OP0223 INHIBITION OF CASEIN KINASE-2 ALLEVIATES THE PROFIBROTIC EFFECTS OF TRANSFORMING GROWTH FACTOR B IN SYSTEMIC SCLEROSIS

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Background: Casein kinase-2 (CK2)is a ubiquitous, highly conserved serine/threonine kinase. CK2 presents as a tetramer composed of 2 catalytic subunits (α orα') and 2β regulatory subunits, whichareessential for cell viability. Meanwhile, JAK-STAT signaling is involved in the regulation of cell survival, proliferation, and differentiation. Recently, we have shown thattargeting of JAK2 might be an interesting molecular approach for the treatment of systemic sclerosis (SSc). However, the role of CK2 in SSc and the functional relationship between CK2 and JAK-STAT signaling in SSc have not been established.
Objectives: The purpose of the study was to characterize whether CK2 contributes to the pathologic activation of fibroblasts in patients with SSc, to evaluate the anti-fibrotic potential of CK2 inhibition for the treatment of SSc, and to investigate the CK2-JAK2-STAT3 signaling interactions in fibrosis.
Methods: Activation of CK2, JAK2, and STAT3 in human skin and in experimental fibrosis were determined by immunohistochemical analysis. CK2 signaling was inhibited by the selective CK2 inhibitor 4, 5, 6, 7-Tetrabromobenzotriazole (TBB). The mouse models of bleomycin-induced and TGF-β receptor I (TBR)-induced dermal fibrosis were used to evaluate the anti-fibrotic potential of specific CK2 inhibition *in vivo*.
Results: Increased expression of CK2 was detected by immunohistochemistry in skin sections of SSc patients, particularly in fibroblasts. Inhibition of CK2 by TBB in cultured fibroblasts completely abrogated the stimulatory effects of TGFβ on collagen release ($p<0.05$). After TBB treatment, stress fiber formation and α-smooth muscle actin (α-SMA) expression in TGFβ-stimulated fibroblasts were significantly reduced by 97% ($p=0.0064$) and 69% ($p=0.0280$). Besides reduced fibroblast activation, western blot analyses showed almost complete normalization of phosphorylated JAK2 (pJAK2) levels in the cytoplasm and of phosphorylated STAT3 (pSTAT3) levels in the nucleus of TGFβ-treated fibroblasts upon pre-incubation with TBB ($p=0.0004$ and $p=0.0214$). In addition, treatment with TBB effectively prevented bleomycin-induced fibrosis in mice with decreased dermal thickness by up to 70% ($p<0.0001$) and efficient reductions in myofibroblast counts by up to 68% ($p=0.0002$). TBR-induced fibrosis in mice was strongly ameliorated by TBB with efficient reductions of dermal thickening by 75% ($p<0.0001$). Myofibroblast counts and hydroxyproline content also decreased by 59% and 40% ($p<0.0001$ and $p=0.0193$), respectively. In both murine models, we observed reduced pJAK2 and pSTAT3 expression as analyzed by immunohistochemistry.
Conclusions: We demonstrate that CK2 is activated in SSc and prove that inhibition of CK2 reduces canonical TGF-β signaling and prevents experimental fibrosis in different preclinical models. Considering the potent anti-fibrotic effects of CK2 inhibition, our study might have direct translational implications. These data provide first evidence that targeting CK2 may be a novel therapeutic approach for fibrotic diseases.
Disclosure of Interest: None Declared

