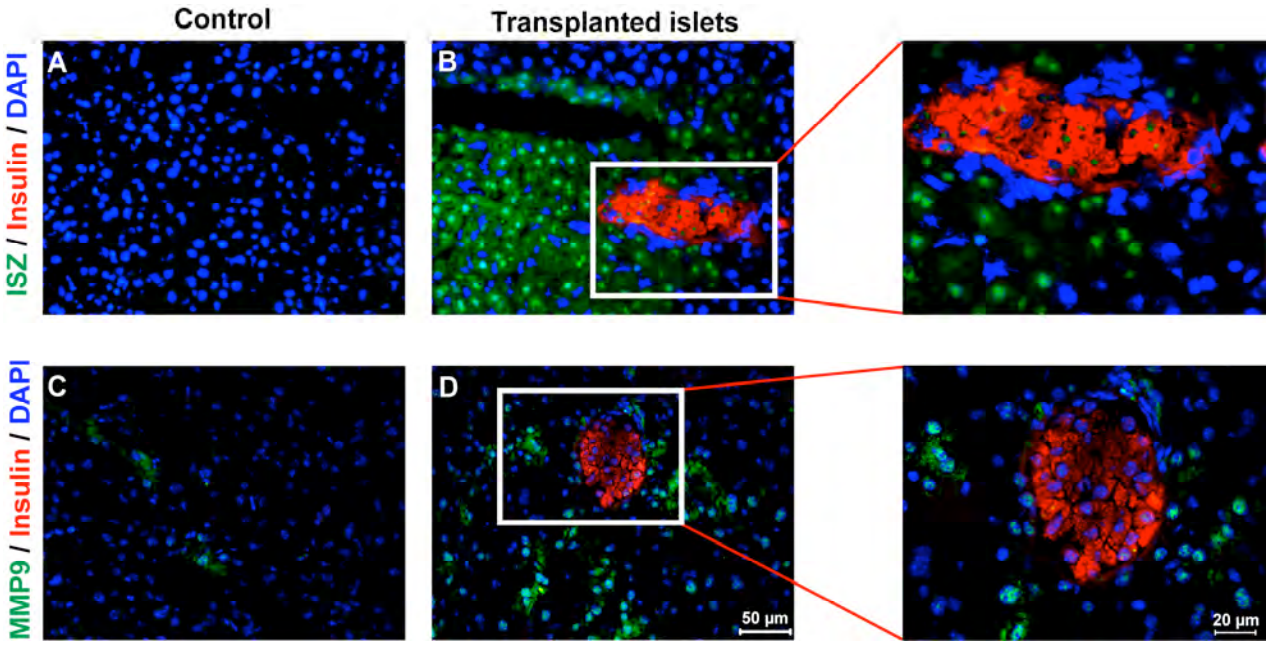


Fig. 6 Gelatinase activity in islet-transplanted liver.
Control liver sections (A, C) and islet-transplanted liver sections (B, D) were analyzed by *in situ* zymography (ISZ) for gelatinase activity (A, B green) as well as stained with specific antibody for MMP-9 immunoreactivity (C, D green). The figures are representative of four independent experiments. (Magnification ×200 inserts ×400). Insulin (red) and DAPI (blue) was stained for localization of islet and nucleus, respectively.

MMP-2 and MMP-9 activation in islet-transplanted liver was detected in association with platelets and thrombus-like material (Fig. 7C) as indicated by staining with an antibody against GpIIb/IIIa (Fig. 7D). Increased gelatinase action was also observed in proximity to CD11b cells (Fig. 7E,F), a prominent cell type that appears within liver tissue after islet transplantation

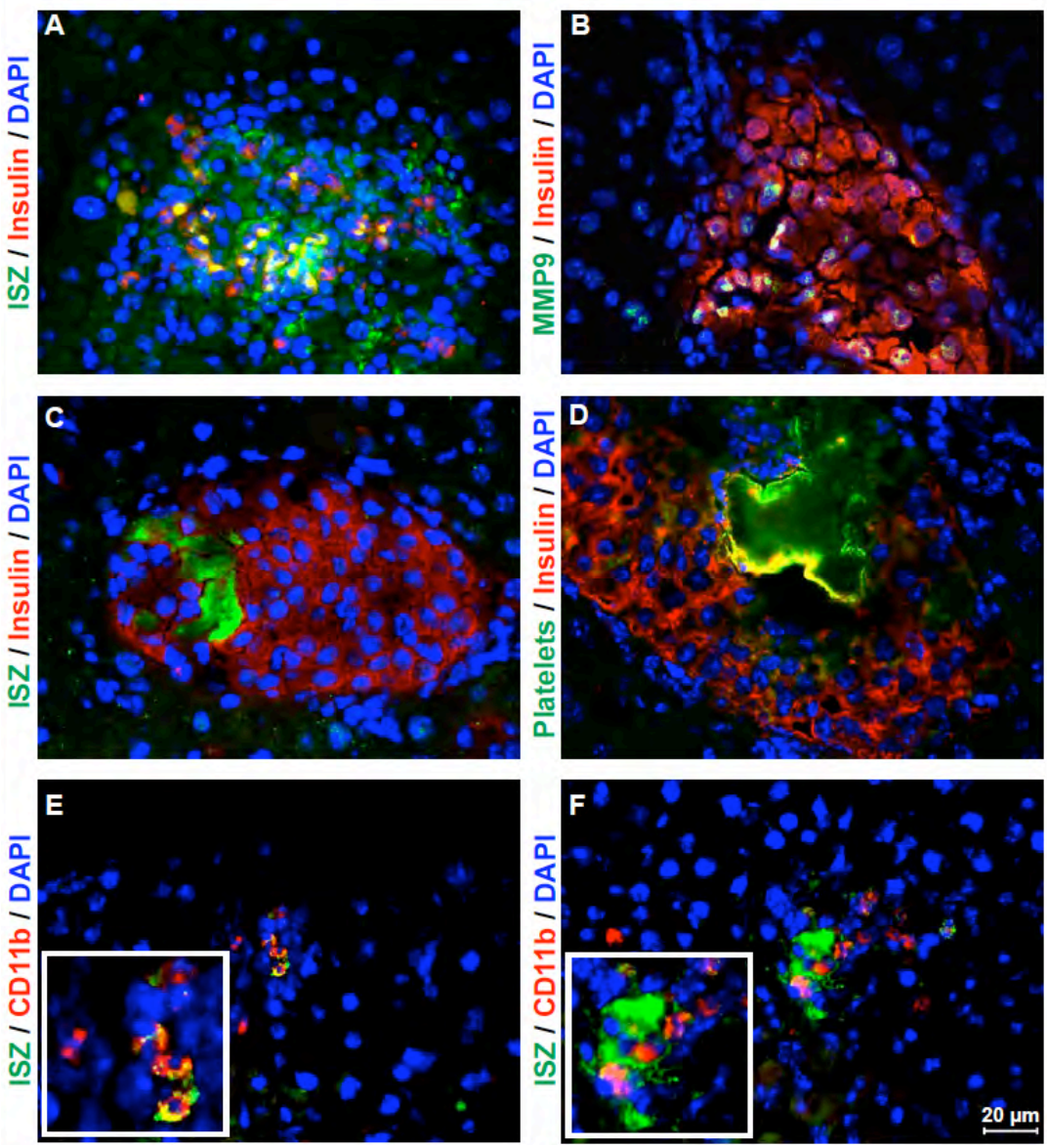


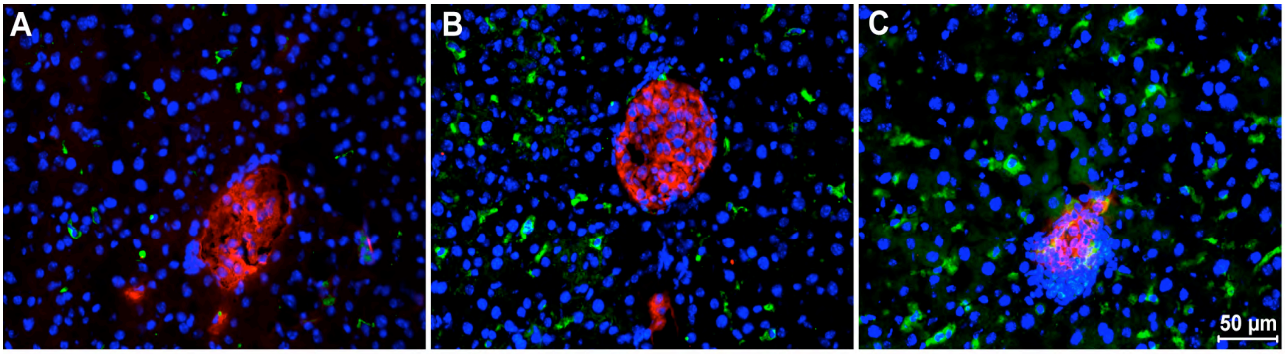
Fig 7 Gelatinase activity associated with islet transplant.
Following transplantation of islet grafts into liver for 4 h, *in situ* zymography (ISZ, green) for gelatinase activity (A) and immunostaining for MMP-9 (B, green) was performed, which showed increased gelatinase activity within a transplanted islet. *In situ* gelatin activity (C, green) was also seen next to thrombus material, which was confirmed by staining platelets aggregates (D, green) with GbIIb/IIIa antibody. Gelatinase activity (E-F, green) was costained with CD11b positive cells (red) in islet-transplanted liver. The figures are representative of three independent experiments. Magnification ×400.

