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Rheumatologie und klinische Immunologie
der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

„Funktionelle Analyse von Autoantikörpern bei Systemischer Sklerose: Rolle in der Pathogenität der Erkrankung“

zur Erlangung des akademischen Grades
Doctor rerum medicinalium (Dr. rer. medic.)

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von

Angela Kill

aus Dshetysaj, Kasachstan

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Einleitung

Vaskulopathie, entzündliche Fibrose und funktionelle Autoantikörper (Ak) sind Hauptmanifestationen der systemischen Sklerose, einer schwerwiegenden rheumatischen Erkrankung mit einer hohen Sterblichkeitsrate, begrenzten therapeutischen Möglichkeiten und ungeklärter Krankheitsursache. Wir haben Ak gerichtet gegen den Angiotensin II Typ 1 Rezeptor (AT_1R) und den Endothelin-1 Typ A Rezeptor (ET_AR) in Patienten mit systemischer Sklerose identifiziert. Klinische Untersuchungen zeigten Assoziationen dieser Ak mit charakteristischen Merkmalen der systemischen Sklerose. Wir stellen die Hypothese auf, dass diese Ak krankheitsverursachende Effekte auslösen können. Der Einfluss von anti-AT_1R und anti-ET_AR Ak zur Initialisierung von Entzündung und Fibrose wurde analysiert.

Methodik

Anti-AT_1R und anti-ET_AR Ak-positives Immunglobulin G (IgG) von Patienten mit systemischer Sklerose (SSc-IgG) wurde verwendet. IgG aus gesunden Spendern (NC-IgG) diente als Negativ-Kontrolle. Die Aktivierung von AT_1R und ET_AR wurde mittels Antagonisten inhibiert. Die Protein-Expression wurde mit ELISA und mRNA-Expression mittels Real Time-PCR gemessen. Die endotheliale Wundheilung wurde mittels einer Wundheilungs-Untersuchung und die Kollagen-Expression mittels Immunocytochemie bestimmt. Transendotheliale Migration von Neutrophilen wurde mittels Zellkultureinsätzen und Aktivierung von reaktiven Sauerstoffspezies mittels Immunfluoreszenz analysiert. Zelluläre Zusammensetzung und die Anzahl der Neutrophilen in bronchoalveolären Lavage-Fluiden (BALF) wurden mittels Durchlichtmikroskopie bestimmt nach passivem Transfer von SSc-IgG oder NC-IgG in naïve C57BL/6J Mäuse. Plasmaspiegel von KC (murines funktionelles Homolog zum humanen Interleukin-8) wurden mittels eines Suspensions-Test-System überprüft. Histologische Analysen wurden mittels Durchlichtmikroskopie durchgeführt.

Ergebnisse

Anti-AT_1R und anti-ET_AR Ak-positives SSc-IgG verursachte eine starke Aktivierung der humanen mikrovaskulären Endothelzellen (human microvascular endothelial cells-1, HMEC-1). Erhöhte Protein- und mRNA-Spiegel des pro-entzündlichen Chemokins IL-8 und erhöhte mRNA-Spiegel des vaskulären Zelladhäsionsmoleküls-1 wurden in HMEC-1 induziert. Darüber

hinaus, erhöhte die Aktivierung von HMEC-1 mit SSc-IgG die Transendotheliale Migration von Neutrophilen. Zudem zeigten Neutrophile, die mit Überständen von aktivierten HMEC-1 behandelt wurden, eine Aktivierung von reaktiven Sauerstoffspezies. SSc-IgG verminderte desweiteren die Wundheilung von HMEC-1 und verursachte unmittelbar eine Kollagenprotein-Typ I-Produktion in dermalen Fibroblasten. Effekte der Migration, Wundheilung und Kollagen-Produktion waren abhängig von Ak-Spiegeln. Passiver Transfer von anti-AT_1R und anti-ET_AR Ak-positiven SSc-IgG in naïve C57BL/6J Mäuse erhöhte die Neutrophilen-Anzahl in BALF. Parallel dazu, wurden erhöhte Spiegel von KC im Plasma von SSc-IgG-behandelten Mäusen gefunden ebenso wie strukturelle Veränderungen der Lungen.

Schlussfolgerungen

Wir kommen zum Schluss, dass die Aktivierung von Angiotensin- und Endothelin-Rezeptoren durch anti-AT_1R und anti-ET_AR Ak pathogene Effekte vermittelt und auf den Beitrag dieser Ak an der Pathogenese der SSc hinweist. Demzufolge können anti-AT_1R und anti-ET_AR Ak dabei helfen unser derzeitiges Verständnis dieser Erkrankung zu verbessern und dadurch neue Angriffsziele für therapeutische Intervention in der Behandlung von SSc zu erforschen.

Introduction

Vasculopathy, inflammatory fibrosis and functional autoantibodies (Abs) are major manifestations of systemic sclerosis (SSc) a severe rheumatic disease with high mortality rates, limited therapeutic options and unclear etiology. We have identified Abs directed against the angiotensin II type 1 receptor (AT_1R) and endothelin-1 type A receptor (ET_AR) in patients with SSc. Clinical analyses revealed an association with characteristic SSc features. We hypothesized that these Abs could facilitate pathogenic effects. The impact of anti-AT_1R and anti-ET_AR Abs on initiation of inflammation and fibrosis was analyzed.

Methods

Anti-AT_1R and anti-ET_AR Ab positive immunoglobulin G (IgG) from SSc patients (SSc-IgG) was used. Healthy donor IgG (NC-IgG) served as a normal control. AT_1R and ET_AR activation was inhibited by antagonists. Protein expression was measured by ELISA, mRNA expression by Real Time-PCR. Endothelial repair was measure by scratch assay and collagen expression by immunocytochemistry. Transendothelial migration of neutrophils was measured by a culture-insert-system and reactive oxygen species (ROS) activation by immunofluorescence. Cellular composition and neutrophil counts in bronchoalveolar lavage fluids (BALF) were analyzed microscopically after passive transfer of SSc-IgG or NC-IgG into naïve C57BL/6J mice. The plasma levels of KC (murine functional homologue to human interleukin-8), were quantified by a suspension array system. Histological analyses were performed using light microscopy.

Results

Anti-AT_1R and anti-ET_AR Ab positive SSc-IgG induced a strong activation of human microvascular endothelial cells (HMEC-1). Elevated protein and mRNA levels of the proinflammatory chemokine IL-8 and elevated mRNA levels of the vascular cell adhesion molecule-1 were induced in HMEC-1. Furthermore, activation of HMEC-1 with SSc-IgG increased the transendothelial neutrophil migration. Moreover, neutrophils exposed to supernatants of activated HMEC-1, showed an activation of ROS. SSc-IgG additionally decreased the wound repair of HMEC-1 and directly induced type I collagen production in fibroblasts. Effects of migration, wound repair and collagen expression were dependent on the Ab-levels. Passive transfer of anti-AT_1R and anti-ET_AR Ab positive SSc-IgG into naïve

C57BL/6J mice increased neutrophil BALF counts. In parallel, increased levels of the chemokine KC, were found in the plasma of SSc-IgG treated mice as well as structural alterations of the lungs.

Conclusions

We conclude that angiotensin and endothelin receptor activation via anti-AT_1R and anti-ET_AR Abs mediate pathogenic effects, indicating their contribution to pathogenesis of SSc. Therefore, anti-AT_1R and anti-ET_AR Abs could help to improve our current understanding of this disease and thereby provide novel targets for therapeutic intervention in the treatment of SSc.

Eidesstattliche Versicherung

„Ich, Angela Kill, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Funktionelle Analyse von Autoantikörpern bei Systemischer Sklerose: Rolle in der Pathogenität der Erkrankung“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

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Ausführliche Anteilserklärung an der erfolgten Publikation

Publikation : Kill A, Tabeling C, Undeutsch R, Kühl AA, Günther J, Radic M, Becker MO, Heidecke H, Worm M, Witzenrath M, Burmester GR, Dragun D, Riemekasten G. Autoantibodies to angiotensin and endothelin receptors in systemic sclerosis induce cellular and systemic events associated with disease pathogenesis. Arthritis Res Ther. 2014 Jan 28;16(1):R29.

Beitrag im Einzelnen:

i) Konzept und Planung: Diese wurden für die Erforschung der Fragestellung hauptsächlich gemeinsam mit Prof. Dr. med. Gabriela Riemekasten und mir erstellt. Desweiteren haben andere Ko-Autoren am Konzept und Planung mitgewirkt (für genaue Auflistung siehe „Author's contribution" in der Publikation). *ii) Durchführung der Experimente:* Für die experimentellen Untersuchung von Autoantikörpern reaktiv zu Angiotensin- und Endothelin-Rezeptoren aus Patienten mit systemischer Sklerose, habe ich erstmals ein System zur *in vitro* Analyse dieser Autoantikörper an humanen Endothelzellen, humanen Neutrophilen und humanen Fibroblasten, erfolgreich etabliert. Hierzu wurden vielfältige Techniken, die sich als Methoden in der Publikation wiederspiegeln, von mir erfolgreich etabliert und zu Beantwortung der Fragestellung angewendet. Alle beschriebenen *in vitro* Untersuchungen in der Publikation wurden von mir durchgeführt (siehe Fig. 1-4). *In vivo* Untersuchungen an Mäusen habe ich gemeinsam mit Ko-Autoren (C. Tabeling, R. Undeutsch, A. Kühl) durchgeführt (siehe Fig. 5). *iii) Aufarbeitung der Ergebnisse, statistische Auswertung und Interpretation:* Alle Daten zu *in vitro* Analysen wurden von mir aufgearbeitet und alle statistischen Untersuchungen von mir durchgeführt. Die Analyse der histologischen Ergebnisse aus *in vivo* Versuchen, wurden von mir durchgeführt. Die Interpretation aller Daten erfolgte durch mich überwiegend gemeinsam mit G. Riemekasten und zu einem geringen Anteil mit anderen Ko-Autoren. *iv) Erstellung der Publikation:* Das Manuskript zur Publikation wurde von mir erstellt und Korrekturen wurden vorwiegend durchgeführt durch Prof. Dr. med. Gabriela Riemekasten und in einem geringeren Anteil durch andere Ko-Autoren.

