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“Pathogenicity of functional autoantibodies against angiotensin II receptor type 1 (AT1R) in experimental systemic sclerosis”

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Declaration

Hereby I declare that I have written this dissertation completely on my own. Furthermore, I confirm that no other sources have been used than those specified in the dissertation itself. This dissertation, in same or similar form, has not been submitted to any other doctoral degree committee yet.

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Zusammenfassung

Hintergrund: Die systemische Sklerose (SSc) ist eine chronische, multisystemische Bindegewebeerkrankung, welche durch Autoimmunität, Entzündung, Fibrose und Vaskulopathie gekennzeichnet ist. Obwohl viele verschiedene Autoantikörper im Blut von SSc-Patienten nachgewiesen wurden, ist es derzeit unklar, ob diese bei der Pathogenese der Krankheit eine Rolle spielen. Eine besondere Bedeutung scheint hier funktionellen (rezeptoraktivierenden) Autoantikörpern gegen den Angiotensin-II-Rezeptor Typ 1 (AT1R) zuzukommen. SSc-Patienten weisen nicht nur erhöhte Spiegel dieser Antikörper auf, sondern die Konzentration der Autoantikörper korreliert auch mit Intensität und Verlauf der Erkrankung sowie mit der durch die Krankheit vermittelten Mortalität Ob diese Antikörper jedoch neben ihren diagnostischen und prognostischen Eigenschaften auch kausal an der Pathogenese der SSc beteiligt sind, ist derzeit noch unklar. Zur Klärung dieser Frage wurde kürzlich in unserer Gruppe ein neuartiges Mausmodell dieser Erkrankung entwickelt, in welchem Mäuse mit einem Membranextrakt aus humanem AT1R-überexprimierenden Zellen immunisiert werden. Immunisierte Tiere entwickeln nicht nur selbst-reaktive funktionelle Antikörper gegen AT1R sondern auch eine Reihe SSc-ähnlicher Symptome wie Hautfibrosen und Entzündungen in Haut und Lunge. Dieses Modell ermöglicht erstmals die direkte Untersuchung der Rolle von Autoantikörpern gegen AT1R bei der Pathogenese der SSc.

Zielsetzungen: In dieser Studie sollte geklärt werden, ob anti-AT1R-Autoantikörper eine propathogene Funktion im AT1R-induzierten Mausmodells der SSc besitzen, welche Zellpopulationen für die Vermittlung einer solchen Pathogenität relevant sind und welche Mechanismen dieser Pathogenität zugrunde liegen.

Methoden: Um die Rolle von T-Zellen, B-Zellen und Komplementsystem im AT1R-induzierten Mausmodell von SSc zu untersuchen, wurden B6.129S2-Cd4^{tm1Mak}/J, B6.129S2-Cd8a^{tm1Mak}/J, B6.129S2-Ighm^{tm1Cgn}/J, B6.129S4-C3^{tm1Crr}/J und Wildtyp- Kontrollmäuse mit hAT1R immunisiert. Neun Wochen nach der ersten Immunisierung wurden die Mäuse abgetötet und der Krankheitsverlauf in den Tieren anhand von Serums und Gewebsproben bestimmt. Um die Pathogenität von anti-AT1R-Antikörpern direkt zu untersuchen, wurden monoklonale Autoantikörper gegen hAT1R in das Ohr von C57BL/6J-Mäusen injiziert und deren Kapazität zur Induktion von Entzündung und Fibrosen im Gewebe der Tiere histopathologisch analysiert. Abschließend wurden potentielle Zielzellen der anti-AT1R-Antikörper im Gewebe und auf einzelnen Zellen mittels Immunfluoreszenzfärbung und Durchflusszytometrie identifiziert.

Ergebnisse: B-Zell-defiziente und $CD4^+$-T-Zell-defiziente Tiere erwiesen sich als resistent im AT1R-induzierten Modell der SSc. Im Gegensatz zu Wildtyp-C57BL/6J-Mäusen und $CD8^+$-T-Zell-defiziente Mäusen, war bei den ersteren weder eine Produktion von AT1R-Antikörpern noch die Ausbildung von Fibrosen und Entzündungen in Haut und Lunge nachweisbar. Eine den Wildtyp-Kontrollen vergleichbare Suszeptibilität gegenüber der experimentellen SSc zeigten Komplement C3-defiziente Mäuse. Die direkte wiederholte Applikation von funktionellen monoklonalen Antikörpern gegen AT1R induzierte nicht nur eine lokale Entzündung in der Haut sondern vermittelte ebenfalls die Infiltration von Entzündungszellen in die Lunge. Mit Hilfe von Immunfluoreszenzhistologie und Durchflusszytometrie konnten Fibroblasten als dominante anti-AT1R-bindende Zellpopulation in der Haut identifiziert werden.

Schlussfolgerungen: In dieser Studie konnte erstmals direkt eine pathogene Wirkung von AT1R-Antikörpern in der experimentellen SSc nachgewiesen werden, wobei dermale Fibroblasten eine dominante Zielzelle dieser Antikörper repräsentieren. Die Bildung von AT1R-Antikörper und die Pathogenese der Erkrankung ist dabei sowohl von $CD4^+$-T-Zellen als auch von B-Zellen abhängig während $CD8^+$-T-Zellen daran nicht beteiligt sind. Das Fehlen einer Beteiligung des Komplementsystems in diesem Modell lässt indirekt auf eine zentrale Rolle der durch die Antikörper vermittelten funktionellen Aktivierung von AT1R schließen. Die Ergebnisse dieser Arbeit erklären nicht nur den Erfolg der B-Zelltherapie in der Behandlung der SSc sondern stellen auch einen Ausgangspunkt für die Identifikation neuer therapeutischer Angriffspunkte in der Erkrankung dar.

Summary

Background: Systemic sclerosis (SSc) is a chronic, multisystemic connective tissue disease characterized by autoimmunity, inflammation, fibrosis and vasculopathy. Although many different autoantibodies have been detected in the blood of SSc patients, it is currently unclear whether they play a role in the pathogenesis of the disease. Functional (receptor activating) autoantibodies against the angiotensin II receptor type 1 (AT1R) seem to be of particular importance. SSc patients do not only show elevated levels of these antibodies, but the concentration of autoantibodies correlates with the intensity and course of the disease as well as with its mortality. However, it is currently unclear whether these antibodies, in addition to their diagnostic and prognostic properties, are also causally involved in the pathogenesis of SSc. To address this question, our group recently developed a novel mouse model of the disease in which mice are immunized with a membrane extract derived from cells overexpressing human AT1R. Immunized animals develop beside self-reactive functional antibodies against AT1R also several SSc-like symptoms such as skin fibrosis and inflammation of skin and lung. This model allows for the first time the direct investigation of the role of autoantibodies against AT1R in the pathogenesis of SSc.

Objectives: The aim of this study was to clarify whether anti-AT1R autoantibodies have a pathogenic function in the AT1R-induced mouse model of SSc, which cell populations are relevant for the mediation of such pathogenicity, and which mechanisms underlie this pathogenicity.

Methods: To study the role of T cells, B cells, and the complement system in the AT1R-induced mouse model of SSc, B6.129S2-Cd4^{tm1Mak}/J, B6.129S2-Cd8a^{tm1Mak}/J, B6.129S2-Ighm^{tm1Cgn}/J, B6.129S4-C3^{tm1Crr}/J and wild-type control mice were immunized with hAT1R. Nine weeks after the first immunization, the mice were sacrificed and disease development in the animals was evaluated in serum and tissue samples. To directly investigate the pathogenicity of anti-AT1R antibodies, monoclonal autoantibodies against hAT1R were injected into the ear of C57BL/6J mice and their capacity to induce inflammation and fibrosis in the animal tissues was analyzed by histopathology. Finally, potential target cells of anti-AT1R antibodies in the tissue and on individual cells were identified by immunofluorescence staining and flow cytometry.

Results: B-cell-deficient and CD4^{+}-T-cell-deficient animals were found to be resistant in the AT1R-induced model of SSc. In contrast to wild-type C57BL/6J mice and CD8^{+}-T-cell-deficient mice, the former did not show production of AT1R antibodies or the development of fibrosis and inflammation in skin and lung. Complement C3-deficient mice showed a

susceptibility to the experimental SSc comparable to that of wild-type controls. The direct repeated application of functional monoclonal antibodies against AT1R not only induced local inflammation in the skin but also mediated the infiltration of inflammatory cells into the lung. By means of immunofluorescence histology and flow cytometry, fibroblasts could be identified as the dominant anti-AT1R-antibodies binding cell population in the skin.

Conclusion: In this study for the first time a pathogenic effect of AT1R-antibodies could be directly shown in an animal model of SSc, in which dermal fibroblasts represent a dominant cellular target of these antibodies. AT1R antibody production and disease pathogenesis is dependent on both $CD4^+$ T cells and B cells, whereas $CD8^+$ T cells are not involved. The absence of complement involvement in this model indirectly suggests a central pathogenic role of antibody-mediated functional activation of AT1R. These results do not only explain the success of B-cell therapy in the treatment of SSc but also provide a starting point for the identification of new therapeutic targets in the disease.

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Abbreviations

ACA	Anti-centromere autoantibody
ACE	Angiotensin converting enzyme
ACR	American College of Rheumatology
ADCC	Antibody-dependent cell-mediated cytotoxicity
AECA	Anti-endothelial cell antibodies
Ang II	Angiotensin II
AT1R	Angiotensin II receptor type 1
AT1R KO	AT1R deficient mice (B6.129P2-Agtr1a^{tm1Unc}/Agtr1b^{tm1Cof}/J)
ATA	Anti-topoisomerase I autoantibody
BAFF	B-cell activating factor
BCL	B-cell lymphoma
BSA	Bovine serum albumin
C3	Complement component 3
C3 KO	Complement C3-deficient mice (B6.129S4-C3^{tm1Crr}/J)
CCL18	Chemokine (C-C motif) ligand 18
CCL2	Chemokine (C-C motif) ligand 2
CD	Cluster of differentiation
CD4 KO	CD4^{+} T cell-deficient mice (B6.129S2-Cd4^{tm1Mak}/J)
CD8 KO	CD8^{+} T cell-deficient mice (B6.129S2-Cd8a^{tm1Mak}/J)
CDR	Complementary-determining region
CFA	Complete Freund adjuvant
CHO cell	Chinese Hamster Ovary cell line
COL7	Collagen type VII
CTGF	Connective tissue growth factor
CXCL4	Chemokine (C-X-C motif) ligand 4
CYC	Cyclophosphamide
dcSSc	Diffuse cutaneous SSc

DMEM	Dulbecco's modified Eagle's medium
EBA	Epidermolysis bullosa acquista
EBV	Epstein-Barr virus
ECL	Extracellular loop
ECM	Extracellular matrix
ELISA	Enzyme linked immunosorbent assay
ET-1	Endothelin 1
ETAR	Endothelin type A receptor
EULAR	European League Against Rheumatism
EUSTAR	EULAR scleroderma trials and research
FCS	Fetal calf serum
FLI1	Friend leukemia integration 1
FSC	Forward scatter
Fv	Variable fragment
GATA3	GATA binding protein 3
GPCR	G-protein-coupled receptor
GVHD	Chronic graft-versus-host disease
GWAS	Genome-wide association study
hAT1R	Human AT1R
hCMV	Human cytomegalovirus
H&E	Haemotoxylin and Eosin staining
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
ICAM	Intercellular adhesion molecule
IF	Immunofluorescence
IFA	Incomplete Freund adjuvant
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin

IL2RB	Interleukin 2 receptor subunit beta
ILD	Interstitial lung disease
IRF	Interferon regulatory factor
JAK	Janus kinase
KLF5	Krueppel-like factor 5
lcSSc	Limited cutaneous SSc
M3R	Muscarinic M3 receptors
MCP-1	Monocyte chemotactic protein 1
ME	Membrane extract
miR	microRNA
MTX	Methotrexate
muMT	B cell-deficient mice (B6.129S2-Ighm^{tm1Cgn}/J)
NGS	Next generation sequencing
NO	Nitric oxide
OD	Optical density
OPIG	Oxford Protein Informatics Group
PAH	Pulmonary arterial hypertension
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cells
PDGF	Platelet derived growth factor
PDGFR	Platelet-derived growth factor receptor
pSS	Primary sjögren's syndrome
PTPN22	Protein tyrosine phosphatase non-receptor type 22
RA	Rheumatoid arthritis
ROS	Reactive oxygen species
RT	Room temperature
SLE	Systemic lupus erythematosus
SRC	Scleroderma renal crisis

SSc	Systemic sclerosis
SSC	Side scatter
ssSSc	SSc sine scleroderma
STAT4	Signal transducer and activator of transcription 4
TCR	T cell receptor
Th cell	T helper cell
Tfh cell	T follicular helper cell
TGF	Transforming growth factor
TLO	Tertiary lymphoid organ
TLR	Toll like receptors
TNF	Tumor necrosis factor
Treg cell	Regulatory T cell
TSAb	Thyroid-stimulating autoantibodies
TSH	Thyroid-stimulating hormone
TSHR	TSH receptor
VCAM-1	Vascular cell adhesion molecule 1
WT	Wild type
β2-AR	β2-adrenergic receptors
β-AR	β-adrenergic receptors

1. Introduction

1.1. Systemic sclerosis

Systemic sclerosis (SSc) is an autoimmune connective tissue disorder with multiple organ involvement and heterogeneous clinical manifestations. It is characterized by autoimmunity, inflammation, vasculopathy and fibrosis in skin and visceral organs [1], [2]. SSc is a rare disease, with a worldwide prevalence of approximately 100 cases per million [3]. Although the disease prevalence is relatively low compared to other autoimmune disorders, SSc is featured by a high mortality rate due to some fatal organ complications [2], [4].

Typical and early symptoms of SSc are skin manifestations, including Raynaud's phenomenon, digital ulcers and hardening and tightening of the skin [2], [3]. Beside skin, several internal organs including the lung, heart and kidney are affected, leading to the development of interstitial lung disease (ILD), pulmonary arterial hypertension (PAH), cardiovascular diseases and renal crisis [3], [5]. Among these complications, interstitial lung disease and pulmonary arterial hypertension are the two leading causes of SSc-related death [2], [6]. Although there are many treatments for patients with SSc, none of them is able to cure disease or reverse the disease progression [3], [5].

1.1.1. Epidemiology

The prevalence and incidence of SSc vary considerably between geographical locations [7]. In East Asia and North Europe, lower prevalence (less than 100 per million) and incidence (less than 8 per million per year) have been reported, while higher prevalences (around 150 to 700 per million) and incidences (around 10 to 25 per million per year) have been observed in North America, South Europe and Australia [7]. However, the abovementioned surveys of incidence and prevalence were performed before 2010. In 2013, European League Against Rheumatism (EULAR) and American College of Rheumatology (ACR) revised the diagnostic and classification criteria which is more sensitive than the criteria proposed by ACR in 1980 [8], [9]. Consequently, according to the updated criteria, prevalence and incidence of SSc could be higher than previously estimated.