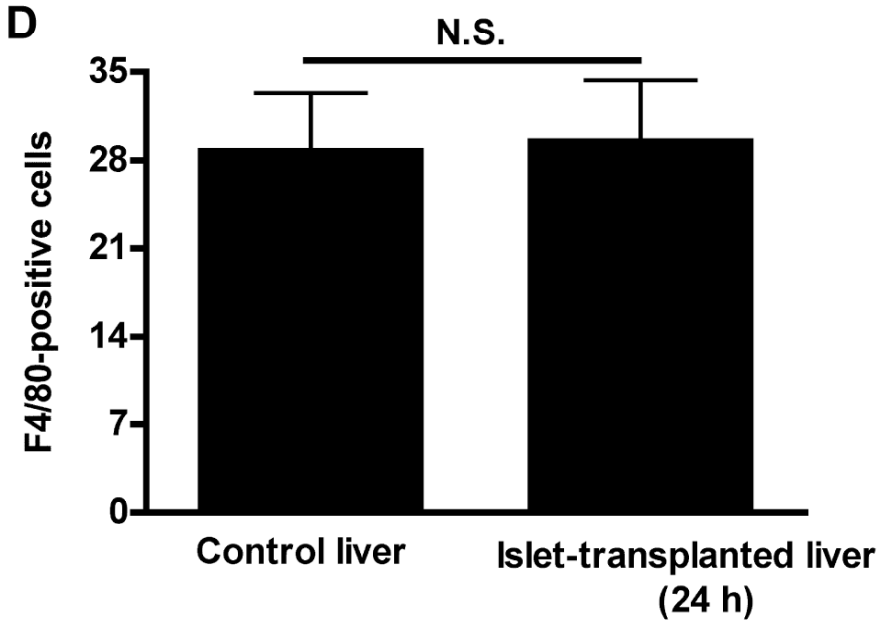
3. Immune cell migration in islet transplanted liver

3.1 F4/80 positive cell migration

Islets were transplanted (Tx) in mice liver and after 30min, 4h and 24h liver was removed and frozen immediately. Cryosections were prepared and stained for F4/80 positive cells. More F4/80 positive cells migrated towards islet transplanted liver after 4h and 24h compared to

30min (Fig 8 B,C,A). Cells were count by using Image J Software and statistic analysis was done with GraphPad prism software, which showed significant difference in F4/80 cell migration in islet vicinity after transplantation (Fig 8 E).





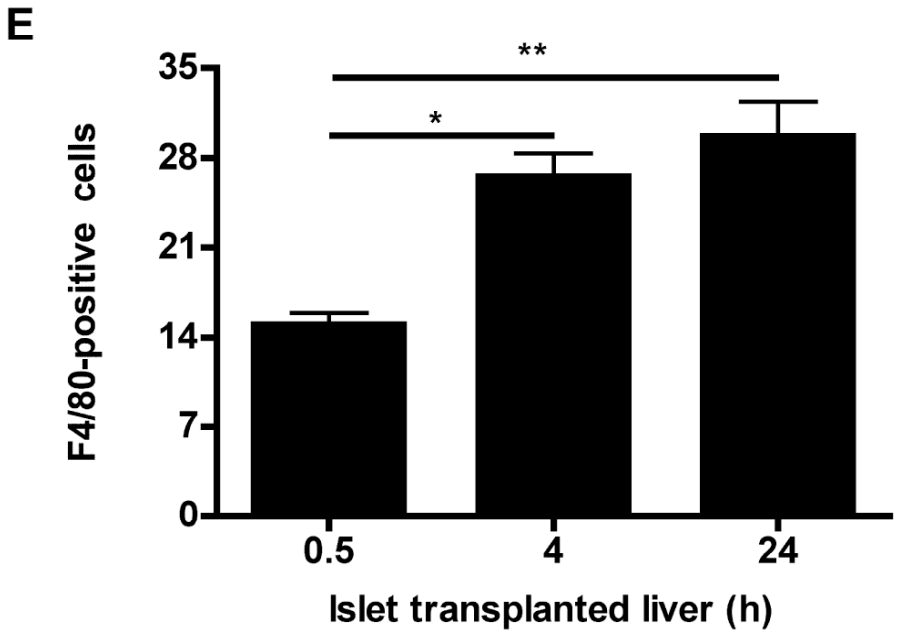
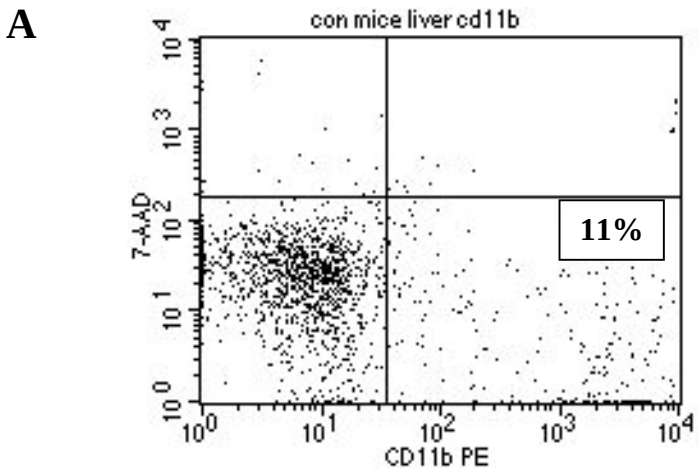


Fig.8 F4/80 cell migration. Increased number of F4/80 positive cells were observed after 4h (B, *P<0.05) and 24h (C, **P<0.001) compared to 30min (A) in islet-transplanted liver. Red color is showing islets stained with insulin antibody. Nuclei were stained with DAPI (blue) (200× magnification). (D) Cells were counted by using Image J Software and statistic analysis (GraphPad prism software) was done for comparing control and transplanted liver F4/80 positive cells. (E) Same method was used for counting F4/80 positive cell migration in vicinity of islet at different time point after transplantation. Color of islet and F4/80 cells were interchanged for creating a comparable color with other figures.

3.2 CD11b positive cell migration

Liver leukocytes cells were prepared after 4h of islets transplantation and stained with CD11b. FACS data showed 52% CD11b positive cells cells in islet-transplanted mice liver in comparison with 11% non-transplanted control liver (Fig 9).



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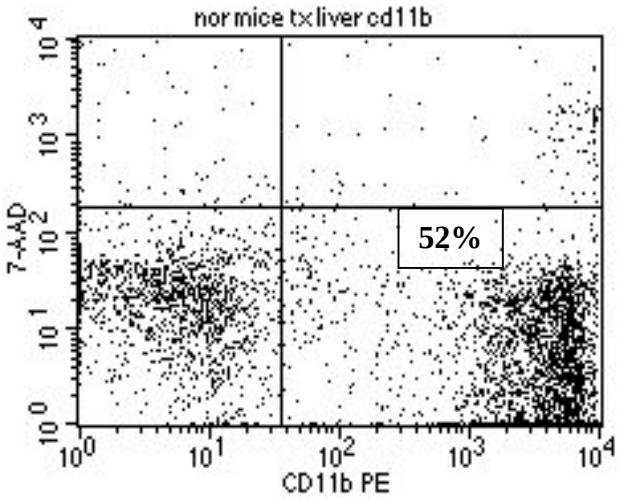


Fig.9 CD11b cell migration. FACS data showed more CD11b positive cells in islet-transplanted mice liver compared to non-transplanted control mice.

