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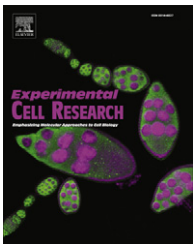

Research Article

Concanavalin-A triggers inflammatory response through JAK/STAT3 signalling and modulates MT1-MMP regulation of COX-2 in mesenchymal stromal cells

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ABSTRACT

Pharmacological targeting of inflammation through STAT3 and NF-κB signaling pathways is, among other inflammatory biomarkers, associated with cyclooxygenase (COX)-2 inhibition and is believed to play a crucial role in prevention and therapy of cancer. Recently, inflammatory factors were found to impact on mesenchymal stromal cells (MSC) contribution to tumor angiogenesis. Given MSC chemotaxis and cell survival are regulated, in part, by the membrane type-1 matrix metalloproteinase (MT1-MMP), an MMP also involved in transducing NF-κB intracellular signaling pathways, we tested whether STAT3 regulation by MT1-MMP may also contribute to the expression balance of COX-2 in MSC. We demonstrate that STAT3 phosphorylation was triggered in MSC treated with the MT1-MMP inducer lectin Concanavalin-A (ConA), and that this phosphorylation was abrogated by the JAK2 inhibitor AG490. MT1-MMP gene silencing significantly inhibited ConA-induced STAT3 phosphorylation and this was correlated with reduced proMMP-2 activation and COX-2 expression. On the other hand, STAT3 gene silencing potentiated ConA-induced COX-2 expression, providing evidence for a new MT1-MMP/JAK/STAT3 signaling axis that may, in part, explain how MT1-MMP contributes to proinflammatory intracellular signaling. Given that MSC are avidly recruited within inflammatory microenvironments and within experimental vascularizing tumors, these mechanistic observations support a possible dual control of cell adaptation to inflammation by MT1-MMP and that may enable MSC to be active participants within inflamed tissues.

Introduction

Cells present in the tumor microenvironment include fibroblasts, vascular, smooth muscle, adipocytes, immune cells and mesenchymal stromal cells (MSC). Most commonly isolated from the bone marrow, MSC are a population of pluripotent adult stem cells that can differentiate into many mesenchymal phenotypes [1,2], allowing MSC to appear either as pro- or anti-tumorigenic [3]. In fact, recruitment of MSC by experimental vascularizing tumours resulted in the incorporation of MSC within the tumor architecture [4,5] which, combined with intrinsic immunomodulatory mechanisms, implies that they must also respond to inflammation- and

Abbreviations: ConA, concanavalin-A; COX, cyclooxygenase; ECM, extracellular matrix; JAK, janus kinase; MSC, mesenchymal stromal cells; MT1-MMP, membrane type-1 matrix metalloproteinase; NF-κB, nuclear factor-kappa B; STAT, signal transducers and activators of transcription

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tumor-derived growth factor cues [6,7]. While chronic inflammation has been found to mediate a wide variety of diseases including neoplasms [8], our understanding of the oncogenic adaptation of MSC within an inflammatory microenvironment lacks the identification of molecular contributors and characterization of inflammation-mediated signaling pathways. Among the gene products involved in the induction of the inflammatory process, cyclooxygenase (COX)-2 has been shown to have a close relationship with tumorigenesis [9] and with altered extracellular matrix (ECM) proteolysis that has been linked to matrix metalloproteinase (MMP)-mediated events [10]. The signaling contributors that link MMP to inflammation and that may lead to unregulated tumor growth, angiogenesis, tissue invasion and metastasis remain poorly documented.

While most MMP are secreted, MT1-MMP is a membrane-associated MMP regulated by hypoxia [11] which, aside from its well-known role in the activation of proMMP-2 and intrinsic proteolytic activity towards ECM molecules, drives MSC mobilization [12,13]. More importantly, phosphorylation of its cytoplasmic domain was recently linked to crucial signal transducing functions, and was shown to interact with several adaptor proteins including p130Cas [14], MTCBP-1 [15], 3BP2 [16], and Src-mediated events [17]. In light of such signaling scaffolds taking place in MT1-MMP cellular signaling, MT1-MMP functions were further found associated with platelet-mediated calcium mobilization [18], regulation of cell death/survival bioswitch [19,20], and regulation of proinflammatory signaling including COX-2 [21,22].

