FIBRONECTIN-α4β1 INTERACTIONS IN HEPATIC COLD ISCHEMIA REPERFUSION INJURY: REGULATION OF MMP-9 AND MT1-MMP VIA THE p38 MAPK PATHWAY

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Abstract

Liver ischemia-reperfusion injury (IRI) remains a challenging problem in clinical settings. The expression of fibronectin (FN) by endothelial cells is a prominent feature of the hepatic response to injury. Here we investigate the effects of the connecting segment-1 (CS-1) peptide therapy, which blocks fibronectin (FN)-α4β1 integrin leukocyte interactions, in a well-established model of 24-hour cold liver IRI. CS-1 peptides significantly inhibited leukocyte recruitment and local release of proinflammatory mediators (COX-2, iNOS, and TNF-α), ameliorating liver IRI and improving recipient survival rate. CS1 therapy inhibited the phosphorylation of p38 MAPK, a kinase linked to inflammatory processes. Moreover, in addition to downregulating the expression of matrix metalloproteinase-9 (MMP-9) in hepatic IRI, CS-1 peptide therapy depressed the expression of membrane type 1-matrix metalloproteinase (MT1-MMP/MMP-14) by macrophages, a membrane-tethered MMP important for focal matrix proteolysis. Inhibition of p38 MAPK activity, with its pharmacological antagonist SB203580, downregulated MMP-9 and MT1-MMP/MMP-14 expressions by fibronectin-stimulated macrophages, suggesting that p38 MAPK kinase pathway controls fibronectin mediated inductions of MMP-9 and MT1-MMP/MMP-14. Hence, this study provides new insights on the role of fibronectin in liver injury, which can potentially be applied to the development of new pharmacological strategies for the successful protection against hepatic IRI.

Keywords

Leukocyte migration; fibronectin; matrix metalloproteinases; liver cold ischemia; reperfusion injury

INTRODUCTION

Ischemia-reperfusion injury (IRI) represents a major problem in orthotopic liver transplantation (OLT). IRI is a multifactorial antigen-independent inflammatory process that can lead to early graft failure and to a higher incidence of both acute and chronic organ dysfunction after transplantation [1, 2].

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The migration of leukocytes into tissues is a central event in inflammatory processes [3], including in acute inflammatory liver injury [4]. Leukocyte transmigration across endothelial and extracellular matrix (ECM) protein barriers is dependent on complex series of adhesion and focal matrix degradation events [5, 6]. Fibronectin (FN) is a ECM glycoprotein implicated in a variety of pathological conditions associated with cell turnover and migration such as tumor metastasis [7], rheumatoid arthritis [8], multiple sclerosis [9], and organ transplantation [10]. Moreover, clinical trials using humanized antibodies against the α4 integrin, a receptor for the connecting segment-1 (CS-1) region of fibronectin [11], have been effective in controlling inflammatory conditions like multiple sclerosis (MS) [12] and inflammatory bowel disease [13]. The role of fibronectin in leukocyte adhesion, migration and activation has been extensively reported [14]. Indeed, it was recently demonstrated that adhesion of leukocytes from MS patients to brain microvascular endothelial cells under flow conditions is preferentially mediated by the a4 integrin/FN-CS1 interactions [15].

We have previously shown that CS-1 peptides, which are FN-specific peptides that interact with the α4β1-integrin and inhibit its binding to FN [16], profoundly depressed leukocyte recruitment and improved liver function of steatotic liver transplants in a model of ex vivo 4-hour cold ischemia followed by isotransplantation [17, 18]. In the present study, we evaluated the effects of the CS-1 peptide therapy in a well-established rat liver model of prolonged cold hepatic IRI, in which normal livers are cold stored for 24 hours prior to being transplanted in syngeneic recipients. Our results show a beneficial role for CS-1 peptides in ameliorating prolonged cold hepatic IRI. Moreover, they provide evidence that FN regulates the expression of both matrix metalloproteinase-9 (MMP-9) and membrane type 1-matrix metalloproteinase (MT1-MMP/MMP-14) through activation of the p38 MAP kinase cell signaling pathway.

MATERIALS AND METHODS

Animals, grafting techniques and CS-1 peptide therapy

Male Sprague Dawley rats (250–300 g) were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN). Syngeneic OLTs were performed at the University of California, Los Angeles under a protocol approved by the UCLA-Animal Research Committee (ARC-2000-177). Briefly, after skeletonization of the liver, the portal vein, bile duct, and inferior vena cava were cannulated, and the liver was flushed with University of Wisconsin (UW) solution. Livers were stored for 24 hours at 4°C in UW solution before being transplanted in syngeneic recipients with revascularization without hepatic artery reconstruction with an anhepatic phase of 16–20 min [19, 20]. Cellular FN was significantly up-regulated in the liver vasculature after 24h of cold storage followed by OLT (Fig. 1). Based on these observations and in our previous studies [17, 18], CS-1 peptides (500 μg/rat) were dissolved in saline and administered ex vivo via portal vein to livers before cold storage and immediately prior to reperfusion; OLT recipients received an additional peptide dose (1 mg/rat i.p.) 1h post-transplantation. Controls received vehicle and were subjected to the same surgical procedures. Rat recipients of liver transplants were sacrificed at 6 hours (n=6/group) and 24 hours (n=5–7/group) after OLT or followed for survival studies (n=8/group).

Assessment of liver Damage

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in blood samples with an auto analyzer by ANTECH Diagnostics (Los Angeles, CA). Liver paraffin sections were stained with H&E and the histological severity was graded from 0 to 4 as described [17]. No necrosis, congestion, or centrilobular ballooning was

given a score of 0, while severe congestion, ballooning degeneration and >60% lobular necrosis was given a value of 4.

Immunohistochemistry

Immunostaining was performed in cryostat sections, as described [18, 21]. Appropriate primary antibodies against rat T-Cells (R73), NK-cells (CD161), monocyte/macrophages (ED1) (AbD Serotec), cellular FN (IST-9) (Accurate Chemical), MMP-9 (gelatinase-B) (EMDchemicals) and MMP-14 (H-72) (Santa Cruz Biotech) were added at optimal dilutions. Sections were evaluated blindly by counting the labeled cells in triplicates; results are presented as number of cells/10 high-power fields. Dual Staining was achieved by immunofluorescence with Alexa Fluor 594-red anti-rabbit IgG (H+L) and Alexa Fluor 488-green IgG (H+L) antibodies (Molecular probes, Carlsbad, CA). Slides were analyzed using a Leica Confocal Microscope (UCLA, Confocal Microscope Core Facility).