Besides geographic locations, gender and ethnicity also play a role in the development of SSc development. According to a large epidemiologic study conducted by the Johns Hopkins Scleroderma Center in 2013 [10], the mean age of African American patients with SSc at the first visit to Hospital is significantly younger than that of white SSc patients. In addition, the

majority of African American SSc patients develop diffuse SSc, while around two-third of white patients manifest the limited SSc [10]. Similar to other rheumatic diseases, SSc affects more females than males. It has been reported that more than 80% of patients with SSc are women [11], suggesting a strong sex-based bias. Besides the disease prevalence, clinical manifestations and prognosis of SSc are also associated with gender. According to a large prospective study from the EULAR scleroderma trials and research (EUSTAR) cohort with 9182 SSc patients including 7861 females and 1321 males [12], male is independently associated with a higher risk of diffuse SSc, a higher proportion of digital ulcers and pulmonary hypertension. In addition, male sex is predictive of new onset of pulmonary hypertension, heart failure and mortality [12]. Taken together, although females are more prone to SSc, male patients with SSc suffer more severe manifestations and show a higher mortality rate than females [12], [13].

1.1.2. Diagnosis and classification

In 1980, a preliminary classification criteria for SSc was proposed by American College of Rheumatology (ACR) as a standard for defining disease to permit a proper comparison of patients of different origin [9]. Although the ACR classification criteria had been used by clinicians to diagnose SSc for several decades, they are featured by a low sensitivity for patients with early SSc and patients with no or weak skin fibrosis [14]. To overcome these weaknesses, the European League Against Rheumatism (EULAR) and ACR jointed together and proposed the diagnosis and classification criteria of SSc in 2013 [8]. According to the 2013 EULAR/ACR criteria, patients with a total score of no less than 9 are classified as having SSc (Table 1) [8]. Compared with previous ACR classification criteria, the EULAR/ACR criteria show better sensitivity and specificity [8], and this improvement has been confirmed by another study using the Canadian Scleroderma Research Group cohort [15].

Table 1: 2013 EULAR-ACR SSc diagnosis and classification criteria.

Items	Subitems	Score
Skin thickening of the fingers of both hands extending proximal to the meta-carpophalangeal joints (sufficient criterion)	-	9
Skin thickening of the fingers (only count the higher score)	Puffy fingers	2
	Sclerodactyly of the fingers (distal to the metacarpophalangeal joints but proximal to the proximal interphalangeal joints)	4
Fingertip lesions (only count the higher score)	Digital tip ulcers	2
	Fingertip pitting scars	3
Telangiectasia	-	2
Abnormal nailfold capillaries	-	2
Lung involvement (maximum score is 2)	Pulmonary arterial hypertension	2
	Interstitial lung disease	2
Raynaud's phenomenon	-	3
SSc-related autoantibodies (maximum score is 3)	Anti-centromere	3
	Anti-topoisomerase I [anti-Scl-70]	3
	Anti-RNA polymerase III	3

According to the extent of skin involvement, SSc is classified into three groups: limited cutaneous SSc (lcSSc), diffuse cutaneous SSc (dcSSc) and SSc sine scleroderma (ssSSc) [3]. Skin involvement in lcSSc is restricted to fingers, distal limb and face, while the proximal and the trunk are also affected in dcSSc. In lcSSc, the appearance of Raynaud's phenomenon normally precedes other skin manifestations and internal organ involvement several months or even years. By contrast, dcSSc is characterized by rapid progression of extensive skin involvement and early development of visceral complications [3]. Apart from lcSSc and dcSSc, ssSSc represents an infrequent variant of SSc featured by visceral and immunological manifestations of SSc in the absence of skin thickening [16].

1.1.3. Clinical manifestations

SSc is an autoimmune connective tissue disease which affects multiple tissues and organs including skin, lung, gastrointestinal tract, heart and kidney [2]. As a heterogeneous disease, SSc exhibits an extensive patient-to-patient variability in immunological features, organ manifestations, clinical courses and disease prognosis [2], [3] (Figure 1).

Despite the heterogeneity of SSc, more than 90% of SSc patients develop skin symptoms [2]. Skin manifestations are the main and, in most times, the earliest clinical symptoms of SSc [17]. Notably, more severe skin symptoms coincide with more extensive internal organ manifestations, poor prognosis and higher disability [18]. Skin thickening is the cardinal feature of cutaneous manifestations of SSc and is regarded as a sufficient criterion of the diagnosis of SSc [8]. Lung manifestations in patients with SSc include interstitial lung disease which is featured by pulmonary fibrosis and pulmonary arterial hypertension which is associated with the obstruction of pulmonary arteries [2]. In the clinic, obvious ILD and PAH have been observed in up to 40% and approximately 15% of patients with SSc, respectively [19]. Notably, approximately 50% of SSc-related mortality was caused by the lung involvements, including ILD and PAH [2]. It has been estimated that approximately 5% of SSc patients develop scleroderma renal crisis (SRC) during the first 4 years after the onset of the disease [2], [20]. Before the use of angiotensin-converting enzyme (ACE) inhibitors in the treatment of SSc, SRC was the leading cause of the disease-related death [21]

Gastrointestinal tract it is the second most commonly affected organs following skin. As the most common internal organ complication, gastrointestinal tract involvements is observed in approximately 90% of patients with SSc [22]. Cardiac involvement in SSc can develop as a direct consequence of SSc or a secondary phenomenon due to pulmonary arterial hypertension and scleroderma renal crisis [23]. Cardiac involvement has been shown to be associated with poor prognosis and increased mortality, thus early diagnosis and management of the complication is of importance [24]. Musculoskeletal system is commonly affected in rheumatic diseases and it occurs much more frequently than expected in SSc patients [25]. Although musculoskeletal involvement is not a life-threatening organ complication, it is the major cause for the disability, which severely affects the quality of life [2].

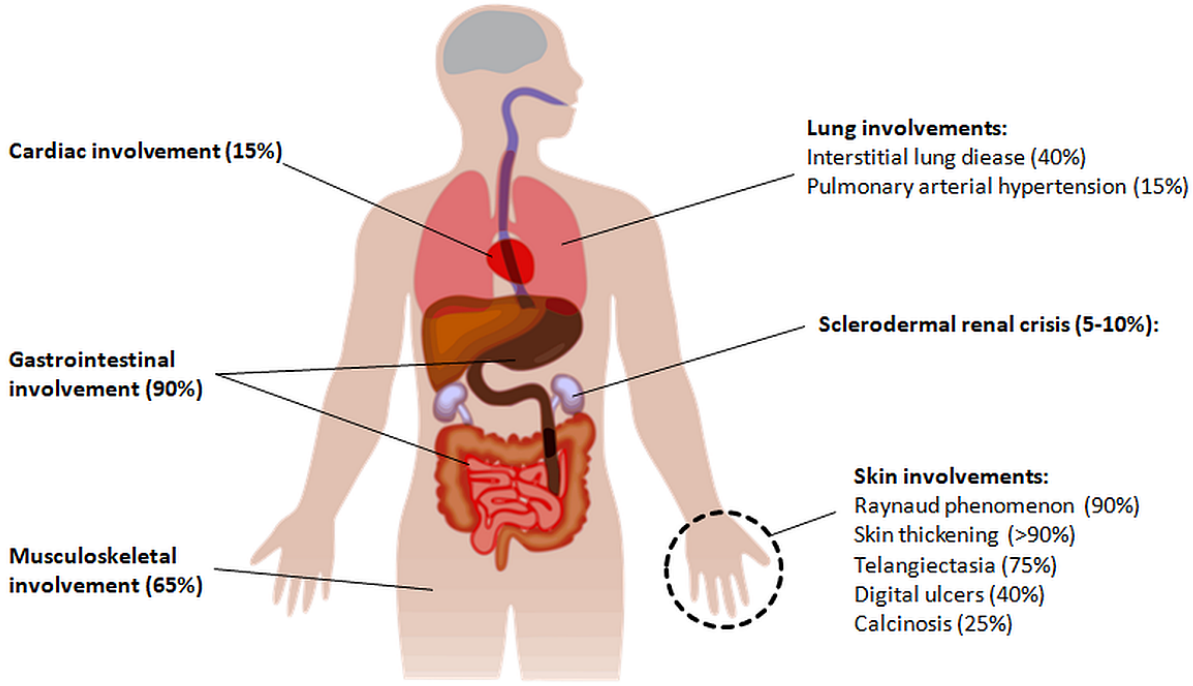

Figure 1. An overview of the frequency of organ manifestations of SSc. The clinical manifestations in the skin and internal organs are depicted. The numbers in the brackets indicate the average frequencies of corresponding manifestations. The figure was adopted and modified from *Allanore Yannick et al. Nat Rev Dis Primers. 2015;1:15002.*

1.1.4. Management of systemic sclerosis

Management of SSc consists of early diagnosis of disease and internal organ involvement, evaluation of risks of development of new organ complications as well as pharmacological and non-pharmacological treatment [26]. Due to the complexity and heterogeneity of clinical manifestations and disease process of SSc, therapeutic strategies of SSc focus on the treatment of organ-specific complications, while no disease-modifying treatments are available [2], [27]. However, early diagnosis and proper treatments of internal organ involvement are likely to modulate tissue damage and eventually improve morbidity and mortality [28], [29]. Currently, strategies used to manage SSc include general immune suppression and complication-targeted therapies based on the systematic assessment of patients and the guidance from experts [2], [3], [28].

The initial step of management of SSc is always to perform a systemic assessment on the clinical phenotypes, the presence and severity of organ complications and disease stage, which is crucial for the classification of patients with SSc and subsequent treatment [2], [3], [28]. For

instance, immunosuppressive agents are generally most effective at the earlier stages of SSc, while for patients with SSc at late stages of SSc when the fibrosis of tissue has been established, antifibrotic agents, disease-specific supportive care and physical therapy are recommended [28], [30]. In the EULAR Scleroderma Trials and Research group (EUSTAR) at 2017, 16 evidence-based recommendations regarding pharmacological treatment of specific organ complications were presented, including cyclophosphamide (CYC) and methotrexate (MTX) [26]. Besides traditional pharmacological treatments, biological treatments have been developed due to advances in the understanding of biological basis of SSc achieved over the past few decades, such as the application of autologous hematopoietic stem cell (HSC) transplantation and Rituximab depleting B lymphocytes have been reported efficient improvement for SSc [30], [31]. Besides, some other biological agents targeting transforming growth factor (TGF) -β, type I interferons, IL-17, platelet-derived growth factor receptor (PDGFR), T lymphocytes and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway are evaluated in ongoing clinical trials [30], [31].

1.2. Etiology of systemic sclerosis

Understanding of the etiology and pathogenesis of SSc is the key for exploring novel therapeutic targets and developing biological and pharmacological treatment approaches [30]. Although the etiology and pathogenesis of SSc are still not completely understood, a widely accepted hypothesis is that SSc is a consequence of interaction between susceptible genetic background and environmental triggers (Figure 2).

1.2.1. Genetic susceptibility

A positive familial history represents one of major risks for SSc, which suggests that genetic factors contribute to the development of the disease [32], [33]. By using of emerging genetic research technologies including candidate gene studies, next generation sequencing (NGS), genome-wide association studies (GWAS) and immunochip studies, many genes have been identified to be associated with susceptibility to SSc during recent decades [34]. Interestingly, the majority of susceptibility genes to SSc is also associated with other autoimmune diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE), suggesting common pathways between multiple diseases [2], [35].

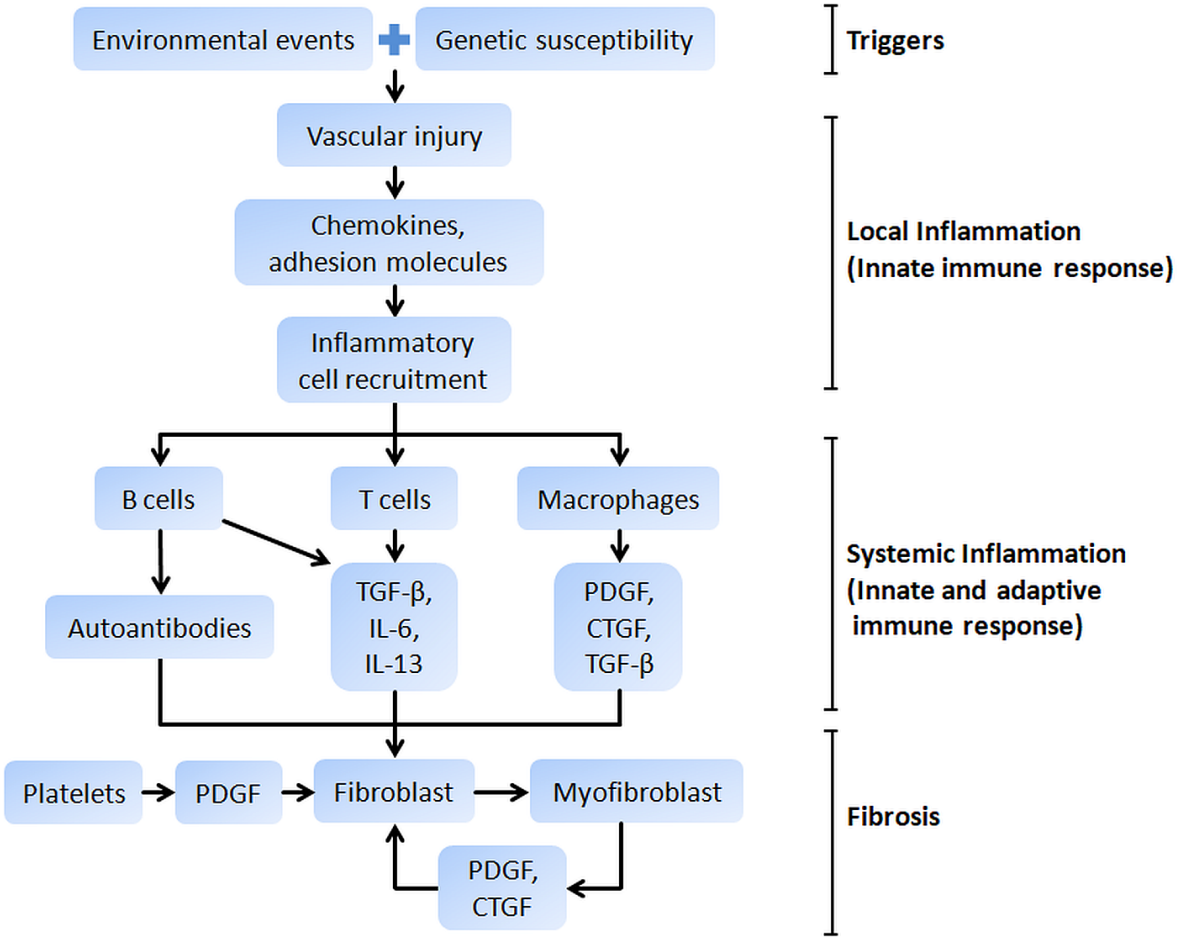

Figure 2. A schematic diagram of the etiology and pathogenesis of SSc. Interaction of environmental factors and genetic susceptibility resulted in the injury of vessels, leading to elevated production of chemokines and expression of adhesion molecules. In response to the chemokines and adhesion molecules, inflammatory cells including B cells, T cells and macrophages are recruited to the organs. Eventually, cytokines and autoantibodies produced by the dysregulated immune system lead to activation of fibroblast, formation of myofibroblast and tissue fibrosis. TGF-β: transforming growth factor β, PDGF: platelet-derived growth factor, CTGF: connective tissue growth factor. The figure was adopted and modified from *Allanore Yannick et al. Nat Rev Dis Primers. 2015;1:15002.*

Like most autoimmune diseases, SSc is strongly associated with HLA. In 1975, Rabin and colleagues for the first time reported the association of HLA with susceptibility to SSc [36]. The association of HLA, both HLA class I and class II genes, and SSc has been confirmed and further characterized in multiple GWAS with large cohort size [34], [37]–[43]. Of note, the association of HLA with SSc varies considerably among populations. For example, DQB1*03:01 has been reported to be associated with a decreased risk of SSc in the Japanese and Mexican patients [44], [45]. However, in Caucasian, African-Americans and Hispanics, DQB1*03:01 is associated with an increased risk of SSc [46]. Apart from the HLA genes, many

non-HLA loci have been shown to be associated with susceptibility to SSc, including interferon regulatory factor 5 (*IRF5*), signal transducer and activator of transcription 4 (*STAT4*), and protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) [34]. Notably, most of these genes are functionally involved in immunity, inflammation, apoptosis, autophagy and fibrosis, indicating that those biological processes contribute to the development of SSc [34], [35]. Interestingly, some disease susceptibility genes are also associated with clinical phenotypes of SSc, including severity, organ manifestations and mortality. For instance, it was reported that IRF5 was associated with mild manifestations of interstitial lung disease and longer survival in SSc patients [47].