Early proinflammatory cellular signaling processes triggered by ConA resulted in the MT1-MMP-mediated regulation of COX-2 in gliomas through an IKK/NF-κB-dependent pathway [22]. Given that JAK/STAT is also involved in COX-2 regulation, we hypothesized that MT1-MMP may in part signal the inflammation molecular adaptation of MSC within solid tumours, and that an inflammatory signaling balance ultimately accounts for some of the therapy resistance phenotype. In order to specifically induce MT1-MMP, for which the biomarker expression correlated with high COX-2 levels in gliomas with increasing histological grade [23], we used the lectin from *Canavalia ensiformis* (Concanavalin-A, ConA), which is well known to provoke and mimic the biological lectin/carbohydrate interactions that regulate ECM protein recognition.

Materials and methods

Materials

Sodium dodecylsulfate (SDS), bovine serum albumin (BSA) and Actinonin were purchased from Sigma (Oakville, ON). Cell culture media were obtained from Life Technologies (Burlington, ON). Electrophoresis reagents were purchased from Bio-Rad (Mississauga, ON). The enhanced chemiluminescence (ECL) reagents were from Amersham Pharmacia Biotech (Baie d'Urfé, QC). Micro bicinchoninic acid protein assay reagents were from Pierce (Rockford, IL). The PI3K inhibitor LY294002, the p38/MAPK inhibitor SB203580, the MEK kinase inhibitor U0126 and the JAK family tyrosine kinase inhibitor AG490 were from EMD Millipore (Toronto, ON). The anti-ERK-1/2 (extracellular signal-regulated kinase 1 and 2) (K-23) polyclonal antibody was from Santa Cruz Biotechnologies (Santa Cruz, CA). Anti-STAT3 (79D7) and anti-phospho-STAT3 (Tyr 705) polyclonal antibodies were from Cell Signaling Technology (Beverly, MA). The polyclonal antibody against COX-2 was from Cayman Chemical (Ann Arbor, MI).

Cell cultures

This study was approved by the "Comité Institutionnel des Risques Biologiques" (certificate #10-CIRB-53.3.5). Bone marrow-derived mesenchymal stromal cells (MSC) were isolated from the whole femur and tibia bone marrow of C57BL/6 female mice; cells were cultured and characterized by flow cytometry as previously described [24]. Serum starvation is classically performed by culturing the cells in high glucose Dulbecco's modified Eagle's medium (DMEM; GibcoBRL) and 100 units/ml Penicillin/Streptomycin from which the 10% inactivated fetal bovine serum (iFBS) (Hyclone Laboratories, Logan, UT) was omitted.

Total RNA isolation, cDNA synthesis and real-time quantitative RT-PCR

Total RNA was extracted from MSC monolayers using TRIzol reagent (Life Technologies, Gaithersburg, MD). For cDNA synthesis, 1 μg of total RNA was reverse-transcribed into cDNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA). cDNA was stored at −80 °C prior to PCR. Gene expression was quantified by real-time quantitative PCR using iQ SYBR Green Supermix (BIO-RAD, Hercules, CA). DNA amplification was carried out using an Icycler iQ5 (BIO-RAD, Hercules, CA) and product detection was performed by measuring binding of the fluorescent dye SYBR Green I to double-stranded DNA. The following primer sets were provided by QIAGEN (Valencia, CA): MT1-MMP (Mm_Mmp14_1_SG QT01064308), β-Actin (Mm_Actb_2_SG QT01136772). The relative quantities of target gene mRNA against an internal control, β-Actin RNA, were measured by following a ΔC_T method employing an amplification plot (fluorescence signal vs. cycle number). The difference (ΔC_T) between the mean values in the triplicate samples of target gene and those of β-Actin RNA were calculated by iQ5 Optical System Software version 2.0 (BIO-RAD, Hercules, CA) and the relative quantified value (RQV) was expressed as $2_T^{-\Delta C}$.

Transfection method and RNA interference

MSC were transiently transfected with 20 nM siRNA against STAT3 (Mm_Stat3_1 FlexiTube siRNA, SI01435287), MT1-MMP (Mm_Mmp14_2 HP siRNA, SI00177800), or scrambled sequences (AllStar Negative Control siRNA, 1027281) using Lipofectamine 2000 transfection reagent (Invitrogen, CA). Small interfering RNA and mismatch siRNA were synthesized by QIAGEN and annealed to form duplexes.