Myeloperoxidase (MPO) Assay

Myeloperoxidase activity was evaluated in frozen tissue homogenized in an iced solution of 0.5% hexadecyltrimethyl-ammonium and 50 mmol/L of potassium phosphate buffer solution [22]. After centrifugation, the supernatants were mixed in a solution of hydrogen peroxide-sodium acetate and tetramethyl benzidine (Sigma). The quantity of enzyme degrading 1 µmol/L of peroxide/minute at 25°C per gram of tissue was defined as 1U of MPO activity.

Western Blot and Zymography Analyses

Western blots and Zymography were performed as described [18]. Proteins (40 µg/sample) in sodium dodecyl sulfate (SDS)-loading buffer were electrophoresed through 10%–12% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to PVDF membranes (Thermo Scientific). Membranes were incubated with specific primary antibodies against MMP-9 (Millipore, Billerica, MA), MT1-MMP (Sigma), phospho-p38, phospho-p44/42, p38, p44/42 (Cell Signaling Technology), COX-2 (Cayman), and iNOS (Santa Cruz). After development, membranes were striped and re-blotted with an antibody against β-actin (Abcam). Gelatinolytic activity was detected in liver extracts (100µg) by 10% SDS-PAGE contained 1mg/ml of gelatin (Invitrogen), under non-reducing conditions. After incubation in development buffer (50 mmol/L Tris-HCl, 5 mmol/L CaCl2, and 0.02% NaN3, pH 7.5), gels were stained with Coomassie brilliant blue R-250 (Bio-Rad), and destained with methanol/acetic acid/water (20:10:70). Prestained molecular weight markers (Fermentas) and MMP-9 (BIOMOL International) served as standards. Relative quantities of protein were determined using a densitometer (Image J, NIH software).

ELISA

TNF-α protein content in liver samples was determined using a commercially available ELISA kit (eBioscience, San Diego, CA) following the manufacturer's instructions, with final results expressed in picograms of TNF-α per milliliter of serum.

RNA Extraction and Reverse Transcriptase PCR

RNA was extracted from livers with Trizol (Life Technologies) as described [18, 22]. Reverse transcription was performed using 5 µg of total RNA in a first-strand cDNA synthesis reaction with SuperScript III RNaseH Reverse Transcriptase (Life Technologies), as recommended by the manufacturer. The cDNA product was amplified by PCR using primers specific for each target cDNA. Densitometric quantification of band intensity was performed using NIH Image J software.

Leukocyte Isolation

Murine neutrophils and macrophages were isolated as previously described [22, 23]. Neutrophil isolation: bone marrow was flushed from harvested femurs and tibias with Hanks' balanced salt solution (HBSS) containing 0.1% (wt/vol) bovine serum albumin (BSA) and 1% (wt/vol) glucose on ice. Cells were pelleted and erythrocytes removed by hypotonic lysis. The bone marrow preparation was resuspended at 5×10^7 cells/mL in HBSS and layered on a Percoll (Sigma) gradient (55% Percoll, top; 65% Percoll, middle; 80% Percoll, bottom). After centrifugation, mature neutrophils were recovered at the interface of the 65% and 80% fractions and were >90% pure. Macrophage isolation: 1 ml of 3% thioglycollate medium was injected into the peritoneal cavity 72 hours before collecting macrophages. The peritoneal cavities were lavaged with HBSS, and the aspirate was placed on ice. After centrifugation, the pellets were resuspended in DMEM. Cell viability was determined by trypan blue exclusion.

Leukocyte Migration Assay

Transmigration of isolated macrophages and neutrophils through fibronectin was performed using a commercially available in vitro cell migration assay kit (BD Bioscience) and as previously described [21, 23]. Leukocytes were resuspended in DMEM without fetal bovine serum at a final concentration of 1.0×10^6 cells/ml. Transwell inserts with 3-μm pore size coated with fibronectin were placed in the 24-well plates, and then macrophages, or neutrophils, (5×10^5 cells/well) were added to the upper chambers. Where indicated, cells were pretreated with anti-MT1-MMP antibody (20 μg/ml; LEM-2/63.1) or control IgG (20 μg/ml) for 30m prior addition to the upper chambers, MMP-9 inhibitor-I (10 nmol/L; $C27H33N3O5S$; Calbiochem) and TNF-α (10 ng/ml; eBioscience) were included in the DMEM medium of the lower chambers. Cells were incubated at 37°C and 5% CO2 for 6 hours, and the leukocytes that had migrated into the lower chambers were collected, stained and counted.

Cell Culture

Isolated macrophages were cultured in medium without fetal bovine serum overnight and pretreated for 30 min with the p38 inhibitor SB203580 (10 μM) prior to being plated on FN-coated plates (Biocoat, BD Biosciences). Controls included combination of cells cultured on polylysine-coated plates, cells stimulated with lipopolysaccharide (10 ng/ml, LPS, Sigma), and cells treated with a specific LPS inhibitor, Polymixin B (10 ng/ml). Cells were cultured on 24-well plates at a concentration of 5×10^5 cells/well and incubated at 37°C, 5% CO2 for 12 hours. After, incubation, cells and supernatants were collected for RT-PCR and zymography, respectively.

Data Analysis

Data are shown as means +/− SD. Statistical comparisons between groups were performed by Student's t-test using the statistical package SPSS (SPSS Inc., Chicago, IL, USA). Kaplan-Meier analysis was used to determine statistical significance of the differences in rat survival. P values of <0.05 were considered statistically significant.

RESULTS

CS-1 peptide therapy ameliorates hepatocellular damage and increases recipient survival in 24h cold liver IRI

We examined the effects of the CS-1 peptide therapy in a well-established model of 24-hour liver cold ischemia followed by OLT. Recipients of livers that had been treated with CS-1 peptides were characterized by improved liver function, as shown by the decreased serum

transaminase levels (IU/L) at 6h (sAST: 1412±420 vs. 2866±864, p<0.05; sALT: 728 ± 428 vs.1690 ± 211, p<0.05) and 24h (sAST: 1350±142 vs. 4000±1358, p<0.05; sALT: 1261±233 vs. 3051±958, p<0.05) post-OLT (Fig. 2A). CS-1 treated OLTs showed relatively good histological preservation, contrasting with high vascular congestion, extensive necrosis, and significant disruption of lobular architecture observed in control livers, (score: 6h/1.5±0.5 vs. 2.7±0.6, p<0.05; 24h/1.7±0.4 vs. 3.1±0.5, p<0.05) (Fig. 2B). Moreover, CS-1 peptide therapy significantly increased the 14-day survival rate (100% vs. 50%, p<0.05; n=8/group) post-OLT (Fig 2C). Therefore, our results agree with previous observations in a 4h model of steatotic liver IRI [17] and support a broadly beneficial role of FN-α4β1 integrin blockade in cold hepatic IRI.