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☐	1	NAT REV RHEUMATOL	1759-4790	1921	9.745	9.318	1.446	65	2.4	0.01196	3.513
☐	2	ANN RHEUM DIS	0003-4967	27020	9.111	8.351	2.308	325	5.5	0.07203	2.510
☐	3	ARTHRITIS RHEUM-US	0004-3591	45200	7.477	7.630	1.659	411	8.0	0.08833	2.507
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☐	7	RHEUMATOLOGY	1462-0324	13184	4.212	4.558	1.107	298	5.5	0.03780	1.420
☐	8	SEMIN ARTHRITIS RHEU	0049-0172	3185	3.806	4.054	0.622	74	8.0	0.00599	1.234
☐	9	ARTHRIT CARE RES	2151-464X	8784	3.731	4.777	0.874	238	4.8	0.03122	1.549
☐	10	BEST PRACT RES CL RH	1521-6942	2138	3.550	3.693	0.373	59	5.5	0.00631	1.143
☐	11	J RHEUMATOL	0315-162X	21050	3.258	3.544	0.921	330	9.5	0.03286	1.070
☐	12	LUPUS	0961-2033	5089	2.783	2.736	0.608	237	5.8	0.01165	0.742
☐	13	JOINT BONE SPINE	1297-319X	2436	2.748	2.395	0.748	107	4.7	0.00700	0.663
☐	14	CLIN EXP RHEUMATOL	0392-856X	5909	2.655	2.312	0.655	252	6.2	0.01170	0.575
☐	15	SCAND J RHEUMATOL	0300-9742	2714	2.216	2.379	0.551	69	9.0	0.00426	0.685
☐	16	RHEUMATOL INT	0172-8172	3781	2.214	1.866	0.318	632	4.3	0.00984	0.474
☐	17	RHEUM DIS CLIN N AM	0889-857X	1728	2.096	2.697	0.170	47	9.1	0.00374	0.915
☐	18	CLIN RHEUMATOL	0770-3198	4559	2.037	1.866	0.373	249	5.3	0.01321	0.519
☐	19	BMC MUSCULOSKEL DIS	1471-2474	3196	1.875	2.305	0.149	261	4.1	0.01362	0.752
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Autoantibodies to angiotensin and endothelin receptors in systemic sclerosis induce cellular and systemic events associated with disease pathogenesis

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Angela Kill (angela.kill@charite.de)
Christoph Tabeling (Christoph.Tabeling@charite.de)
Reinmar Undeutsch (undeutsch@drfz.de)
Anja A Kühl (Anja.Kuehl@charite.de)
Jeannine Günther (guenther@drfz.de)
Mislav Radic (mislavradic@gmail.com)
Mike O Becker (Mike.Becker@charite.de)
Harald Heidecke (heidecke@celltrend.de)
Margitta Worm (margitta.worm@charite.de)
Martin Witzenrath (Martin.Witzenrath@charite.de)
Gerd-Rüdiger Burmester (gerd.burmester@charite.de)
Duska Dragun (Duska.Dragun@charite.de)
Gabriela Riemekasten (Gabriela.Riemekasten@charite.de)

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Autoantibodies to angiotensin and endothelin receptors in systemic sclerosis induce cellular and systemic events associated with disease pathogenesis

Angela Kill[1,2]
Email: angela.kill@charite.de

Christoph Tabeling[3]
Email: Christoph.Tabeling@charite.de

Reinmar Undeutsch[1,2]
Email: undeutsch@drfz.de

Anja A Kühl[4]
Email: Anja.Kuehl@charite.de

Jeannine Günther[1,2]
Email: guenther@drfz.de

Mislav Radic[2,5]
Email: mislavradic@gmail.com

Mike O Becker[2]
Email: Mike.Becker@charite.de

Harald Heidecke[6]
Email: heidecke@celltrend.de

Margitta Worm[7]
Email: margitta.worm@charite.de

Martin Witzenrath[3]
Email: Martin.Witzenrath@charite.de

Gerd-Rüdiger Burmester[2]
Email: gerd.burmester@charite.de

Duska Dragun[8]
Email: Duska.Dragun@charite.de

Gabriela Riemekasten[1,2,*]
Email: Gabriela.Riemekasten@charite.de

[1] German Rheumatism Research Centre (DRFZ), A Leibniz Institute, Berlin, Germany

[2] Department of Rheumatology and Clinical Immunology, University Hospital Charité, Luisenstraße 13, Berlin 10117, Germany

[3] Department of Infectious Diseases and Pulmonary Medicine, University Hospital Charité, Berlin, Germany

[4] Department of Inner Medicine, University Hospital Charité, Berlin, Germany

[5] Department of Rheumatology and Clinical Immunology, University Hospital Split, Split, Croatia

[6] CellTrend GmbH, Luckenwalde, Germany

[7] Department of Dermatology, University Hospital Charité, Berlin, Germany

[8] Department of Nephrology and Intensive Care Medicine, University Hospital Charité, Berlin, Germany

[*] Corresponding author. Department of Rheumatology and Clinical Immunology, University Hospital Charité, Luisenstraße 13, Berlin 10117, Germany

Abstract

Introduction

Vasculopathy, inflammatory fibrosis and functional autoantibodies (Abs) are major manifestations of systemic sclerosis (SSc). Abs directed against the angiotensin II type 1 receptor (AT_1R) and endothelin-1 type A receptor (ET_AR) are associated with characteristic disease features including vascular, inflammatory and fibrotic complications indicating their role in SSc pathogenesis. Therefore, the impact of anti-AT_1R and anti-ET_AR Abs on initiation of inflammation and fibrosis was analyzed.

Methods

Anti-AT_1R and anti-ET_AR Ab positive immunoglobulin G (IgG) from SSc patients (SSc-IgG) was used for experiments. Healthy donor IgG served as a normal control and AT_1R and ET_AR activation was inhibited by antagonists. Protein expression was measured by ELISA, mRNA expression by Real Time-PCR, endothelial repair by a scratch assay and collagen expression by immunocytochemistry. Transendothelial neutrophil migration was measured by a culture insert system and neutrophil ROS activation by immunofluorescence. Neutrophils in bronchoalveolar lavage fluids (BALF) were analyzed microscopically after passive transfer of SSc-IgG or NC-IgG into naïve C57BL/6J mice. KC plasma levels were quantified by a suspension array system. Histological analyses were performed using light microscopy.

Results

Anti-AT_1R and anti-ET_AR Ab positive SSc-IgG induced activation of human microvascular endothelial cells (HMEC-1). Elevated protein and mRNA levels of the proinflammatory chemokine interleukin-8 (IL-8, CXCL8) and elevated mRNA levels of the vascular cell adhesion molecule-1 (VCAM-1) were induced in HMEC-1. Furthermore, activation of HMEC-1 with SSc-IgG increased neutrophil migration through an endothelial cell layer and

activation of reactive oxygen species (ROS). SSc-IgG decreased HMEC-1 wound repair and induced type I collagen production in healthy donor skin fibroblasts. Effects of migration, wound repair and collagen expression were dependent on the Ab-levels. Passive transfer of anti-AT_1R and anti-ET_AR Ab positive SSc-IgG into naïve C57BL/6J mice increased neutrophil BALF counts. In parallel, increased levels of the murine functional IL-8 homologue, chemokine KC, were found in the plasma of SSc-IgG treated mice as well as structural alterations of the lungs.

Conclusions

We conclude that angiotensin and endothelin receptor activation via anti-AT_1R and anti-ET_AR Abs mediate pathogenic effects, indicating their contribution to pathogenesis of SSc. Therefore, anti-AT_1R and anti-ET_AR Abs could provide novel targets for therapeutic intervention in the treatment of SSc.

Introduction

Systemic sclerosis (SSc) is an autoimmune disorder with severe clinical manifestations, high mortality, and limited therapeutic options. Autoimmunity, vasculopathy, and fibrosis are hallmarks of the disease [1,2]. So far, mechanisms by which these hallmarks may be linked together are not well understood. Recent work from our group has shown that anti-AT_1R and anti-ET_AR Abs are present in SSc [3], and that elevated Ab levels in sera correlated with major disease manifestations, emphasizing their potential role in SSc pathogenesis. It is well established that microvascular damage, featuring endothelial cell dysfunction and perivascular infiltrates, is a key event in SSc pathogenesis, that appears early in the course of the disease and precedes fibrosis [4-6]. Inflammation also is a crucial event in SSc development and is reflected by abnormal chemokine and cytokine levels in sera and BALF [7-9], as well as by inflammatory infiltrates [2,4]. Of note are elevated levels of IL-8, both in sera and in BALF [7,9,10]. Furthermore, the latter were connected to neutrophilic alveolitis in SSc related interstitial lung disease [7,11], demonstrating a link between increased IL-8 levels and neutrophil accumulation.

Progressive fibrosis is characterized by amplified production of extracellular matrix (ECM) components including increased collagen synthesis by fibroblasts. SSc skin fibroblasts have been demonstrated to produce higher amounts of collagen when compared to skin fibroblasts from healthy donors [12,13]. Similarly, increased collagen expression was found in an animal model of SSc [14].

Lastly, an increased activation of the angiotensin and endothelin axis has been reported for in SSc [15-17]. Accordingly, we reasoned that anti-AT_1R and anti-ET_AR Abs could directly contribute to the initiation of inflammation and fibrosis *in vitro* and *in vivo* by activation of endothelial cells, fibroblasts and neutrophils and thus contribute to the key pathogenic manifestations of SSc. The objective of this study was to analyze the impact of functional anti-AT_1R and anti-ET_AR Abs on inflammatory and fibrotic events to help understand their role in disease pathogenesis.