4. MMP-9 inhibition - Impact on leukocyte migration and islet function after transplantation

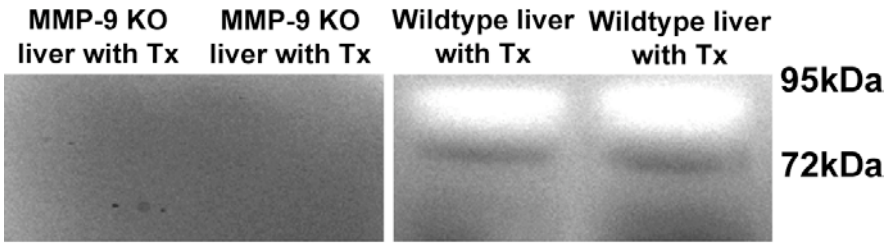
We observed that pancreatic islets had gelatinase activity in normal pancreas. Yet, they were not able to produce the same amount after transplantation. On the other hand, there was increased gelatinase activity in liver after transplantation, which was associated with other cell type. Since MMP-9 secretion was increased in transplanted as compared to non-transplanted liver, the role of MMP-9 after transplantation was investigated. CD11b cell migration was examined in two transplantation models using MMP-9 KO mice as recipients and captopril as gelatinase inhibitor.

4.1 MMP-9 KO mice and migration of CD11b positive cells

To confirm the absence of MMP-9 activity in MMP-9 KO mice liver after islet transplantation substrate zymography was performed (Fig 10 I). Gel zymography is based on degradation of gelatin giving precise information about MMP-2 and MMP-9 activity with their pro and active forms. We did not observe activity of MMP-9 in MMP-9 KO islet- transplanted liver

though MMP-9 activity was present in wild type islet-transplanted liver. Since MMP-9 secretion was inducible in the islet-transplanted liver but not in control liver, the role of MMP-9 after transplantation was further investigated by analyzing the distribution of CD11b-positive (neutrophils and macrophages) cells using MMP-9 KO and respective control mice as recipients. It is known from previous studies that MMP-9 is actively involved in recruitment of CD11b-positive cells driving inflammatory processes such as in post-ischemic liver and neutrophil migration (77; 78). Thus, we examined the involvement of MMP-9 in migration of CD11b-positive cells to the pancreatic islet transplants.

I



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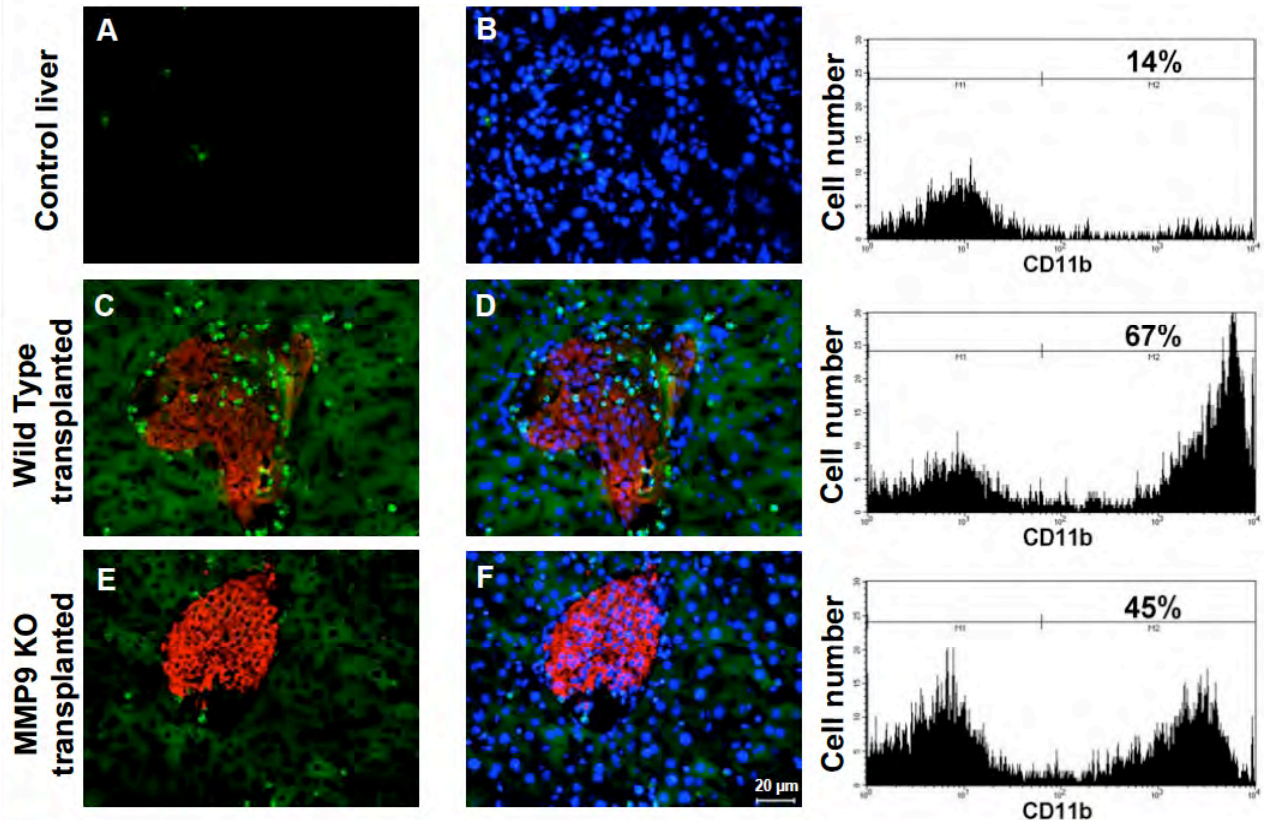


Fig 10. MMP-9 in CD11b cell migration after islet transplantation. (I) Islets were transplanted in mice liver and gelatinase activity was compared in MMP-9KO and normal mice. (II) Cryosections from islet transplanted liver were prepared and stained for CD11b positive cells (green) in wild type transplanted, MMP-9 KO transplanted mice and non-transplanted control mice liver. (A,B) Sections from wild type mouse liver without islet transplantation showed few CD11b positive cells. (C, D) More CD11b positive cells migrated in wild type mouse liver with transplanted islets (red). (E, F) MMP-9 KO liver with transplanted islets (red) showed less CD11b positive cells. Nuclei were stained with DAPI (blue). Magnification 200×. FACS data, which is placed next to respective immunostaining figures showed 67% CD11b cells in wild type transplanted mice compared to 45% in MMP-9 KO transplanted mice.

Only very few leukocytes were observed in non-transplanted control liver (Fig. 10II,A-B). Their number was increased in sections of wild-type recipient mice with hepatic islet grafts (Fig. 10II,C-D) compared to MMP-9 KO recipients (Fig. 10II,E-F). These results were confirmed by FACS analysis of liver leukocyte cells, where we found 67% CD11b-positive cells in wild-type islet-transplanted liver as opposed to 45% in MMP-9 KO mice.

4.2 Inhibition of gelatinase activity by captopril by direct inhibition not via angiotensin II

Next we examined the effect of captopril on gelatinase inhibition. Islets were transplanted into C57Bl/6 mice liver in the absence or presence of captopril. Livers were removed after 4 h of transplantation and explored for gelatinase activity by substrate zymography (Fig. 11A). Increase in MMP-9 activity (pro~92KDa and active~86KDa) was observed in wild type islet transplanted liver, which was inhibited in captopril treated mice (Fig 11).