In addition to genetic variants mentioned above, a growing body of evidence suggests that epigenetic factors are associated with a variety of disease phenotypes of SSc, including onset, severity and response to medication [34]. Epigenetics is heritable alterations which influence the expression of gene without altering DNA sequence, including DNA methylation, histone modification and non-coding RNAs [48]. Of particular interest, the transcript factors Krueppel-like factor 5 (KLF5) and Friend leukemia integration 1 (FLI1) which are involved in fibrotic process have been observed to be epigenetically suppressed in dermal fibroblasts from SSc patients [49]. Moreover, mice with double heterozygous deficiency of *Klf5* and *Fli1* spontaneously develop three hallmark features of SSc, including autoimmunity, tissue fibrosis and vasculopathy [50].

1.2.2. Environmental factors

Environmental factors also contribute to the development of the disease in subjects genetically susceptible to SSc [51]. So far, many environmental risk factors in SSc have been proposed, including infectious agents, drugs, chemicals and occupational exposures to silica, vinyl chloride and organic solvents [1], [2]. Among those environmental factors, viral infection is of particular interest. For example, antibodies against protein UL94 derived from human cytomegalovirus (hCMV) are able to induce apoptosis of endothelial cells and activate fibroblasts [52], implicating that immune responses against hCMV may contribute to the pathogenesis of SSc. In line with this finding, Farina et al. reported that Epstein-Barr virus (EBV) established infection in the majority of skin fibroblasts and endothelial cells of SSc patients and the infection of EBV in dermal fibroblasts induced innate immune response and fibroblast-myofibroblast conversion [53].

1.3. Pathogenesis of systemic sclerosis

Disease manifestations of SSc often start with vascular injury and endothelial activation which leads to an uncontrolled inflammatory responses and subsequent tissue alternations (Figure 2) [2]. Generally, the disease process of SSc consists of three steps: 1) vascular injury, which is likely triggered by environmental factors in a susceptible genetic background, leads to a dysregulated secretion of endothelin and chemokines and expression of adhesion molecules on surface of endothelial cells; 2) in response to chemokines and adhesion molecules, several types of immune cells are recruited, resulting in the production of autoantibodies and profibrotic cytokines such as TGF-β, interleukin (IL) -13 and IL-6; 3) finally, this cytokines cocktail activates resident fibroblasts to generate reactive oxygen species (ROS) and to differentiate into myofibroblasts, which are responsible for the generation of excessive extracellular matrix. Many types of immune cells as well as resident tissue cells including macrophages, platelets, T cells, B cells, endothelial cells, fibroblasts, and myofibroblasts are involved in the disease process. In addition, TGF-β, platelet derived growth factor (PDGF), connective tissue growth factor (CTGF), IL-6, IL-13, endothelin-1 (ET-1), ROS and autoantibodies have been implicated as prominent mediators in driving the pathogenesis of SSc.

1.3.1. Endothelial injury and vascular dysfunction

Endothelial injury and microvascular dysfunction are usually observed in early stages of SSc, and they might play a fundamental role in the development of SSc [2], [54]. To date, the earliest detectable vascular changes include decreased storage vesicles in endothelial, cytoskeletal rearrangement and perivascular edema [55]. It has been shown in a large prospective study of Raynaud's phenomenon evolving to SSc that microvascular abnormalities develop dynamically and sequentially, starting with capillary enlargement, followed by reduction of the number of capillaries and capillary telangiectasias [56]. This study also demonstrates that coexistence of vascular abnormalities and SSc-specific autoantibodies is highly predictive for the probability of evolving to definite SSc, suggesting an essential role of microvascular abnormality in the development of SSc [56].

The underlying mechanisms of the microvascular abnormalities remain largely unknown. Dermal vessels of SSc are characterized by loss of vascular endothelial cadherin and overexpression of interferon α (IFN-α) in endothelial cells, which suggests the existence of endothelial injury and dysfunction [55]. Although factors which cause endothelial injury and dysfunction in SSc remain unidentified, some potential triggers have been suggested. For

instance, infectious agents such as hCMV could be a putative candidate, as antibodies against hCMV are able to induce apoptosis of endothelial cells [52]. Another potential trigger of endothelial injury and dysfunction is the anti-endothelial cell antibodies (AECA), which are detected in sera of 22%-86% of SSc patients, depending on the detection method and on patient selection [57]. In line with this observation, it has been shown that AECA is able to induce the apoptosis of human dermal microvascular endothelial cells by antibody-dependent cell-mediated cytotoxicity (ADCC) via the Fas/FasL pathway [58]. Besides virus infection and AECA, T cells have also been suggested to be involved in endothelial injury since both γδT cells [59] and activated cytotoxic T cells [60] are able to bind and mediate injury to endothelial cells via releasing granular enzyme.

Injury and dysfunction of endothelial cells could contribute to the development of SSc via multiple ways. First of all, expression of adhesion molecules such as intercellular adhesion molecule (ICAM), vascular cell adhesion molecule 1 (VCAM-1), P-selectin and E-selectin, are increased in endothelial cells, which mediate the firm adhesion of immune cells to endothelium [61], [62]. Second, release of chemokines and cytokine facilitate the infiltration of immune cells into sites of inflammation [2]. Third, enhanced secretion of vasoconstrictors such as endothelin 1 and decreased secretion of vasodilators like prostacyclin and nitric oxide (NO) contribute to the further vascular damage [54]. Finally, platelet activation, another response secondary to endothelial injury and activation [54], releases a variety of inflammatory factors and profibrotic molecules such as TGF-β and PDGF, mediating inflammatory cell recruitment, activation of fibroblast and consequent deposition of extracellular matrix [63].

1.3.2. Tissue fibrosis

Fibrosis is the most distinguishing pathological feature of SSc, especially in dcSSc [2]. It is caused by activation of fibroblasts, which leads to the consequent differentiation into myofibroblast and subsequent excessive production and accumulation of extracellular matrix composed of collagen, elastin, fibronectin and proteoglycans [64]. This process results in permanent scarring and disruption of normal architecture of the skin and visceral organs including lung, heart, kidney, gastrointestinal tract and endocrine glands and eventually tissue damage and dysfunction of organs, which accounts for much of SSc associated morbidity and mortality [5], [65], [66]. Therefore, anti-fibrotic therapy is one of treatment strategies to improve the manifestations and quality of life of SSc patients [3]. Better understanding of the

underlying mechanisms of fibrotic process would identify novel targets for the development of biological or pharmacological treatments [30].

Activated fibroblasts are a central effector cells in fibrotic process [5]. It is generally accepted that fibroblast activation is a consequence of vascular injury and inappropriate immune responses [51]. Vascular injury leads to vasculopathy and reduction of blood supply, resulting in tissue hypoxia and excessive production of reactive oxygen species [67], which are able to activate fibroblast and potently stimulate the production of extracellular matrix like collagen and fibronectin [65]. Besides, aberrant immune responses induce a complex mixture of potent profibrotic mediators released from inflammatory cells, such as IL-6, IL-13 and TGF-β, which promote their myofibroblast differentiation and the production of excessive ECM [64].

Under physiological conditions, fibroblast activation a well-controlled process which plays a key role in wound healing, while pathological fibroblast activation in SSc is a sustained and self-amplifying process leading to the excessive production of ECM [5]. Interestingly, fibroblasts derived from SSc patients express receptor of several profibrotic mediators such as TGF-β, PDGF and CCL2 [68]–[70], and these profibrotic features of fibroblasts from patients with SSc persist in several passages in vitro, even in absence of any profibrotic mediators or cells [69], which suggesting an endogenous mechanism underlying the profibrotic features. Indeed, blocking of endogenous TGF-β signaling could abolishes the profibrotic features of fibroblasts from patients with SSc [71]. In addition to the autocrine TGF-β signaling loop, alterations of epigenetic and microRNA has also been suggested to contribute to the abnormal activation of fibroblasts [72].

The major consequence of activated fibroblasts is differentiation into α-SMA positive myofibroblasts, which produce a large amount of extracellular matrix and leads to fibrosis [73]. In addition to fibroblasts, tissue resident cells including endothelial cells, epithelial cells, pericytes, adipocytes, smooth muscle cells and blood borne fibrocytes are able to differentiate into myofibroblast and thus potentially contribute to the high heterogeneity of myofibroblasts in affected tissue of SSc [73], [74]. However, the contribution and therapeutic relevance to the disease pathogenesis of these myofibroblasts with diverse origins remain unclear [74].

Rather than the increased formation of myofibroblasts, the persistent presence of myofibroblasts is likely the key reason of tissue fibrosis in SSc [75]. In healthy tissues, the presence of myofibroblasts is rare as they often undergo apoptosis when wound healing process is completed [76]. Compared with healthy subjects, significantly increased number of myofibroblasts can be found in the fibrotic skin and visceral organs of patients with SSc [74],

indicating a prolonged presence of myofibroblasts. The mechanism underlying the persistent presence of myofibroblasts in SSc has been partially explored [75]. One hypothesis is that myofibroblasts are capable to evade apoptosis in the context of SSc [73], [75], [77], which is supported by the fact that less pro-apoptotic factors are found in SSc myofibroblasts compared to control subjects [78]. Very recently, it has been reported that mitochondria in activated myofibroblasts but not quiescent fibroblasts are primed by death signals, which creates a requirement for tonic expression of the antiapoptotic protein B-cell lymphoma (BCL) -X_L to ensure myofibroblast survival [79]. These death signal-primed myofibroblasts do not tend to die but still survive in specific microenvironment featured by increased tissue stiffness due to excessive ECM production [73], [75]. The increased tissue stiffness is able to directly promote the survival of myofibroblasts through elevating of pro-survival BCL-2 family of proteins, and blockade of these pro-survival pathways could effectively induce the apoptosis of myofibroblasts and eventually reverse the tissue fibrosis [79], [80]. In addition, the specific microenvironment in SSc could promote the proliferation and survival of myofibroblasts by upregulating the activation of TGF-β [81], [82] and the expression of anti-apoptotic micro RNA such as miR-29a and miR-21 [83], [84]. Taken together, pro-survival signals in activated myofibroblasts in SSc enable them evade apoptosis and thus lead to the persistent presence of myofibroblasts in the affected tissue [75].

1.4. Immunopathomechanisms of systemic sclerosis

1.4.1. Immune dysfunction and autoimmunity

Autoimmunity is one of hallmarks of SSc and plays a prominent role in the pathogenesis of the disease [85]. The best evidence of autoimmunity in SSc is the presence of multiple autoantibodies in sera of patients, including anti-centromere autoantibody (ACA), anti-topoisomerase I autoantibody (ATA) and anti-RNA polymerase III autoantibody which have been used for the diagnosis of SSc [8]. Furthermore, genetic studies have shown that genes functionally involved in immune responses are significantly associated with SSc [35]. In addition, affected tissue in SSc such as skin and lung of SSc patients are featured by infiltration of inflammatory cells including lymphocytes [86], [87], further supporting a role of immune dysfunction and autoimmunity in the pathogenesis of SSc.

T cells are the main cells in the infiltrate in affected tissues and organs in SSc [88][89]. According to the activation status, T cells can be categorized into two groups, naïve T cells which have not encountered their cognate antigens and memory T cells which have been

activated by antigens. Notably, the majority of infiltrated T cells in affected tissues of SSc patients are activated T cells [88]. Analysis of the skin biopsies of SSc patients revealed that infiltrated inflammatory cells contain mostly T cells, and these cells express early T cell activation antigen CD69 [90]. Of note, CD69 plays a role in the cell interaction between T cells and tissue resident cells like fibroblast, which suggests that T cells might actively participate in the progression of fibrosis [90]. This notion is supported by in-situ hybridization studies which showed that infiltrating T cells are adjacent to myofibroblasts [87], [91]. In addition to the increased expression of activation markers, another interesting feature of infiltrated T cells in SSc is that they exhibit oligoclonal expansion [92]. It has been shown in an *in vitro* co-culture experimental system that autologous fibroblasts are able to trigger the expansion of T cells from SSc patients, indicating that the antigen driving oligoclonal expansion of T cell might from fibroblast [93]. Taken together, the presence of activated T cells in affected tissues, together with their interaction with tissue resident cells, implicates that T cells might actively participate in the contribution to the development of disease manifestations.

After being activated, $CD4^+$ T cells differentiate into distinct effector subtypes, including type 1 T helper (Th1) cells, Th2 cells, Th9 cells, Th17 cells, regulatory T (Treg) cells, and T follicular helper (Tfh) cells [88], [91]. Generally, it is believed that Th2-type autoimmune response played a crucial role in the pathogenesis of the disease [2], [88], [94]. Besides Th2 response, imbalance of Th17 cells and regulatory T cells appears to alter the immune homeostasis and thus contribute to the pathogenesis of SSc [95]. Th17 cells promote inflammation, autoimmunity and fibrosis in SSc, while regulatory T cells show immunosuppressive function [95]. In addition, IL-9 released by Th9 cells and IL-21 released by T follicular helper cells have also been suggested to be associated with SSc [96], [97].