OP0224 ANTI-AT1R AND ANTI-ETAR AUTOANTIBODIES FROM PATIENTS WITH SSC AND THEIR AGONISTIC EFFECTS

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Background: Autoantibodies reactive to angiotensin II type 1 receptor (AT_1R) and endothelin 1 type A receptor (ET_AR), anti-AT_1R and anti-ET_AR autoantibodies, are found in patients with Systemic sclerosis (SSc) as a prototypic connective tissue disease with limited therapeutic options. SSc is characterized by three major hallmarks: autoimmunity, vasculopathy and fibrosis. Anti-AT_1R and anti-ET_AR autoantibodies (Ab) have been demonstrated in association with clinical symptoms of SSc including vascular and fibrotic complications. Therefore, direct agonistic effects of anti-AT_1R and anti-ET_AR Ab were studied.
Objectives: To analyse anti-AT_1R and anti-ET_AR Ab-mediated agonistic effects in *in vitro* and *in vivo* experimental settings.
Methods: Human microdermal endothelial cells-1 (HMEC-1) were treated with IgG from SSc patients positive for anti-AT_1R and anti-ET_AR Ab, or with IgG of healthy donors (NC-IgG). To demonstrate angiotensin- and/or endothelin-receptor activation, cells were pre-treated with specific receptor inhibitors, either alone or in combination. Cell activation was measured by IL-8 chemokine expression, adhesion molecule VCAM-1 expression, trans-endothelial neutrophil migration and ROS activation using qRT-PCR, sandwich ELISA, cell culture inserts and fluorescence analysis. *In vivo* effects were measured by autoantibody transfer into healthy C57Bl/6J mice and analysis of cellular BALF composition.
Results: Upon treatment with anti-AT_1R and anti-ET_AR Ab positive SSc-IgG, HMEC-1 cells increased IL-8 mRNA levels and IL-8 protein secretion into culture supernatants compared to cells treated with NC-IgG. Levels of IL-8 mRNA and protein were decreased in samples that were pre-treated with specific angiotensin- and endothelin-receptor blockers. Culture supernatants with increased IL-8 protein levels due to SSc-IgG treatment, showed an increased potential to recruit neutrophils through an endothelial cell layer, compared to supernatants of NC-IgG treated samples. Again, specific angiotensin- and endothelin-receptor inhibition showed a reduction in neutrophil recruitment. Culture supernatants conditioned with SSc-IgG increased reactive oxygen species (ROS) production in healthy donor neutrophils compared to supernatants with control treatment. Furthermore, HMEC-1 cells showed also increased mRNA levels of VCAM-1 with significant reduction by receptor inhibition. Moreover, mice that received anti-AT_1R and anti-ET_AR Ab positive SSc-IgG showed significantly increased neutrophil number in BALF compared to NC-IgG.
Conclusions: Our findings demonstrate the potential of anti-AT_1R and anti-ET_AR Ab to induce pathogenic effects *in vitro* and *in vivo*. Activation of endothelial cells by anti-AT_1R and anti-ET_AR Ab might reflect in some aspects the situation as seen *in vivo* and could thereby be an important factor in disease pathogenesis. Furthermore, cell activation by these autoantibodies induced neutrophil recruitment *in vitro.* Finally, activation of neutrophil recruitment by anti-AT_1R and anti-ET_AR Ab positive SSc-IgG, demonstrated the potential to directly induce pathogenic effects in healthy mice *in vivo*. Thus, angiotensin- and endothelin-receptor activation by anti-AT_1R and anti-ET_AR Ab could significantly contribute to pathogenesis of SSc.
Disclosure of Interest: None Declared