CS-1 peptide therapy disrupts leukocyte infiltration in 24h cold liver IRI

CS-1 peptide therapy significantly depressed leukocyte infiltration (/10HPF) in prolonged cold liver IRI. The numbers of T lymphocytes (31±8 vs. 64±3, p<0.05), NK cells (19±2 vs. 41±3, p<0.05) and ED1 monocyte/macrophages (21±6 vs. 32± 1, p<0.05) were decreased in the CS-1 peptide treated livers at 6h post-transplantation (Fig. 3). T-cell (30±3 vs. 83±15, p<0.05), NK cell (16±3 vs. 30±8, p<0.05) and ED1 macrophage (35±7 vs. 57±16, p<0.05) infiltration was also lower in the CS-1 treated recipients 24h post-OLT (Fig. 3). MPO activity (U/g), an index of neutrophil infiltration, was significantly reduced in the CS-1 treated rats at 6h (1.22 ± 0.48 vs. 2.93 ± 0.57 p<0.05) and 24h (0.48 ± 0.02 vs. 3.18 ± 0.94, p<0.05) post-OLT (Fig. 3E).

CS-1 peptide therapy decreases iNOS, COX-2 and proinflammatory cytokine expression in prolonged cold liver IRI

Inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are two of the most prominent inflammatory mediators [24] and their expressions have been linked to liver IRI [23, 25]. As shown in figure 4, CS1 peptide therapy reduced the intragraft mRNA expression of both iNOS (0.06±0.02 vs. 1.40±0.41, p<0.05) and COX-2 (0.80±0.11 vs. 2.20±0.61, p<0.05) at 6h post-OLT (Fig. 4A). Other pro-inflammatory mediators, such as TNF-α(0.03±0.04 vs. 0.83±0.26, p<0.05), IL1β (0.35 ± 0.13 vs. 0.74 ± 0.30, p<0.05), IL-6 (0.45±0.17 vs. 1.10±0.32, p<0.05), and IFN-γ(0.41±0.11 vs. 0.58±0.06, p<0.05) were also markedly depressed in the CS-1 peptide treated OLTs at mRNA level (Fig. 4A). Evaluations of iNOS, COX-2, and TNF-α at protein level confirmed the beneficial effect of the CS-1 peptide therapy in the reduction of these major inflammatory mediators in prolonged cold liver IRI (Fig. 4B and C).

CS-1 peptide therapy downregulates MMP-9 and MT1-MMP/MMP-14 expressions in 24h cold liver IRI

Our earlier studies have shown that MMP-9 is induced upon leukocyte attachment to fibronectin in damaged steatotic livers [18] and that MMP-9 mediates leukocyte migration in liver IRI [21]. Here, we evaluated whether CS-1 peptide mediated therapy affected the expressions of MMP-9 and MT1-MMP/MMP-14 in the liver model of prolonged cold IRI. CS-1 peptide therapy reduced the intragraft MMP-9 expression at mRNA (0.10±0.12 vs. 0.50±0.35, p<0.05), protein (0.03±0.01 vs. 0.23±0.14, p<0.05), and activity (~2.5-fold decrease, p<0.05) levels at 6h post-OLT (Fig. 5). MMP-9 expression was also depressed at mRNA (0.33±0.19 vs. 1.13±0.22, p<0.05), protein (0.15±0.07 vs. 0.70±0.14, p<0.05), and activity (~4.7-fold decrease, p<0.05) levels in CS-1 peptide treated OLTs at 24h post-IRI, compared to controls. Moreover, while control OLTs were characterized by significant MMP-9+ leukocyte infiltration, CS-1 peptide treated livers showed only very few intragraft MMP-9+ leukocytes, (Fig. 5C). MT1-MMP/MMP-14 was virtually absent from naïve livers and upregulated in livers post-OLT; however, its expression was significantly depressed in CS-1 peptide treated OLTs at mRNA (0.27±0.20 vs. 0.76±0.09; p<0.05) and protein

(0.32±0.07 vs. 0.61±0.04; p<0.05) levels at 6h post-OLT, compared to respective controls (Fig. 5E and F). MT1-MMP/MMP-14 expression was similar in CS-1 peptide treated and control OLTs at 24h post-transplantation (data not shown), raising the possibility that the MT1-MMP/MMP-14 expression could perhaps be more relevant during the initial phase of liver IRI. Double immunofluorescence staining was performed in serial liver sections to identify the sources of MT1-MMP/MMP-14, which detected MT1-MMP/MMP-14 staining predominantly in liver infiltrating monocyte/macrophages at 6h post-cold IRI (Fig. 5G).

MMP-9 and MT1-MMP/MMP-14 induce distinct patterns of leukocyte migration across fibronectin

We have shown that MMP-9 regulates the migration of neutrophils [21, 23], others have shown that MT1-MMP/MMP-14 controls the migration of macrophages [26] *in vitro*. Therefore, to complement these earlier studies, we assessed the role of MMP-9 and MT1-MMP/MMP-14 on the migration of both TNF-α-stimulated neutrophils and macrophages across fibronectin. Migration of both macrophages and neutrophils across fibronectin was markedly increased in the presence of TNF-α stimulation. However, MMP-9 inhibition significantly depressed TNF-α-stimulated macrophage (~2.0-fold decrease, p<0.05) and neutrophil (~1.8-fold decrease, p<0.05) migration (Fig. 6). In contrast, MMP/MMP-14 inhibition, while significantly reducing TNF-α-stimulated macrophage migration (~1.7-fold decrease, p<0.05), it was virtually ineffective in preventing stimulated neutrophils to migrate across fibronectin. Therefore, these data suggest a rather selective role for MT1-MMP/MMP-14 on macrophage migration (Fig. 6).

CS-1 peptide therapy inhibits the phosphorylation of p38 mitogen-activated protein kinase in 24h cold liver IRI

Mitogen-activated protein kinases (MAPKs) are a family of serine/threonine protein kinases implied in the regulation of cellular responses to the environment [27]. In an attempt to elucidate the significance of CS-1 peptide therapy upon MAPK cell signaling, we evaluated the activation of p38 MAPK and p44/42 MAPK signaling pathways in our rat liver model of ex vivo 24h cold ischemia followed by transplantation. The phosphorylation of the p38 MAPK threonine and tyrosine residues (Thr180/Tyr182), which was virtually undetected in naïve livers, was slightly upregulated in CS-1 treated livers (0.06±0.01 vs. 0.27±0.13; p<0.05) at 6h post-reperfusion, contrasting with the strong p38 phosphorylation levels detected in the respective control OLTs, (Fig. 7). On the other hand, significant differences in p44/42 MAPK threonine and tyrosine phosphorylation (Thr202/Tyr204) were not detected between CS-1 peptide treated and control OLTs (0.60±0.28 vs. 0.57±0.27) at 6h post-reperfusion, (Fig. 7). Thus, our data suggest that CS-1 peptide therapy preferentially inhibits the p38 MAPK signaling pathway in cold liver IRI.