In addition to $CD4^+$ T cells, $CD8^+$ T cells and γδT cells have also been suggested to contribute to the development of SSc. $CD8^+$ T cells in SSc patients produce abnormally high levels of IL-13 which is associated with increased skin fibrosis [98], suggesting that they might contribute to the fibrotic process. γδT cells, a subset of T cells expressing γδ T cell receptor (TCR), represent a small subset of T cells in the peripheral blood while account for up to 50% of T cells in the skin and mucosal tissues [99]. Interestingly, the absolute number of circulating γδT cells in SSc patients is decreased compared with healthy controls, which might due to increased recruitment of γδT cells in the affected tissues [100]–[102]. In comparison with healthy controls, SSc patients are featured by larger fractions of γδT cells expressing activation antigen CD49d that promotes the adhesion of γδT cells to endothelial cells through binding to ICAM molecules

[103], γδT cells with Vδ1 chain, and $CD27^+$ γδT cells [102]. The differences between SSc patients and healthy controls in γδT cells regarding their distribution and constitution implicate a role of γδT cells are involved in the development of SSc [102], [103].

B cells are at the center of the adaptive humoral immune system and are responsible for the production of antibodies, including autoantibodies. There is accumulating evidence showing that SSc is characterized by dysregulation of B cell homeostasis. First of all, patients with SSc is often characterized by hypergammaglobulinemia and polyclonal B cell activation [104]. Secondly, B cells are found in the infiltrate of the skin in patients with SSc [88], and genes associated with $CD20^+$ B cells are significantly increased in the skin of SSc patients compared to healthy controls [105], [106]. Finally, The circulating memory B cells in patients with SSc express higher levels of activation markers such as CD80, CD95, HLA-DR and B-cell activating factor (BAFF) than those from healthy controls [107], [108]. The dysregulated B cell homeostasis suggests that B cells play a key role in the pathogenesis of SSc. In line with this notion, treatment of Rituximab, a B cell-depletion monoclonal antibody, has been shown to be able to improve the skin and lung involvement in patients with SSc [109]–[111].

Macrophages play essential roles in tissue homeostasis and immune surveillance. Considering the important role of macrophage in responding to microbial invasion and promoting wound healing, failure to resolve macrophage activation can lead to chronic inflammation and fibrosis [112]. As one of the main cell types in inflammatory infiltrates in affected tissues of SSc, a role of macrophage in the disease has been suggested several decades ago [64], [113]. However, the precise role of macrophages in the pathogenesis is still largely unknown [113]. Given that activated macrophages are the primary source of potent profibrotic cytokine TGF-β, it is conceivable that macrophages contribute to the fibrotic process in SSc [64]. In line with this notion, the progressive lung fibrosis in patients with SSc are correlated with increased expression of TGF-β gene and activation of macrophage [114], and inhibition of macrophage activation by Nintedanib in the *Fra2* transgenic mice could attenuate dermal and pulmonary fibrosis [115]. Regarding the underlying molecular mechanism of the role the macrophages in SSc, it has been suggested macrophages are activated through toll like receptors (TLR) such as TLR2, TLR4 and TLR9, resulting in release of pro-fibrotic cytokines and thus promote development of the disease [116].

Activated macrophages are generally categorized into two types, classically activated pro-inflammatory type macrophages (M1) and alternatively activated pro-fibrotic/anti-inflammatory type macrophages (M2) [117]. Both M1 and M2 are presented in the infiltrates

of affected skin and lung of patients with SSc [113]. By using a novel multi-network approach, Taroni et al demonstrated that expression of multiple markers of alternatively activated macrophage are elevated in skin and lung of patients with SSc compared with healthy controls [118]. In addition, macrophages from SSc patients express higher level of pro-fibrotic cytokines, indicating a significant contribution of M2 in mediating fibrosis and disease pathogenesis of SSc [118]. In line with this observation, multiple studies reported that M2 macrophages expressing CD169 are significantly increased and activated in the affected skin of SSc patients, and soluble CD169 molecules are elevated in the sera of SSc patients [119]–[121]. The increased expression of CD169 and activation of macrophages are likely to be induced by type I IFN and Toll like receptor (TLR) agonists [122].

Besides macrophages, mast cells are also observed in the skin infiltrate of patients with early SSc [123]. Mast cells have been identified as a source of TGF-β, a potent profibrotic cytokine [124]. In addition to releasing TGF-β, mast cells are able to augment the proliferation of fibroblast by heterotypic cell-cell adhesion and secretion of IL-4, and thus stimulate the production of collagen [125]. This cell-to-cell contact of activated mast cells and fibroblasts has also been observed in the skin biopsy from SSc patients [126], further supporting a role of mast cells in the development of SSc.

Infiltrated plasmacytoid dendritic cells (pDC) are present in the skin of SSc patients but not in healthy subjects [127], and pDCs isolated from SSc patients are chronically activated and spontaneously release cytokines and chemokines such as IFN-α and CXCL4 [127]. Consistently, serum levels of IFN-α and CXCL4 in SSc patients are associated with disease severity [128]. In line with observations in patients, experimental evidences show that depletion of pDCs attenuates multiple molecular and histological phenotypes, including skin and lung fibrosis, inflammation in affected tissues as well as expression of genes involved in chemotaxis, inflammation and fibrosis in affected tissues in the bleomycin-induced mouse model for SSc [129].

1.4.2. Autoantibodies and functional autoantibodies in SSc

1.4.2.1. Autoantibodies in SSc

The presence of autoantibodies against multiple intracellular antigens including anti-topoisomerase I autoantibodies, anti-centromere autoantibodies and anti-RNA polymerase III autoantibodies in sera of patients have been frequently observed in systemic sclerosis. Some of

these autoantibodies are very specific for SSc, whereas other autoantibodies are observed in a variety of other autoimmune diseases such as SSc, RA, SLE and primary Sjögren's syndrome (pSS) as well [130], [131]. Most of these autoantibodies are not treated as pathogenic driver but diagnostic biomarkers in the clinic including anti-centromere, anti-topoisomerase I and anti-RNA polymerase III autoantibodies [8]. Limited SSc is commonly associated with anti-centromere autoantibody, by contrast, diffuse SSc is more often related with anti-topoisomerase I and anti-RNA polymerase III autoantibodies [3]. Generation of autoantibodies suggests a breach of central and/or peripheral immune tolerance which results in the maturation of autoreactive B cells [132], [133].

1.4.2.2. Functional autoantibodies in SSc

Functional autoantibodies represent a group of autoantibodies which are able to bind to their target proteins such as cell surface receptors and subsequently excite stimulatory or inhibitory effects [134]. A well-known example of functional autoantibodies is thyroid-stimulating autoantibodies (TSAb), which are the direct cause of Grave's disease [135]. TSAb are able to mimic the thyroid-stimulating hormone (TSH), bind and activate TSH receptor (TSHR), inducing hyperthyroidism and Grave's disease [135].

As the TSHR is a member of G-protein-coupled receptors (GPCRs) family, it is conceivable that there are functional autoantibodies against other GPCRs. This concept has been validated by several studies which demonstrated the presence of functional autoantibodies against different types of GPCRs such as beta-adrenergic receptors (β-AR) in cardiomyopathy [136] and muscarinic M3 receptors (M3R) in pSS [137]. The first functional autoantibody identified in the patients with SSc was autoantibody against PDGFR as it was showed that autoantibody purified from SSc patients was able to stimulate the Ras-ERK1/2 signaling pathway, expression of collagen type 1 and production of ROS from fibroblasts expressing PDGFR. Following studies have identified more functional autoantibodies against different type of antigens or cells including estrogen receptor, endothelin type A receptor (ETAR), endothelial cells and fibroblasts [134].

Although mimicking the ligand binding, functional autoantibodies differ greatly from ligands in terms of receptor activation. As each IgG molecule carries two antigen-binding fragments, which endows autoantibodies an unique ability of binding and cross linking two receptor molecules (Figure 3) [136]. Receptor dimerization induced by functional autoantibodies is able to mediate a different type of receptor activation compared with natural ligand-mediated

activation. This is best exemplified by anti-β2-Adrenoceptor (β2-AR) autoantibodies whose bivalent Fab fragments are able to induce an uncontrolled and sustained activation of β2-AR [138].

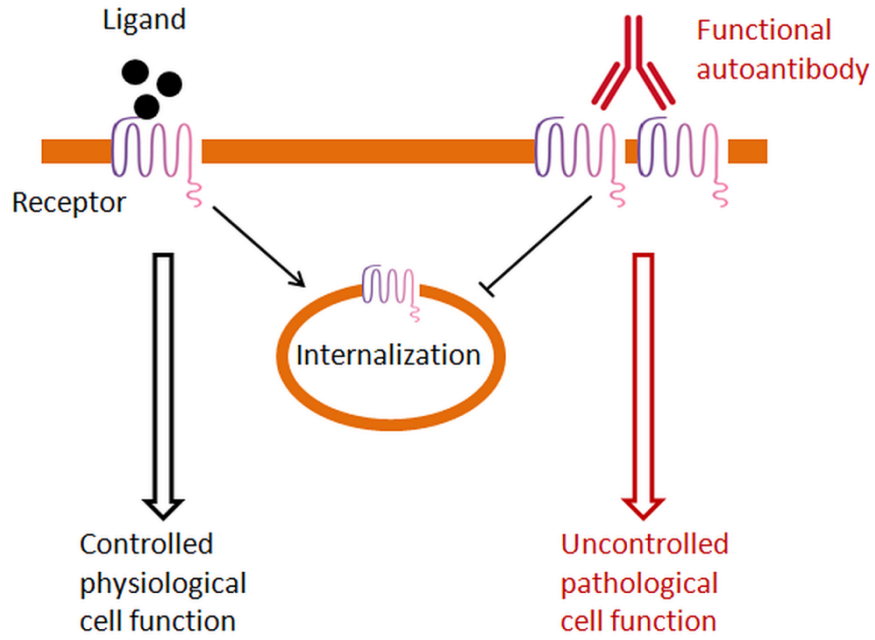

Figure 3. Schematic diagram of GPCR activation. Activation of GPCR by natural ligands induces physiological functions in a well-controlled manner via internalization of GPCR, whereas activation of GPCR by functional autoantibodies induce pathological events in an uncontrolled manner through inhibiting the internalization of GPCR.

One possible explanation for this abnormal receptor activation is that the dimerization of receptor could inhibit the internalization of the receptor, leading to an sustained activation (Figure 3) [134], [136]. Under physiological conditions, the activation of a receptor by its classical ligands results in the internalization and desensitization of receptor, which, in turn, negatively control the receptor mediated response [136]. However, binding of bivalent functional autoantibodies to the receptors inhibits the internalization of receptors, leading to an sustained activation (Figure 3) [134], [136], [139]. Besides abovementioned anti-β2-AR autoantibodies, anti-AT1R autoantibodies are also capable of inducing the sustained receptor activation by inhibiting the internalization of the receptor [140]. Furthermore, the sustained activation of AT1R receptor by angiotensin II due to defective function of internalization of AT1R could result in glomerulosclerosis, kidney failure and significantly increased mortality of mice [141]. Therefore, the functional autoantibody-mediated sustained activation of GPCRs seems to be a general mechanism of anti-GPCR autoantibody-induced pathology [134], [136].

1.4.3. Functional autoantibodies against Angiotensin II receptor type 1

1.4.3.1. Angiotensin II receptor type 1

Angiotensin II (Ang II), a vasoconstricting peptide hormone, is a main effector component of the renin-angiotensin system [142]. It is generated in the circulation and within tissues, via a sequential enzymatic cleavages by renin and angiotensin converting enzyme (ACE) [143]. Ang II is a strong vasoconstrictor which induces vessel constriction and secretion of aldosterone and thus plays a pivotal role in electrolyte homeostasis, blood pressure maintenance and retention of sodium and water [142]–[144]. Overproduction of Ang II and aberrant responses to Ang II have been demonstrated to be associated with hypertension and other cardiovascular diseases [143]. Besides, treatment of patients with cardiovascular diseases using drugs inhibiting Ang II production or blocking its receptor is able to reduce blood pressure and disease-related morbidity and mortality [143].

The biological functions of Ang II are mediated by two distinct receptors, angiotensin II receptor type 1 (AT1R) and type 2 (AT2R) [144], [145]. Both AT1R and AT2R are 7-transmembrane receptor, also known as GPCR which are associated with heterotrimeric G-proteins composed of α, β and γ subunits [143]. Activation of GPCR, such as AT1R, results in dissociation of the Gα and the Gβγ complex and subsequent intracellular signaling events, including calcium influx, production of inositol trisphosphate and diacylglycerol, and activation of tyrosine kinases and serine/threonine kinases [146]. AT1R is widely expressed in the body and is the major receptor mediating the biological functions of Ang II, including vessel constriction, blood pressure elevation and secretion of aldosterone (Figure 4) [144], [145], [147], [148]. AT2R is generally considered to antagonize the functions of AT1R, but the precise physiological function of AT2R is still largely unknown [144], [145], [147]. While human express one AT1R protein, mice express two version of the receptor, AT1A and AT1B receptors [145]. Murine AT1RA is mainly expressed in kidney, heart, liver, vascular smooth muscle and other tissues, whereas AT1RB is primarily expressed in the anterior pituitary and zona glomerulosa [145]. With regard to their function, AT1A receptor confers most of classical actions of Ang II including blood pressure increase, aldosterone release and sodium retention, while AT1B receptor is able to regulate the blood pressure when AT1A receptor is absent [145].