OP0225 MICRORNAS UNDERLIE PLASMACYTOID DENTRITIC CELL DYSFUNCTION IN SYSTEMIC SCLEROSIS

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Background: Systemic sclerosis (SSc) is an autoimmune disease characterized by fibrosis of the skin and internal organs that carries a high burden of morbidity and mortality. Immune system dysfunction has been undisputedly demonstrated to underlie SSc pathogenesis. In particular, plasmacytoid dendritic cells (pDCs) not only circulate in very high number in SSc patients but also secrete high concentrations of specific chemokines that directly drive various features of SSc, including endothelial dysfunction and fibroblast activation.
Objectives: The aim of the study was to identify the molecular basis underlying pDC dysfunction and increased number in SSc patients.
Methods: pDCs were isolated from whole blood of early diffuse SSc, late diffuse SSc, limited SSc patients or healthy controls. The genome-wide profiling of microRNAs (miRNA) was performed on pDC total RNA by using the Illumina miRNA Profiling Array. Genes targeted by differentially expressed miRNAs were identified thanks to a combination of Miranda, Mirtar and MirPath prediction algorithms.
Results: miRNA screening demonstrated that seven miRNAs were significantly over- and two were under-expressed in pDCs from patients with early diffuse SSc compared to late diffuse disease, limited SSc or healthy controls and this profile perfectly correlated with pDC abundance in the different disease stages. According to Miranda, Mirtar and MirPath prediction algorithms, this set of 9 miRNAs is predicted to influence a vast number of target genes, mostly involved in three molecular pathways: 1) leukocyte apoptosis, 2) epidermal growth factor EGFR (ErbB) signaling and 3) WNT signaling. Interestingly, the emergence of the leukocyte apoptosis pathway might provide a preliminary hint on why pDCs are expanded early in SSc. On the other side, ErbB and WNT signaling are ultimately involved in immune response regulation and often linked to the malfunctioning of the immune system in SSc.
Conclusions: Altogether these unique observations suggest that SSc-associated miRNAs could strongly impact on cellular pathways involved in SSc pathology, and therefore they would provide an important target for disease prediction and therapeutic approaches.
Acknowledgements: This research was kindly supported by the ERC Starting European grant.
Disclosure of Interest: None Declared

OP0226 WIF1, A WNT PATHWAY INHIBITOR, IS SILENCED IN SYSTEMIC SCLEROSIS BY DNA DAMAGE: A MECHANISM LINKING DNA DAMAGE TO WNT AND FIBROSIS

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Background: Dysregulation of Wnt signaling is common in a variety of human malignancies, carcinogenesis, aging and fibrosis. Wnt signaling is tightly controlled by several negative regulators, such as WIF1 (Wnt inhibitor factor 1). Activation of canonical Wnt signaling has been recently found in fibrotic diseases included Systemic Sclerosis (SSc).
Objectives: The objective of the present work is to identify the mechanism responsible for the silencing of WIF1 in SSc.
Methods: Skin fibroblasts from SSc patients and normal controls were treated with bleomycin or ATM-HDAC inhibitors. Cells were transiently transfected with the siRNA against c-jun and ATF-3 with Lipofectamine (Invitrogen). Total

RNA was isolated and reverse-transcribed, according to the manufacturer's instructions (Bio-Rad). Quantitative real-time PCR reactions were performed using SYBR-Green PCR Master Mix (Bio-Rad). The relative expression levels were calculated using the $2^{-\Delta\Delta CT}$ method. To analyzed protein expression, cells were lysed with RIPA buffer and subjected to western blot with specific antibodies.

Results: Our data indicate that WIF1 is silenced by DNA damage and the check point kinase, ATM. Cell derived from SSc patients reactivate WIF1 expression if exposed to ATM or HDACI-III inhibitors. ROS and SSc immunoglobulins silence WIF1 expression via PDGF receptor, stimulate b-catenin accumulation by inducing ROS-dependent DNA damage. Bleomycin, a drug widely used to induce local skin fibrosis in vivo, silences WIF1 and stimulates Wnt signaling and its effects are suppressed by ATM or HDAC inhibitors. Silencing of WIF1 in normal cells amplifies Wnt signaling and increases collagen expression. As molecular actors that silence WIF1 in DNA damaged cells, we report that the knocking down of the expression of transcription factors ATF-3 and c-jun relieves WIF1 inhibition and dowregulates collagen expression in SSc cells. Bleomycin profibrotic phenotype is caused by activation of ATF-3 which silences WIF1 and amplifies Wnt signaling and by c-jun which cooperates with Wnt and stimulates collagen expression.

Conclusions: These results explain Wnt signaling hypertrophy in fibrotic disease, unveil a direct link between DNA damage and Wnt, and pave a novel route to treat fibrosis.