Methods

Reagents

All reagents were purchased from Sigma Aldrich (Germany) if not otherwise stated.

Ethical admission for patient sample collection and performance of animal experiments

Serum was collected from venous blood after written informed consent and local ethics committee approval (EA1/013/705). Healthy donor skin was obtained by biopsy after written informed consent and approval by University Hospital Charité ethics committee (EA1/168/06). C57BL/6J mice were obtained from Charles River (Sulzfeld, Germany). Experiments were performed according to institutional and federal guidelines (Landesamt für Gesundheit und Soziales, Berlin, Germany).

Patients and healthy control donors

SSc patients with diffuse or limited SSc were classified according to LeRoy and ACR criteria [1,18]. Patients with established vasculopathy and/or fibrosis including pulmonary arterial hypertension (PAH), lung and skin fibrosis were chosen for IgG isolation that subsequently was used for experiments. Healthy control subjects served as negative controls. Identical IgG processing was used for SSc patients and healthy donors for serum collection and IgG isolation. For functional assays, individual IgG samples were used that were isolated from one serum sample as described below, followed by measurement of anti-AT_1R and anti-ET_AR Abs levels of each sample as previously reported [3]. For animal experiments, the same methods were used, exept that IgG was isolated from a pool of sera (from several patients or healthy donors) to provide enough material. Detailed patient and healthy donor control characteristics are summarized in Table 1.

Table 1 Patient and healthy donor characteristics

Parameter	SSc patients (n = 33)	Healthy donors (n = 13)
Mean age, years (SD)	55 (13)	43 (7)
Females / males, n (%)	24 / 9 (73 / 27)	9 / 4 (69 / 31)
Mean anti-AT_1R Abs, units (SD)	16 (8)	9 (4)
Mean anti-ET_AR Abs, units (SD)	15 (10)	6 (4)
Diffuse cutaneous form, limited cutaneous form, other, n (%)	21 (64), 10 (30), 2 (6)	n.a.
Scl 70 positive, n (%), n.d. n (%)	13 (39), 3 (90)	n.a.
Anti-Centromer positive, n (%), n.d. n (%)	4 (12), 4 (12)	n.a.
Duration since Raynaud's phenomenon, years (+/-SD)	10 (11)	n.a.
Duration since skin involvement onset, years (+/-SD)	9 (7)	n.a.
Duration since internal organ onset, years (+/-SD)	8 (7)	n.a.
mRSS median (IQR)	8 (4-18)	n.a.
Pulmonary arterial hypertension, n (%) *	14 (42)	n.a.
Lung fibrosis, n (%) **	18 (55)	n.a.
Mean DLCO% (SD)	50 (20)	n.a.
Mean FVC% (SD)	85 (18)	n.a.
animal experiments	**SSc patients (n = 14)**	**Healthy donors (n = 15)**
Mean age, years (SD)	57 (14)	47 (8)
Females / males, n (%)	11 / 3 (79 / 21)	12 / 3 (80 / 20)
Mean anti-AT_1R Abs, units (SD)	18 (10)	6 (4)
Mean anti-ET_AR Abs, units (SD)	17 (10)	4 (3)
Diffuse cutaneous form, limited cutaneous form, other, n (%)	10 (71), 3 (21), 0 (0)	n.a.
Scl 70 positive, n (%), n.d. n (%)	10 (71), 1 (7)	n.a.
Anti-Centromer positive, n (%), n.d. n (%)	2 (14), 3 (21)	n.a.
Duration since Raynaud's phenomenon, years (+/-SD)	11 (8)	n.a.
Duration since skin involvement onset, years (+/-SD)	10 (7)	n.a.
Duration since internal organ onset, years (+/-SD)	10 (9)	n.a.
mRSS median (IQR)	8.5 (5.3-11)	n.a.
Pulmonary arterial hypertension, n (%) *	8 (57)	n.a.
Lung fibrosis, n (%) **	8 (57)	n.a.
Mean DLCO% (SD)	42 (18)	n.a.
Mean FVC% (SD)	77 (19)	n.a.

n.d. = not defined, n.a. = not applicable.
* by > 35 mm Hg sPAP in echocardiography and/or 25 mm Hg mPAP in right heart catheterization.
** by HR-CT or X-ray.

Isolation of IgG and detection of anti-AT_1R and anti-ET_AR Abs

IgG was isolated by protein-G sepharose chromatography in 20 mM phosphate buffer pH 7.0. IgG was eluted with 0.1 M glycin/HCl, pH 2.7 and pH was neutralized with 1 M Tris/HCl, pH 9.0. Eluted IgG was dialyzed against PBS and absorbance was measured at 280 nm (Emax, Molecular Devices, U.S.A.). Anti-AT_1R and anti-ET_AR Abs were detected in purified IgG in cooperation with CellTrend GmbH (Germany) by a commercially available solid phase assay (One Lambda, Inc., U.S.A.) as described previously [3].

Cultivation and treatment of cells

Human microvascular endothelial cells-1 (HMEC-1) were serum-starved prior to all experiments in endothelial cell medium with IgG-free fetal calf serum (FCS) 0.5%, penicillin 100 U/mL, streptomycin 100 µg/mL, hydrocortisone 25 µM, epidermal growth factor 0.01 µg/mL and L-glutamine 10 mM. Cells were incubated in a humidified atmosphere at 5% CO_2

and 37°C. Human fibroblasts were isolated from healthy donor skin. Dermis was removed by dispase (4 mg/mL) and epidermis was digested with collagenase type 1A (1 mg/mL). Fibroblasts were cultivated in DMEM with IgG-free FCS 10%, penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin B 2.5 µg/mL. For all experiments passages three to eight were serum-starved prior to experiments in DMEM with IgG-free FCS 1%, penicillin 100 U/mL, streptomycin 100 µg/mL and amphotericin B 2.5 µg/mL. For transendothelial migration, neutrophils were freshly isolated from healthy donor blood as described before [19]. Isolated neutrophils were added to phosphate buffered saline (PBS)/IgG-free FCS 10% and their migration capacity was assessed by transwell culture inserts as described below. All reagents were purchased from PAA Laboratories (Germany) and Invitrogen (Europe). For all experiments that included receptor antagonism, inhibitors were added to cell cultures 18 and 3 hours prior to IgG treatment. The most effective antagonist concentration was determined in serial experiments. For individual receptor antagonism AT_1R was inhibited by valsartan and the ET_AR by the selective inhibitor sitaxentan. In parallel the ET_AR was inhibited by the dual antagonist bosentan. All antagonists were used at 10^{-5} M concentration, as described before [16,20]. Antagonists were also applied simultaneously by combination of valsartan and sitaxentan (each at 10^{-7} M), or valsartan and bosentan (at 10^{-5} M and 5×10^{-7} M, respectively). For inactivation of NFκB, tosyl-L-phenylalanine chlormethyl ketone (TPCK) was used (3×10^{-6} M) 30 min before IgG treatment. All antagonists were tested non-toxic, individually or simultaneously, in a cell-viability test (WST-8, Dojindo, Japan). Dose-dependent experiments were performed for IL-8 protein expression as described below (range of 0.125 mg/mL to 1.5 mg/mL IgG). An IgG concentration of 1 mg/mL was used in all experiments described. Of note, a similar IgG concentration has already been used previously [21]. Angiotensin II (Ang II) and endothelin-1 (ET-1) were used at 10^{-6} M and 10^{-8} M concentrations, respectively, and incubation times were the same as for IgG [16,20].

Scratch assay

For analysis of endothelial repair, uniform scratches were made by 1 ml pipette tip in confluent HMEC-1 layers as described before [22]. HMEC-1 cells were allowed to migrate into scratch areas to close wounds for 24 hours in the presence of SSc-IgG or NC-IgG. Cells were fixed in 96% ethanol, stained with haematoxylin and eosin (Merck, Germany), and light microscopy pictures were taken (Leica DMIL LED, LAS-EZ 2.0, Germany). Scratch areas were semi-quantified with ImageJ software by measuring relative scratch areas.

RNA, cDNA and Real-Time PCR

RNA was isolated from HMEC-1 48 hours after IgG treatment by NucleoSpin® RNA II (Macherey-Nagel, Germany) and cDNA was generated by M-MLV reverse transcriptase (Promega, Germany), each according to manufacturer's instructions. Real-Time PCR reactions contained 5 µL of cDNA, 0.25 mM dNTP (Bioline, Germany), 12 µg/mL bovine serum albumin, 1 × SYBR Green-I (Molecular Probes, Germany), 1 U Immolase (Bioline, Germany), 500 mM TRIS pH 8.8, 6 mM $MgCl_2$, 0.5 nmol/mL primer mix (TIB MOLBIOL, Germany) and were performed in MX3000P cycler (Stratagene, Europe). Primers were designed by Primer3 [23]. IL-8 forward 5′CAA-GAG-CCA-GGA-AGA-AAC-CA3′, reverse 5′ACT-CCT-TGG-CAA-AAC-TGC-AC3′. VCAM-1 forward 5′AAG-ATG-GTC-GTG-ATC-CTT-GG3′, reverse 5′GGT-GCT-GCA-AGT-CAA-TGA-GA3′. Eukaryotic translation elongation factor 1-alpha 1 (EEF1A1) was used as housekeeping gene, forward 5′GTT-GAT-ATG-GTT-CCT-GGC-AAG-C3′, reverse 5′GCC-AGC-TCC-AGC-AGC-CTT-C3′. Samples

were analyzed by MxPro-Mx3005P (Stratagene, Europe) and expression levels were normalized to the housekeeping gene.