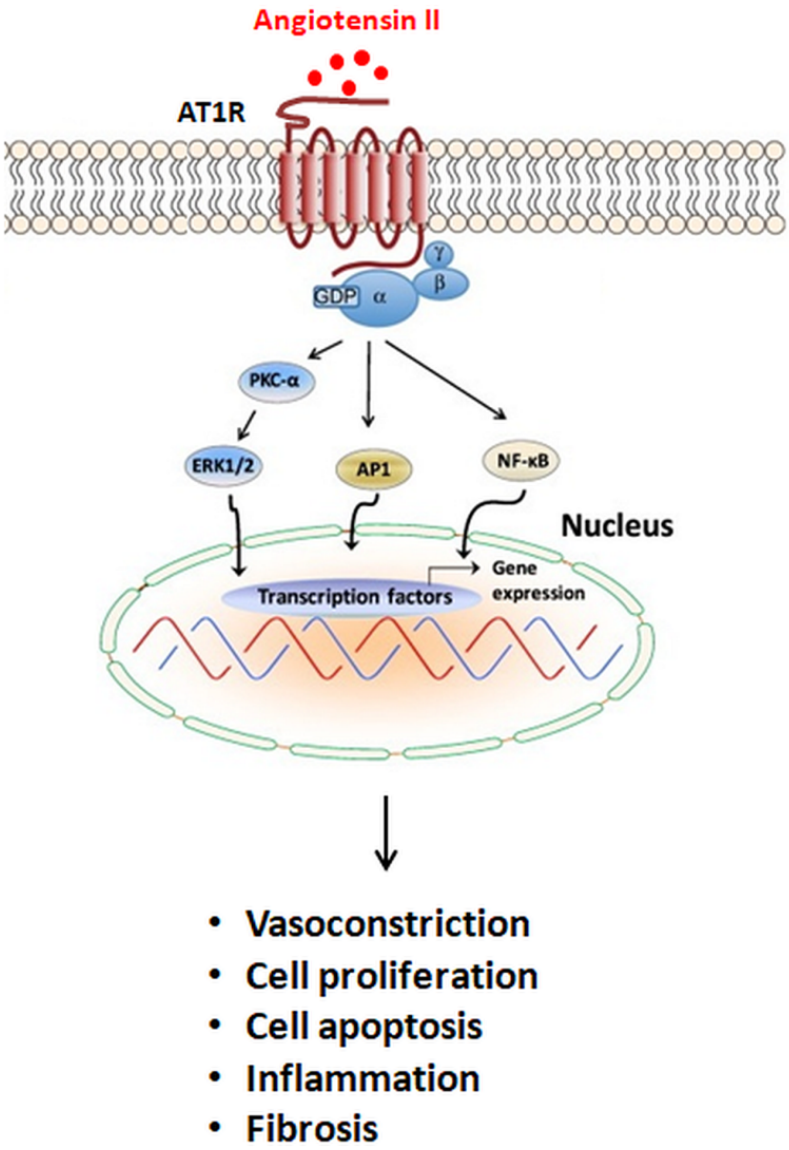

Figure 4. Physiological and pathological effects induced by activation of AT1R. Binding of angiotensin II with AT1R stimulate the activation of ERK1/2 signaling pathway and the activation of transcription factors NF-kb and AP1, and eventually induced physiological and pathological effects which described in the bottom of figure. Figure was adopted and modified from *Cabral-Marques O, Riemekasten G. Autoimmun Rev. 2016 Jul;15(7):690-4. doi:10.1016/j.autrev. 2016.03.005.*

Beyond physiological functions of AT1R in regulating blood pressure and controlling circulatory homeostasis, there are increasing evidences indicating that AT1R contributes to inflammation, and tissue fibrosis which are hallmarks of SSc (Figure 4) [144], [149], [150]. Activation of AT1R is able to mediate multiple events of inflammatory responses, leading to enhanced migration of inflammatory cells from blood to tissues, including T cells, NK cells, monocytes and dendritic cells [151]. For instance, AT1R activation leads to the upregulation of

adhesion molecules such as ICAM, VCAM-1 and selectin on endothelial cells, which enhances the migration of leukocytes from peripheral blood to inflammation sites. In addition, two other proinflammatory effects of AT1R activation, namely stimulating the release of chemokines such as monocyte chemotactic protein 1 (MCP-1) and increasing cell permeability of vessels, have also been suggested [144], [151], [152]. Besides its proinflammatory effect, activation of AT1R promotes the production and secretion of collagen from rat cardiac fibroblasts in vitro, and this effect can be specifically blocked by AT1R antagonist [153]–[155], suggesting a profibrotic effect of AT1R activation. In line with this notion, consistent infusion of angiotensin II into mice using osmotic mini pump successfully induced inflammation and tissue fibrosis in the heart and skin [156]–[158], providing direct experimental evidence for a role of AT1R in tissue fibrosis.

1.4.3.2. Functional autoantibodies against AT1R

The presence of a wide range of autoantibodies is a hallmark of SSc and correlated with clinical manifestations, disease severity and the risk of mortality [159], suggesting that those autoantibodies are involved in the pathogenesis of SSc. Among the autoantibodies associated with SSc, autoantibodies against angiotensin II receptor type 1 (AT1R) have been suggested to be pathogenic in the development of SSc by several clinical evidence. Firstly, it has been reported that autoantibodies against AT1R is significantly elevated in sera of SSc patients, compared to healthy subjects and patients with other autoimmune diseases such as primary Sjögren's syndrome and rheumatoid arthritis (RA) [160]. Secondly, higher levels of anti-AT1R autoantibodies are associated with more severe disease manifestations such as lung fibrosis and PAH and could predict SSc-related mortality [160]. Thirdly, *in vitro* studies have demonstrated that IgG from SSc patients are capable of promoting the release of chemokine and cytokines from endothelial cells and the production of collagen from fibroblasts, and the promoting effect could be specifically blocked by using antagonists of AT1R, suggesting that anit-AT1R autoantibodies are functional [161], [162]. Collectively, autoantibodies against AT1R might act as functional autoantibodies and contribute to the pathogenesis of SSc *via* agonizing AT1R.

Besides clinical evidence, experimental studies with animals also support the hypothesis that autoantibodies against AT1R contribute to the pathogenesis of SSc. It has been shown that repetitive transfer of SSc IgG containing higher level of AT1R autoantibodies purified from patients could induce inflammation in the lung and abundant expression of α-SMA in the vessel wall and airway epithelium, indicating that autoantibodies against AT1R are pathogenic [163]. However, since the transferred antibodies are total IgG purified from patients comprising

antibodies targeting numerous antigens, the specific role of anti-AT1R autoantibodies need to be further elucidated.

Recently, in order to determine the pathogenic role of anti-AT1R autoantibodies, we immunized C57BL/6J mice with membrane extract isolated from Chinese Hamster Ovary (CHO) cells overexpressing human AT1R or control membrane extract isolated from CHO cells. Since cell membrane extract can maintain conformational structure of AT1R, such immunization therefore is able to induce the production of autoantibodies against native conformational epitopes of AT1R in mice. Nine weeks after immunization, mice immunized with AT1R membrane extract developed autoantibodies against AT1R, inflammation in the lung and skin, and fibrosis in the skin, which resembles multiple hallmarks of SSc (unpublished data). Therefore, immunization with human AT1R is able to induce a novel mouse model for SSc, and it also suggests that autoimmunity against AT1R is pathogenic. Furthermore, to prove that anti-AT1R autoantibodies induced by immunization with AT1R are functional, we generated one murine monoclonal antibody against AT1R from AT1R-immunized mice. *In vitro* experiments have shown that this monoclonal antibody is able to increase the beating rate of rat cardiomyocytes, suggesting that it is a functional monoclonal antibody (unpublished data).

1.5. Aims of this study

Taken together, systemic sclerosis is a multi-organ involved autoimmune disease with complex pathogenesis and unclear mechanisms. It is believed that dysregulation of immune system including T lymphocytes, B lymphocytes and production of massive autoantibodies play important role in the pathogenesis of systemic sclerosis. However, the pathomechanisms of immune dysregulation especially production of autoantibodies in the pathogenesis of SSc are largely unknown. The mouse model of SSc recently established in our group is initiated by immunization of AT1R and provides us an ideal tool to investigate the pathomechanisms of immune dysregulation and autoantibodies induced disease. In this thesis, I hypothesized that immunization of AT1R activates the autoreactive lymphocytes, which results in cytokines release and production of functional anti-AT1R autoantibodies, and the functional anti-AT1R autoantibodies mediate an abnormal activation of AT1R and thus induce pathological changes.

To prove my hypothesis, I primarily address two goals. First, as described above, dysregulation and activation of immune cells including $CD4^+$ T cells, $CD8^+$ T cells and B cells are a hallmark pathological feature of SSc and might be implicated in the pathogenesis [88]. Thus, the role of $CD4^+$ T cells, $CD8^+$ T cells and B cells in the pathogenesis of AT1R immunization induced

mouse model of SSc was investigated. Second, it was demonstrated that functional autoantibodies against AT1R were present in SSc patients [160] and in our novel mouse model of SSc. However, the pathogenicity of those functional autoantibodies against AT1R as well as the underlying mechanisms are unclear. To address these questions, the pathogenicity of these antibodies *in vivo* by transfer experiments in mice was determined. Moreover, as the autoantibodies against AT1R are able to stimulate the receptor, it is reasonable to believe that autoantibodies against AT1R in the mouse are able to induce pathological changes through agonizing AT1R but not through activation of complement system. To prove this, complement C3 deficient mice were immunized with AT1R membrane extract.

2. Materials and Methods

2.1. Materials

2.1.1. List of chemicals and reagents

Products	Source	Catalogue number
0.9% NaCl	BBraun	1511675
30% H_2O_2	Sigma	H1009
70um cell strainer	Greiner bio one	542070
Absolute ethanol	Walter CMP	WAL6425000
Acetic acid	Merck Millipore	1.00063.1000
Bouin's solution	Sigma	HT10132
Bovine serum albumin (BSA)	Sigma	A4503
Citric acid	Merck Millipore	1.00244.1000
Collagenase type 4	Worthington	LS004188
Complete freund's adjuvant	Sigma	F5881
Cryomold	Sakura	4566
Dulbecco's Modified Eagle Medium (DMEM)	Merck Millipore	FG0445
Eosin G solution 1%	Carl Roth	3137.2
Fetal calf serum (FCS)	Gibco	10270
Giemsa stain solution	Sigma	GS1L
Goat serum	PAN Biotech	P30-1001
H_2SO_4	Merck	1.00731.1000
Hematoxylin Gill II solution	Carl Roth	T864.2
Histological embedding cassette	Simport	M516
Incomplete freund's adjuvant	Sigma	F5506
KCl	Merck Millipore	1.04936.0500
Ketamin	WDT	0916603AA
KH_2PO_4	Merck Millipore	4873

Live-dead blue fluorescence solution	Invitrogen	L34962
May-Grünwald stain solution	Sigma	MG1L
Microscope slide	R.Langenbrinck	03-0060
Mounting medium	Merck Millipore	1.07961.0500
Na_2HPO_4	Merck Millipore	1.06346.1000
NaCl	Merck Millipore	1.06404.5000
Paraffin	DCS innovative diagnostic systeme	PL00352K
Porcine pepsin	Sigma	P7012
Penicillin/Streptomycin (PEST)	PAN Biotech	P06-07700
ProLong Gold Antifade mountant	Invitrogen	P36934
Proteinase inhibitor	Roche	11836145001
RNAlater solution	Invitrogen	AM7020
Roti ImmunoBlock	Carl Roth	T144.1
Roti Histofix	Carl Roth	P087.1
Sodium citrate dihydrate	Merck Millipore	6448
Tetramethylbenzidine (TMB)	Sigma	T2885
Tissue-Tek cryo gel	Sakura	4583
TMB	Sigma	T2885
Trypsin/EDTA	PAN Biotech	P10-020100
Tween-20	Sigma	P1379
Xylazin	CP Pharma	16D257
Xylene	Walter CMP	WAL12401

2.1.2. List of solutions and buffers

Solution	**Recipe**
0.1 mg/ml pepsin solution	3.6mg was dissolved in 36ml 0.5M acetic acid solution
0.5M Acetic acid solution	1ml Acetic acid + 35ml MilliQ water, vortex mix
1% Acetic acid solution	1ml Acetic acid + 100ml MilliQ water, vortex mix

10mM Sodium citrate buffer	2.94g, Sodium citrate (dihydrate), add 800ml MilliQ water, adjust PH to 6.0 with HCl, fill to 1L with MilliQ water.
1M H_2SO_4 solution	Slowly add 53.2 ml to 900ml MilliQ water (stir during adding), fill up to 1L with MilliQ water
1x proteinase inhibitor solution	1 proteinase inhibitor tablet in 50ml PBS
2mg/ml Collagenase type 4 solution	40mg Collagenase type 4 in 20ml PBS
Phosphate buffered saline (PBS)	8g NaCl, 0.2g KCl, 1.44g NaHPO4•2H2O, 0.2g KH2PO4, add 800ml MilliQ water, adjust the PH to 7.4 with HCl, fill to 1L with MilliQ water.
TMB substrate solution	480mg TMB + 10ml Aceton, add EtOH up to 100ml, mixed well, add 600ul 30% H_2O_2, mixed well and stored in dark at 4ºC for up to 6 months
TMB substrate buffer	6.3g Citric acid, add 900ml MilliQ water, adjust PH with KOH to 4.1, add MilliQ water up to 1L, filter with 0.2um filter and store at RT

2.1.3. List of consumables

Products	**Source**	**Catalogue number**
96 well cell culture plates	Costar	3596
Blood collection tubes	BD	365968
Cannulas	BBraun	4252136B
Culture flasks	Sarstedt	83.3911.002
ELISA plates	NUNC	442404
FACS tubes	FALCON	352054
Falcon tubes	Greiner bio one	227261
Forceps	Schreiber	50-2000
IBIDI µ-Slides 8 Well	IBIDI	80826
Luer lock syringes	BD	309628
Micro tubes	Sarstedt	72.706.600
Micro-emulsifying needles	Cadence Science	7976
Microtome blades	pfm medical	02.075.00.001
Needles	BD	301300

Punch	Schreiber	45-0201
RNase free micro tubes	Sarstedt	72.706.400
Scalpels	BBraun	5518040
Scissors	Schreiber	50-4140
Serological pipettes	Costar	4487
Sterile syringe filters 0.22 um	Sarstedt	83.1826.001
Syringes	BBraun	9166017v
Tips	Sarstedt	70.760

2.1.4. List of kits

Kits	**Source**	**Cat. number**
AT1R ELISA kit	CellTrend	AT1R-MTP
Avidin-Biotin blocking kit	Vector laboratories	SP-2001
DAB Peroxidase (HRP) Substrate Kit	Vector laboratories	SK-4100
High Pure RNA Isolation Kit	Roche	11828665001
Masson staining kit	Sigma	HT15-1KT
Sircol collagen kit	Biocolor	S1000
Transcriptor First Strand cDNA Synthesis Kit	Roche	04897030001
VECTASTAIN Elite ABC HRP Kit (Peroxidase, Standard)	Vector laboratories	PK-6100
Murine IL-1α Mini ABTS ELISA Development Kit	PeproTech	900-M82
Murine IL-4 Mini ABTS ELISA Development Kit	PeproTech	900-M49
Murine TNF-a Mini ABTS ELISA Development Kit	PeproTech	900-M54
Murine IFN-g Mini ABTS ELISA Development Kit	PeproTech	900-M98