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Disclosure of Interest: T. Spadoni: None Declared, S. svegliati: None Declared, A. pezone: None Declared, G. marrone: None Declared, A. grieco: None Declared, G. moroncini: None Declared, A. Jüngel: None Declared, O. distler Grant/research support from: Actelion, Pfizer, Ergonex, BMS, Sanofi-Aventis, United BioSource Corporation, Roche/Genentech, medac, Biovitrium, Boehringer Ingelheim Pharma, Novartis, 4 D Science, Active Biotec and Sinoxa, Consultant for: Actelion, Pfizer, Ergonex, BMS, Sanofi-Aventis, United BioSource Corporation, Roche/Genentech, medac, Biovitrium, Boehringer Ingelheim Pharma, Novartis, 4 D Science, Active Biotec and Sinoxa, E. avvedimento: None Declared, A. gabrielli Grant/research support from: Roche, Actelion

OP0227 CRITICAL ROLE OF THE ADHESION RECEPTOR DNAX ACCESSORY MOLECULE-1 (DNAM-1) IN THE DEVELOPMENT OF INFLAMMATION-DRIVEN DERMAL FIBROSIS IN MOUSE MODEL OF SYSTEMIC SCLEROSIS

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Background: DNAX accessory molecule 1 (DNAM-1) is an adhesion factor involved in the adhesion and co-stimulation of T cells. DNAM-1 has been recently identified as a genetic susceptibility factor to systemic sclerosis (SSc) and also to other autoimmune diseases.

Objectives: Our aim was to investigate the contribution of DNAM-1 in the development of dermal fibrosis upon gene inactivation and targeted molecular strategies.

Methods: Human skin expression of DNAM-1 was determined by immunohistochemistry. Mice deficient for DNAM-1 ($dnam1^{-/-}$) and wildtype controls ($dnam1^{+/+}$) were injected with bleomycin or NaCl. Infiltrating leukocytes, T cells, B cells and monocytes were quantified respectively on hematoxylin and eosin stained sections and by immunohistochemistry for CD3, CD22 and CD68. Inflammatory cytokines were measured in lesional skin of $dnam1^{-/-}$ and $dnam1^{+/+}$ mice by flow cytometry. The anti-fibrotic potential of a DNAM-1 neutralizing monoclonal antibody (mAb) was also evaluated in the mouse model of bleomycin-induced dermal fibrosis.

Results: Overexpression of DNAM-1 was detected in the lesional skin of SSc patients, especially in perivascular inflammatory cells. $Dnam1^{-/-}$ mice were protected from bleomycin-induced dermal fibrosis with reduced dermal thickening (75±5% reduction, p=0.02), hydroxyproline content (46±8% decrease, p=0.02) and myofibroblast counts (39±5% reduction, p=0.04). The numbers infiltrating T cells were decreased in lesional skin of $dnam1^{-/-}$ mice by 69±15% (p=0.009). The number of B cells and monocytes was not significantly different in $dnam1^{-/-}$ and $dnam1^{+/+}$ mice upon bleomycin challenge. Moreover, $dnam1^{-/-}$ mice displayed in lesional skin decreased levels of inflammatory cytokines, such as IL-6 (59±12%, p=0.001 decrease), and TNFα (60±15%, p=0.03). Treatment with anti-DNAM-1 mAb significantly reduced dermal thickening by 64±6% (p=0.01), hydroxyproline content by 61±8% (p=0.001), and myofibroblast counts by 83±12% (P=0.03). These results were similar to those observed in DNAM-1 deficient mice.

Conclusions: We demonstrate with two complementary approaches that inhibition of DNAM-1 significantly ameliorates dermal inflammation-driven fibrosis. DNAM-1 displays profibrotic effects by promoting the infiltration of T cells, into lesional skin and by stimulating the release of inflammatory cytokines. In addition, molecular targeting strategy using a DNAM-1 neutralizing mAb confirmed potent antifibrotic properties of DNAM-1 inhibition. Our findings might have direct translational implications and inhibition of DNAM-1 might be a promising new approach for the treatment of SSc and potentially other fibrotic diseases.