Induction of MMP-9 and MT1-MMP/MMP-14 by fibronectin is mediated by p38 mitogen-activated protein kinase

We have previously shown that fibronectin-leukocyte interactions regulate MMP-9 expression in a macrophage cell line [18]. Here, we tested whether fibronectin was able to upregulate the expressions of both MMP-9 and MT1-MMP/MMP-14 in isolated macrophages. Indeed, in addition to MMP-9 upregulation (0.80±0.24 vs. 0.36±0.11; p<0.05), we observed that FN is also capable of upregulating MT1-MMP/MMP-14 expression in cultured macrophages (0.97±0.16 vs. 0.48±0.11; p<0.05) (Fig 8A). It has been shown that human monocytes stimulated with LPS express MT1-MMP/MMP-14 [28]; polymyxin B was added to some cultures to eliminate the effect of potential endotoxin contamination. We further determined the role of the p38 MAP kinase pathway in fibronectin-stimulated MMP-9 and MT1-MMP/MMP-14 expressions by culturing isolated macrophages in fibronectin in the absence or presence of the pharmacological inhibitor of

p38 MAPK SB203580. As shown in figure 8, SB203580 significantly inhibited fibronectin-stimulated MMP-9 (0.36 ± 0.07 vs. 0.80 ± 0.24; $p<0.05$) and MT1-MMP/MMP-14 (0.62 ± 0.04 vs. 0.97 ± 0.16; $p<0.05$) mRNA expressions. Moreover, gelatin zymography carried out on protein extracts from the cultures of fibronectin-stimulated macrophages confirmed a significant decrease in MMP-9 activity ($p<0.05$) in the presence of SB203580. Thus, our data suggest that fibronectin-stimulated MMP-9 and MT1-MMP/MMP-14 expressions are p38 MAPK signaling pathway-dependent.

DISCUSSION

It is generally accepted that hepatic IRI associated with leukocyte recruitment and release of cytokines and free radicals plays a major role in liver dysfunction after OLT [29]; nevertheless, the goal of improving therapies for liver IRI has been hindered by the need to develop a thorough understanding about which factors drive leukocyte recruitment and production of inflammatory cytokines. This study provides new insights on the role of fibronectin in the pathophysiology of hepatic IRI. FN can exist in two forms, plasma, which lacks the EDA sequence, and cellular FN; plasma FN circulates in the blood in a closed (allegedly) non-active form, while cellular FN, which exists as part of the extracellular matrix, has been linked to most of the FN activities in the body [30, 31]. EDA-containing cellular fibronectin is virtually absent in normal adult tissues and is expressed by endothelial cells very early after injury [10, 32]. Its expression has been associated to both physiologic wound healing and pathologic tissue fibrosis. While EDA[+] FN expression by sinusoidal endothelial cells mediates TGF-β induced wound repair [33], its absence results in abnormal wound healing [34]. The dysregulation of tissue-repair processes can lead to fibrosis [35]; cellular FN, which stimulates the conversion of hepatic stellate cells into fibrogenic myofibroblasts [36], may play an important role in the development of this condition. Indeed, EDA-null mice fail to significantly develop pulmonary fibrosis. [37].

We have previously shown that cellular fibronectin is upregulated in the liver vasculature preceding leukocyte recruitment and that CS-1 peptide facilitate blockade of $\alpha4\beta1$-FN interactions disrupted leukocyte infiltration and ameliorated steatotic hepatic IRI [17]. Comparable beneficial effects of the CS-1 peptide therapy, on both leukocyte recruitment and recipient survival rate, were observed in prolonged cold liver IRI. In 24-hour cold liver IRI, CS-1 peptides significantly improved liver histological preservation and increased recipient survival. CS-1 peptide therapy disrupted the recruitment of T cells, NK cells, macrophages, and neutrophils, which are leukocytes associated with the development of liver IRI [38, 39]. Moreover, the CS-1 peptide therapy suppressed the release of several pro-inflammatory mediators, such as TNF-α, IFN-γ, COX-2 and iNOS. TNF-α as well as IFN-γ are critical mediators of hepatic IRI [38]. COX-2 and iNOS have been shown by us [23, 25] and by others [40, 41] to have deleterious effects on liver IRI.

Leukocyte transmigration across vascular barriers is dependent on both adhesive and matrix degradation mechanisms. Whereas adhesion molecules are important to leukocyte transmigration by providing leukocyte attachment to the vascular endothelium, matrix metalloproteinases are critical for facilitating leukocyte movement across vascular barriers. Among different MMPs, MMP-9, an inducible gelatinase expressed by leukocytes during hepatic IRI, is emerging as an important mediator of leukocyte traffic to inflamed liver [5]. Fibronectin-$\alpha4\beta1$ interactions are capable of upregulating MMP-9 expression by infiltrating leukocytes [18], which is a critical mediator of leukocyte recruitment in liver IRI [21]. There is growing evidence supporting a complex spatiotemporal regulation of the proteolytic activity is involved in focal matrix degradation during extravasation [26]. In addition to downregulating the expression of MMP-9, CS-1 peptide therapy also depressed the expression of MT1-MMP/MMP-14 in hepatic IRI. The membrane-anchored MT1-MMP/

MMP-14 is a MMP involved in the breakdown of several adhesion molecules, including fibronectin [42]. Unlike soluble MMPs, MT1-MMP/MMP-14 has a stretch of hydrophobic amino acids that anchors the enzyme to the plasma membrane and restricts its activity to the cell surface [43, 44]. We show for the first time that MT1-MMP/MMP-14 expression, which was undetectable in naïve livers, was upregulated by infiltrating monocyte/macrophages after prolonged liver IRI, suggesting a potential role for MT1-MMP/MMP-14 in cold liver IRI. MT1-MMP/MMP-14 has been associated to focalized ECM degradation and to migration of a variety of cell types, including macrophages, endothelial, and tumor cells [26, 45]. Indeed, it has been recently demonstrated that MT1-MMP/MMP-14 is needed to increase the migration of cancer cells in mammary tumors [38]. In our settings, MT1-MMP/MMP-14 appears to preferentially regulate macrophage migration as its inhibition didn't affect neutrophil migration across fibronectin. All together, these observations support the view that MT1-MMP/MMP-14 may act as an amplifier in the recruitment of macrophages, important regulators of inflammation and fibrosis [46], in liver IRI; additional experimentation is warranted to further unveil the role of MT1-MMP/MMP-14 in inflamed livers.