Detection of IL-8 protein

HMEC-1 supernatants were collected 48 hours after IgG treatment. IL-8 was measured by sandwich ELISA. The coating antibody and the biotinylated detection antibody were obtained as a matched pairs kit from ImmunoTools (Germany). Recombinant human IL-8 was obtained from Biolegend (Germany) to generate a standard curve and streptavidin conjugated HRP were obtained from Biolegend (Germany). All reagents were used according to manufacturer's instructions. Absorbance was measured at 450 nm using the Emax microplate reader (Molecular Devices, U.S.A.). Data were analyzed by SoftMax Pro v5 (Molecular Devices, U.S.A).

Transendothelial neutrophil migration and measurement of neutrophil derived ROS

Supernatants from IgG-treated HMEC-1 (SSc-IgG or NC-IgG, 48 hours) were placed in multiwell TM 24 plates followed by confluent HMEC-1 on transwell culture inserts (3 µM pore size, all Becton Dickinson, Europe). Anti-IL-8 antibody (AB-208-NA, R&D Systems, Germany) was added to supernatants (10 µg/mL) 30 min before migration was assessed. Neutrophils were freshly isolated from healthy donor blood as described above and added to inserts (2×10^6 cells/insert). Migrated cells were counted automatically after 4 hours (CASY, Schärfe Systems, Germany). In parallel, neutrophils were isolated as described above and were loaded with DCFH-DA (2′,7′-Dichlorofluorescin diacetate, 25 µM in PBS/ IgG-free FCS 1%) in a humidified atmosphere at 5% CO_2 and 37°C. Loaded neutrophils were washed with 37°C warm PBS and $1x10^5$ neutrophils were added to 200 µL of HMEC-1 supernatants treated with IgG in a 96-well round bottom cell culture plate. After 30 min at 5% CO_2 and 37°C, cells were washed with PBS and fixed in 2% paraformaldehyde for 30 min at 4°C, washed again and taken up in 150 µL PBS. Cells were transferred into a white 96-well plate suitable for colorimetric analysis (F96 MicroWell^{TM}, Nunc, Germany) and generation of reactive oxygen species (ROS) was analyzed at an excitation wavelength of 485 nm and at an emission wavelength of 538 nm using a fluorescence reader (Fluoroskan Ascent, Thermo Labsystems, Germany).

Collagen detection

Confluent fibroblasts isolated from healthy donor skin were grown on glass chamber slides (Iwaki, Japan) and treated with SSc-IgG or NC-IgG for five days for maximum collagen expression. Cells were fixed in paraformaldehyde (2%) and triton X-100 (0.1%). Type I collagen immunocytochemistry was performed using a monoclonal primary antibody reactive to human collagen protein 1A (sc-59772, Santa Cruz, U.S.A.) and a secondary Cy3-conjugated antibody (C2181, Sigma-Aldrich, Germany). Nucleic DNA was stained by 4′,6-diamidino-2-phenylindole dihydrochlodride (DAPI). Monochrome fluorescence pictures were taken (Axioplan, Carl Zeiss MicroImaging GmbH, Germany) at identical illumination times and fluorescence intensity signals were analyzed relative to cell number by ImageJ software (NIH, U.S.A.).

Antibody transfer into naïve mice

Female 7-week-old C57BL/6J mice (n = 7/group) were maintained under specific pathogen-free conditions and received intravenously endotoxin-free pooled NC-IgG or SSc-IgG (800 µg, dissolved in 100 µl NaCl 0.9%) at day 0, as previously described for anti-AT_1R Abs from pre-eclampsia patients to study systemic events [24]. Pooled IgG-fractions were tested for anti-AT_1R and anti-ET_AR Abs as described above. NC-IgG fraction of low Ab levels (anti-AT_1R Abs units of 3.85 and anti-ET_AR Abs units of 2.5) and SSc-IgG fraction of high Ab levels (anti-AT_1R Abs units of 21.8 and anti-ET_AR Abs units of 17.91) were used for transfer. At day 7 mice were sacrificed, blood was harvested, and BALF of the right lung was collected using 2× 650 µl PBS. Leukocytes were counted and differentiated by means of microscopic analysis in a blinded fashion (800 cells counted/individual). KC plasma levels were quantified by Bio-Plex® array according to the manufacturer's guide. For repeated IgG-treatment female 8-week-old C57BL/6J mice (n = 7/group) were maintained as described above. Mice were treated with NC-IgG or SSc-IgG intravenously with pooled IgG at day 1, day 17, day 30 and 7 days before analysis for a total of 100 days (200µg, dissolved in 100 µl NaCl 0.9%). Histology analysis of the lungs was performed by paraffin embedding, Hematoxylin and Eosin (H&E) staining and light microscopy (Axioplan 2, Carl Zeiss MicroImaging GmbH, Germany).

Statistical analysis

Results were analyzed by GraphPad Prism® software (version 5.02) using Mann-Whitney U test (NC-IgG compared to SSc-IgG) and Wilcoxon signed-rank test (SSc-IgG compared to SSc-IgG with blockers). Correlation analyses were performed by nonparametric correlation (Spearman) and linear regression correlation. $^{**}p < 0.01$ and $^{*}p < 0.05$.

Results

Induction of IL-8 expression and release by endothelial cells

Analysis of HMEC-1 activation by anti-AT_1R and anti-ET_AR Abs positive SSc-IgG showed a secretion of the pro-inflammatory and pro-fibrotic chemokine IL-8 into culture supernatants. A dose-dependent pattern of IL-8 protein levels was found in HMEC-1 cells upon stimulation with 0.125 mg/mL to 1.5 mg/mL SSc-IgG with the highest response between 0.5-1.5 mg/mL IgG (Figure 1A), that was not present with NC-IgG. A comparison of SSc-IgG versus NC-IgG treatment revealed increased IL-8 levels with SSc-IgG treatment, with high variability in individual IgG samples ($p < 0.05$, Figure 1B). Increased IL-8 protein levels were reduced by individual as well as simultaneous receptor antagonism (ETR-A and ATR-A/ETR-A, each $p < 0.05$, Figure 1B). We further analyzed IL-8 expression on mRNA level and found a significant increase in SSc-IgG- over NC-IgG-treated cells ($p < 0.05$, Figure 1C). Elevated IL-8 mRNA levels were reduced by receptor antagonism as indicated (ATR-A and AT1R-A/ETR-A, $p < 0.05$ and $p < 0.01$ respectively, Figure 1C). Similarly, significantly increased mRNA levels of VCAM-1 were induced by SSc-IgG treatment compared to NC-IgG ($p < 0.01$, Figure 1D) and reduced by receptor antagonism (ETR-A and ATR-A/ETR-A, each $p < 0.05$ Figure 1D).

Figure 1 Activation of HMEC-1 by anti-AT_1R and anti-ET_AR Ab positive SSc-IgG on protein and mRNA levels. (A) Dose-dependent IL-8 secretion upon different doses of anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. Same treatment with NC-IgG fails to demonstrate a dose-dependent IL-8 secretion pattern. **(B)** Significant increase in IL-8 secretion with SSc-IgG versus NC-IgG and decrease with receptor antagonism (NC-IgG $n = 9$, SSc-IgG $n = 13$, $p < 0.05$). **(C)** Increase in IL-8 mRNA levels by SSc-IgG versus NC-IgG (NC-IgG $n = 7$, SSc-IgG $n = 18$, $p < 0.05$). Receptor antagonism leads to inhibition of IL-8 secretion as indicated (SSc-IgG $n = 13$, $p < 0.05$, $p < 0.01$). **(D)** Increase in VCAM-1 mRNA levels with SSc-IgG compared to NC-IgG (NC-IgG $n = 5$ and SSc-IgG $n = 6$, $p < 0.01$) and inhibition by receptor antagonism as indicated ($p < 0.05$). Mean and SEM, ** $p < 0.01$ and * $p < 0.05$.

Induction of IL-8 and Ab-level-dependent neutrophil transendothelial migration and ROS activation

Neutrophil recruitment and migration were analyzed by transendothelial migration and ROS generation. Supernatants of SSc-IgG treated HMEC-1 increased healthy donor neutrophil migration through an endothelial cell layer compared to supernatants of NC-IgG treated HMEC-1 ($p < 0.05$, Figure 2A). Neutrophil migration towards supernatants was significantly reduced by receptor inhibitors (ATR-A, ETR-A and ATR-A/ETR-A, all $p < 0.05$, Figure 2A). Addition of an IL-8 neutralizing antibody to SSc-IgG treated samples, as well as addition of NFκB inactivator TPCK, significantly decreased neutrophil transendothelial migration (each $p < 0.05$, Figure 2B). Finally, SSc-IgG conditioned HMEC-1 supernatants significantly increased generation of ROS in healthy donor neutrophils compared to NC-IgG conditioned supernatants or untreated controls ($p < 0.05$ and $p < 0.01$ respectively, Figure 2C). Statistical analyses revealed a significant correlation between neutrophil migration and anti-AT_1R and anti-ET_AR Ab levels ($r = 0.5849$ and $r = 0.7461$ respectively, $p < 0.05$ and $p < 0.01$ respectively, Figure 2D).

Figure 2 Neutrophil migration and ROS activation induced by anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. Supernatants of HMEC-1 conditioned with anti-AT_1R and anti-ET_AR Ab positive SSc-IgG induce activation and recruitment of neutrophils, measured by transendothelial migration and ROS generation. **(A)** Significantly increased transendothelial neutrophil migration with SSc-IgG conditioned supernatants compared to NC-IgG and decrease in samples with receptor antagonism as indicated (NC-IgG $n = 5$, SSc-IgG $n = 7$, $p < 0.05$). **(B)** Neutrophil migration is induced by IL-8 in supernatants shown by IL-8 neutralization (anti-IL-8 Ab) and NFκB inactivation (TPCK) (SSc-IgG $n = 7$, $p < 0.05$). **(C)** Increased ROS activation in neutrophils treated with SSc-IgG conditioned HMEC-1 supernatants compared to NC-IgG or untreated (NC-IgG $n = 8$, SSc-IgG $n = 12$, $p < 0.05$, $p < 0.01$). Mean and SEM, ** $p < 0.01$ and * $p < 0.05$. **(D)** Neutrophil migration shows a positive correlation to anti-AT1R Abs and anti-ETAR Abs in the IgG samples used (NC-IgG $n = 5$, SSc-IgG $n = 7$, total $n = 12$). Spearman correlation.