Disclosure of Interest: None Declared

OP0228 PROTECTIVE EFFECT OF LPA1 AND 3 RECEPTOR ANTAGONISM IN EXPERIMENTAL SKIN FIBROSIS IS LINKED TO LPA ACTIVITY IN DERMAL FIBROBLASTS OF SSC PATIENTS

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Background: Lysophosphatidic acid (LPA) is a phospholipid generated by phospholipase D-mediated cleavage of phosphatidylcholine, mainly in adipocytes, fibroblasts or activated platelets. Knock down studies of the LPA1 receptor in lung and kidney fibrosis suggest a role of LPA in fibrosis.

Objectives: To investigate the potential of LPA1/3 receptor antagonism in Systemic Sclerosis (SSc) using both in vitro and in vivo approaches.

Methods: Primary cultures of dermal fibroblasts from patients with SSc and healthy controls were used for expression analysis and functional (Ca^{2+} + cytokines measurement) in vitro studies (n=5 each). In some experiments, cells were pre-treated overnight with Pertussis Toxin (50 ng/ml) to evaluate Gi protein involvement in LPA-induced Ca^{2+} response. The bleomycin skin model and the tight skin mouse model (Tsk-1) were used to assess the effects of the LPA1/3 antagonist SAR100842 (30 mg/kg BID) in vivo (n= 7 for each group) using established endpoints.

Results: SSc skin fibroblasts expressed mainly LPA1 receptors and LPA4 receptors to a lower extent. The SSc fibroblasts demonstrated a greater sensitivity to LPA-induced increase in cytosolic Ca2+ than normal dermal fibroblasts. The LPA-induced increase in cytosolic Ca2+ was inhibited by Pertussis Toxin (supporting the evidence of a Gi coupling) and was also fully antagonized with SAR100842 (confirming the role of LPA1 receptors). LPA also induced expression of aSMA in SSc as well as markers of fibrosis like PAI-1, cytokines (IL-6, IL-8, CXCL-1, CCL-2) and markers of the Wnt pathway (SFRP4 and WNT2). In the mouse model of bleomycin-induced skin fibrosis, using a therapeutic protocol, LPA1/3 antagonism by SAR100842 reversed significantly ($p<0.001$) dermal thickness, inhibited myofibroblast differentiation and skin collagen content. These effects of SAR100842 were comparable to that of imatinib which was used as a positive control. In the Tsk-1 model, SAR100842 significantly decreased dermal thickness, myofibroblast differentiation and skin collagen content comparable with those obtained after treatment with imatinib ($p < 0.05$). In addition, SFRP4 and WNT2 gene expressions were also inhibited in the skin of TSK1 mice. Interestingly, the latter two markers were both induced by LPA in dermal fibroblasts from SSc patients and have been reported to be increased in the skin of SSc patients. SAR100842 also significantly ($p<0.05$) reduced the expression of IL-13, associated with the TH-2 cytokine milieu found in SSc, as well as CXCL-1.

Conclusions: These results strongly implicate a role for LPA1 in the pathogenesis of skin manifestation of SSc and support the clinical development of SAR100842 in treating skin manifestation of SSc.

Disclosure of Interest: S. Illiano Employee of: Sanofi, L. Ledein Employee of: Sanofi, J.-P. Bidouard Employee of: Sanofi, M. Schaefer Employee of: Sanofi, H. Ruetten Employee of: Sanofi, P. Janiak Employee of: Sanofi, C. Beyer: None Declared, A. Distler: None Declared, C. Dees: None Declared, J. Distler Shareholder of: 4 D Science, Consultant for: Boehringer Ingelheim, Celgene, Bayer Pharma, Actelion, Pfizer, Ergonex, BMS, JB Therapeutics, Anaphore, Inc, Sanofi-Aventis, Novartis, Array Biopharma and Active Biotec in the area of potential treatments of scleroderma, O. Distler Consultant for: Actelion, Pfizer, Ergonex, BMS, Sanofi-Aventis, United BioSource Corporation, Roche/Genentech, medac, Biovitrium, Boehringer Ingelheim Pharma, Novartis, 4 D Science, Active Biotec and Sinoxa in the area of systemic sclerosis and related conditions