Gelatin zymography

Gelatin zymography was used to assess the extracellular levels of secreted proMMP-2 and MMP-2 activities. Briefly, an aliquot (20 μl) of the culture medium was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in a gel containing 0.1 mg/ml gelatin (Sigma-Aldrich Canada, G2625). The gels were then incubated in 2.5% Triton X-100 (Bioshop, TRX506.500) and rinsed in nanopure

distilled water. Gels were further incubated at 37 °C for 20 h in 20 mM NaCl, 5 mM CaCl2, 0.02% Brij-35, 50 mM Tris-HCl buffer, pH 7.6 and then stained with 0.1% Coomassie Brilliant blue R-250 (Bioshop, CBB250) and destained in 10% acetic acid, 30% methanol in water. Gelatinolytic activity was detected as unstained bands on a blue background.

Immunoblotting procedures

The following electrophoresis reagents were used: Sodium dodecyl-sulfate (SDS; Sigma-Aldrich Canada, L3771), acrylamide (Bioshop, ACR001.1), and bis-acrylamide (Bioshop, BIS001.100). Proteins from control and treated cells were separated by SDS-PAGE. After electrophoresis, proteins were electrotransferred to polyvinylidene difluoride membranes (Millipore, IPVH00010) which were then blocked for 1 h at room temperature with 5% non-fat dry milk in Tris-buffered saline (150 mM NaCl, 20 mM Tris-HCl, pH 7.5) containing 0.3% Tween-20 (TBST; Bioshop, TWN510-500). Membranes were further washed in TBST and incubated with the above mentioned primary antibodies (1/1000 dilution) in TBST containing 3% bovine serum albumin and 0.1% sodium azide (Sigma-Aldrich Canada, S2002), followed by a 1 h incubation with horseradish peroxidase-conjugated donkey anti-rabbit (Jackson ImmunoResearch Laboratories, 711-035-152) or goat anti-mouse IgG (Jackson ImmunoResearch Laboratories, 115-035-062) at 1/2500 dilutions in TBST containing 5% non-fat dry milk. Immunoreactive material was