Influence on endothelial repair function

As we observed endothelial cell activation and neutrophil recruitment, we also analyzed the influence of anti-AT_1R and anti-ET_AR Abs on endothelial repair function. Artificially generated wounds in HMEC-1 layers were analyzed in a scratch assay. Reduced cell layer repair was reflected by a larger wound area in HMEC-1 treated with SSc-IgG compared to NC-IgG ($p < 0.01$, Figure 3A and 3B). Individual and simultaneous receptor antagonism

improved endothelial repair of SSc-IgG treated cells with significant scratch area reduction (ATR-A, ATR-A/ETR-A, all $p < 0.05$, Figure 3B). Correlation analyses showed a significant relationship between impaired endothelial repair, reflected by the wound area and anti-AT_1R and anti-ET_AR Ab levels ($r = 0.4111$ and $r = 0.4273$ respectively, $p < 0.05$, Figure 3C).

Figure 3 Diminished endothelial repair by treatment with anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. HMEC-1 treated with anti-AT_1R and anti-ET_AR Ab positive SSc-IgG reduce wound areas measured by a scratch assay. **(A)** Representative pictures of NC-IgG and of SSc-IgG treated cells and indicated inhibitors. **(B)** After 24 hours wound areas are significantly greater in SSc-IgG versus NC-IgG treatment (NC-IgG n = 6, SSc-IgG n = 6, $p < 0.01$) and are significantly reduced by antagonists as indicated ($p < 0.05$). Bar indicates 250 micron. Independent experiments were performed at least twice. Mean and SEM, ** $p < 0.01$ and * $p < 0.05$. **(C)** Wound area as measurement of endothelial repair shows a correlation to levels of anti-AT1R Abs and anti-ETAR Abs (NC-IgG n = 11, SSc-IgG n = 14, total n = 25). Spearman correlation.

Induction of collagen expression in healthy donor skin fibroblasts

Because pro-fibrotic events could be induced by anti-AT_1R and anti-ET_AR Abs positive SSc-IgG in HMEC-1 and neutrophil recruitment by IL-8, we additionally investigated pro-fibrotic effects on fibroblasts as major collagen expressing cells. Human fibroblasts were isolated from healthy donor skin and expression of type I collagen was measured by immunocytochemistry upon treatment with anti-AT_1R and anti-ET_AR Abs positive SSc-IgG or NC-IgG. Increased type I collagen expression was found with SSc-IgG treatment compared to NC-IgG (Figure 4A). Measurement of collagen intensity relative to cell number showed significantly increased collagen content in SSc-IgG treated cells over NC-IgG treated cells ($p < 0.05$, Figure 4B). Antagonism of ATR-A and ETR-A resulted in a marked, not significant reduction of collagen due to high variability in the tested samples. Statistical tests demonstrated a significant correlation between collagen induction and anti-ETAR Ab levels, while only a marked tendency was observed to anti-AT1R Ab levels ($r = 0.7619$ and $r = 0.6905$ respectively, $p < 0.05$ and $p = 0.0694$ respectively, Figure 4C).

Figure 4 Induction of collagen expression in fibroblasts by anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. Skin fibroblasts of healthy donors increase expression of type I collagen with anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. **(A)** Expression of type I collagen analyzed by immunocytochemistry. Collagen expression is shown in green, nucleic acid DAPI stain in blue. Bar indicates 50 micron, shown are representative pictures. **(B)** Significant increase in type I collagen with SSc-IgG versus NC-IgG (NC-IgG n = 4 and SSc-IgG n = 5, $p < 0.05$). Relative fluorescence intensity was analyzed and normalized to cell number for each sample. Mean and SEM, * $p < 0.05$. **(C)** Collagen expression (type I collagen) shows a significant correlation to anti-ETAR Ab levels and a trend to anti-AT1R Ab levels (NC-IgG n = 3, SSc-IgG n = 5, total n = 8, $p < 0.05$). Spearman correlation.

Induction of pulmonary neutrophil recruitment, increased plasma levels of murine IL-8 analogue KC and structural alterations in lungs of naïve C57BL/6J mice

To analyze systemic effects of anti-AT_1R and anti-ET_AR Abs *in vivo*, naïve C57BL/6J mice were subjected to passive transfer of pooled SSc-IgG or pooled NC-IgG as previously

described [24]. Seven days after the transfer, increased numbers of neutrophils were found in BALF of SSc-IgG treated mice as compared to NC-IgG-treated mice ($p < 0.01$, Figure 5A), while no differences were observed for macrophages or lymphocytes. Eosinophils were not detectable. Structural alteration of the lungs were not observed by a single IgG-treatment.

Figure 5 Systemic effects in naïve mice induced by anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. Anti-AT_1R and anti-ET_AR Ab positive SSc-IgG transfer increases neutrophil recruitment and KC levels (murine IL-8 analogue) in mice. **(A)** Significantly increased neutrophil counts detected in BALF of mice treated with SSc-IgG compared to NC-IgG ($n = 7$, $p < 0.01$). **(B)** KC plasma levels in SSc-IgG treated mice as compared to NC-IgG-treated mice are increased by trend (NC-IgG $n = 5$, SSc-IgG $n = 6$, $p = 0.05$). **(C)** KC plasma levels significantly correlate with neutrophil counts in BALF (NC-IgG $n = 5$, SSc-IgG $n = 6$, $r^2 = 0.6756$, $p = 0.0019$, linear regression correlation). Mean and SEM, ** $p < 0.01$. **(D)** Repeated treatment with IgG result in marked alterations of the lung structure of SSc-IgG treated mice compared to NC-IgG or untreated mice. Shown are representative light microscopy pictures, H&E staining, 12.5× magnification, bar indicates 500 micron. Asterisks indicate examples of airway vessels, arrows indicate elevated cell density in interstitial tissue.

However, plasma levels of the murine IL-8 analogue KC were found to be increased after SSc-IgG treatment compared to NC-IgG treatment ($p = 0.05$, Figure 5B). Moreover, correlation analysis of KC plasma levels and BALF neutrophil counts showed a strong positive correlation ($r^2 = 0.6756$ and $p = 0.0019$, Figure 5C). Repeated IgG-treatment resulted in profound structural alteration of the lungs including increased cellular density and interstitial cellular infiltrations in mice treated with SSc-IgG compared to NC-IgG or untreated mice (Figure 5D).

Discussion

The purpose of this study was to analyze the impact of anti-AT_1R and anti-ET_AR Abs on the induction of vascular inflammation and fibrosis, the key features of SSc. The presence of elevated anti-AT_1R and anti-ET_AR Ab levels in sera of SSc patients correlates with an increased risk for the development of lung fibrosis, pulmonary arterial hypertension (PAH) as well as with mortality, as demonstrated previousl [3]. Furthermore, these Abs induced the expression of transforming growth factor-β (TGF-β) in HMEC-1, suggesting a potential involvement in fibrosis [3]. Therefore, we sought to analyze the actions of anti-AT_1R and anti-ET_AR Abs with a focus on inflammation and fibrosis. Here, we demonstrate that IgG samples from SSc patients positive for anti-AT_1R and anti-ET_AR Abs induce proinflammatory and fibrotic events in endothelial cells and healthy donor fibroblasts via angiotensin and endothelin receptor activation. Besides the activation of fibroblasts, possible pathogenic effects mediated by SSc-IgG were reflected by endothelial dysfunction, expression of IL-8 and increased neutrophil migration into target tissues.

Involvement of both the angiotensin and the endothelin system in SSc pathogenesis has been demonstrated previously: Elevated serum levels of Ang II and ET-1 in SSc patients were reported, as well as increased ET-1 levels in SSc lung fibrotic tissue, indicating their central role in SSc pathogenesis [15,25,26]. Also, a link between angiotensin and endothelin receptor activation and fibrosis, perhaps the most prominent feature of SSc, was also suggested in the literature [26-28]. Activation of AT_1R by anti-AT_1R Abs was reported previously for pre-eclampsia and renal-allograft rejection [24,29]. Overexpression of extracellular matrix

components (ECM), of which collagen represents an important element, is a key aspect in fibrosis development [12,14]. Angiotensin and endothelin mediated collagen expression was demonstrated [17,27]. Accordingly, we found increased expression of collagen in healthy donor dermal fibroblasts after exposure to anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. The intensity of collagen expression was significantly dependent on levels of anti-ET_AR Abs, showing an Ab-dependent effect.

Besides the importance of fibrosis in SSc, many studies propose that microvascular damage and inflammation can precede fibrosis [4,30]. Additionally, autoimmune-mediated damage to endothelial cells has been demonstrated to cause endothelial dysfunction [31], which can lead to vessel leaks and lymphocyte infiltration [4,32]. In this regard, anti-AT_1R and anti-ET_AR Abs induced microvascular endothelial cell (HMEC-1) dysfunction after exposure to positive SSc-IgG, resulting in reduced endothelial repair in an Ab-level dependent manner. Moreover, endothelial dysfunction was further reflected by VCAM-1 expression on endothelial cells. Recently, the concept of vascular leak was proposed to be a central feature of SSc pathogenesis, highlighting the importance of changes in the microvasculature in disease progression [33]. Our findings indicate a general disturbance of endothelial functions by SSc-IgG *in vitro,* which could probably also occur *in vivo*. However, this hypothesis will need to be tested in more detail in future experiments.