Integrins transmit information from the ECM to the cell resulting in activation of cell signaling pathways important for regulating different cell functions, including adhesion, migration, and proliferation [47]. The p38 MAPK signaling transduction pathway, which is activated through extracellular stimuli, plays an essential role in regulating inflammatory processes [48], including hepatic IRI [49]. Moreover, it has been suggested that the main biological response of p38 MAPK activation has been linked to initiation of leukocyte recruitment and activation [49]. Indeed, the results of this study show that CS-1 mediated blockade of the FN-α4β1 interactions, which disrupted leukocyte recruitment, markedly depressed the phosphorylation of p38 MAPK. We next investigated whether treatment with the pharmacological inhibitor of p38 MAPK SB203580 would affect the expressions of MMP-9 and MT1-MMP/MMP-14 in fibronectin-stimulated macrophages. Macrophages are major sources of MMP-9 in cold liver IRI [18] and, as we report here, of MT1-MMP/MMP-14 as well. Indeed, inhibition of p38 MAPK activity with the antagonist SB203580 depressed the expressions of MMP-9 and MT1-MMP/MMP-14 by fibronectin-stimulated macrophages in culture, suggesting that the p38 MAPK kinase pathway controls fibronectin MMP-9 and MT1-MMP/MMP-14 inductions. In this regard, activation of p38 MAPK has been implied in TNF-α mediated MMP-9 induction [50] and in promoting cancer cell invasion via regulation of MMP mRNA stability [51]. In fact, inhibition of the p38 MAPK pathway significantly depressed TNF-α stimulated leukocyte migration across fibronectin (data not shown).

In summary, the findings we report here further emphasize an important role for the FN-α4β1 integrin interactions in cold hepatic IRI. Our results show that CS-1 peptide therapy down-regulated the expressions of MMP-9 and MT1-MMP/MMP-14, disrupted leukocyte recruitment, and decreased the release of pro-inflammatory mediators, resulting in protection against prolonged cold liver IRI and increased OLT recipient survival. Additionally, the CS-1 peptide facilitated blockade of the FN-α4β1integrin interactions depressed the phosphorylation of p38 MAPK, which is considered to be an attractive target for pharmacologic intervention [52], and therefore implying a regulatory role for fibronectin on the activation of p38 MAPK in hepatic IRI. Furthermore, we provide evidence that p38 MAPK kinase pathway controls the fibronectin mediated induction of both MMP-9 and MT1-MMP/MMP-14 by macrophages.

Acknowledgments

Abbreviations

ALT	alanine aminotransferase
AST	aspartate aminotransferase
CS-1	connecting segment-1
COX-2	cyclooxygenase-2
FN	fibronectin
ECM	extracellular matrix
iNOS	inducible nitric oxide synthase
IRI	ischemia-reperfusion injury
MMP	matrix metalloproteinase
MT1-MMP	membrane type 1-matrix metalloproteinase
MAPK	mitogen-activated protein kinase
MPO	myeloperoxidase
OLT	orthotopic liver transplantation