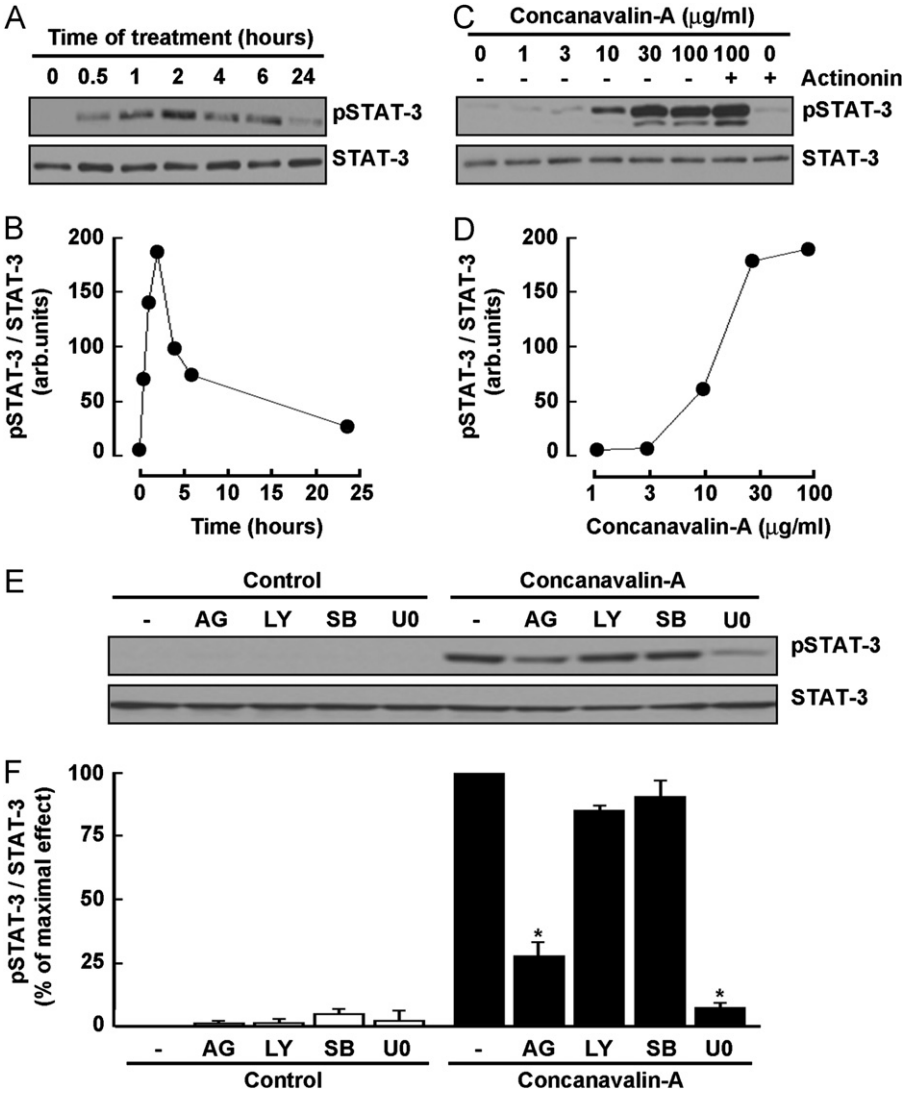

Fig. 1 – Concanavalin-A triggers transient and dose-dependent STAT3 phosphorylation. Subconfluent MSC were serum-starved and treated with either 30 μg/ml Concanavalin-A for up to 24 h (A), with various Concanavalin-A concentrations for 2 h (C), or pre-treated for 30 min with 10 μM of the JAK family tyrosine kinase inhibitor (AG490), the p38/MAPK inhibitor (SB203580), MEK kinase inhibitor (U0126) or the PI3K inhibitor (LY294002), followed by a 2 h treatment with 30 μg/ml Concanavalin-A. (A, C and E) Cell lysates were isolated, western blotting and immunodetection were performed with anti-phospho-STAT3 and anti-STAT3 antibodies as described in the Methods section. (B, D and F) Densitometric measurements were performed and represent the values for pSTAT3/STAT3 ratios. A representative blot, out of 3 independent experiments, and corresponding densitometric analysis are shown. Statistical significance was assessed using Student's unpaired *t*-test. Probability values of less than 0.05 were considered significant and an asterisk identifies such significance.

visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, RPN3004) [25].

Statistical data analysis

Data are representative of three or more independent experiments. Statistical significance was assessed using Student's unpaired *t*-test. Probability values of less than 0.05 were considered significant and an asterisk identifies such significance in the figures.

Results

Concanavalin-A triggers STAT3 phosphorylation. In order to first assess whether any JAK/STAT3 signaling pathway is inducible, MSC were serum-starved and then cultured in the presence of 30 μg/ml Concanavalin-A (ConA) for up to 24 h. Cells were harvested and lysates used to evaluate the extent of STAT3 phosphorylation (Fig. 1A). We found that ConA significantly triggered STAT3 phosphorylation, reaching a maximal value at 2 h of treatment (Fig. 1B). When various ConA concentrations were tested on MSC (Fig. 1C), a dose-dependent increase in STAT3 phosphorylation was observed which reached a plateau at 30 μg/ml (Fig. 1D). Given part of ConA cell surface action involves membrane bound biomarker proteases including transmembrane protease aminopeptidase N (APN)/CD13 [26] as well as MT1-MMP [20], we tested whether Actinonin, which was reported to target both biomarkers catalytic functions [27–29], reversed ConA signaling. Interestingly, treatment with Actinonin was unable to reverse ConA-induced STAT3 phosphorylation, suggesting that induction of the JAK/STAT pathway requires alternate specific intracellular cell surface-mediated signaling (Fig. 1C).

STAT3 phosphorylation by Concanavalin-A is controlled through JAK and MAPK signaling pathways

We next moved on to delineate the potential signaling pathways and kinases involved in ConA-mediated STAT3 phosphorylation. MSC were serum-starved, pre-treated for 30 min with the JAK family tyrosine kinase inhibitor (AG490), the p38/MAPK inhibitor (SB203580), MEK kinase inhibitor (U0126) or the PI3K inhibitor (LY294002) and were then stimulated with ConA for 2 h. STAT3 phosphorylation (Fig. 1E) was significantly diminished by AG490 and U0126 (Fig. 1F). This supports that JAK and MEK transduce ConA-mediated STAT3 phosphorylation.

MT1-MMP gene silencing abrogates Concanavalin-A-mediated STAT3 phosphorylation, proMMP-2 activation and COX-2 expression

Given the crucial intracellular signaling role that MT1-MMP exerts in ConA-mediated cell surface binding, we next assessed its possible contribution to STAT3 phosphorylation. MT1-MMP gene silencing was first validated by qRT-PCR (Fig. 2A), and then cells were treated with various concentrations of ConA (Fig. 2B). While total STAT3 levels did not change, STAT3 phosphorylation was significantly abrogated when MT1-MMP expression was silenced (Fig. 2C). Silencing of MT1-MMP did not alter Interleukin-6-induced STAT3 phosphorylation confirming a specific link in ConA/MT1-MMP interaction (data not shown). Abrogation of the MT1-MMP/STAT3 signaling axis was further correlated to MT1-MMP-dependent proMMP-2 activation and to ConA-induced intracellular COX-2 expression (Fig. 2D). Indeed, functional downregulation of MT1-MMP at the cell surface was found to efficiently inhibit proMMP-2 activation as assessed by gelatin zymography and confirms the efficiency of MT1-MMP silencing (Fig. 2E). Moreover, ConA-induced intracellular COX-2 expression was also inhibited when MT1-MMP expression was silenced (Fig. 2F).