Furthermore, expression of the chemokine IL-8 with proinflammatory and profibrotic properties has been reported to be increased in sera, BALF and fibroblasts in SSc [7,8,10]. Accordingly, we found increased mRNA and protein levels of IL-8 in HMEC-1 cells after exposure to anti-AT_1R and anti-ET_AR Ab positive SSc-IgG. In line with this, the murine IL-8 functional homolog KC was found to be increased in plasma of naïve mice treated with SSc-IgG. Given the chemotactic abilities of IL-8, we congruously found increased neutrophil transendothelial migration towards supernatants of SSc-IgG activated endothelial cells, which was dependent on IL-8. Increased neutrophil counts were also detected *in vivo* in BALF of naïve mice treated with SSc-IgG where neutrophil counts correlated with KC plasma levels. In addition to signs of an inflammatory fibrosis, repeated passive transfer of anti-AT_1R and anti-ET_AR Ab positive SSc-IgG resulted in marked structural alterations of lungs with increased cellular density in interstitial tissue. Our data suggest, furthermore, that these Abs can also activate angiotensin and endothelin receptors across both species due to high receptor homology [34,35].

Our study has some limitations, of which the most prominent is the use of total purified IgG instead of specifically purified anti-AT_1R and anti-ET_AR Abs. Instead, we here used receptor antagonists to demonstrate receptor-mediated activation as previously reported [21,29]. Therefore, measured effects could partly result from other Abs, suggested by incomplete effect inhibition by receptor antagonists. Also, we cannot exclude the participation of other Abs present in IgG on the measured effects. However, we have focused on effects that have already been associated to angiotensin and endothelin receptor activation. Another shortcoming is that the observed effects showed sometimes very high variability within tested samples, indicating the very complex nature of these Abs.

Conclusions

In summary, our *in vitro* results indicate an induction of proinflammatory and profibrotic events by anti-AT_1R and anti-ET_AR Ab positive SSc-IgG that might also be present *in vivo*.

Our experimental data complements the association of anti-AT_1R and anti-ET_AR Ab to clinical features of SSc, especially with interstitial lung disease. On the basis of these findings, we conclude that anti-AT_1R and anti-ET_AR Abs can activate angiotensin and endothelin receptor-expressing cells, among them some of the key players of SSc pathogenesis, and thus affect mechanisms of inflammation and fibrosis. Therefore, anti-AT_1R and anti-ET_AR Abs may present a novel future target in SSc therapeutic intervention.

Abbreviations

Ab, Autoantibody; Abs, Autoantibodies; AT_1R, Angiotensin II type 1 receptor; ATR-A, Angiotensin receptor antagonism; BALF, Bronchoalveolar lavage fluid; ECM, Extracellular matrix; ET_AR, Endothelin-1 type A receptor; ETR-A, Endothelin receptor antagonism; HMEC-1, Human microvascular endothelial cells; IL-8, Interleukin-8; NC-IgG, IgG from healthy donors; ROS, Reactive oxygen species; SSc, Systemic sclerosis; SSc-IgG, Anti-AT_1R and anti-ET_AR Ab positive IgG of SSc patients

Competing interests

Study was supported by an unrestricted grant from Actelion Pharmaceuticals Germany GmbH. There are no other competing interests.

Authors' contributions

AK, CT, MOB, JG, MR, DD and GR participated in study design and data interpretation. AK participated in *in vitro* experiments. AK, CT and RU participated in animal experiments. AK and CT performed statistical analyses. HH provided measurements of anti-AT_1R and anti-ET_AR Ab. MWo provided healthy donor skin samples. MWi provided equipment for BALF analysis. AKü performed histological preparation of lungs. G-RB, DD and GR participated in study coordination. AK participated in manuscript preparation with support of all other authors, who read and approved of manuscript.

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References

1. LeRoy EC, Black C, Fleischmajer R, Jablonska S, Krieg T, Medsger TA Jr, Rowell N, Wollheim F: **Scleroderma (systemic sclerosis): classification, subsets and pathogenesis.** *J Rheumatol* 1988, **15:**202–205.

2. Varga J, Abraham D: **Systemic sclerosis: a prototypic multisystem fibrotic disorder.** *J Clin Invest* 2007, **117:**557–567.

3. Riemekasten G, Philippe A, Nather M, Slowinski T, Muller DN, Heidecke H, Matucci-Cerinic M, Czirjak L, Lukitsch I, Becker M, *et al*: **Involvement of functional autoantibodies against vascular receptors in systemic sclerosis.** *Ann Rheum Dis* 2011, **70:**530–536.

4. Fleischmajer R, Perlish JS: **Capillary alterations in scleroderma.** *J Am Acad Dermatol* 1980, **2:**161–170.

5. Prescott RJ, Freemont AJ, Jones CJ, Hoyland J, Fielding P: **Sequential dermal microvascular and perivascular changes in the development of scleroderma.** *J Pathol* 1992, **166:**255–263.

6. Kahaleh MB, Sherer GK, LeRoy EC: **Endothelial injury in scleroderma.** *J Exp Med* 1979, **149:**1326–1335.

7. Schmidt K, Martinez-Gamboa L, Meier S, Witt C, Meisel C, Hanitsch LG, Becker MO, Huscher D, Burmester GR, Riemekasten G: **Bronchoalveoloar lavage fluid cytokines and chemokines as markers and predictors for the outcome of interstitial lung disease in systemic sclerosis patients.** *Arthritis Res Ther* 2009, **11:**R111.

8. Kadono T, Kikuchi K, Ihn H, Takehara K, Tamaki K: **Increased production of interleukin 6 and interleukin 8 in scleroderma fibroblasts.** *J Rheumatol* 1998, **25:**296–301.

9. Furuse S, Fujii H, Kaburagi Y, Fujimoto M, Hasegawa M, Takehara K, Sato S: **Serum concentrations of the CXC chemokines interleukin 8 and growth-regulated oncogene-alpha are elevated in patients with systemic sclerosis.** *J Rheumatol* 2003, **30:**1524–1528.

10. Codullo V, Baldwin HM, Singh MD, Fraser AR, Wilson C, Gilmour A, Hueber AJ, Bonino C, McInnes IB, Montecucco C, *et al*: **An investigation of the inflammatory cytokine and chemokine network in systemic sclerosis.** *Ann Rheum Dis* 2011, **70:**1115–1121.

11. Crestani B, Seta N, Palazzo E, Rolland C, Venembre P, Dehoux M, Boutten A, Soler P, Dombret MC, Kahn MF, *et al*: **Interleukin-8 and neutrophils in systemic sclerosis with lung involvement.** *Am J Respir Crit Care Med* 1994, **150:**1363–1367.

12. LeRoy EC: **Increased collagen synthesis by scleroderma skin fibroblasts in vitro: a possible defect in the regulation or activation of the scleroderma fibroblast.** *J Clin Invest* 1974, **54:**880–889.

13. Jimenez SA, Feldman G, Bashey RI, Bienkowski R, Rosenbloom J: **Co-ordinate increase in the expression of type I and type III collagen genes in progressive systemic sclerosis fibroblasts.** *Biochem J* 1986, **237:**837–843.

14. Bocchieri MH, Christner PJ, Henriksen PD, Jimenez SA: **Immunological characterization of (tight skin/NZB)F1 hybrid mice with connective tissue and autoimmune features resembling human systemic sclerosis.** *J Autoimmun* 1993, **6:**337–351.

15. Kawaguchi Y, Takagi K, Hara M, Fukasawa C, Sugiura T, Nishimagi E, Harigai M, Kamatani N: **Angiotensin II in the lesional skin of systemic sclerosis patients contributes to tissue fibrosis via angiotensin II type 1 receptors.** *Arthritis Rheum* 2004, **50:**216–226.

16. Shi-Wen X, Chen Y, Denton CP, Eastwood M, Renzoni EA, Bou-Gharios G, Pearson JD, Dashwood M, du Bois RM, Black CM, *et al*: **Endothelin-1 promotes myofibroblast induction through the ETA receptor via a rac/phosphoinositide 3-kinase/Akt-dependent pathway and is essential for the enhanced contractile phenotype of fibrotic fibroblasts.** *Mol Biol Cell* 2004, **15:**2707–2719.

17. Kawaguchi Y, Suzuki K, Hara M, Hidaka T, Ishizuka T, Kawagoe M, Nakamura H: **Increased endothelin-1 production in fibroblasts derived from patients with systemic sclerosis.** *Ann Rheum Dis* 1994, **53:**506–510.

18. van den Hoogen F, Khanna D, Fransen J, Johnson SR, Baron M, Tyndall A, Matucci-Cerinic M, Naden RP, Medsger TA Jr, Carreira PE, *et al*: **2013 classification criteria for systemic sclerosis: an american college of rheumatology/european league against rheumatism collaborative initiative.** *Arthritis Rheum* 2013, **65:**2737–2747.

19. Heit B, Liu L, Colarusso P, Puri KD, Kubes P: **PI3K accelerates, but is not required for, neutrophil chemotaxis to fMLP.** *J Cell Sci* 2008, **121:**205–214.

20. Sironi L, Calvio AM, Arnaboldi L, Corsini A, Parolari A, de Gasparo M, Tremoli E, Mussoni L: **Effect of valsartan on angiotensin II-induced plasminogen activator inhibitor-1 biosynthesis in arterial smooth muscle cells.** *Hypertension* 2001, **37:**961–966.

21. Dechend R, Viedt C, Muller DN, Ugele B, Brandes RP, Wallukat G, Park JK, Janke J, Barta P, Theuer J, *et al*: **AT1 receptor agonistic antibodies from preeclamptic patients stimulate NADPH oxidase.** *Circulation* 2003, **107:**1632–1639.

