Session 8: *Mechanism of Fibrosis*

S.8.2

SIGNAL TRANSDUCER AND ACTIVATOR OF TRANSCRIPTION 3 (STAT3) REGULATES TRANSFORMING GROWTH FACTOR-BETA INDUCED FIBROSIS IN SYSTEMIC SCLEROSIS

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Background. Systemic sclerosis (SSc) is an autoimmune disease characterized by uncontrolled activation of fibroblasts resulting in excessive accumulation of collagen. TGFβ is considered as a crucial participant in the pathogenesis of SSc. Signal transducer and activator of transcription 3 (STAT3) is a transcription factor modulating the expression of targeted genes. STAT3 is activated by phosphorylation by several receptors tyrosine kinases, particularly by Janus kinase 2 (JAK2). The aim of this study was to evaluate the role of STAT3 in TGFβ signaling and its potential as a novel anti-fibrotic target.
Methods. Activation of STAT3 in human skin and murine models was analyzed by IF staining for STAT3 and phosphorylated STAT3 (pSTAT3). Specific inhibitors of JAK2 and STAT3 and knockdown strategies were used to study the STAT3 signaling *in vitro* and *in vivo*. The potential anti-fibrotic effect of STAT3 inhibition was evaluated in two mouse models of SSc: bleomycin-induced fibrosis and fibrosis induced by overexpression of a constitutively active TGFβ receptor type I (TBR).
Results. Increased activation of STAT3 signaling with accumulation of pSTAT3 was observed in the skin of SSc patients and in murine models of SSc. Stimulation with TGFβ increased the expression of STAT3 protein and induced nuclear accumulation of pSTAT3 in human fibroblasts. Inhibition of JAK2 by selective inhibitor TG101209 abrogated the TGFβ induced activation of STAT3 as well as nuclear accumulation of pSTAT3, demonstrating that TGFβ activates STAT3 in a JAK2 dependent manner. Inactivation of STAT3 with the selective STAT3 inhibitor S3I-201 significantly abrogated the TGFβ induced activation of human fibroblasts by reduction of Col1a1 (-71 %, p=0.0095) and Col1a2 (-35 %, p=0.0095) mRNA levels, collagen release (-56 %, p=0.05) and myofibroblast differentiation. The same results were observed when STAT3 was inactivated by conditional knockout in murine fibroblasts. In the model of bleomycin induced fibrosis, treatment with S3I-201 decreased dermal thickening by 33 % p=0.0009), hydroxyproline content (HP) by 51 % (p=0.001) and myofibroblast counts by 55 % (p=0.0009). Anti-fibrotic effects with reduced dermal thickening, decreased HP content and reduced myofibroblast differentiation were also observed in TBR induced fibrosis.
Conclusion. We demonstrate for the first time the role of STAT3 in SSc. We showed that STAT3 serves as a downstream mediator of TGFβ. Inhibition of STAT3 prevented fibroblast activation and demonstrated potent anti-fibrotic effect in two different preclinical models of SSc. Our findings may have direct translational implications as several STAT3 inhibitors are currently in clinical trails.

S.8.3

INVESTIGATING THE ROLE OF MYOCARDIN RELATED TRANSCRIPTION FACTOR (MRTF) INSYSTEMIC SCLEROSIS (SSc)