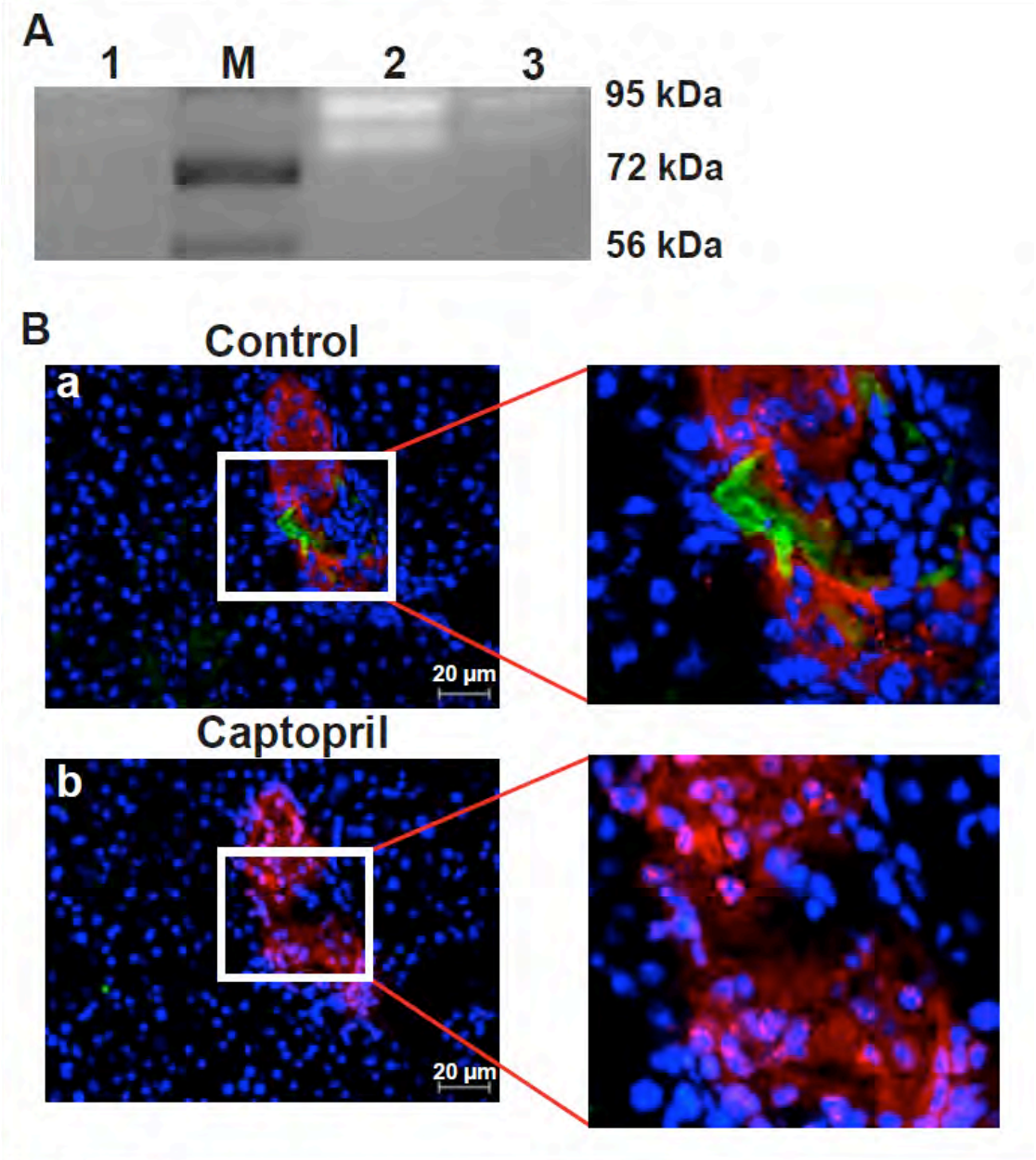


Fig 11. Zymography with and without captopril treatment. Islet-transplanted liver isolated from mice injected with captopril was used for gel gelatin zymography (A). Gelatinase activity was not present in control liver (A, lane 1; M = Marker) though it was increased 4 h after islet transplantation (A, lane 2) and was inhibited by captopril injection (A, lane 3). *In situ* gelatin zymography (B, green) was performed without (a) or with (b) addition of captopril in islet-transplanted liver sections. Captopril inhibited MMP activity (green) in islet (red) surrounding (b). Nucleus was stained with DAPI.

There was no detectable MMP-2 activity in islet-transplanted liver. Captopril was used to inhibit gelatinase activity and its effect was confirmed by *in situ* zymography. Administration of 10 mM captopril completely abrogated enzymatic activity on tissue sections (Fig. 11B).

As captopril can inhibit MMP either by direct Zn^{2+} inhibition or angiotensin II inhibition. We performed EMMPRIN immunostaining for dissecting captopril function, which is a downstream molecule of the angiotensin II system. From this experiment we observed complete lack of CD147 expression in islet-transplanted liver (Fig12).

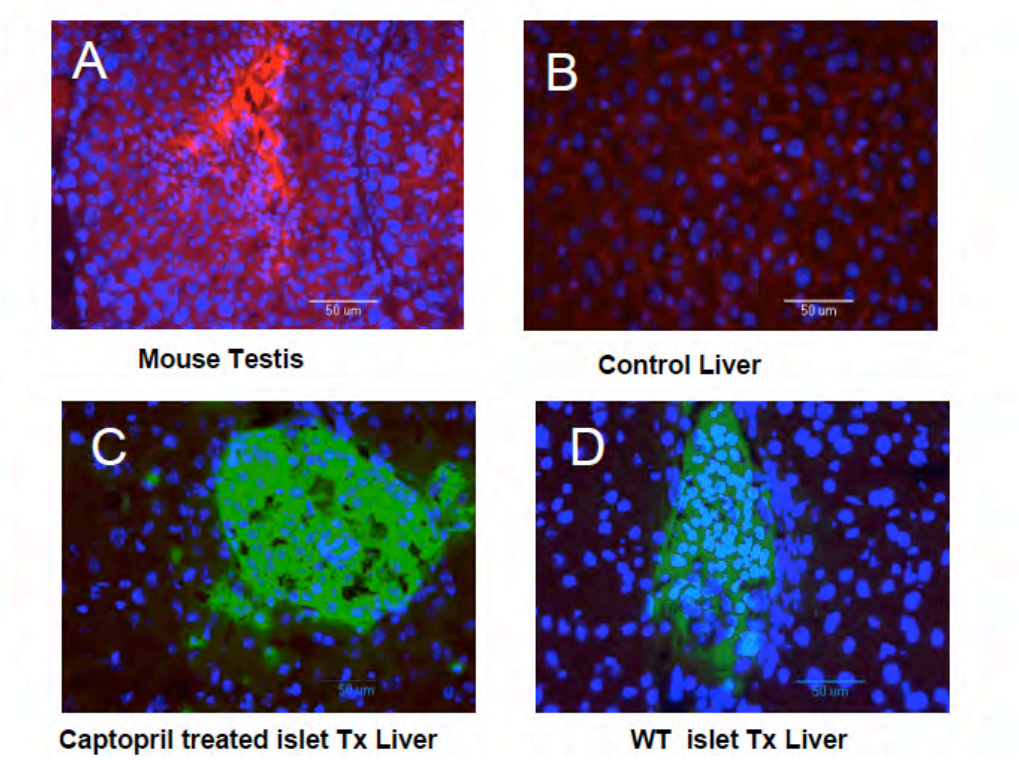


Fig 12.EMMPRIN staining in islet transplanted liver. Sections were stained with antibody against extracellular matrix metalloproteinase inducer (EMMPRIN, CD147, red), anti-insulin antibody (green) for islets and DAPI for nucleus. Mouse testis section (A) was used as positive control for antibody CD147. Control Liver section without islet transplantation (B) was also stained for CD147. Pig islets were transplanted into mice liver with (C) and without (D) captopril treatment. Four hours later islet-transplanted livers were retrieved and snap-frozen in liquid nitrogen for further processing. While transplanted islets showed significant insulin staining, CD147 was expressed neither in beta cells nor other types of cells.

4.3 Mouse macrophage invasion was inhibited by Captopril and GM6001 *in vitro*

Macrophages are known to migrate towards transplanted islets (20). We observed that there was a significant increase in F4/80-positive cell migration towards islet grafts after 4 h and 24 h after transplantation. To determine the specificity of captopril treatment, macrophage invasion was performed *in vitro* in the absence and presence of GM6001 (10 μM), a well-known gelatinase inhibitor, and captopril (5 mM).