STAT3 gene silencing potentiates Concanavalin-A-induced COX-2 expression

We next analyzed the direct impact of STAT3 on ConA-mediated COX-2 expression and proMMP-2 activation. STAT3 gene expression was transiently silenced and MSC treated with various concentrations of ConA (Fig. 3A). While STAT3 protein expression was abrogated, we found that ConA-induced COX-2 expression was significantly potentiated within cells that had low STAT3 expresion (Fig. 3B). STAT3 silencing did not affect ConA-induced proMMP-2 activation, suggesting that MT1-MMP cell surface expression/function was not altered (Fig. 3C).

Discussion

Recent work in understanding inflammatory signaling cascades has identified a series of novel promising targets, notably in pathways involving NF-κB and JAK/STAT transcription factors [24]. Being the two most important transcription factors associated with inflammation-mediated tumor promotion, NF-κB and STAT3 are also aberrantly activated in glioma where they regulate the expression of genes crucial for tumorigenesis [30]. Given the roles that MSC potentially play in brain tumour development, and since inflammation is crucial for glioblastoma progression, it is further believed that a subset of primary glioblastomas, derived from transformed stem cells that possess MSC-like properties [31], retain partial phenotypic aspects of the MSC nature within the tumours' hypoxic and inflammatory environment [32]. Accordingly, exogenously delivered human MSC were found to be recruited and to adapt within human gliomas after intravascular delivery [5]. More recently, molecular markers associated with MSC were found to characterize the brain tumour-initiating cells involved in the development of hypoxic solid tumours such as glioblastomas [32].

Clinical applications for MSC have been suggested given their immunomodulatory functions and ability to home in and to adapt within sites of inflammation following tissue injury [33]. However, homing of MSC to tumours is also believed to be among the earliest phenomena in MSC-cancer interactions and was recently reported in a mouse model where injected human MSC preferentially migrated to implanted human tumours [4,6], and where cotransplantation of MSC with melanoma cells enhanced tumour engraftment and growth [11]. This suggests that MSC are active participants in the development of solid tumours, and that adaptive cellular conditions have significant pathological implications towards hypoxic solid tumour development involving a potential complex interrelationship and crosstalk between JAK/STAT- and NF-κB-regulated expression of COX-2 in inflammation [34]. In support, isolation of cancer