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MRTF-A is a 120kDa transcription factor widely expressed and normally sequestered in the cytosol by binding to G actin. Following actin polymerisation downstream of Rho signalling, MRTF-A is released and functions as a signalling molecule partnering serum response factor, influencing gene transcription via CARG elements. Genes expressing CARG like elements induced by MRTF-A/SRF include CTGF and type I collagen. The MRTF-A/SRF axis is highly relevant to SSc.
MRTF-A signal transduction was studied in healthy control and SSc fibroblasts. The MRTF-A/SRF small molecule inhibitor CCG1423 was used to block MRTF-A in vitro. SSc fibrotic responses were modelled by collagen gel contraction, CTGF, and type I collagen expression. MRTF-A signalling was assayed by Western blotting of nuclear and cytoplasmic extracts. Wound healing and fibrosis was studied in an MRTF-A knockout mouse and wild type. Immunochemistry looking for nuclear localisation of MRTF-A was used to determine presence of active signalling in SSc involved skin and healthy control.
SSc fibroblasts showed enhanced nuclear localisation of MRTF-A at 8 hours following exposure to TGFβ (4ng/ml) not seen in control fibroblasts. Immunochemistry of SSc skin biopsy revealed enhanced nuclear localisation in dermal fibroblast like cells, keratinocytes within the epidermis, as well as in perivascular cells. Following excisional wounding MRTF-A mice wounds failed to close normally and increased in size during days 1-7, wound area decreasing by day 11. When compared to wild type, MRTF-A knockout wounds were enlarged at day 7 (wild type area 6mm2, knockout area 12.4 mm2, p<0.03), and at day 11 (wild type area 0.42 mm2, knockout area 3.4 mm2, p<0.01). Day 11 wounds were extracted and found to exhibit abnormal histology,showing reduced scar formation, and altered vasculogenesis. Small blood vessels within the granulation tissue were dilated, and exhibited extravasation of red blood cells. Gel contraction by wild type fibroblasts was enhanced by TGFβ and blocked by CCG1423 1μM (basal conditions mean gel mass =0.176g, TGFβ treated = 0.118g, TGFβ+CCG1423 =0.238g, p<0.002). Dermal fibroblasts from MRTF-A knockout mice showed reduced basal gel contraction, and impaired response to TGFβ, (basal conditions mean gel mass =-0.349g, TGFβ treated =0.259g, TGFβ+CCG1423 =0.313g (p<0.05 basal vs wild type). Studies of belomycin induced skin fibrosis in MRTF-A -/- mice are ongoing.
MRTF-A signalling is abnormal in SSc involved skin. MRTF-A knockout mice fail to contract wounds adequately and show reduced scar formation, as well as abnormal vasculogenesis. CCG1423 and derivatives may be potential anti-fibrotics and of benefit in SSc.

S.8.4

EPHRIN B2 IS OVEREXPRESSED IN HUMAN SCLERODERMA SKIN AND MEDIATES FIBROBLAST TO MYOFIBROBLAST DIFFERENTIATION, AND INDUCES FIBROSIS IN MICE

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Ephrin B2 is a member of ephrin family belonging to the largest sub-family of membranous receptor protein-tyrosine kinases. The role of ephrin B2 in the pathophysiology of scleroderma (SSc) disease is largely unknown. In the present study we explored the potential of ephrin B2 in mediating fibroblast-myofibroblast differentiation and fibrosis associated with the pathophysiology of SSc disease.
Our immunohistochemistry, Real-Time PCR and western blot analysis show that Ephrin B2 expression was elevated in SSc skin compared to normal human skin. Further, ELISA results showed enhanced ephrin B2 production in SSc skin fibroblasts compared to fibroblasts isolated from healthy donors. In addition, the expression of ephrin B2 receptor, ephB4, is elevated in SSc skin compared to normal human skin. Interestingly, we identified that *in vitro* treatment of normal human skin fibroblasts with recombinant ephrin B2 is able to transform fibroblasts into myofibroblastic cells exhibiting all typical myofibroblastic-characteristics including increased stress fibre formation, increased cell spreading and focal adhesions, increased activation of focal adhesion kinase (FAK, a critical mediator of fibroblast to myofibroblast differentiation) and increased expression of alpha-smooth muscle actin (alpha-SMA) expressing myofibroblasts. In addition, treatment with the recombinant ephrin B2 is able to enhance fibroblast functions including increased rate of fibroblast migration and adhesion to fibronectin in both normal and SSc skin fibroblasts.
Mice were then injected subcutaneously with recombinant mouse ephrin B2/Fc (100ug/Kg/mouse) daily for two weeks and degree of fibrosis was determined. Mice treated with recombinant mouse ephrin B2/Fc exhibited significant skin fibrosis associated with enhanced collagen deposition, dermal thickness, hydroxyproline content, alpha-SMA-expressing myofibroblasts and increased expression of p-FAK, type I collagen and CTGF. We then generated fibroblast-specific ephrin B2 knockout mice (KO) mice in which Cre is under the control of a fibroblast-specific regulatory sequence from the pro-alpha-2(I) collagen gene to achieve ephrin B2 inactivation specifically in the fibroblasts. Wild type mice and ephrin B2 mice were subjected to bleomycin-induced skin and lung fibrosis. Results showed that all ephrin B2 KO mice showed significant protection from bleomycin-induced skin and lung fibrosis associated with significant reduction

in dermal thickness, skin fibrosis, lung fibrosis, collagen synthesis, alpha-SMA expression and phosphorylation of FAK.
Our study, for the first time, provides compelling evidence that ephrin B2 is a key mediator of fibroblast to myofibroblast differentiation and targeting ephrin B2 could open up new potential therapeutic avenues to counteract fibrotic and adhesive signalling associated with SSc and related diseases.