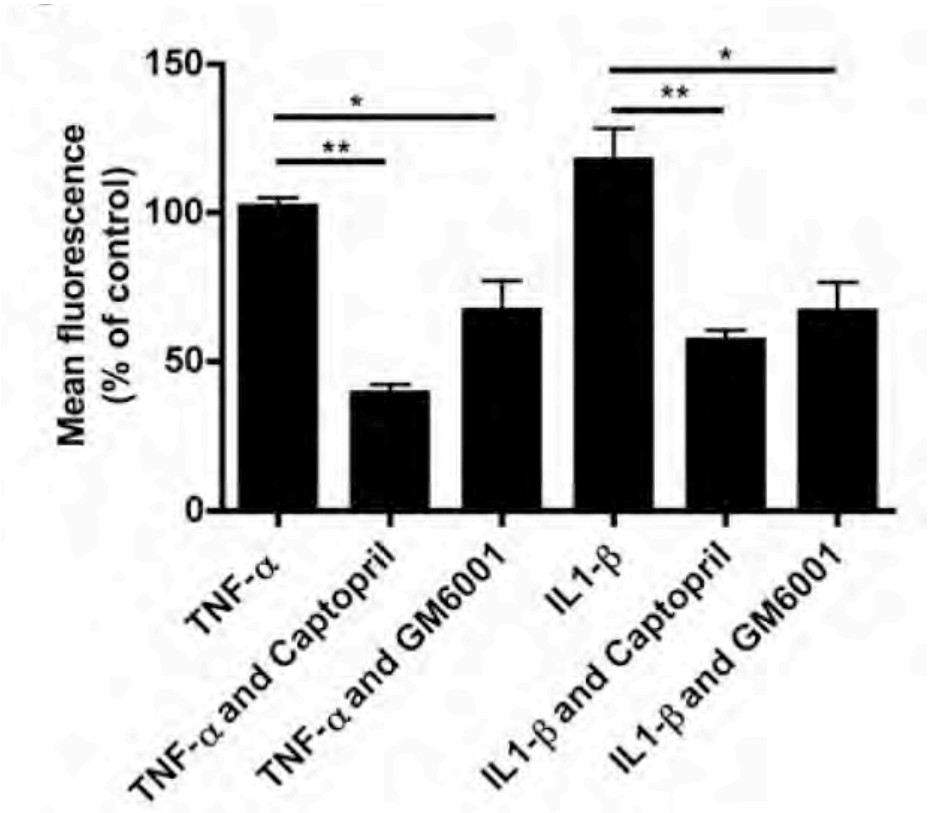


Fig 13. Murine peritoneal macrophages were allowed to migrate with or without MMP inhibitors . Numbers are expressed as the percentage of macrophage migrated towards islet-conditioned medium without inhibitor. Macrophage migration was significantly inhibited by captopril in TNF- alpha (**P< 0.001, n=3) and IL1- beta treated cell-conditioned medium (**P<0.001, n=3). The effect was also significant when macrophages were treated with GM6001 (*P<0.05, *P<0.05, B, n=3).

To determine the specificity of captopril treatment, macrophage migration was measured in the absence or presence of the specific gelatinase inhibitor GM6001 (10 μM) and captopril (5 mM). As shown in Fig. 13, significant inhibition of macrophage migration towards TNF-alpha treated islet cell-conditioned medium occurred in the presence of captopril or GM6001 (Fig. 13). A similar effect was observed when macrophages were subjected to IL1- beta treated islet cell-conditioned medium in the presence of captopril or GM6001.

4.4 Neutrophil and macrophage migration after MMP-9 inhibition *in vivo*

The influence of MMP-9 inhibition on *in vivo* macrophage and neutrophil migration was studied. Following islet transplantation, immunostaining for CD11b and Ly6G was performed (Fig. 14). CD11b and Ly6G-positive cell numbers were increased in wild type transplanted mice as opposed to those animals treated with captopril or MMP-2/9 inhibitor and in

transplanted MMP-9KO mice, altogether demonstrating that this effect was mediated mainly by inhibition of MMP-9**.**

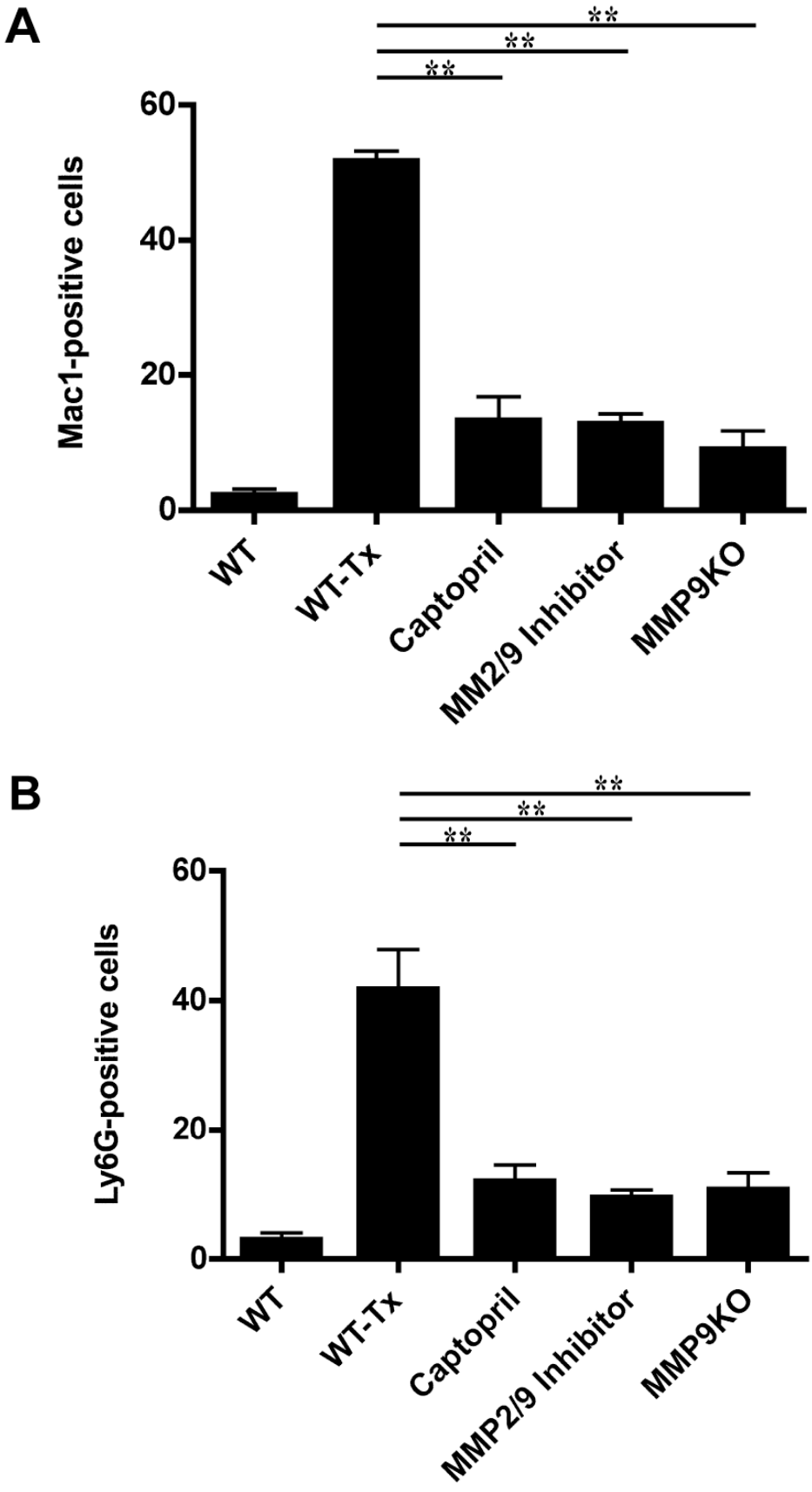


Fig. 14 MMP-9 inhibition and reduction of leukocyte migration to the transplanted islets.
Leukocyte migration was quantitated in islet-transplanted liver sections with and without *in vivo* MMP-9 inhibition. CD11b (Mac-1) staining revealed significant inhibition of CD11b positive cell migration towards islet-transplanted liver in captopril or MMP-2/9 inhibitor-treated and MMP-9 KO mice compared to wild type islet-transplanted mice (WT-Tx) (A, **P<0.001, n=3 mice). Number of Ly-6G positive cells was also reduced in captopril, MMP-2/9 inhibitor treated and MMP-9 KO animals alike compared to WT-Tx. (B, **P<0.001, n=3, three fields per section).

4.5 Islet transplantation *in vivo*

When pig islets were transplanted to C57Bl/6 recipients with streptozotocin-induced diabetes, blood glucose was initially normalized, but diabetes recurred within five days as expected.