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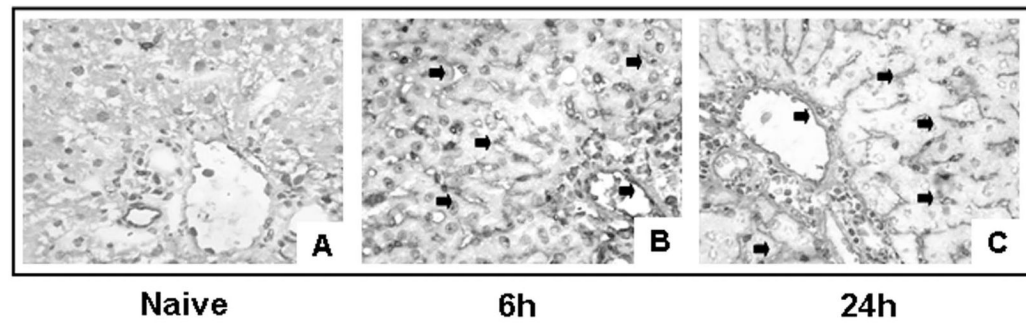
Figure 1.
Representative cellular fibronectin staining in 24h cold liver IRI. Cellular fibronectin was virtually absent in naïve livers (A) and it was abundantly upregulated in livers after 24h of cold storage followed by 6h (B) and 24h (C) of reperfusion (n=4/group).
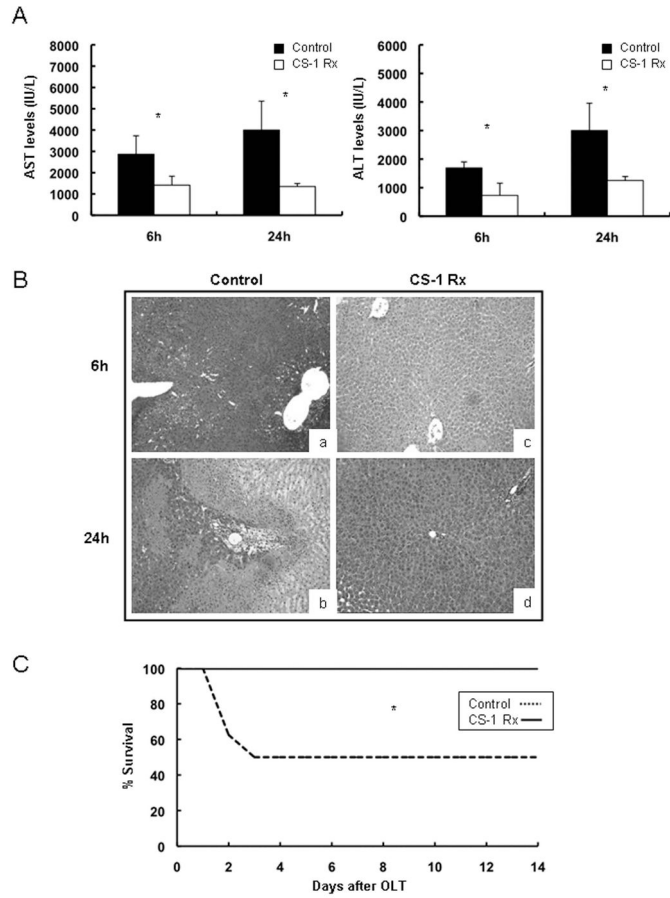
Figure 2.
Transaminase levels, histology, and recipient survival in 24h cold liver IRI. CS-1peptide therapy significantly improved liver function as evidenced by the lower AST and ALT levels (panel A) in the CS-1 peptide treated recipients at 6h and 24h post-cold liver IRI. Hematoxylin and eosin staining of liver grafts (panel, B) indicated a better histological preservation in the CS-1 peptide treated liver OLTs (c, and d) as compared with respective controls (a, and b), at 6h (a, and c) and 24h (b, and d) post-OLT. Moreover, CS-1 peptide treated OLTs had a significantly prolonged survival rate (panel C) as compared to respective controls at 14-day post-OLT (*$p < 0.05$; ×100 H&E; panels A and B n=5–7 rats/group; panel C n=8 rats/group).
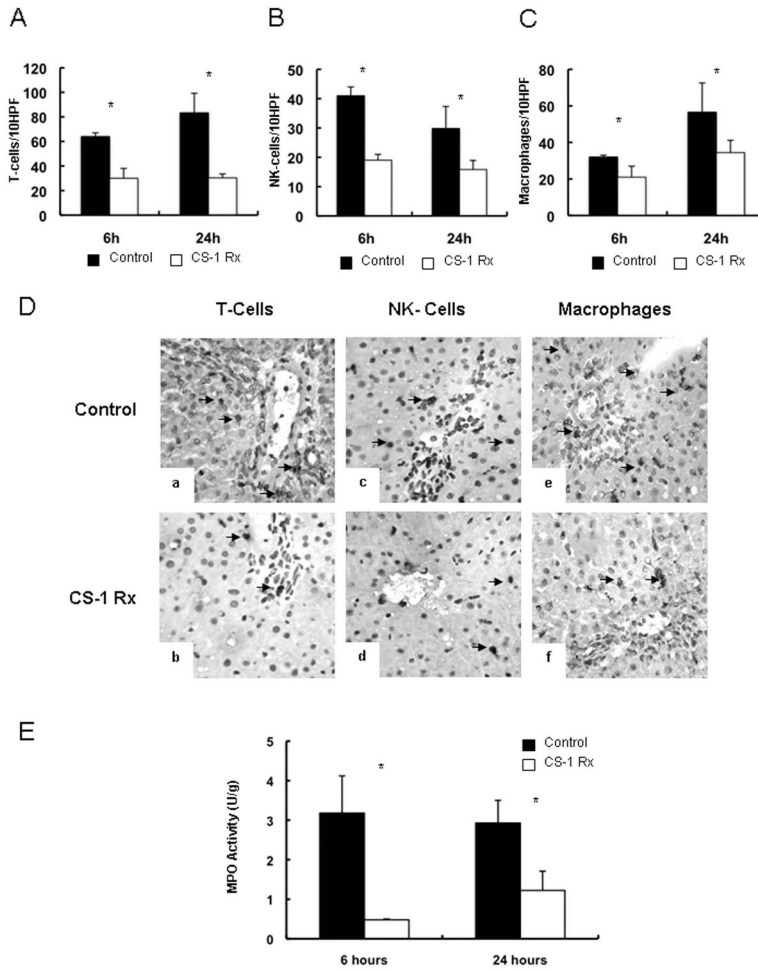
Figure 3.
Leukocyte infiltration in 24h cold liver IRI. The infiltration of T lymphocytes (panel A), NK cells (panel B), and monocyte/ macrophages (panel C) was significantly depressed in CS-1 peptide treated OLTs at 6h and 24h post-reperfusion. Panel D illustrates immunoperoxidase staining of T cells (a, and b), NK lymphocytes (c, and d), and macrophages (e, and f) in CS-1 (b, d, and f) and control (a, c, and e) OLTs at 6h post-transplantation. MPO activity (panel E), an index of neutrophil infiltration, was significantly depressed in CS-1 peptide treated OLTs at 6h and 24h post-transplantation as compared to respective controls (*$p < 0.05$; ×200; n=5–6 rats/group).
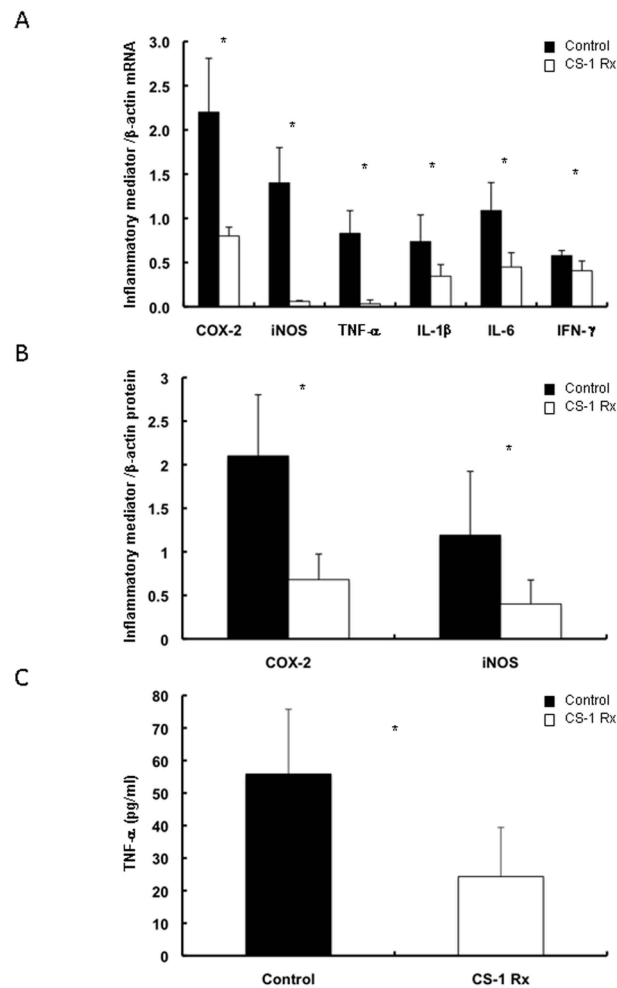
Figure 4.