22. Maurer B, Busch N, Jungel A, Pileckyte M, Gay RE, Michel BA, Schett G, Gay S, Distler J, Distler O: **Transcription factor fos-related antigen-2 induces progressive peripheral vasculopathy in mice closely resembling human systemic sclerosis.** *Circulation* 2009, **120:**2367–2376.

23. . http://frodo.wi.mit.edu/primer3/.

24. Zhou CC, Zhang Y, Irani RA, Zhang H, Mi T, Popek EJ, Hicks MJ, Ramin SM, Kellems RE, Xia Y: **Angiotensin receptor agonistic autoantibodies induce pre-eclampsia in pregnant mice.** *Nat Med* 2008, **14:**855–862.

25. Coral-Alvarado P, Quintana G, Garces MF, Cepeda LA, Caminos JE, Rondon F, Iglesias-Gamarra A, Restrepo JF: **Potential biomarkers for detecting pulmonary arterial hypertension in patients with systemic sclerosis.** *Rheumatol Int* 2009, **29:**1017–1024.

26. Abraham DJ, Vancheeswaran R, Dashwood MR, Rajkumar VS, Pantelides P, Xu SW, du Bois RM, Black CM: **Increased levels of endothelin-1 and differential endothelin type A and B receptor expression in scleroderma-associated fibrotic lung disease.** *Am J Pathol* 1997, **151:**831–841.

27. Stawski L, Han R, Bujor AM, Trojanowska M: **Angiotensin II induces skin fibrosis: a novel mouse model of dermal fibrosis.** *Arthritis Res Ther* 2012, **14:**R194.

28. Denton CP, Black CM, Abraham DJ: **Mechanisms and consequences of fibrosis in systemic sclerosis.** *Nat Clin Pract Rheumatol* 2006, **2:**134–144.

29. Dragun D, Muller DN, Brasen JH, Fritsche L, Nieminen-Kelha M, Dechend R, Kintscher U, Rudolph B, Hoebeke J, Eckert D, *et al*: **Angiotensin II type 1-receptor activating antibodies in renal-allograft rejection.** *N Engl J Med* 2005, **352:**558–569.

30. Nguyen VA, Sgonc R, Dietrich H, Wick G: **Endothelial injury in internal organs of University of California at Davis line 200 (UCD 200) chickens, an animal model for systemic sclerosis (Scleroderma).** *J Autoimmun* 2000, **14:**143–149.

31. Sgonc R, Gruschwitz MS, Boeck G, Sepp N, Gruber J, Wick G: **Endothelial cell apoptosis in systemic sclerosis is induced by antibody-dependent cell-mediated cytotoxicity via CD95.** *Arthritis Rheum* 2000, **43:**2550–2562.

32. Katsumoto TR, Whitfield ML, Connolly MK: **The pathogenesis of systemic sclerosis.** *Annu Rev Pathol* 2011, **6:**509–537.

33. Frech TM, Revelo MP, Drakos SG, Murtaugh MA, Markewitz BA, Sawitzke AD, Li DY: **Vascular leak is a central feature in the pathogenesis of systemic sclerosis.** *J Rheumatol* 2012, **39:**1385–1391.

34. Richard V, Solans V, Favre J, Henry JP, Lallemand F, Thuillez C, Marie I: **Role of endogenous endothelin in endothelial dysfunction in murine model of systemic sclerosis: tight skin mice 1.** *Fundam Clin Pharmacol* 2008, **22:**649–655.

35. Inaba S, Iwai M, Furuno M, Kanno H, Senba I, Okayama H, Mogi M, Higaki J, Horiuchi M: **Temporary treatment with AT1 receptor blocker, valsartan, from early stage of hypertension prevented vascular remodeling.** *Am J Hypertens* 2011, **24:**550–556.

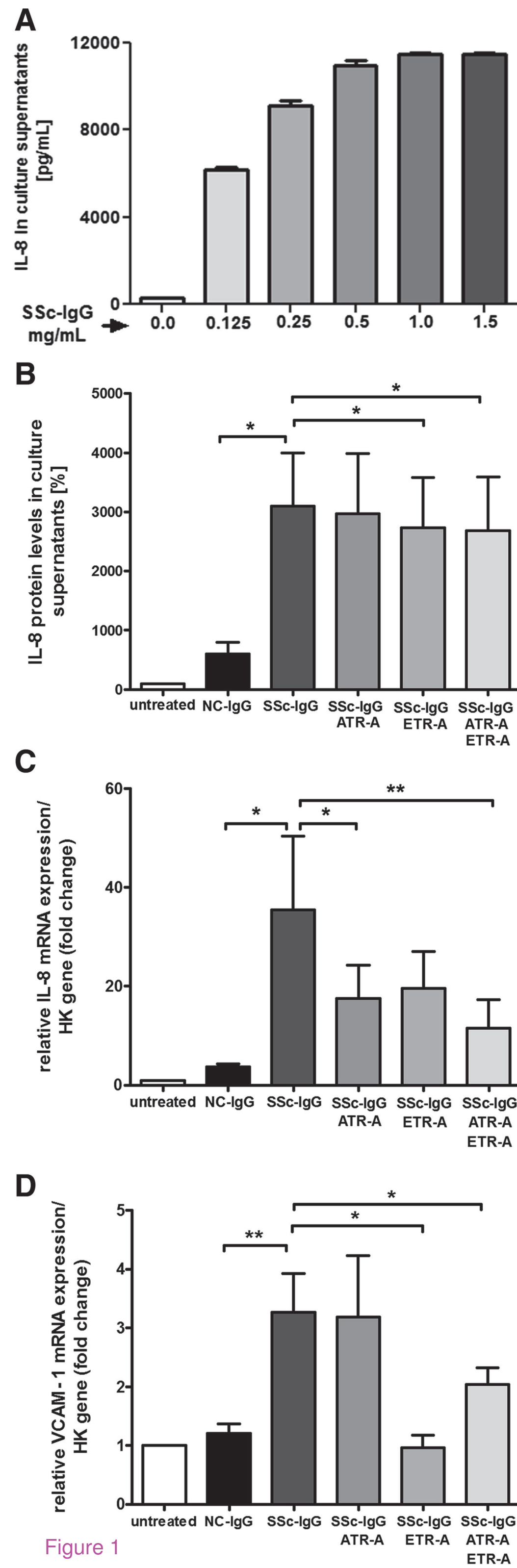
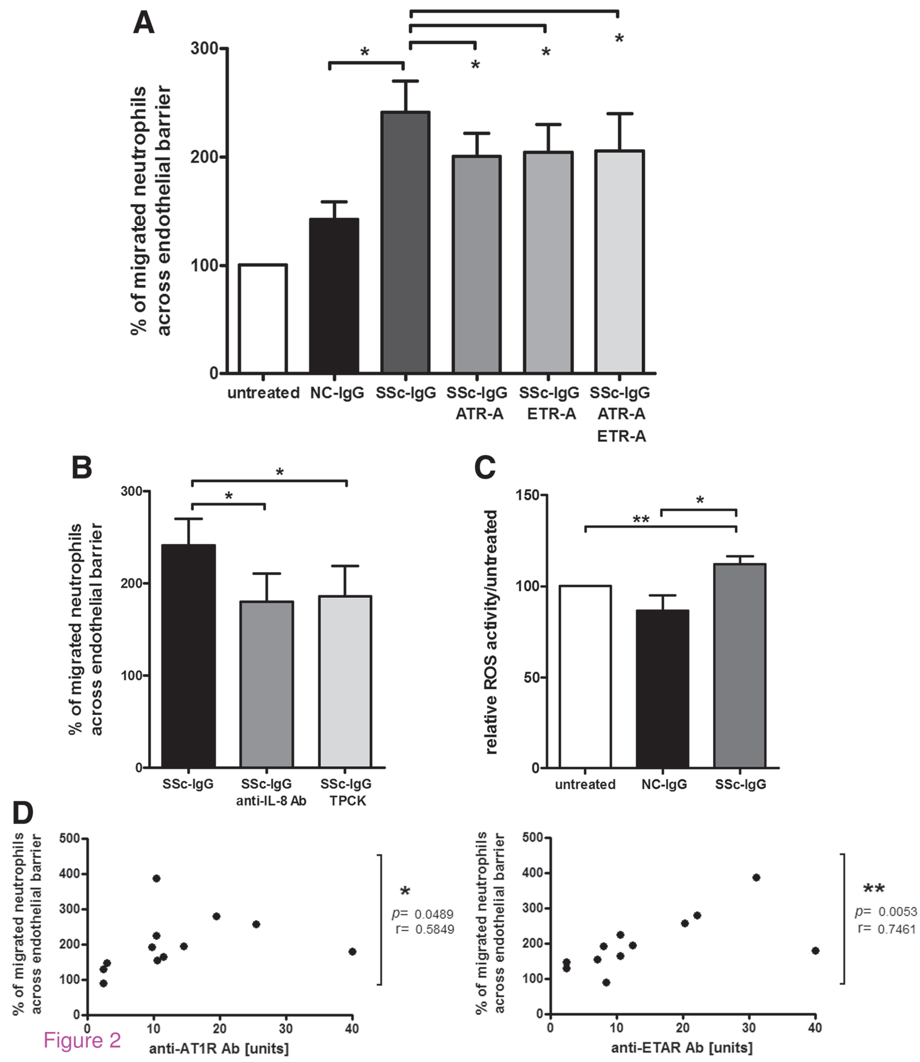