2.1.5. List of antibodies

Antibodies	**Clone**	**Source**	**Cat. number**	**Class**
Unlabeled antibodies				

Mouse anti-Human AT1R mAb5.2a	5.2a	Borstel	-	Mouse IgG2a
Mouse IgG2a isotype	MG2a-53	BioLegend	401504	Mouse IgG2a
TruStain Fc-block	93	BioLegend	101320	Rat IgG2a
Rabbit anti-Mouse CD3	polyclone	Abcam	ab5690	Rabbit IgG
Rat anti-Mouse CD45R (B220)	RA3-6B2	eBioscience	14-0452	Rat IgG2a
Rat anti-Mouse neutrophil	7/4	Cedarlane	CL8993AP	Rat IgG2a
Labeled antibodies				
Percp/Cy5.5 conjugated Rat anti-Mouse F4/80	BM8	eBioscience	45-4801	Rat IgG2a
Percp/Cy5.5 conjugated Rat IgG2a	eBR2a	eBioscience	45-4321	Rat IgG2a
FITC conjugated Hamster anti-Mouse CD11c	HL3	eBioscience	557400	Armenian Hamster IgG
FITC conjugated Hamster IgG	eBio299Arm	eBioscience	11-4888	Armenian Hamster IgG
PE conjugated Rat anti-Mouse CD45	30-F11	BioLegend	103105	Rat IgG2b
PE conjugated Rat IgG2b	A95-1	BDPharmingen	553989	Rat IgG2b
APC conjugated Rat anti-Mouse CD140a	APA5	BioLegend	135907	Rat IgG2a
APC conjugated Rat IgG2a	RTK2758	BioLegend	400511	Rat IgG2a
Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Polyclone	Invitrogen	A-11029	Goat IgG
DyLight™ 649 Goat anti-mouse IgG (minimal x-reactivity) Antibody	Poly4053	BioLegend	405312	Goat IgG
Peroxidase AffiniPure Goat Anti-Mouse IgG, Fcγ fragment specific	Polyclone	Jackson ImmunoResearch	115-035-071	Goat IgG
Biotin-SP (long spacer) AffiniPure $F(ab')_2$ Fragment Goat Anti-Rat IgG (H+L)	Polyclone	Jackson ImmunoResearch	112-066-003	Goat IgG
Biotin-SP (long spacer) AffiniPure Goat Anti-Rabbit IgG (H+L)	Polyclone	Jackson ImmunoResearch	111-065-144	Goat IgG

2.1.6. List of equipment and instruments

Equipment	Source	Model
37ºC incubator	Memmert	30-750
Advanced analytical balance	Sartorius	R300S
Biological safety cabinet	Heraeus	HERA safe
CO2 incubator	Heraeus	HERA cell 150
Confocal microscope	Leica	TCS SP5
Cryostat system	Leica	CM3050S
Embedding workstation	Thermo scientific	HistoStar
Flow cytometer	BD	LSRII
Fluid aspiration system	Vacuubrand	BVC control
Hemocytometer	Marienfeld	NA
High speed centrifuge	Hettich	Mikro 22R
Light microscope I	Carl Zeiss	Primovert
Light microscope II	Nikon	NIS-Elements
Liquid blocker PAP pen	Kisker biotech	NA
Low speed centrifuge	Hettich	Rotixa 50RS
Microplate reader	TECAN	Sunrise
Microtome	Leica	RM2125RT
Mini centrifuge	Neolab	D-6015
Multichannel pipette	Biohit	e1200
PH meter	Knick	PH-meter 766
Pipette	Starlab	Transferpette
Precision balance	Kern	EG4200-2NM
Pressure pot	Instant pot	IP-DUO60
Repeater pipette	Eppendorf	Multipette plus
Roller	Phonix instrument	RS-TR05
Serological pipette gun	ABIMED	swiftpet
Shaker 1	Heidolph	MR Hei-standard
Shaker 2	Stuart scientific	STR8

Spectrophotometer	Nanodrop	1000
Tissue processor	Leica	TP1020
Vortex mixer	Scientific industries	vortex genie 2
Water bath	Julabo	12B

2.2. Mice and handling of mice

2.2.1. Mice

Seven-week old female wild type C57BL/6J mice, $CD4^+$ T cell-deficient mice (B6.129S2-$Cd4^{tm1Mak}$/J, CD4 KO), $CD8^+$ T cell-deficient mice (B6.129S2-$Cd8a^{tm1Mak}$/J, CD8 KO) and B cell-deficient mice (B6.129S2-$Ighm^{tm1Cgn}$/J, muMT) were purchased from the Jackson Laboratory (Bar Harbor, ME, US). Complement C3-deficient mice (B6.129S4-$C3^{tm1Crr}$/J, C3 KO), were kindly provided by Prof. Dr. Admar Verschoor in University of Lübeck, Lübeck, Germany. AT1R deficient mice (B6.129P2- $Agtr1a^{tm1Unc}$/$Agtr1b^{tm1Cof}$/J, AT1R KO) and their littermate controls were kindly provided by Prof. Thomas Walther from University College Cork, Ireland and bred at the animal facility of Research Center Borstel. All mice were housed under specific pathogen free conditions with 12-hour light/darkness cycles. All animal experiments were reviewed and approved by the Animal Research Ethics Board of the Ministry of Energy Change, Agriculture, Environment, Nature, and Digitalization, Kiel, Germany.

2.2.2 Immunization with membrane extract

Cell membrane extract (ME) isolated from Chinese Hamster Ovary (CHO) cells overexpressing AT1R or untransfected CHO cells were kindly provided by CellTrend (Luckenwalde, Berlin, Germany). Eight to ten weeks old female mice were anesthetized by injection i.p. with 120 µl anesthetic consisted of 2.156 ml 0.9% NaCl, 312.5ul ketamin and 31.25ul xylazin. After anesthetization, mice were immunized with 0.2 mg of membrane extracts prepared from CHO cells overexpressing human AT1R in 50 µl PBS emulsified with an equal volume of complete Freund adjuvant (CFA, Sigma-Aldrich, USA) via subcutaneous injection into the footpad. Three weeks after the primary immunization, mice were boosted with same amount of the membrane extracts emulsified with incomplete Freund adjuvant (IFA, Sigma-Aldrich, USA). In the control group, mice were treated with same amount of membrane extracts isolated from untransfected CHO cells. Nine weeks after the first immunization, mice were euthanized via

inhalation of CO2, and peripheral blood, dorsal skin and the lung were collected for the evaluation of disease characteristics.

2.2.3 Transfer of monoclonal antibodies

Mouse monoclonal antibody against AT1R (Clone: mAb5.2a) was generated through hybridoma technology by Dr. Xiaoqing Wang in our laboratory. To transfer anti-AT1R monoclonal IgG into C57BL/6J mice, AT1R deficient mice or their littermate controls, mice were anesthetized by i.p. injection with 120ul anesthetic consisted of 2.156ml 0.9% NaCl, 312.5 µl ketamin and 31.25 µl xylazin, then 100 µg mAb5.2a (2 mg/ml in PBS) was injected intradermally into each mouse ear. The injection of monoclonal antibodies was repeated at day 2, 4, 6, 8, 10, and 12 after the first injection. Mouse IgG2a antibodies (Clone MG2a-53, Biolegend, USA) were used as isotype control for the transfer. Fourteen days after the first injection, mice were euthanized via inhalation of CO2 and the ears and lung were collected for further evaluation.

2.2.4 Preparation of serum

Peripheral blood of mice was collected from the inferior vena cava of mice and stored in BD Microtainer® blood collection tubes. Freshly collected blood samples were left to stand at RT for one hour, centrifuged at 6000 g for 10 min, and then the supernatant (sera) were collected. Serum samples were transferred to sterilized microtubes and stored at -80 °C.

2.2.5 Preparation of murine tissues

Lung and dorsal skin were collected from mice immunized with membrane extract for further evaluation. One lobe of the lung was used to prepare paraffin-embedded sections, another lobe was used for preparing Tissue-Tek Cryo gel-embedded sections, and the rests were frozen and stored at -80 °C. The dorsal skin samples were cut into strips. Part of skin samples were used to prepare paraffin-embedded and Tissue-Tek Cryo gel-embedded sections, and the rests were stored at -80 °C.

Lung and ear skin biopsies were collected from mice transferred with monoclonal antibodies against AT1R. The lung tissue was fixed in 4% formalin solution and then dehydrated at room temperature (RT) for 24 hours. Mouse ear tissue was embedded in paraffin or Tissue-Tek Cryo gel or stored at -80 °C.

2.3. Histological methods

2.3.1. Preparation of paraffin-embedded sections

Tissue samples were fixed in 4% formalin for 24 hours. After dehydration and paraffinization, tissue samples are embedded in paraffin and sectioned at thickness of 5 µm. A detailed procedure is summarized in the table 8.

Table 8: Procedures of dehydration and paraffinization

Steps	Solutions	Time
1	4%formalin	1 hour
2	70% ethanol	1 hour
3	80% ethanol	1 hour
4	90% ethanol	1 hour
5	96% ethanol	1 hour
6	100% ethanol	1 hour
7	100% ethanol	1 hour
8	100% ethanol	1 hour
9	Xylene I	1 hour
10	Xylene II	1 hour
11	Paraffin I	1.5 hours
12	Paraffin II	1.5 hours

2.3.2. H&E staining

To determine the inflammation in tissues, H&E staining was performed using following procedures. Briefly, paraffin-embedded sections were first deparaffinized in xylene and rehydrated in ethanol series with decreasing concentrations, then stained with hematoxylin and eosin solutions. Thereafter, the stained sections were dehydrated and then mounted with mount medium. A detailed procedure is summarized in the table 9.

Table 9: Procedures of H&E staining

Step	Reagent	Incubation
Deparaffinization	Xylene I	5 min

	Xylene II	5 min
	Xylene III	5 min
Rehydration	Absolute ethanol I	5 min
	Absolute ethanol II	5 min
	Ethanol 96%	5 min
	Ethanol 70%	5 min
	Tap water	5 min
Staining	Gill's II hematoxylin solution	20 min
Wash	Running tap water	10 min
Staining	Eosin (1%, acidic) counterstain	3 min
Wash	Tap water	10 seconds
Dehydration	Ethanol 70%	10 seconds
	Ethanol 96% I	10 seconds
	Ethanol 96% II	10 seconds
	Absolute ethanol I	10 seconds
	Absolute ethanol II	3 min
Transparentization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Mounting	Entellan mounting medium	-

2.3.3. Immunohistochemistry

Immunohistochemistry staining was performed on paraffin-embedded skin and lung sections. Tissue sections were deparaffinized in xylene and rehydrated in ethanol series with decreasing concentrations. Subsequently, antigen retrieval was performed by heating slides in a high-pressure pot at 121°C for 50 min in 10mM citrate buffer (PH=6.0). After cooling down at RT for 15min, sections were then blocked with 3% H_2O_2 for 15 min, with biotin blocking solution (Vector Laboratories, USA) for the 15 min and with 5% BSA solution for 50 min. After the blocking, sections were incubated with rat anti-mouse neutrophil (Clone: 7/4, Cedarlane, Canada), rabbit anti-mouse CD3 (Clone: polyclone, Abcam, UK) or rat anti-mouse B220 (Clone: RA3-6B2, eBioscience, USA) at 4°C overnight. On the next day, after washing with PBS to remove unbound primary antibodies, sections were incubated with biotin-conjugated goat anti-rat IgG (H+L) secondary antibody (Clone: polyclone, Jackson ImmunoResearch, USA) or biotin-conjugated goat anti-rabbit IgG (H+L) secondary antibody (Clone: polyclone,

Jackson ImmunoResearch, USA) for 50 min at RT, followed by incubation with well-mixed avidin and biotinylated-HRP solution (Vector Laboratories, USA) for 30 minutes. Diaminobenzidine (DAB, Vector Laboratories, USA) was applied to visualize immunoreactivity. Afterwards, the sections were counterstained with hematoxylin for 5 minutes. When the staining process was finished, sections were dehydrated and mounted with mount medium. A detailed procedure is summarized in the table 10.

Table 10: Procedures of immunohistochemistry

Step	Reagent	Incubation
Deparaffinization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Rehydration	Absolute ethanol I	5 min
	Absolute ethanol II	5 min
	Ethanol 96%	3 min
	Ethanol 70%	3 min
	Deionized water	5 min
Antigen retrieval	10Mm citrate buffer (PH＝6.0)	121°C, 4 min (50 min in high pressure pot)
Cool down	10Mm citrate buffer (PH＝6.0)	15 min
Blocking endogenous peroxidase	3% H_2O_2 solution	15 min
Washing	PBS	5 min, three times
Blocking endogenous biotin	Avidin solution	15 min
Washing	PBS	5 min
Blocking endogenous avidin	Biotin solution	15 min
Washing	PBS	5 min, three times
Blocking unspecific binding sites	5% BSA	50 min
Incubation with primary antibody	Primary antibodies diluted in 5% BSA	4°C, overnight
Washing	PBS	5 min, three times
Incubation with biotinylated secondary antibody	Biotinylated secondary antibodies diluted in 5% BSA	50 min, RT
Washing	PBS	5 min, three times
Preparation of avidin and biotinylated HRP complex (ABC solution)	100 ul of avidin solution and 100 ul of biotinylated HRP solution in 5 ml of PBS	30 min, RT
Incubation with ABC solution	ABC solution	30 min, RT

Washing	PBS	5 min, three times
Incubation with DAB solution	84 ul of buffer stock solution, 100 ul of DAB reagent and 80 ul of H_2O_2 in 5 ml of Deionized water	2 min, RT
Washing	Tap water	5 min, three times
Counterstaining	Gill's II hematoxylin solution	1 min
Washing	Running tap water	5 min
Dehydration	Ethanol 70%	10 seconds
	Ethanol 96% I	10 seconds
	Ethanol 96% II	3 min
	Absolute ethanol I	3 min
	Absolute ethanol II	3 min
Transparentization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Mounting	Entellan mounting medium	-

2.3.4. Masson's Trichrome staining

To evaluate tissue fibrosis, paraffin-embedded tissue sections were stained with Masson's Trichrome staining kit (Sigma-Aldrich, USA) which is able to stain collagen fibers as bright blue color, nuclei as dark blue color, and muscle fibers as red color. Briefly, tissue sections were deparaffinized in xylene and rehydrated in ethanol series with decreasing concentrations. Rehydrated sections were fixed in Bouin's solution at RT overnight, counterstained with Hematoxylin Gill II solution, washed and incubated with Biebrich scarlet-Acid Fuchsin, and subjected to phosphotungstic/phosphomolybdic acid solution. Phospho acid treated sections were then incubated with aniline blue solution, differentiated in 1% acetic acid, rinsed with deionized water, dehydrated in ethanol, and finally mounted with mounting medium. A detailed procedure is summarized in the table 11.

Table 11: Procedures of masson's trichrome staining

Step	Reagent	Incubation
Deparaffinization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Rehydration	Absolute ethanol I	5 min
	Absolute ethanol II	5 min
	Ethanol 96%	5 min
	Ethanol 70%	5 min
	Deionized water	5 min
Fixation	Bouin's solution	Overnight, RT
Washing	Running tap water	5 min
Staining	Gill's II hematoxylin solution	5 min
Washing	Running tap water	5 min
Washing	Deionized water	10 seconds
Staining	Biebrich scarlet-Acid Fuchsin	5 min
Washing	Deionized water	10 seconds, twice
Incubation with phospho acid solution	Phosphotungstic/Phosphomolybdic Acid solution	5 min
Staining	Aniline Blue solution	5 min
Removal of extra binding	1% Acetic Acid	1.5 min
Washing	Deionized water	10 seconds, twice
Dehydration	Absolute ethanol I	5 min
	Absolute ethanol II	5 min
Transparentization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Mounting	Entellan mounting medium	-

2.4. Cell preparation and culture

2.4.1 Preparation of single cells from murine skin

To prepare single cell suspension from murine skin, 1 cm^2 dorsal skin and 2 mouse ears were cut into small pieces, put into Falcon tubes containing 20 ml sterile PBS supplemented with 2

mg/ml collagenase IV and incubated on a shaker at 37°C incubator for 1.5 hours. Thereafter, the solution was filtered using a 70-um cell strainer, and the flow through was centrifuged at 300 g for 10 min at RT. Cell pellets were washed one time with warm PBS and then resuspended in the PBS solution.