S.8.5

EXPERIMENTAL RENAL INJURY IN A TGFBETA DEPENDENT MOUSE MODEL OF SCLERODERMA

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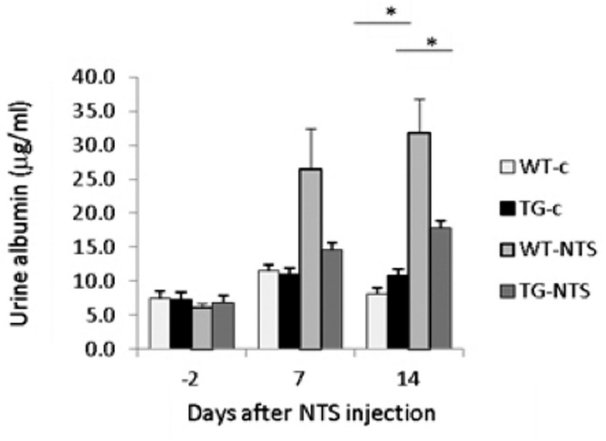
Purpose. Accelerated hypertension and rapidly progressive renal dysfunction are hallmarks of scleroderma renal crisis, a predominantly vascular and fibrotic condition without major inflammatory features compared with other autoimmune rheumatic diseases. No current animal model of scleroderma (SSc) develops this complication. The TβRIIδk-fib transgenic mouse model of SSc constitutively develops hypertension and large vessel adventitial fibrosis without renal disease. The response to long-term elevation of blood pressure or an inflammatory renal insult has not been studied. We have therefore explored the link between altered TGFβ bioactivity, vasospasm and inflammatory stress on the systemic vascular endothelium using NO synthase inhibition and the nephrotoxic nephritis model in this strain.
Methods. Histological assessment of cardiac and renal architecture, immunostaining for microvessel density and inflammatory cells and assessment of microalbuminuria by ELISA were performed on adult transgenic (TG) animals following treatment with either L-NAME or a single dose of nephrotoxic serum with pre-immunisation. Biochemical analysis of the TGFβ signalling pathway was performed assessing RNA and protein using whole organ isolates, and by immunostaining of tissue sections. Results were compared to appropriate TG and WT control groups.
Results. Increased cardiac mass and cardiac collagen measured by qPCR and Sircol® assay in TG and WT treated groups demonstrated that L-NAME treatment successfully induced hypertensive stress in this strain. Whole kidney lysates from L-NAME treated TG animals showed upregulated expression of Col1a1 (TG untreated copy number 3937±315, TG treated 6319±48, $p<0.05$) and Pai-1 (TG untreated 410±57, TG treated 740±74, $p<0.05$), and glomerulosclerosis was present on sirius red staining in the TG treated group, suggesting that these animals exhibited an enhanced renal fibrotic response when compared to WT treated animals. No other structural vascular changes were identified. In contrast, by day 14, TG animals had developed significantly less proteinuria following treatment with nephrotoxic serum (NTS) when compared with WT littermates. Examination of PAS stained samples showed glomerular damage in both WT and TG animals treated with NTS, with increased severity and number of damaged glomeruli in WT treated mice compared with TG.



Conclusions. This mouse model of scleroderma demonstrates exaggerated fibrotic response to hypertensive injury and is relatively resistant to experimental glomerulonephritis. This is likely to be a consequence of increased tissue levels of TGFβ. Both of these processes may underpin the unique vascular pathology seen in scleroderma renal crisis, this mouse strain provides a platform for further studies of renal injury in scleroderma.