Figure 1



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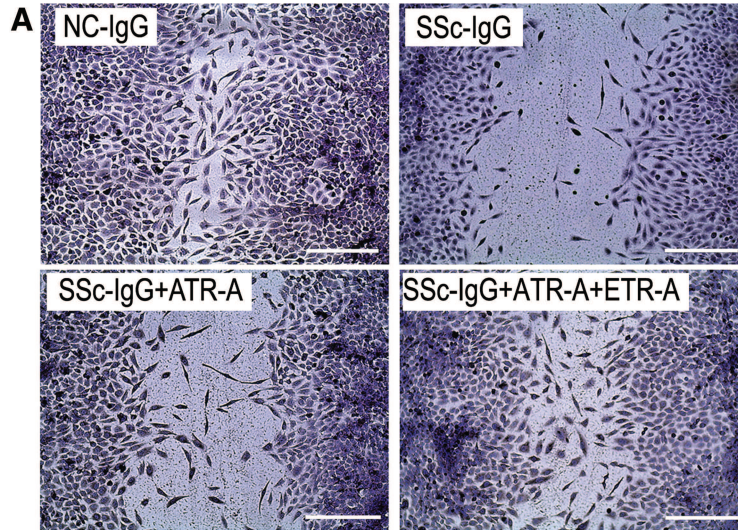
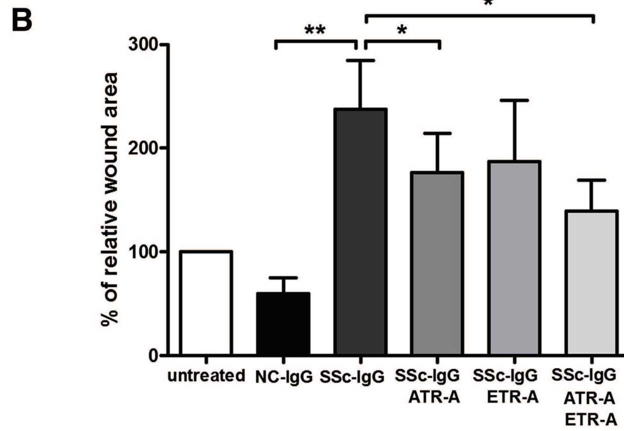




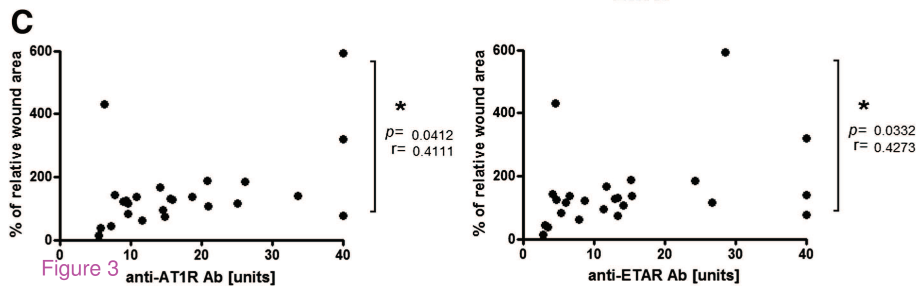
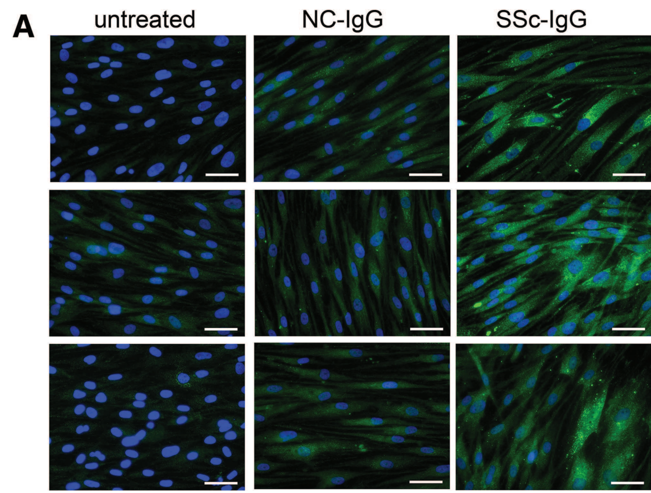
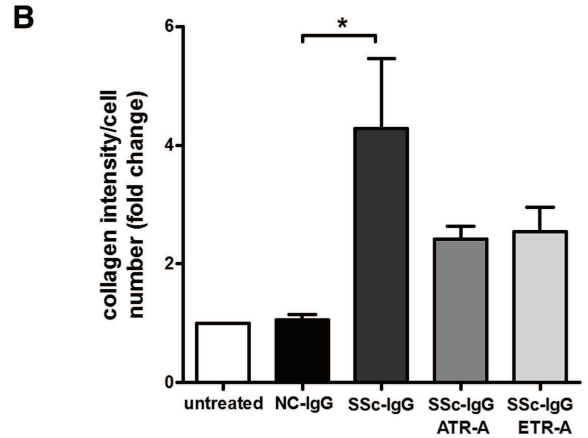


Figure 3





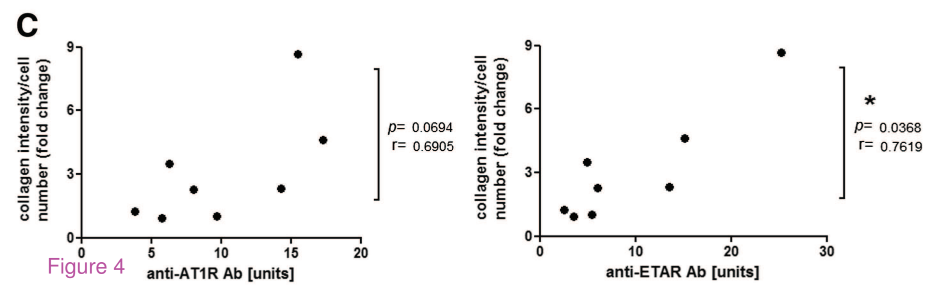
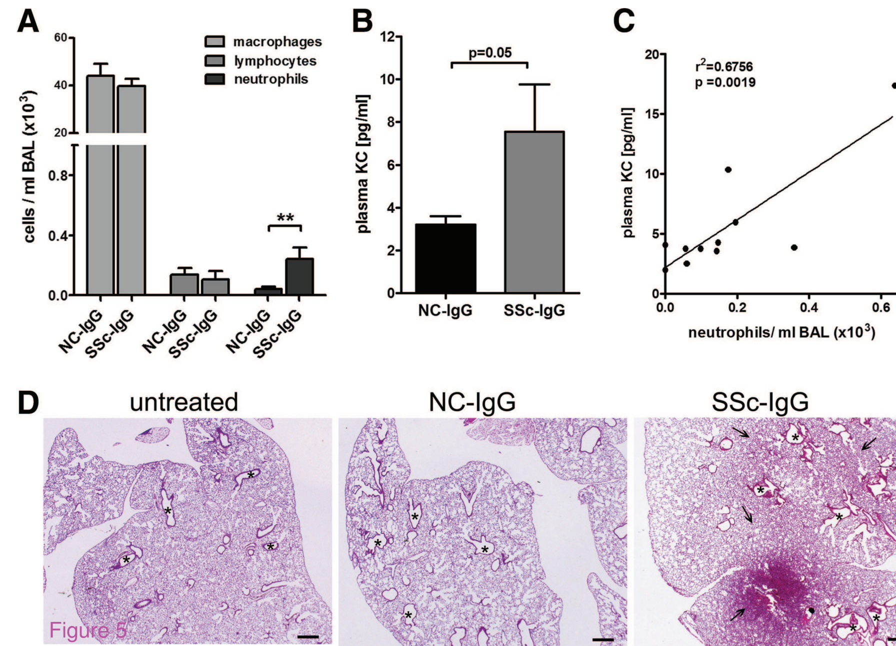


Figure 4


A
macrophages
lymphocytes
neutrophils
cells / ml BAL (x10³)
60
40
20
0.8
0.4
0.0
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NC-IgG
SSc-IgG
NC-IgG
SSc-IgG
NC-IgG
SSc-IgG
B
plasma KC [pg/ml]
12
10
8
6
4
2
0
p=0.05
NC-IgG
SSc-IgG
C
plasma KC [pg/ml]
r²=0.6756
p =0.0019
20
15
10
5
0
0.0
0.2
0.4
0.6
neutrophils/ ml BAL (x10³)
D
untreated
NC-IgG
SSc-IgG
Figure 5

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

"Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht."

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1 Kill A, Tabeling C, Undeutsch R, Kühl AA, Günther J, Radic M, Becker MO, Heidecke H, Worm M, Witzenrath M, Burmester GR, Dragun D, Riemekasten G. Autoantibodies to angiotensin and endothelin receptors in systemic sclerosis induce cellular and systemic events associated with disease pathogenesis. Arthritis Res Ther. 2014 Jan 28;16(1):R29.

2 Günther J, Kill A, Becker MO, Heidecke H, Rademacher J, Siegert E, Radic M, Burmester GR, Dragun D, Riemekasten G. Angiotensin receptor type 1 and endothelin receptor type A on immune cells mediate migration and the expression of IL-8 and CCL18 when stimulated by autoantibodies from systemic sclerosis patients. Arthritis Res Ther. 2014. Accepted in principle.

3 Riemekasten G, Philippe A, Näther M, Slowinski T, Müller DN, Heidecke H, Matucci-Cerinic M, Czirják L, Lukitsch I, Becker M, Kill A, van Laar JM, Catar R, Luft FC, Burmester GR, Hegner B, Dragun D. Involvement of functional autoantibodies against vascular receptors in systemic sclerosis. Ann Rheum Dis. 2011 Mar;70(3):530-6.

4 Hanke K, Brückner CS, Dähnrich C, Huscher D, Komorowski L, Meyer W, Janssen A, Backhaus M, Becker M, Kill A, Egerer K, Burmester GR, Hiepe F, Schlumberger W, Riemekasten G. Antibodies against PM/Scl-75 and PM/Scl-100 are independent markers for different subsets of systemic sclerosis patients. Arthritis Res Ther. 2009;11(1)

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