2.4.2 Culture of murine fibroblast cell line L929

To detect the binding of autoantibodies against AT1R to murine fibroblasts, L929 cells were seeded in an 8-well IBIDI u slide equipped with a polymer coverslip bottom with highest optical quality enabling their use for confocal microscopy. Prior to cell loading, 200 µl complete culture medium (DMEM + 10% FCS + 1% Penicillin/Streptomycin) was added into each well of the 8-well IBIDI u slide and incubated at 37°C in 5% CO2 incubator for 1 hour. Afterwards, the medium was aspirated and 30,000 cells in 200 µl complete culture medium were seeded into each well of the 8-well IBIDI u slide and incubated overnight at 37°C and 5% CO2 incubator to let the cells attach to the bottom of slides. On the next day, cells were used for detecting the binding of anti-AT1R autoantibodies.

2.5. Immunofluorescence staining

2.5.1 Detection of binding of anti-AT1R monoclonal IgG to the skin

Immunofluorescence staining was used to detect the binding of monoclonal antibody against AT1R to cells on cryosections prepared from murine ear skin. Ear samples from untreated mice were collected and embedded in Tissue-Tek Cryo gel, and 5 µM ear cryosections were prepared using a Leica Cryo microtome. Briefly, cryosections were blocked with 1x ROTI® ImmunoBlock (Carl Roth, Germany) solution for 1 hour at RT, incubated with anti-AT1R monoclonal antibody (mAb5.2a) or mouse IgG2a isotype control antibody solution overnight at 4°C. On the next day, sections were washed with PBS, incubated with Dylight649-conjugated goat anti-mouse IgG (minimal x-reactivity) secondary antibody (Clone: poly4053, Biolegend, USA) at RT for 45 min. After the incubation, sections were washed with PBS, counterstained and mounted with ProLong Gold Antifade mountant containing DAPI (Thermo Fisher, USA). Finally, fluorescence was detected using Leica SP5 confocal microscopy (Leica SP5, Germany).

2.5.2 Detection of binding of anti-AT1R monoclonal IgG to cells

Binding of anti-AT1R monoclonal IgG to cells of the murine fibroblast cell line L929 was determined by immunofluorescence staining. Briefly, L929 cells cultured on ibidi µ-Slides (ibidi GmbH, Germany) were washed with warm PBS and incubated with 5% goat serum for 1 hour at RT to block non-specific binding. After blocking, cells were incubated with monoclonal antibody against AT1R or mouse IgG2a isotype control antibody at 4°C for 2 hours. Subsequently, cells were washed and incubated with DyLight649 conjugated goat anti-mouse IgG (minimal x-reactivity) secondary antibody (Clone: poly4053, Biolegend, USA) at 4°C for 2 hours. Finally, cells were fixed in 4% PFA, counterstained and mounted with ProLong Gold Antifade mountant containing DAPI (Thermo Fisher, USA), and fluorescence was detected using Leica SP5 confocal microscopy (Leica SP5, Germany).

2.6. Enzyme-Linked ImmunoSorbent Assay (ELISA)

A commercial enzyme-linked immunosorbent assay (ELISA) kit (CellTrend, Germany) was used to detect levels of anti-AT1R autoantibodies in the mouse serum samples according to the recommendations from the manufacturer with modification. Briefly, microplates pre-coated with membrane extract from CHO cells overexpressing human AT1R were incubated with serum samples prepared in log dilutions starting from starting from 1:100 to 1:100,000,000 at 4 °C for two hours. After washing with PBS containing 0.05% Tween-20 and incubation with HRP-conjugated goat anti-mouse IgG secondary antibody (Clone: polyclone, Jackson ImmunoResearch, USA) at RT for 1 hour, specific antibody binding was visualized by using TMB solution. After stopping the reaction with 1M H_2SO_4, optical density (OD) value was determined at 450 nm on a TECAN microplate reader, and the OD value at 620 nm was used as the reference. To determine levels of anti-AT1R antibodies, standard curves were generated using serum samples from an AT1R-immunized mouse. Levels of anti-AT1R antibodies were defined as the dilution at which the OD value reached the half of maximal OD values of the curve.

Levels of cytokines in mouse sera were determined by using the commercial ELISA kits purchased from PeproTech and the sandwich ELISA was performed according to the manufacture's protocol. Briefly, NUNC MaxiSorp® flat-bottom 96 well microplates (Thermo Fisher Scientific, USA) were coated with capture antibody in 50 µl PBS overnight at RT. On the next day, plates were washed 4 times with PBS containing 0.05% Tween-20, blocked with PBS containing 1% BSA at RT for 1 hour, and then incubated with 1:10 diluted serum samples or 2-fold serial diluted standard controls at RT for 2 hours. After the incubation, biotinylated detection antibodies were added, followed by HRP-conjugated avidin and assays were

developed by using TMB solution. After stopping the reaction with an equal volume of 1M H_2SO_4, OD value of samples were determined at 450 nm on a TECAN microplate reader, and the OD value at 620 nm was used as the reference. Finally, serum concentrations of cytokines were calculated according to standard curves.

2.7. Quantification of skin fibrosis

Skin fibrosis was quantified using two methods, measuring the thickness of the skin and determining the collagen content of skin. Skin thickness was measured as the thickness of the collagen layer which was defined by Masson's Trichrome staining, while the collagen content of the skin was determined by using the Sircol collagen detection kit (Biocolor, UK) according to the manufacture's instruction. Briefly, punch of skin tissues (9 mm^2) were cut into pieces and digested in in 0.5 M acetic acid solution containing 0.1 mg/ml porcine pepsin (Sigma, USA) at 4 °C overnight on the shaker. On the next day, samples were centrifuged at 12,000 g for 10 min at 4°C, and supernatant were collected for collagen measurement. The 1:10 diluted supernatant samples were mixed with Sircol dye solution and incubated on shaker for 30 minutes at RT. Collagen samples of 5, 10, 15, 20, 25 µg were also mixed and incubated with Sircol dye solution and served as standards. Subsequently, samples were centrifuged at 12000 g for 10 min, and pellets were washed with ice cold acid salt wash reagent to remove unbound dyes. Subsequently, dye bound collagen pellets were dissolved in Alkali buffer was added to each sample to release collagen. To record the OD values, samples were transferred into a plate and measured on TECAN microplate reader at a wavelength of 550 nm. Amount of collagen in samples were calculated according to the standard curve generated with gradient diluted collagen.

2.8. Flow cytometry

In some cases, binding of anti-AT1R monoclonal IgG to cells was determined by flow cytometry. Single cells prepared from ears of untreated mice were used for flow cytometry analysis. Prior to staining with antibodies, cells were washed once with PBS with 0.1% BSA, stained with the LIVE/DEAD blue fluorescence solution (Thermo Fisher, MA, USA) and blocked with TruStain Fc-block (Clone: 93, Biolegend, USA) at 4°C for 15min. Subsequently, cells were washed with PBS with 0.1% BSA, incubated with the monoclonal antibody against AT1R or isotype control IgG (Clone: MG2a-53, Biolegend, USA) at 4°C for 20min. After incubation, cells were washed and incubated with Alexa488-conjugated goat anti-mouse IgG antibody (Polyclone, Invitrogen, USA) or Dylight649-conjugated goat anti-mouse IgG antibody (Clone: poly4053, Biolegend, USA). To discriminate different cell populations, cells

were further stained with fluorescent-conjugated antibodies for identification of fibroblasts, macrophages and dendritic cells, including PE-conjugated rat anti-mouse CD45 (Clone:30-F11, Biolegend, USA), APC-conjugated rat anti-mouse CD140a (PDGFR, Clone: APA5, Biolegend, USA), Percp/Cy5.5-conjugated rat anti-mouse F4/80 (Clone: BM8, eBioscience, USA) and FITC-conjugated hamster anti-mouse CD11c (Clone: HL3, eBioscience, USA). PE-conjugated rat IgG2b (Clone: A95-1, BD Pharmingen, USA), APC-conjugated rat IgG2a (Clone: RTK2758, Biolegend, USA), Percp/Cy5.5-conjugated rat IgG2a (Clone: eBR2a, eBioscience, USA), FITC-conjugated hamster IgG (Clone: eBio299Arm, eBioscience, USA) were used as corresponding isotype controls, respectively. Finally, samples were analyzed by using a LSRII flow cytometer (BD, USA), and acquired data were processed by using FCS Express software (De novo software, version 6).

2.9. Structure modeling and antibody-antigen docking

To model the binding of mAb5.2a and AT1R, sequences of the cDNA of variable fragment of light and heavy chain of mAb5.2a which were cloned and sequenced by Dr. Antje Müller (University of Lübeck, Germany) were loaded into the Primer 5.0 software and translated to protein sequence. The protein sequences of variable fragment of light and heavy chain of mAb5.2a were submitted into SAbPred server [164], a web-based server designed by Oxford Protein Informatics Group (OPIG) for antibody structural modeling. The structure of variable fragment (Fv) portion of mAb5.2a antibody was modeled using the ABodyBuilder application tool in the SAbPred server, and the ANARCI tool and Chothia numbering scheme were used to annotate the model of the Fv portion [165]. To model the structure of AT1R receptor, the protein sequence of human AT1R (P30556, AGTR1_HUMAN) was retrieved from the Uniprot database (https://www.uniprot.org/). The protein sequence of human AT1R was submitted to the SWISS-MODEL server which is a widely used and reliable web-server for protein structure modeling [166]. To model the binding of mAb5.2a with AT1R, outputted structural models of the Fv portion of mAb5.2a and the AT1R receptor were submitted to the ZDOCK server, a widely used web-server for predicting structural model of protein-protein complex [167]. The derived structural model of mAb5.2a-AT1R complex was subsequently loaded in the PyMol open source molecular visualization system for further analysis. Finally, to visualize the contacts in the interface of mAb5.2a and AT1R, the hydrogen bonds and polar contacts at the interface were highlighted using PyMol.

2.10. Statistics

All data was analyzed by using Graphpad Prism software (version: prism 5.0). Quantitative data was first examined by the Kolmogorov-Smirnov normality test. For the quantitative data with normal distribution, statistical differences were determined by two-tail unpaired Student's t test. All the other quantitative data were analyzed by Mann-Whitney U test. Significant differences between qualitative datasets were determined by Fisher-exact test. A p value below 0.05 was considered as statistically significant.

3. Results

3.1. Role of CD4$^+$ T cells, CD8$^+$ T cells and B cells in the active mouse model

As mentioned above, we have established an active mouse model of SSc by immunization of AT1R membrane extract (ME) to the C57BL/6J mice. This active mouse model reproduced several key features of SSc including autoimmunity, inflammation in the skin and lung and skin fibrosis. Given that CD4$^+$ T cells, CD8$^+$ T cells and B cells have been suggested to be involved in the development of human SSc, it is conceivable that all these 3 cells might contribute to the pathogenesis of our active animal models of SSc. Therefore, I determined the role of CD4$^+$ T cells, CD8$^+$ T cells and B cells in the AT1R-induced mouse model for SSc. As shown in Figure 5, CD4$^+$ T cell deficient mice, CD8$^+$ T cell deficient mice, B cell deficient mice and wild type C57BL/6J mice were immunized with either membrane extract of CHO cells overexpressing AT1R (AT1R ME) or membrane extract from control CHO cells (control ME). Nine weeks after the first immunization, mice were sacrificed and key features of this novel mouse model for SSc, including production of anti-AT1R autoantibodies, lung inflammation, skin inflammation and fibrosis, were assessed.

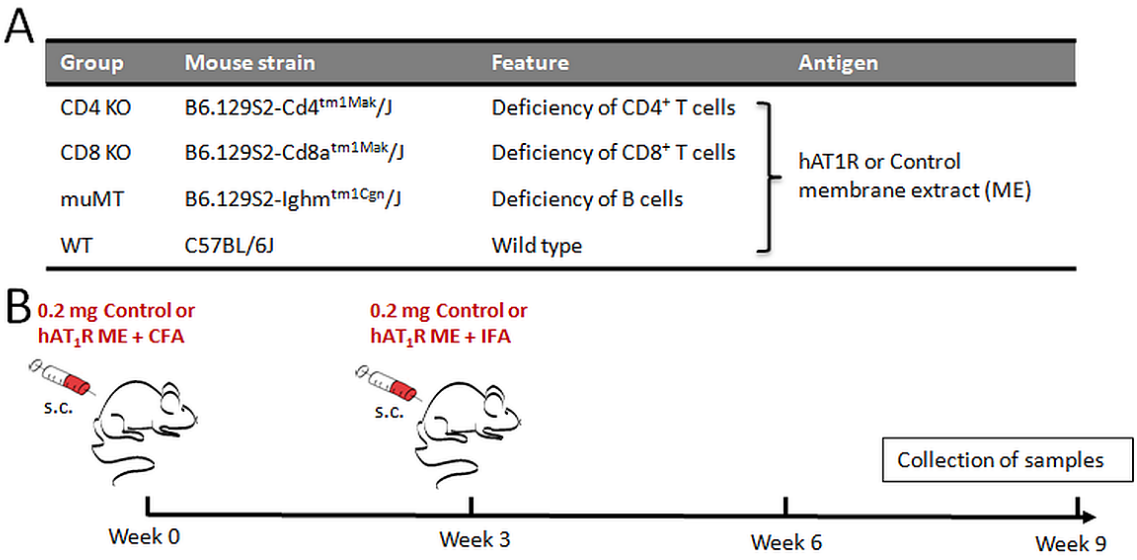

Figure 5. Overview of the experimental design. A. Mouse strains used in this experiment, including CD4$^+$ T cell deficient mice, CD8$^+$ T cell deficient mice, B cell deficient mice and wild type (WT) C57BL/6J mice. **B**. Schematic overview of the experiment of immunization. Mice were immunized at week 0 with membrane extract (AT1R ME or control ME) emulsified with CFA and boosted 3 weeks later with membrane extract emulsified with IFA. Nine weeks after the first immunization, mice were sacrificed, and blood and tissue samples were collected for evaluation. Mouse number: WT (Untreated-

Control-AT1R, 3-6-7), CD4 KO (3-5-11), CD8 KO (3-6-8), muMT (3-6-9). Mouse number used in figure 6, 7, 8, 9, 10 is same with this figure.