COX-2, iNOS and proinflammatory cytokine expression in cold liver IRI. The mRNA expressions (panel A) of COX-2 and iNOS, two major tissue injury mediators, as well as of various proinflammatory cytokines were profoundly depressed in the CS-1 treated grafts at 6h post-IRI. The mRNA expressions correlated with the protein expressions (panels B and C), as iNOS, COX-2, and TNF-α were significantly depressed at protein level in the CS-1 peptide treated OLTs at 6h after reperfusion (*p<0.05; panel A n=4 rats/group; panels B and C n=5 rats/group).
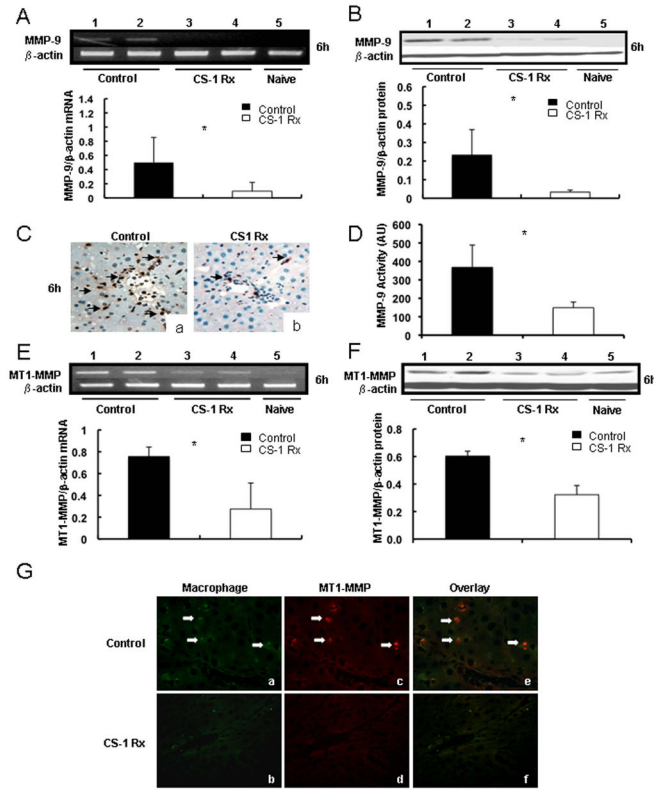
Figure 5.
MMP-9 and MT1-MMP expressions in 24h cold liver IRI. The expressions of MMP-9 (panels A–D) and MT1-MMP (panels E–G) at mRNA (panel A and E), protein (panel B and F), and activity (panel D) levels were readily detected in control OLTs (lanes 1, and 2) and only slightly detected in CS-1 peptide treated livers at 6h post-IRI (lanes 3, and 4); MMP-9 and MT1-MMP expressions were nearly undetectable in naïve livers (lane 5). Panel C shows MMP-9 + leukocyte infiltration in control (a) and CS-1 peptide treated (b) OLTs at 6h post-IRI. Panel G displays MT1-MMP and Mac-1 immunofluorescence staining in control (a, c, and e) and CS-1 peptide treated (b, d, and f) livers at 6h post-OLT. Mac-1 (a, and b) is stained in green (Alexa Fluor 488) and MT1-MMP (c, and d) is labeled in red (Alexa Fluor 594); cell colocalization of Mac-1/MT1-MMP markers is shown in yellow-orange (e, and f) (*p<0.05; arrows denote positive labeling; × 200; n=5–6 rats/group).
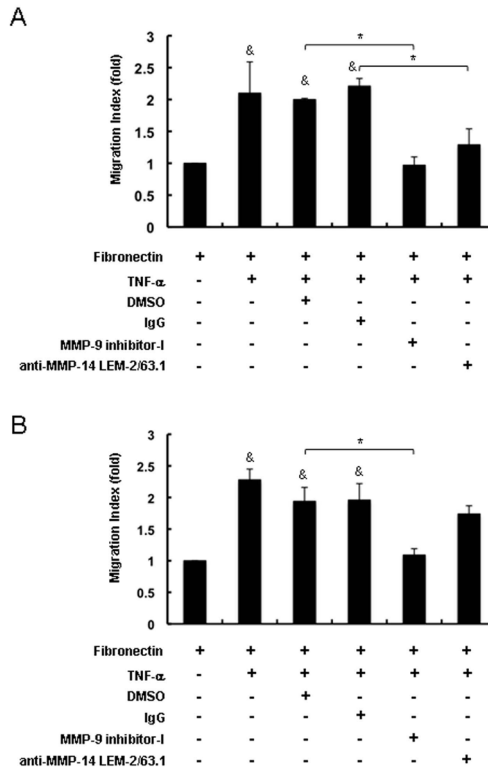
Figure 6.
Regulation of neutrophil and macrophage migration by MMP-9 and MT1-MMP/MMP-14. Migration of macrophages across fibronectin (A) was markedly increased in the presence of TNF-α; MMP-9 inhibition as well as MT1-MMP/MMP-14 inhibition significantly reduced macrophage migration to levels almost comparable with those observed in the absence of TNF-α stimulation. Migration of neutrophils across fibronectin (B) was also markedly increased in the presence of TNF-α stimulation; however, while MMP-9 inhibition markedly reduced neutrophil infiltration, MT1-MMP/MMP-14 