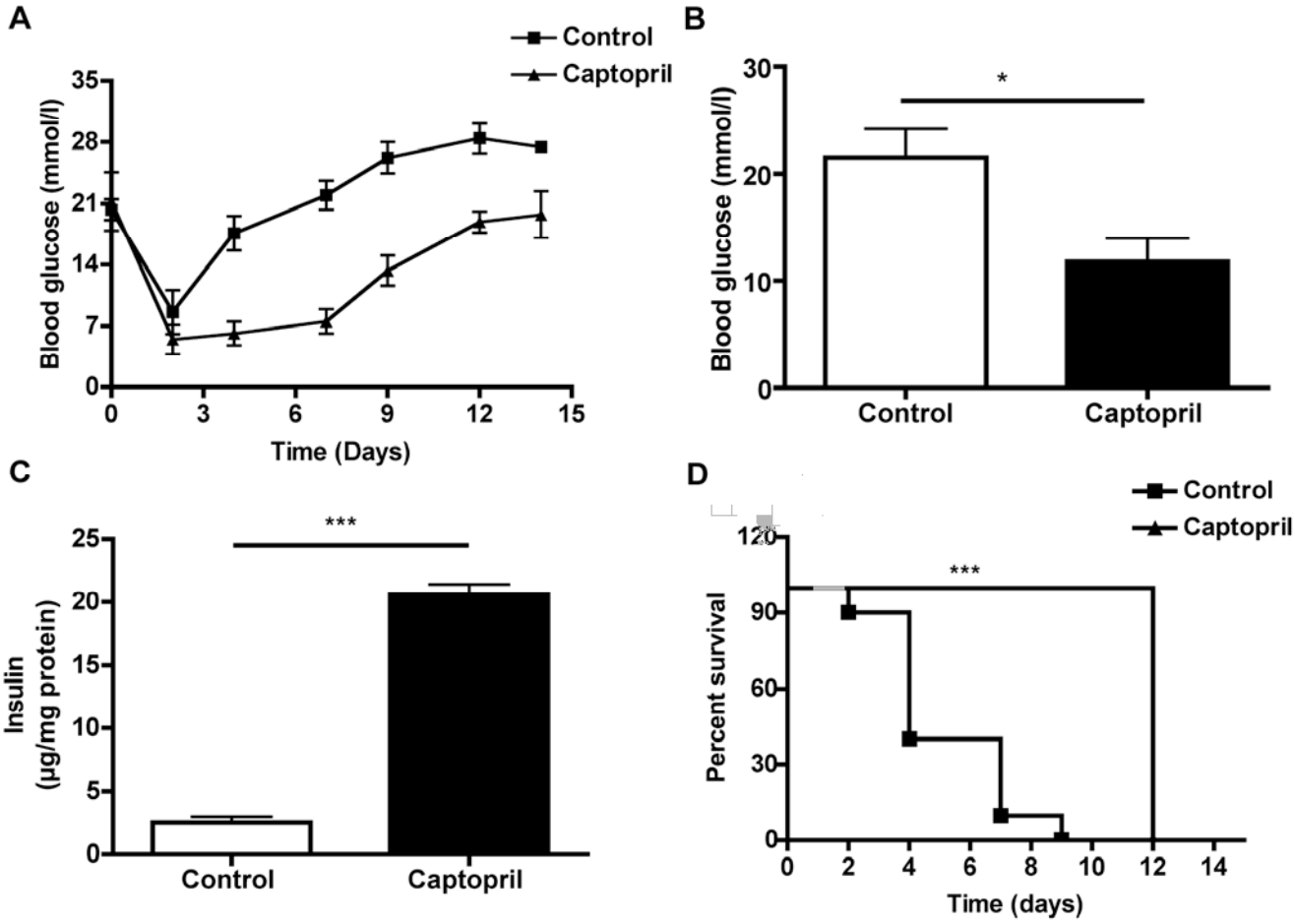


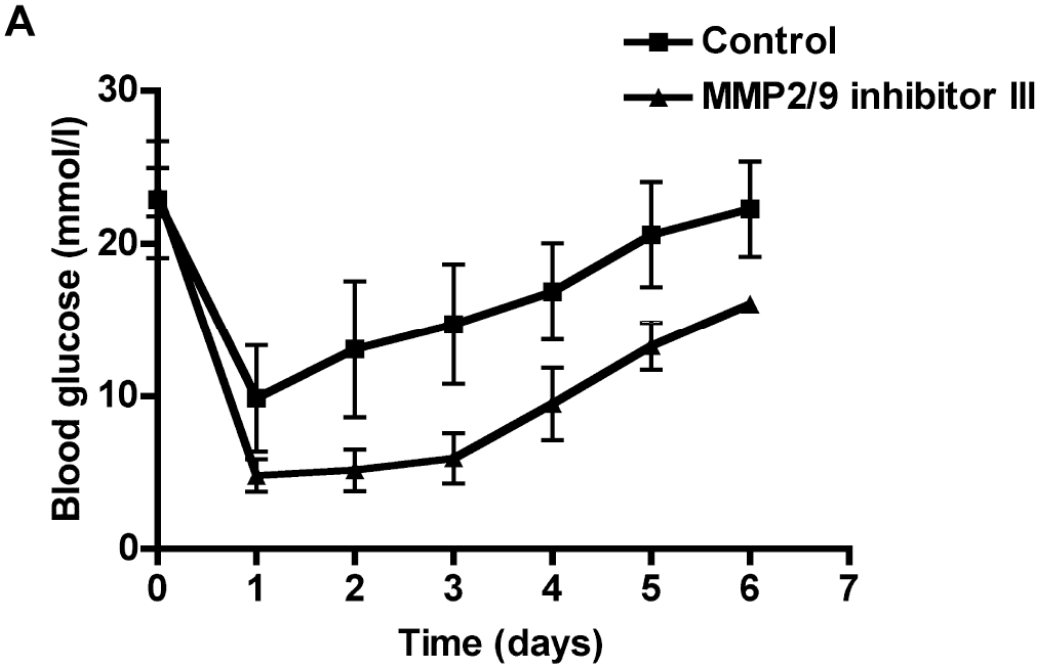
Fig. 15 Captopril and graft function.
Captopril was injected subcutaneously for 7 days to mice transplanted with pig islets (n=10 in each group). Mean blood glucose levels in the captopril-treated group (A, black triangle) were lower then control group (A, black squares). Statistic analysis showed significant difference in mean blood glucose value (B, *P<0.05). Insulin content of liver grafts retrieved from recipients after 14 days of transplantation in the captopril-treated group was increased compared to the untreated liver grafts (C, ***P<0.0001). Log-rank analysis of the graft survival curve determined from daily blood glucose levels showed significant difference (D,

***P<0.0001) of groups of transplanted mice treated with captopril (black triangle) or with solvent (black squares). The day of graft loss was defined as the last one of successive levels of > 16.7 mmol/l glucose.

The rejection was delayed in the captopril-treated group as compared to the control group and mean blood glucose levels in the captopril-treated group were lower (Fig. 15A, B).

At the end of the experiment, insulin contents of the grafts retrieved from recipients treated with captopril were significantly increased as compared to controls (Fig. 15C). Log-rank analysis of the graft survival curve revealed that treatment with captopril significantly extended survival times as compared to controls (Fig. 15D). These data indicated that gelatinase inhibition is beneficial for early islet engraftment.

Improved early islet graft function after captopril treatment was compared with the specific gelatinase inhibitor MMP-2/MMP-9 III. We observed decreased blood glucose level in MMP-2/9 inhibitor-treated mice compared to islet-transplanted mice without MMP inhibitor treatment. The transplantation experiments confirm that inhibition of gelatinases is an effective treatment in early islet transplantation (Fig. 16A,B).



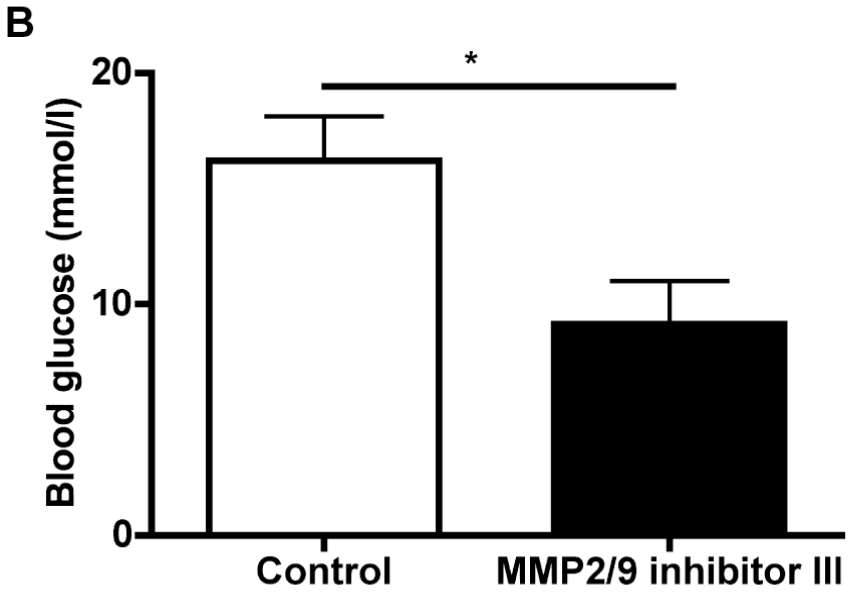


Fig. 16 MMP-9 inhibitor peptide and graft function.
MMP2/9 inhibitor (15 mg/kg) was injected intravenously for 7 days to mice transplanted with pig islets (n=4 in each group). Mean blood glucose levels in the group treated with the inhibitor (A, black triangles) were lower compared to the control group (A, black squares). Statistic analysis showed a significant difference in mean blood glucose value (B, *P<0.05). The day of graft loss was defined as the last one of successive levels of > 16.7 mmol/l glucose.