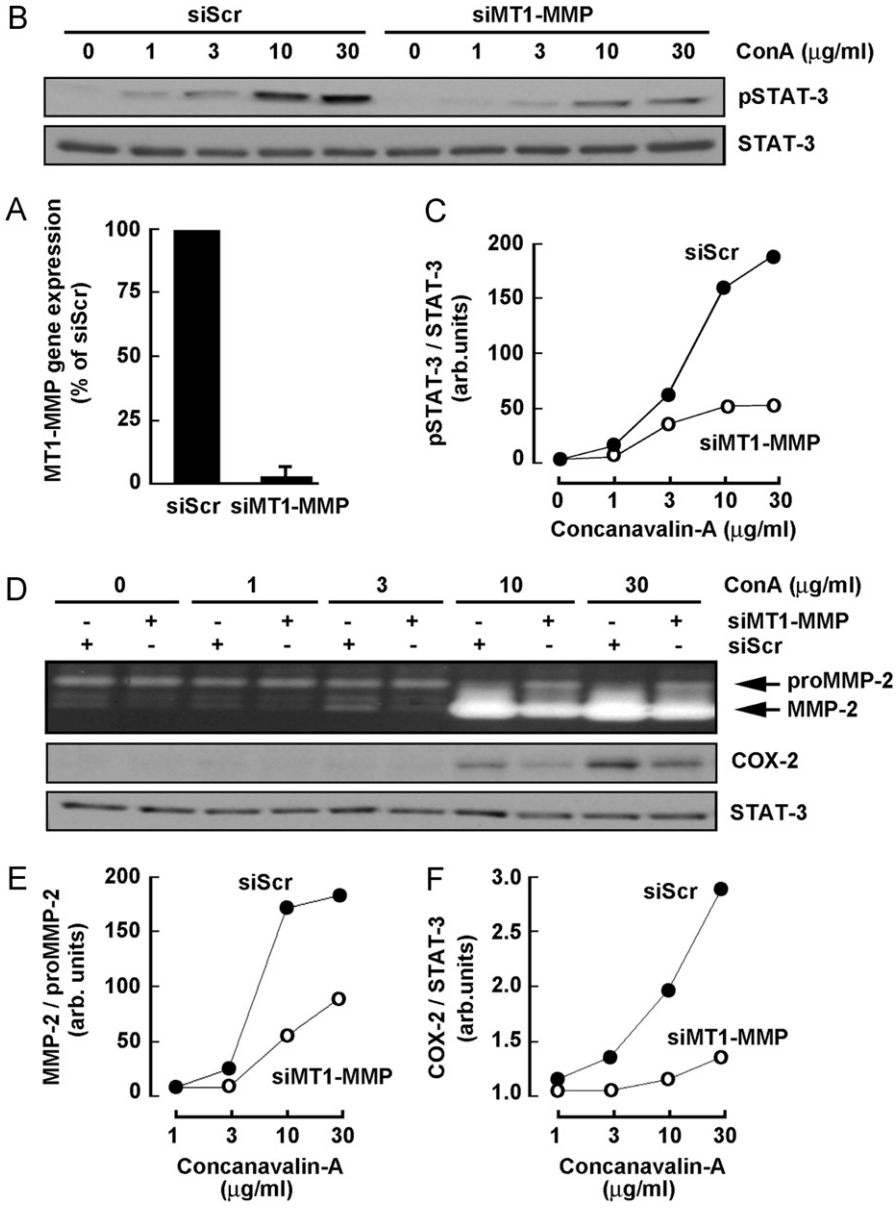

Fig. 2 – MT1-MMP gene silencing abrogates Concanavalin-A-mediated STAT3 phosphorylation, proMMP-2 activation and COX-2 expression. MSC were transiently transfected with scrambled sequences (siScr) or MT1-MMP siRNA (siMT1-MMP) as described in the Methods section. (A) Total RNA was extracted, and qRT-PCR was used to assess MT1-MMP gene expression and confirm gene downregulation. (B, D) Subconfluent transfected MSC were serum-starved and treated with 30 μg/ml of Concanavalin-A for 2 h (B) or 24 h (D). Conditioned media was isolated and gelatin zymography performed as described in the Methods section to assess the extent of proMMP-2 to MMP-2 activation. Cell lysates were isolated, western blotting and immunodetection were performed with anti-phospho-STAT3, anti-STAT3, or anti-COX-2 antibodies as described in the Methods section. (C, E, F) Densitometric measurements were performed and represent the values for pSTAT3/STAT3, MMP-2/proMMP-2, COX-2/STAT3 ratios respectively. A representative blot and zymogram, out of 3 independent experiments, and corresponding densitometric analysis are shown.

stem-like cells from the glioblastoma cell line U87MG has been reported [21,35], and NF-κB-mediated COX-2 regulation by MT1-MMP demonstrated [21].

Given NF-κB can affect STAT3 activity, and that STAT3 can reciprocally contribute to NF-κB activation [36], mutual contribution of NF-κB and STAT3 activation processes may therefore provide rationale for their therapeutic targeting. Accordingly, targeting such dual transcriptional control of one crucial angiogenic and inflammatory biomarker, such as the blood-barrier barrier disruptor MMP-9, may be envisioned [37]. Interestingly in recent years, targeting strategies have explored the chemopreventive properties of diet-derived products including curcumin [38], delphinidin [39], epigallocatechin gallate (EGCG) [40,41], and resveratrol [42] all effective in inhibiting MMP-9 expression through NF-κB and/or STAT3 activity. Therapeutic targeting of such crosstalk signaling may therefore be reasonably considered as an adjunct to existing chemotherapy and radiation treatments [43]. Among the latter molecules, EGCG was also able