inhibition was virtually ineffective in depressing the migration of these cells. The cell migration index was measured as the ratio of cells that migrated into the lower wells in the experimental groups to those in the control groups without TNF-α stimulation. MMP indexes are expressed as mean ± SD of four experiments (&p<0.05, relative to controls without TNF-α stimulation; *p<0.05 relative to TNF-α-stimulated controls).
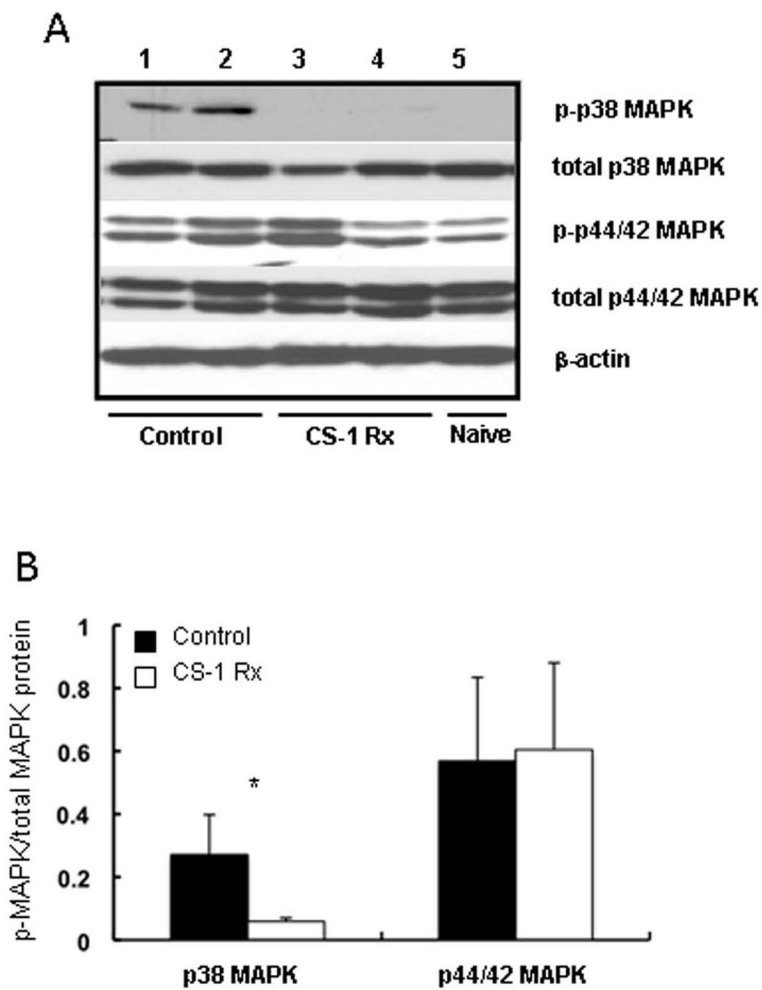
Figure 7.
Mitogen-activated protein kinase pathways in 24h cold liver IRI. Levels of p38 MAPK and p44/42 MAPK phosphorylation (panel A) in control (lanes 1, and 2) and in CS-1 peptide (lanes 3, and 4) treated livers at 6h post-IRI. Densitometric analysis (panel B) revealed a marked decrease in p38 MAPK activation in CS-1 peptide treated OLTs as compared to controls; there were no significant differences on p44/42 MAPK phosphorylation between CS-1 peptide treated and control OLTs (*$p < 0.05$; n=6/group).
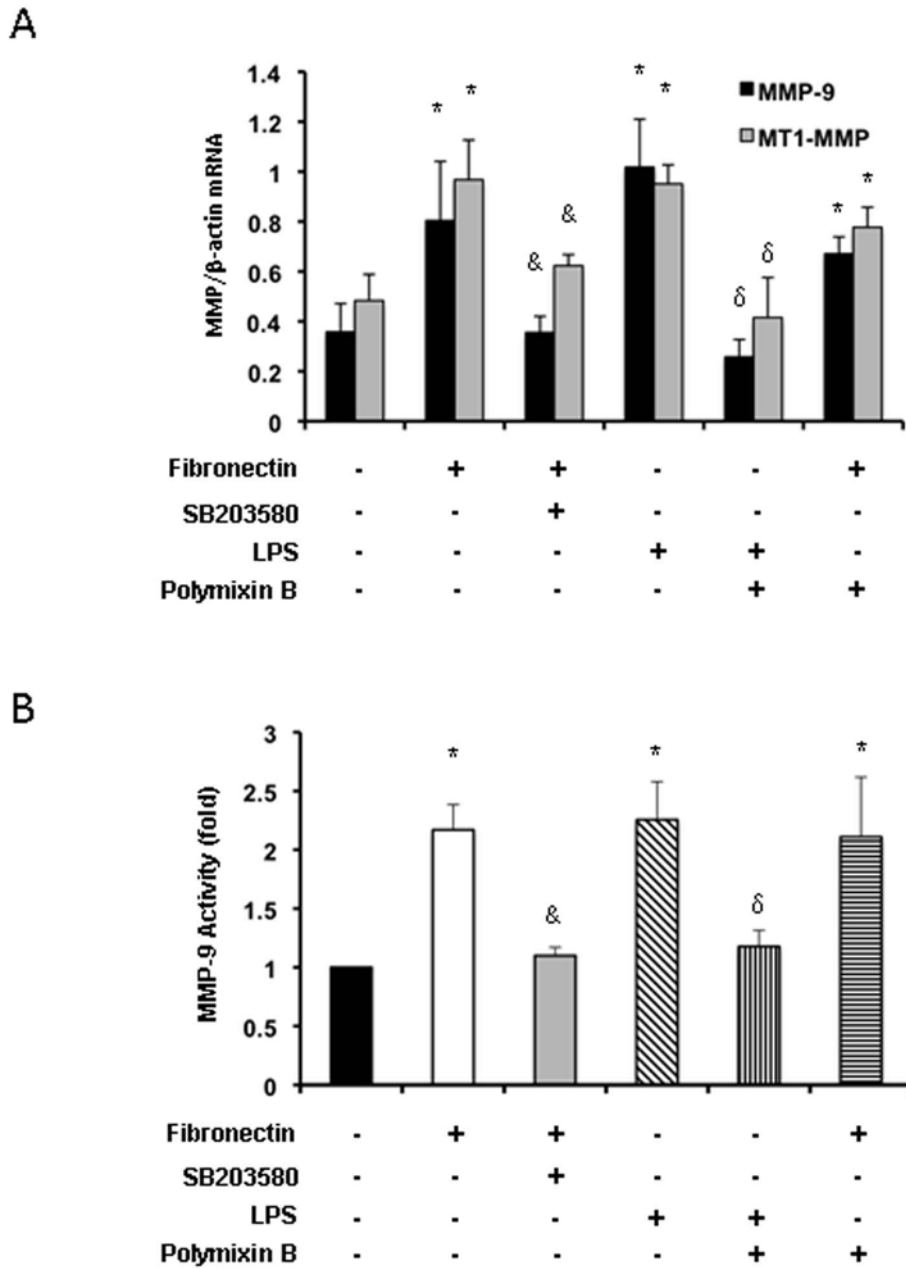
A

B

Figure 8.
Fibronectin-mediated MMP-9 and MT1-MMP expressions are dependent on p38 MAPK activation. Attenuation of FN-mediated MMP-9 and MT1-MMP mRNA expressions (panel A) by inhibition of p38 MAPK in isolated macrophages treated with SB-203580 (10 μg/ml). While polymyxin B (10 ng/ml) profoundly depressed the expression of MMP-9 and MT1-MMP in LPS-stimulated macrophages, it didn't significantly affect the production of MMP-9 or MT1-MMP induced by fibronectin in isolated macrophages. Conditioned media obtained from cultured macrophages was subjected to a gelatin zymography assay (panel B); inhibition of p38 MAPK with SB-203580 significantly depressed the MMP-9 activity in fibronectin-stimulated macrophages (graph represents fold increases in enzymatic activity over unstimulated macrophages). MMP levels expressed as mean ± SD of four experiments

(*p<0.05, relative to unstimulated controls; &p<0.05, relative to fibronectin stimulated controls; δp<0.05, relative to LPS stimulated controls).