S.8.6

DIRECT THROMBIN INHIBITOR DABIGATRAN ETEXILATE PROTECTS ALVEOLAR EPITHELIAL CELLS FROM APOPTOSIS IN A BLEOMYCIN MODEL OF SCLERODERMA-ASSOCIATED INTERSTITIAL LUNG DISEASE

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Background/Aims. Apoptosis of alveolar epithelial cells (AEC) is an important early event implicated in the pathogenesis of scleroderma-associated interstitial lung disease (SSc-ILD). However, the mechanisms underlying AEC apoptosis remain obscure. We previously demonstrated that the direct thrombin inhibitor dabigatran etexilate has marked anti-inflammatory and anti-fibrotic effects in vitro and in vivo in a bleomycin murine model of SSc-ILD. The aim of this study was to investigate the effects of dabigatran etexilate on apoptosis of AEC.
Materials and Methods. Lung injury was induced in 6-8 week old female C57BL/6 mice by a single intratracheal (IT) instillation of bleomycin. Dabigatran etexilate was given as supplemented chow beginning on day one following bleomycin instillation. Mice were euthanized one, two, and three weeks after IT bleomycin instillation and lung tissue, isolated AEC, bronchoalveolar lavage fluid (BALF), and plasma were investigated. Apoptosis was measured by ELISA and in situ cell death detection assay. Caspase-3, CCAAT enhancer-binding homologous protein (CHOP), immunoglobulin-binding protein (BiP), and activating transcription factor 4 (ATF4) were studied by immunoblotting and immunofluorescent staining. Reactive oxygen species (ROS) were measured by flow cytometry. The level of active thrombin in BALF was routinely monitored using thrombin substrate N-Benzoyl-Phe-Val-Arg-p-nitroanilide (Sigma) by a spectrophotometric method.
Results. In control mice receiving IT saline alone or IT saline plus dabigatran etexilate, alveolar structures were composed mostly of elongated type 1 AEC with few cuboidal type 2 AEC expressing surfactant protein C (SPC). Alveoli of bleomycin-treated mice were characterized by presence of multiple AEC type 2 cells similarl to what we have observed in alveoli of SSc-ILD patients. Lung tissue isolated 7 and 14 days after bleomycin treatment exhibited extensive apoptosis of AEC confirmed by TUNEL and caspase-3 positive staining. SPC-positive AEC were characterized by the presence of ROS and ER stress markers (BiP, ATF4, and CHOP). In contrast, significantly less apoptosis, lower amounts of ROS, and reduced ER stress markers were observed in bleomycin treated mice receiving dabigatran etexilate. Primary AEC cells isolated one and two weeks after bleomycin instillation continued to express high amounts of CHOP and caspase-3, which were not detectable by Western blotting in cells isolated from control mice. By contrast, significantly lower expression of CHOP and caspase-3 was detected in bleomycin treated mice receiving dabigatran etexilate.
Conclusions. We conclude that dabigatran etexilate reduces apoptosis of AEC by blocking reactive oxygen species and by decreasing endoplasmic reticulum stress in these cells.

S.8.7

STIMULATION OF THE SOLUBLE GUANYLATE CYCLASE (SGC) INHIBITS DERMAL FIBROSIS BY BLOCKING NON-CANONICAL TGF-BETA-SIGNALING

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Background. The soluble guanylate cyclase (sGC) converts GTP to cGMP to regulate vascular tone and homeostasis. The sGC stimulator riociguat has recently demonstrated high efficacy and excellent tolerability in phase 3 clinical trials for pulmonary arterial hypertension (PAH) and chronic thrombembolic pulmonary hypertension (CTPH). In the current project, we investigated a novel anti-fibrotic role of the sGC in systemic sclerosis (SSc).
Methods. Normal and SSc fibroblasts as well as sGC-knockout fibroblasts were treated with the sGC stimulator BAY41-2272 (lead compound of riociguat) or

the stable cGMP analogue 8-Bromo-cGMP and stimulated with TGFβ. Crosstalk between sGC signaling and TGFβ signaling was studied by levels of phosphorylated SMAD2 and 3 (IF, WB), SMAD-reporter activity and target gene expression. In vivo, we investigated the anti-fibrotic activity and the tolerability of sGC stimulation in bleomycin-induced skin fibrosis, tight skin-1 mice, and mice challenged with an adenovirus expressing a constitutively active TGFβ receptor I (TBR model).
Summary of the results. When assessing the anti-fibrotic activity of the sGC, we observed that sGC stimulation by BAY41-2272 inhibited TGFβ-dependent fibroblast activation and collagen release from SSc and healthy fibroblasts in a dose dependent manner. In addition, sGC stimulation was effective in preventing the development of skin fibrosis and reversing established skin fibrosis in the bleomycin model and in tight skin-1 mice. sGC stimulation was well-tolerated and did not have significant effects on systemic blood pressure and heart rate as indicated by telemetry studies. Mechanistically, sGC knockout fibroblasts confirmed that the sGC is essential for the anti-fibrotic effects of BAY41-2272. Furthermore, we observed that 8-Bromo-cGMP mimicked the effects of BAY41-2272 and reduced TGFβ-dependent collagen release. Nuclear p-SMAD2 and 3 levels, SMAD-reporter activity, and transcription of classical TGFβ target genes remained unchanged upon sGC stimulation, suggesting that the anti-fibrotic sGC activity is independent of canonical TGFβ-signaling. In TGFβ-driven experimental fibrosis (TBR model), sGC stimulation inhibited TGFβ-driven fibroblast activation and collagen release, but did not change p-SMAD2 and 3 levels and TGFβ target gene expression, confirming that non-canonical TGFβ cascades mediate the anti-fibrotic sGC activity.
Conclusions. We identified a novel anti-fibrotic role of the sGC. sGC activity increases cGMP levels, blocks non-canonical TGFβ signaling and inhibits fibrosis in various model systems of SSc. Since sGC stimulators have shown excellent efficacy and tolerability in phase 3 clinical trials for PAH and CTPH, they may be further developed for the simultaneous treatment of fibrosis and vascular disease in SSc.