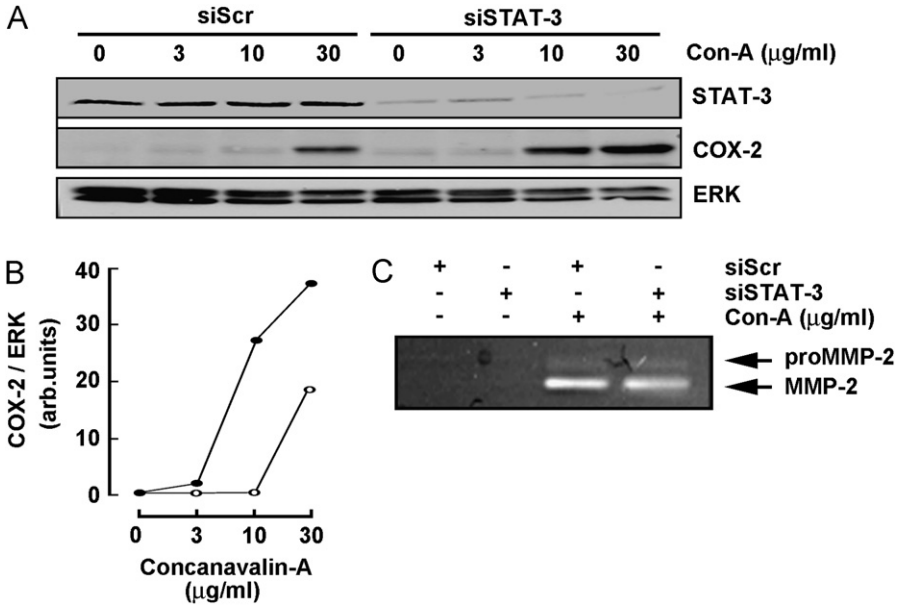

Fig. 3 – STAT3 gene silencing potentiates Concanavalin-A-induced COX-2 expression. MSC were transiently transfected with scrambled sequences (siScr) or STAT3 siRNA (siSTAT3) as described in the Methods section. Subconfluent transfected MSC were serum-starved and treated with various Concanavalin-A concentrations for 24 h. (A) Cell lysates were isolated, western blotting and immunodetection were performed with anti-STAT3, anti-COX-2 and anti-ERK antibodies as described in the Methods section. (B) Densitometric measurements were performed and represent the values for COX-2/ERK ratios. (C) Conditioned media were isolated and gelatin zymography performed as described in the Methods section to assess the extent of proMMP-2 activation. A representative blot, out of 3 independent experiments, and corresponding densitometric analysis are shown.

to inhibit MT1-MMP expression and function [44,45], and showed promising anti-angiogenic efficacy when combined to ionizing radiation [46,47]. Finally, MT1-MMP expression level status was found to dictate the in vitro anti-inflammatory action of lupeol on MMP-9 and COX-2 [48].

Recently, endoplasmic reticulum stress was found to contribute to the activation of STAT3 and NF-κB [49,50]. Given that MT1-MMP-mediated endoplasmic reticulum stress leads to COX-2 expression in human glioblastoma cells [51], and that COX-2 is overexpressed in a majority of gliomas [52,53], targeting MT1-MMP/STAT/COX-2 signaling in tumourigenesis may therefore be considered an attractive therapeutic avenue. Paradoxically, the effectiveness of direct COX-2 inhibitors on glioma cell proliferation and radioresponse enhancement has been shown to be independent from COX-2 protein expression [54]. This evidence suggests that alternate initiator molecules, possibly involving cell surface transducing mechanisms, are associated with therapy resistance and involved in the regulation of COX-2 expression. Whether MT1-MMP, or any cell surface carbohydrate structures, are directly involved in such regulation remains to be confirmed. Nevertheless, given that endoplasmic reticulum stress-induced autophagy mechanisms still remain controversial [55], it becomes tempting to suggest that targeting MT1-MMP pleiotropic intracellular transducing functions that, among other targets, lead to COX-2 expression may help design or optimize current therapeutic strategies.

Finally, studies have indicated the ability of stem cell populations, including MSC, to downregulate immune responses in vitro and in vivo [56]. MSC have recently been reported to inhibit naive and memory antigen-specific T cells [57]. The immunosuppressive qualities of MSC, which may facilitate evasion of the immune system by a tumour, may in part involve major histocompatibility complex (MHC) class I. Interestingly, phenotypic characterization of MSC by flow cytometry showed expression of MHC class I alloantigens, but failed to elicit T cell proliferative responses due to active suppressive mechanisms [58]. Recently, shedding of the tumour cell surface MHC class I chain-related molecule A by MT1-MMP was demonstrated to regulate sensitivity of tumour cells to NK cell killing, a process which may add to tumour immune evasion and contribute to tumour progression [59]. Such cell surface proteolytic activity of MT1-MMP was also shown in MSC to contribute to cleavage of CD44, another cell adhesion molecule expressed at the cell surface of MSC, and to promote cell migration [60,61].