Chapter IV

1.Gelatinase secretion from islet in normal and inflammatory conditions

2.Increased gelatinase activity in islet transplanted liver

3.Immune cell migration increased after early islet transplantation

4.Gelatinase inhibition decreased immune cell migration and increased islet graft survival

5. Gelatinase inhibitors and their impact on clinical islet transplantation

Discussion

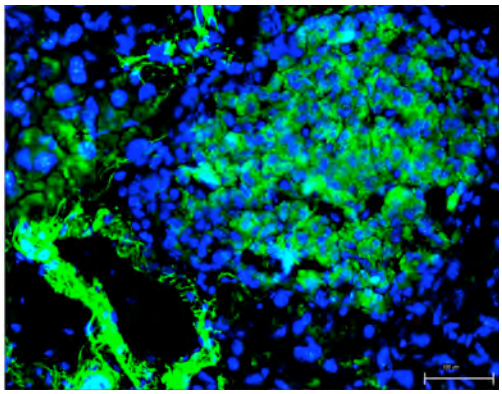
In particular, gelatinases have long been known to enhance inflammatory cell migration or to generate the biological active forms of cytokines (2,3,35). Islet infusion into the portal vein was assumed to induce protease activity in the context of an instant inflammatory or procoagulant response triggered by tissue factor or blood constituents (56) and may potentially be amplified by drainage of endotoxins from the intestine (79). As early islet graft loss is associated with immune mediated destruction, we hypothesized that gelatinase inhibition can inhibit immune cell migration and can increase graft survival.

1. Gelatinase secretion from islet in normal and inflammatory conditions

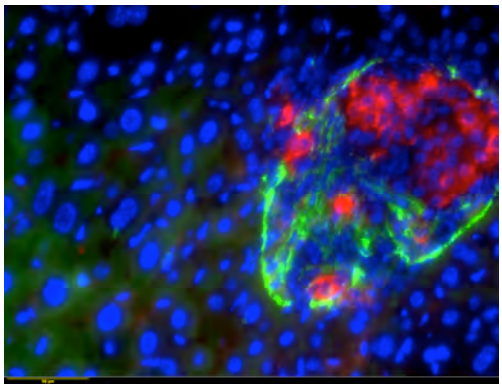
Immune cells infiltrate to liver after islet transplantation and increase proinflammatory cytokines inside liver (80). So firstly, we examined MMP secretion by pancreatic islet and found that MMP-2 and 9 secretions are low, but under tight control of cytokines, such as IL1-beta or TNF- alpha they increased. Though it is well known that MMP-2 is a constitutively regulated protease (81) we also observed that isolated islets are secreting MMP-9 without any stimulation. However, it is already shown that islets are expressing MMP in acute pancreatitis and in type 1 diabetes (71-73). Further we checked gelatinase activity in pancreatic islet inside normal pancreas and found high gelatinase activity within islet, which suggest that MMPs are normally expressed in islets. On the other hand we found very little or no gelatinase activity within transplanted islet, as after transplantation gelatinase activity is mostly associated with other cells in islet surrounding.

We hypothesise from these observations that MMPs are important for islet normal activity, which includes cell cycle and insulin secretion. After transplantation reduced gelatinase activity may influence islet survival though the exact function is not understood.

A Wild Type Pancreas



B Islet Transplanted Liver



C Islet Transplanted Liver

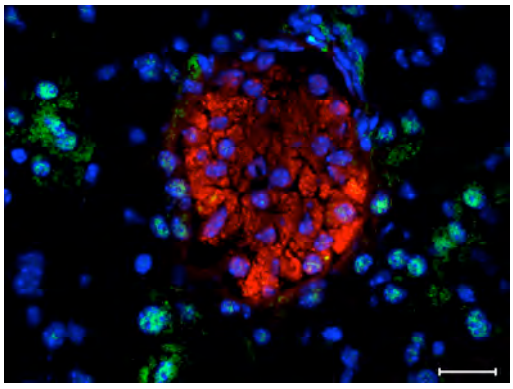


Fig.1 Gelatinase activity decreased after islet transplantation but increased in islet vicinity. Wild-type pancreas section with gelatinase (green) activity within islet (A). Islet (red-insulin staining) transplanted liver showed gelatinase activity (green) in the surroundings of grafted islet (B). MMP-9 was also stained (green) with antibody. Nucleus was stained with DAPI (blue).

2. Increased gelatinase activity in islet transplanted liver

Gelatinase activity in association with transplanted islets was significant in surrounding tissue but was minor within islet grafts. We observed a preferential increase of its activity in non-endocrine cells, such as in neutrophils, platelets and hepatocytes. Accordingly, no morphological reorganization of transplanted islets was observed, such as aggregation or re-arrangement of endocrine cells. In addition elevated levels of MMP-9 mRNA and protein were found in the recipient liver, while MMP-2 mRNA expression was essentially the same in control or transplanted mice liver and not detectable at the protein level. These data indicate that increased MMP-9 expression and activity provides a likely candidate responsible for early islet graft loss.

It has been shown previously that MMP-9 plays an important role in revascularization of transplanted pancreatic islets following culture with stimulators of angiogenesis. Despite improved revascularization, control mice showed better response to glucose challenge than MMP-9 treated mice (82). As Gelatinase B is diabetogenic in acute and chronic pancreatitis by cleaving insulin (71), this could make mice less responsive for glucose challenge after MMP-9 treatment of islet.

3. Gelatinase and immune cell migration after early islet transplantation

Both neutrophils and macrophages express and secret gelatinases (2,3). Previous studies have shown that such cells contribute to the first line of rejection (50; 51). Neutrophils are a key component of the inflammatory response. They can generate chemotactic signals and thereby attract dendritic cells and macrophages (83; 84). Since neutrophil chemotaxis has been reported to occur in islet grafts, the influence of pharmaceutical MMP inhibition on migration of Ly6G-positive cells towards transplanted islets has been investigated. Previous reports showed that neutrophils play a major role in early graft loss by increasing local interferon-gamma production (51). We also confirmed that CD11b and Ly6G-positive cells were the main mediators of the instant inflammatory reaction following islet transplantation. Moreover, by therapeutically targeting these cells with MMP inhibitors the functional damage to the graft observed during the very first hours was reduced. No significant enhancement of F4/80-positive cells was noted in complete liver section 24 h after islet transplantation as compared to non-transplanted liver sections. We observed a slight increase in the number of F4/80 positive cells in surroundings of transplanted islets at 4 h and 24 h as opposed to 30 minutes *post* transplantation. However, within 4 h following transplantation, CD11b accumulation was enhanced in islet-transplanted liver.

Islet transplantation to liver increases proinflammatory cytokines (80). Similarly, it has been observed that inflammatory cytokines can increase MMP-9 secretion from different cell types

such as –macrophages, neutrophils and hepatocytes (85-87) and can aggravate inflammatory reactions.

4. Gelatinase inhibition decreased immune cell migration and increased islet graft survival

The mechanisms for *in vivo* activation of secreted MMPs in transplantation are not well understood. Therefore, we decided to investigate their role in a recently acute *in vivo* inflammatory model of pancreatic islet transplantation. We assumed that a large number of mediators will be released following transplantation and they will interact with each other to make the picture even more complicated. In such a complex situation it was our aim to identify master mediators of the IBMIR.