Session 10: *Cardiovascular Involvement*

S.10.1

CONDUCTION AND RHYTHM DEFECTS IN SCLERODERMA

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Signs or symptoms of arrhythmias or conduction defects are frequently reported in patients with systemic sclerosis. These rhythm disorders may have several origins (i.e. related to primary heart involvement, pericardial disease, valvular regurgitation, pulmonary arterial hypertension…) and may negatively affect the overall prognosis of these patients. It is important to identify patients at high risk for cardiac arrhythmias thanks to a complete cardiologic evaluation, find out the underlying heart disease including SSc related myocardial involvement; in addition, some therapeutics options in SSc patients may differ from that are recommended on other population

S.10.2

IMPROVEMENT OF DIGITAL ULCERATIVE DISEASE IN PATIENTS WITH SYSTEMIC SCLEROSIS IS ASSOCIATED WITH BETTER FUNCTIONAL PROGNOSIS

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Background/Objective. Ischemic digital ulcers (DU) represent a major complication of systemic sclerosis (SSc) leading to hand disability. We investigated the impact of controlling the ulcerative disease on hand disability and quality of life after one year in SSc patients treated with bosentan.
Methods. ECLIPSE is a 2-year prospective, observational study. Patients with SSc who experienced at least one DU in previous year and received bosentan were included between October 2009 and March 2011. Demographical and clinical data were collected at inclusion and at 1 year, as well as disability scores (Cochin hand function scale (CHFS), health assessment questionnaire disability index (HAQ-DI)), pain score (Visual Analog Scale), and quality of life (SF-36). A controlled ulcerative disease was defined by the absence of new ulcer between inclusion and one-year follow-up. Data are presented as means ± standard deviations.
Results. Follow-up data were available at one year for 120 patients out of the 190 included patients. Patients' characteristics were similar to those of the overall cohort. Mean ages at inclusion and SSc diagnosis were 54±15 and 44±15 years, respectively. SSc was diffuse in 42% of the cases. At inclusion, patients had been receiving bosentan for 15.6±22.1 months. During the one-year follow-up, 46 (38%) patients experienced a new DU and the incidence of the event was 0.6 event/patient-year [95% confidence interval: 0.44-0.81]. Nevertheless, the proportion of patients with DU decreased from 61% to 22% and the number of DU per patient decreased from 1.4±1.8 to 0.6±1.6 (p<0.0001). This diminution was associated with a significant decrease in disability scores from 29.4±20.1 to 25.0±20.2 (p=0.005) on the CHFS and from 0.96±0.68 to 0.88±0.73 (p=0.04) for the HAQ-DI; the pain score decreased from 4.3±3.1 to 2.9±2.8 (p<0.0001). Improvements in the physical and mental components of the SF-36 were non-significant except for bodily pain (p=0.04) and mental health (p=0.01).
Patients with a controlled ulcerative disease (n=58) significantly improved CHFS (p=0.04), HAQ-DI (p=0.04), and physical component of the SF-36 (p=0.05) compared with patients with an uncontrolled disease (n=62).
During the one-year follow-up, 21 (17%) patients discontinued bosentan for an adverse event including 5 patients presenting elevated aminotransferases.
Conclusion. In patients with SSc receiving bosentan, a controlled ulcerative disease is associated with a significant attenuation of disability.