In conclusion, our study provides unexpected but original evidence linking the signaling functions of MT1-MMP to the JAK/STAT pathway. No evidence for neither PI3K nor p38/MAPK crosstalk in ConA-mediated STAT3 phosphorylation could be demonstrated in our hands, although some involvement of MEK could be inferred but will require more investigation (Fig. 1F). Given that the cooperativity between STAT3 and NF-κB transcription factors regulates a highly overlapping repertoire of pro-survival, proliferative, and pro-angiogenic genes associated with tumor progression [26], multi-targeted inhibitory approaches should thus offer greater therapeutic efficacy. Our data support a new molecular signaling axis balance between STAT3 and NF-κB, through the intracellular domain-mediated signaling of MT1-MMP and that may impact on COX-2 expression (Fig. 4). How the phosphorylated forms of STAT3 and NF-κB interact in the nucleus has however yet to be elucidated. Interestingly, STAT3 has been implicated in inhibiting IKK activity in normal immune cells [62], and this piece of published evidence strongly supports our present observations on how the

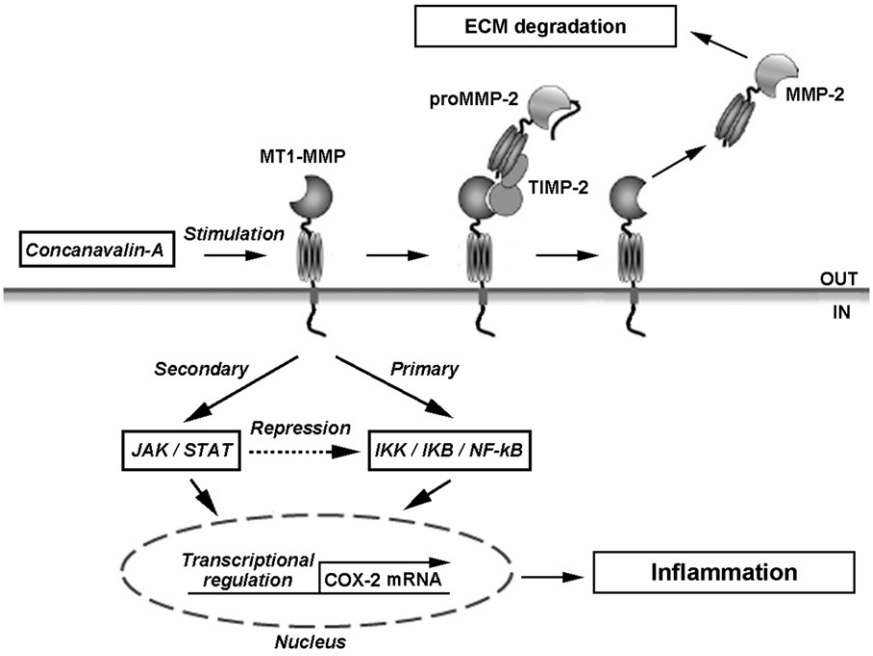

Fig. 4 – Schematic representation of MT1-MMP dual transcriptional regulation roles of lectin-induced inflammation. Concanavalin-A treatment of MSC requires MT1-MMP cell surface functions that ultimately trigger activation of latent proMMP-2 into active MMP-2 and lead to extracellular matrix (ECM) degradation. MT1-MMP is also thought to signal intracellular JAK/STAT- and IKK/IκB/NF-κB-mediated transcriptional regulation of COX-2 gene expression leading to the acquisition of an inflammatory phenotype. According to our current hypothesis, silencing of the JAK/STAT signaling axis amplifies MT1-MMP's ability to trigger COX-2 expression, therefore establishing the IKK/IκB/NF-κB signaling pathway as the primary contributor to MT1-MMP-mediated inflammation while JAK/STAT signaling may serve as a secondary contributor and exert some repressive regulation on IKK/IκB/NF-κB through yet unidentified mechanisms.

relief of JAK/STAT repressive effects on IKK/IκB/NF-κB leads to increased ConA-mediated COX-2 expression. Our study reconciles the roles of STAT3 and NF-κB in mediating the complex interactions between the tumor and its immune microenvironment, and supports some aspects of the oncogenic and immunomodulatory adaptive mechanisms that could eventually be targeted in MSC's contribution to hypoxic tumour development.

Conflict of interest statement

The authors declare no conflict of interest.

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