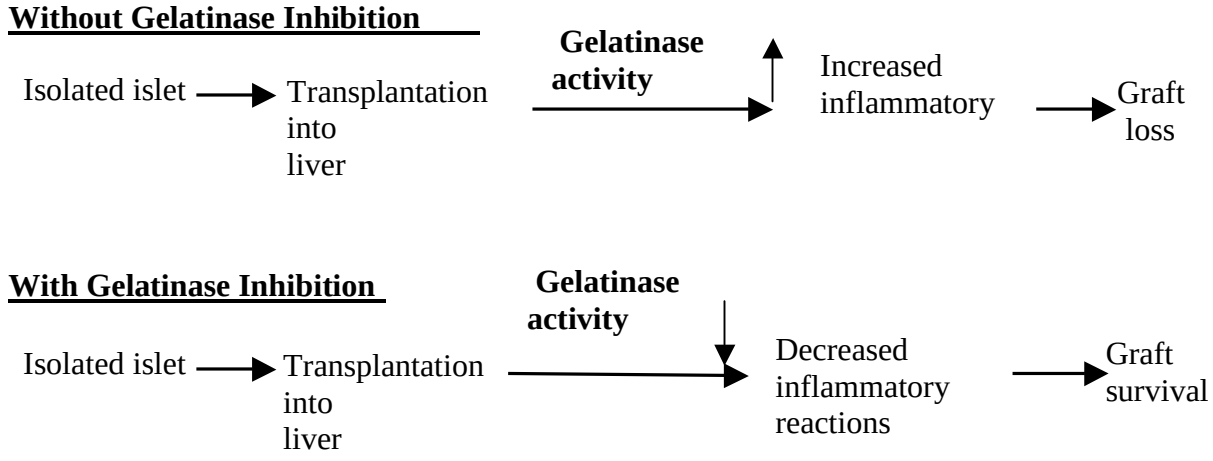


Fig.2 Influence of gelatinase on islet transplantation. Gelatinases are increased in islet vicinity after islet transplantation and they increased immune cell migration towards transplanted islet. Inhibition of gelatinase can inhibit immune cell migration and thereby can influence islet graft survival.

We employed two approaches, namely transplantation into MMP-9 knockout mice and application of the MMP inhibitors captopril and MMP-2/9 inhibitor III to study transplant function. In the first experiment, accumulation of CD11b-positive cells in the islet-transplanted liver was reduced, indicating that MMP-9 supports early islet graft loss by increasing immune cell infiltration to the site of transplantation. From these data we conclude

that the recipient tissue devoid of MMP-9 is apparently protected against inflammatory cell infiltration towards grafted islets, and thus, graft survival is increased.

In the second approach, *in vitro* and *in vivo* functional studies were performed with captopril and MMP-2/9 inhibitor III as prominent gelatinase inhibitors. Captopril forms complexes with the catalytically functional zinc ion present at the active site of angiotensin-converting enzyme (ACE) and gelatinase (88-91). To dissect which of the two zinc-dependent proteases ACE or gelatinase are more prominent in pancreatic islet transplantation we compared EMMPRIN/CD147 (extracellular matrix metalloproteinase inducer) immunostaining in islet transplanted sections, as EMPRIN/CD147 is downstream of angiotensin II pathway. It is known that angiotensin II stimulates EMMPRIN (extracellular matrix metalloproteinase inducer) that leads to upregulation of MMP (92). In confirmation, we were not able to detect significant expression in liver after islet transplantation, not to mention down-regulation by captopril.

Captopril amplified the glucose-lowering function of transplanted islets more effectively than losartan indicating that metalloproteases are activated minutes after intraportal islet transplantation by mechanisms other than the angiotensin receptor. Not only was the drug able to inhibit macrophage migration/invasion towards islet supernatant *in vitro*, similar to the action of the MMP inhibitor GM6001, but also captopril treatment was found to increase insulin content and reduce blood glucose levels when compared to untreated mice. MMP-2/9 inhibitor III also improved blood glucose level in transplanted diabetic mice and in addition, reduced neutrophil and macrophage accumulation in islet-transplanted liver to the same extent as found in captopril treated or MMP-9 KO recipient mice. Both captopril and MMP-2/9 inhibitor III can reduce MMP activity, but as only MMP-9 protein level was increased after islet transplantation to liver, it was assumed that the observed effect was predominantly specific for MMP-9. Together, these data demonstrate that MMP-9 inhibition has an important positive influence on islet graft survival. Thus, inhibition of gelatinase B activity in

early pancreatic islet grafts serves as a protective treatment for the reduction of leukocyte infiltration and for the restoration of inflammation and graft function.

5. Gelatinase inhibitors and their impact on clinical islet transplantation

Till now it has not been convincingly shown that MMP inhibition has an effect on the outcome of islet transplantation. Our results demonstrate that reduction of MMP activity will prevent instant inflammatory reactions during the first hours and days following intraportal islet transplantation. This will result in a long-term benefit for the engrafted islets because it is known that up to half of the total of transplanted islet cells will suffer severe damage from instantaneous attack of neutrophils. Therapies directed at neutrophils and other components of the innate immune system, such as complement and proteins of the coagulation cascade are lacking in human islet transplantation programs at the moment. MMP-inhibiting drugs could be combined with anti-CD3 antibodies in clinical regimens to antagonize both neutrophils and T-lymphocytes.

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Declaration

I declare that I have completed this dissertation single-handedly without the unauthorized help of a second party and only with the assistance acknowledged therein. I have appropriately acknowledged and referenced all text passages that are derived literally from or are based on the content of published or unpublished work of others, and all information that relates to verbal communications. I have abided by the principles of good scientific conduct laid down in the charter of the Justus Liebig University of Giessen in carrying out the investigations described in the dissertation.

_____ _____

Place and Date Neelam Lingwal

Appendix

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

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Inhibition of gelatinase B (MMP-9) activity reduces cellular inflammation and restores function of transplanted pancreatic islets. Neelam Lingwal, Manju Padmasekar, Balaji Samikannu, Reinhard G Bretzel, Klaus T Preissner, Thomas Linn. Diabetes, Vol.61, July 2012. DOI: 10.2337/db11-1143.

2. Adjuvant Properties of Thermal Component of Hyperthermia Enhanced Transdermal Immunization: Effect on Dendritic Cells. Neha Joshi, Vikas Duhan, Neelam Lingwal, Sangeeta Bhaskar, and Pramod Upadhyay.PLoS One. 2012; 7(2): e32067.

3.Exendin-4 protects hypoxic islets from oxidative stress and improves islet transplantation outcome via the PI3-kinase-Akt signalling pathway. Manju Padmasekar , Neelam Lingwal, Balaji Samikannu , Thomas Linn. Endocrinology (under revision).

4. Dipeptidyl Peptidase IV inhibition activates CREB and improves islet re-vascularization through the VEGF-A/VEGFR-2 pathway. Balaji Samikannu, Chunguang Chen, Neelam Lingwal, Manju Padmasekar, Felix B. Engel, Reinhard G Bretzel, Thomas Linn. (Manuscript in preparation).

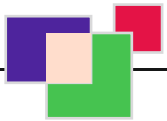
Abstract-

1.Poster Presentation in AIDPIT/EPITA winter symposium. 23rd-25th January 2011, Innsbruck , Austria. Inhibition of gelatinase activity in early islets graft is important to reduce inflammation and for restoration of islet function and its integrity. Neelam Lingwal1 Reinhard G. Bretzel1, Klaus T. Preissner2, and Thomas Linn1 .Medizinische Klinik und Poliklinik III1 and Institute of Biochemistry2 Justus Liebig University, Giessen, Germany.

2.Attended ECCPs symosium ´Remodeling and Reverse Remodeling in the Cardiopulmonary System´. 29th June-2nd July 2008,Max Plank Bad Nauheim,Germany.

3.Poster Presentation in GGL 2009,Giessen, Germany. Contribution of factors of the coagulation system to inflammation of the endocrine pancreas. Neelam Rawat, Klaus T. Preissner, Thomas Linn. Medizinische Klinik und Poliklinic III and Institute of Biochemistry, Justus Liebig University, 35392 Giessen, Germany.

4. Talk in GGL conference 2010,Giessen, Germany. Pancreatic beta cells secreting Matrix Metalloproteinases: Role of extracellular matrix turnover after transplantation. Neelam Lingwal,Klaus T Preissner,Thomas Linn. Medizinische Klinik und Poliklinic III and Institute of Biochemistry, Justus Liebig University, 35392 Giessen, Germany.



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