3.1.1. $CD4^+$ T cells, B cells but not $CD8^+$ T cells are required for the production of anti-AT1R autoantibodies

To determine which types of lymphocytes are required for the generation of autoantibodies, I first determined levels of anti-AT1R autoantibodies in the sera of mice by using ELISA. As expected, AT1R ME-immunized wild type mice produced high levels of anti-AT1R IgG, while untreated or control ME-immunized wild type mice did not. Moreover, levels of AT1R ME immunization induced anti-AT1R IgG in $CD8^+$ T cell deficient mice comparable to those in wild type controls. By contrast, neither AT1R ME-immunized $CD4^+$ T cell deficient mice nor AT1R ME-immunized B cell deficient mice produced any detectable level of anti-AT1R IgG, suggesting that those antibodies are generated in a T cell-dependent manner (Figure 6).

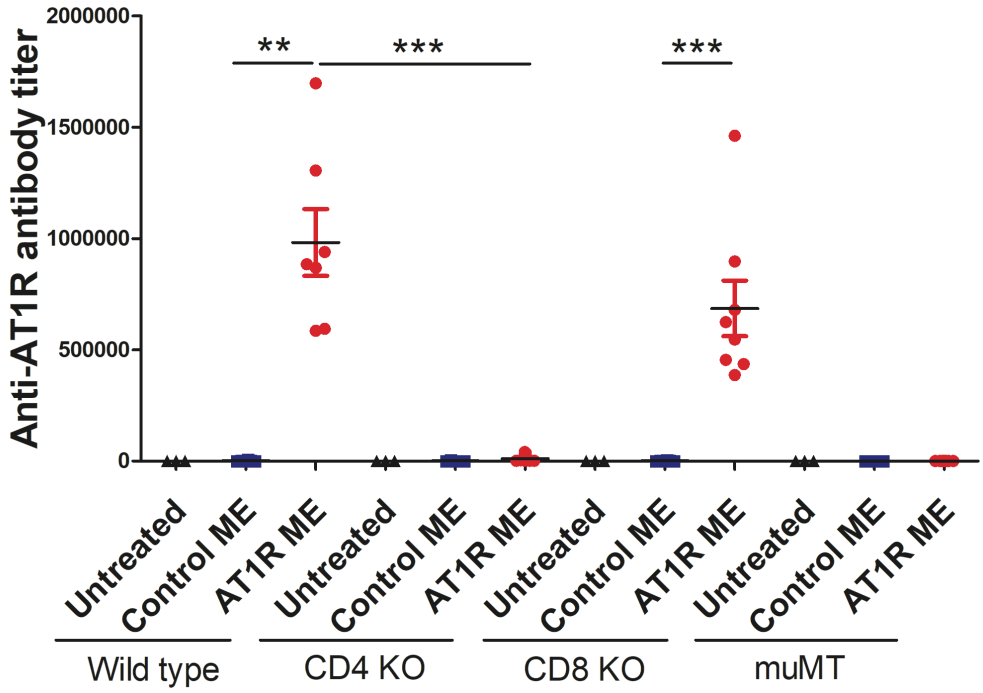

Figure 6. $CD4^+$ T cells and B cells are required for AT1R-induced production of autoantibodies in mice. Wild type (WT), $CD4^+$ T cell deficient (CD4 KO), $CD8^+$ T cell deficient (CD8 KO) and B cell deficient (muMT) mice were immunized with control ME or AT1R ME. Levels of anti-AT1R IgG were detected in the sera of untreated, control ME-immunized or AT1R ME-immunized mice by using ELISA. P values were calculated by Mann Whitney U test. **, $p<0.01$, ***, $p<0.001$.

3.1.2. $CD4^+$ T cells and B cells are required for the elevation of pro-inflammatory cytokines

Since dysregulation of cytokines in the sera of patients is a feature of SSc and correlated with clinical manifestations of patients, I next determined the serum levels of four pro-inflammatory cytokines which have been reported to be implicated in the pathogenesis of SSc, namely IL-1α, IL-4, tumor necrosis factor α (TNF-α) and IFN-γ in mice [88]. As shown in Figure 7, AT1R ME-immunized wild type mice produced significant higher level of IL-1α, IL-4, TNF-α and IFN-γ than control-ME immunized wild type mice. Similarly, significantly increased levels of IL-1α, IL-4, TNF-α and IFN-γ were observed in the AT1R ME immunized $CD8^+$ T cell deficient mice. By contrast, the elevated levels of cytokines were not observed in AT1R ME-immunized $CD4^+$ T cell deficient mice or B cell deficient mice, suggesting that both $CD4^+$ T cells and B cells are required for the dysregulation of cytokines in this mouse model.

3.1.3. $CD4^+$ T cells and B cells are involved in the development of pulmonary inflammation

Next, the contribution of those lymphocytes to the development of histopathology in AT1R ME-induced mouse model for SSc was investigated. By using H&E staining of paraffin sections of the lung, the pulmonary inflammation in those mice was assessed. The severity of pulmonary inflammation was calculated as infiltration score which was quantified according to the number and size of infiltrates. As shown in Figure 8, no inflammation was observed in untreated wild type mice, while immunization with AT1R ME induced more severe inflammation than immunization with control ME in the wild type mice. Similarly, stronger lung inflammation was observed in AT1R ME-immunized $CD8^+$ T cell deficient mice compared with control ME-immunized $CD8^+$ T cell deficient mice (Figure 8). In addition, severity of pulmonary inflammation in AT1R ME immunization induced $CD8^+$ T cell deficient mice was comparable to that in wild type mice. However, in the $CD4^+$ T cell deficient mice, there was no significant difference of the severity of pulmonary inflammation induced by AT1R ME immunization and control ME immunization (Figure 8). Similarly, no significant difference of lung inflammation was observed when compared AT1R ME immunized B cell deficient mice and control ME immunized B cell deficient mice (Figure 8). These data suggest that both $CD4^+$ T cells and B cells are required for the development of pulmonary inflammation in the active mouse model of SSc.

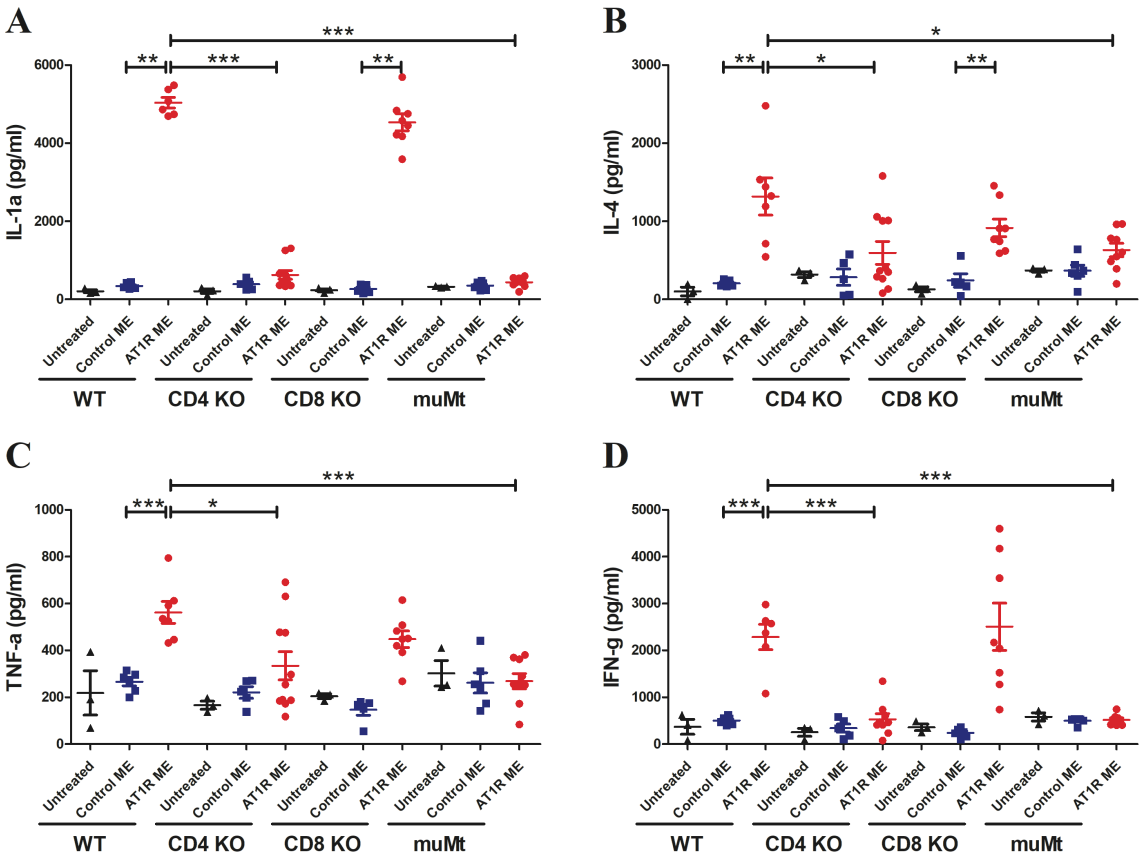

Figure 7. Serum levels of cytokines in mice. Serum levels of IL-1α (A), IL-4 (B), TNF-α (C) and IFN-γ (D) were detected in untreated, control ME-immunized or AT1R ME-immunized wild type (WT), $CD4^+$ T cell deficient (CD4 KO), $CD8^+$ T cell deficient (CD8 KO) and B cell deficient (muMT) mice using ELISA kit purchased from PeproTech. Statistical analysis was performed using Mann Whitney U test or Student t-test depending on the normality of data. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

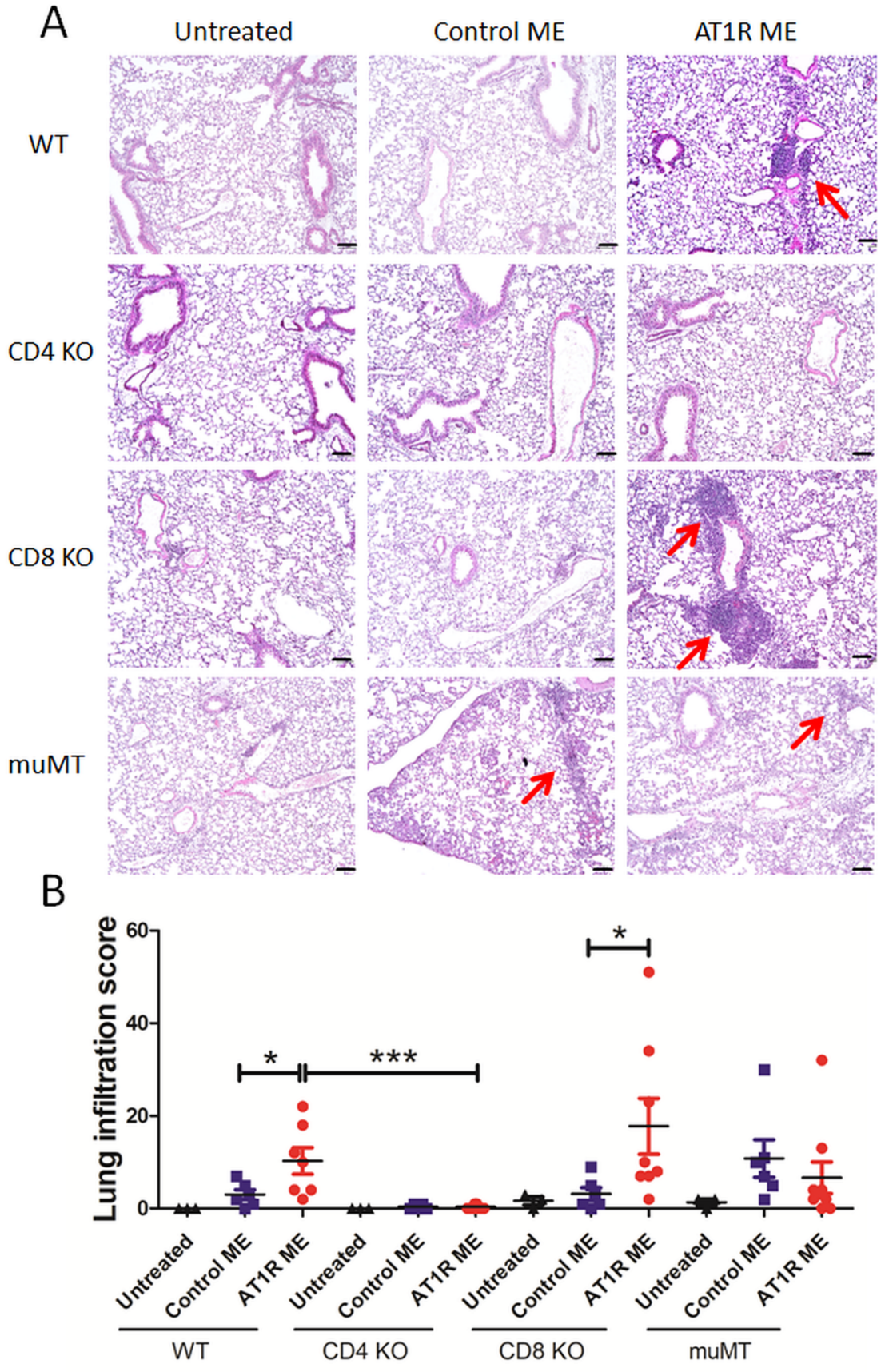

Figure 8. CD4$^+$ T cells and B cells are required for AT1R-induced pulmonary inflammation in mice. Wild type (WT), CD4$^+$ T cell deficient (CD4 KO), CD8$^+$ T cell deficient (CD8 KO) and B cell

deficient (muMT) mice were immunized with control ME or AT1R ME. **A**. Representative micrographs of H&E stained lung sections of untreated, control ME-immunized or AT1R ME-immunized mice. Red arrows indicate inflammatory cell infiltration in the lung. Bar = 100 μm. **B**. Quantitative analysis of pulmonary inflammation in mice. Severity of pulmonary inflammation was quantified based on size and number of infiltrates in the lung and scored in a double-blinded fashion. Statistical analysis was performed using Mann Whitney U test or Student t test depending on the normality of data. *, $p<0.05$, ***, $p<0.001$.

3.1.4. $CD4^+$ T cells and B cells are required for the development of skin inflammation

Since skin inflammation is further a hallmark of the histopathology of the AT1R-induced mouse model for SSc, the involvement of $CD4^+$ T cells, $CD8^+$ T cells and B cells in the development of skin inflammation was investigated. Consistent with our previous findings, obvious perivascular infiltration was observed in the skin of 5 out of 7 AT1R ME-immunized wild type mice, but not in the skin of any untreated or control ME-immunized wild type mice (Figure 9). This AT1R ME immunization-induced skin inflammation was observed in 50% (4 out of 8 mice) of $CD8^+$ T cell deficient mice, 11.1% (1 out of 9 mice) of B cell deficient mice, and 0% (0 out 11 mice) of $CD4^+$ T cell deficient mice (Figure 9). Statistical analysis revealed that incidence of AT1R-induced skin inflammation in both $CD4^+$ T cell deficient mice and B cell deficient mice were significantly lower than that in wild type controls (Figure 9).

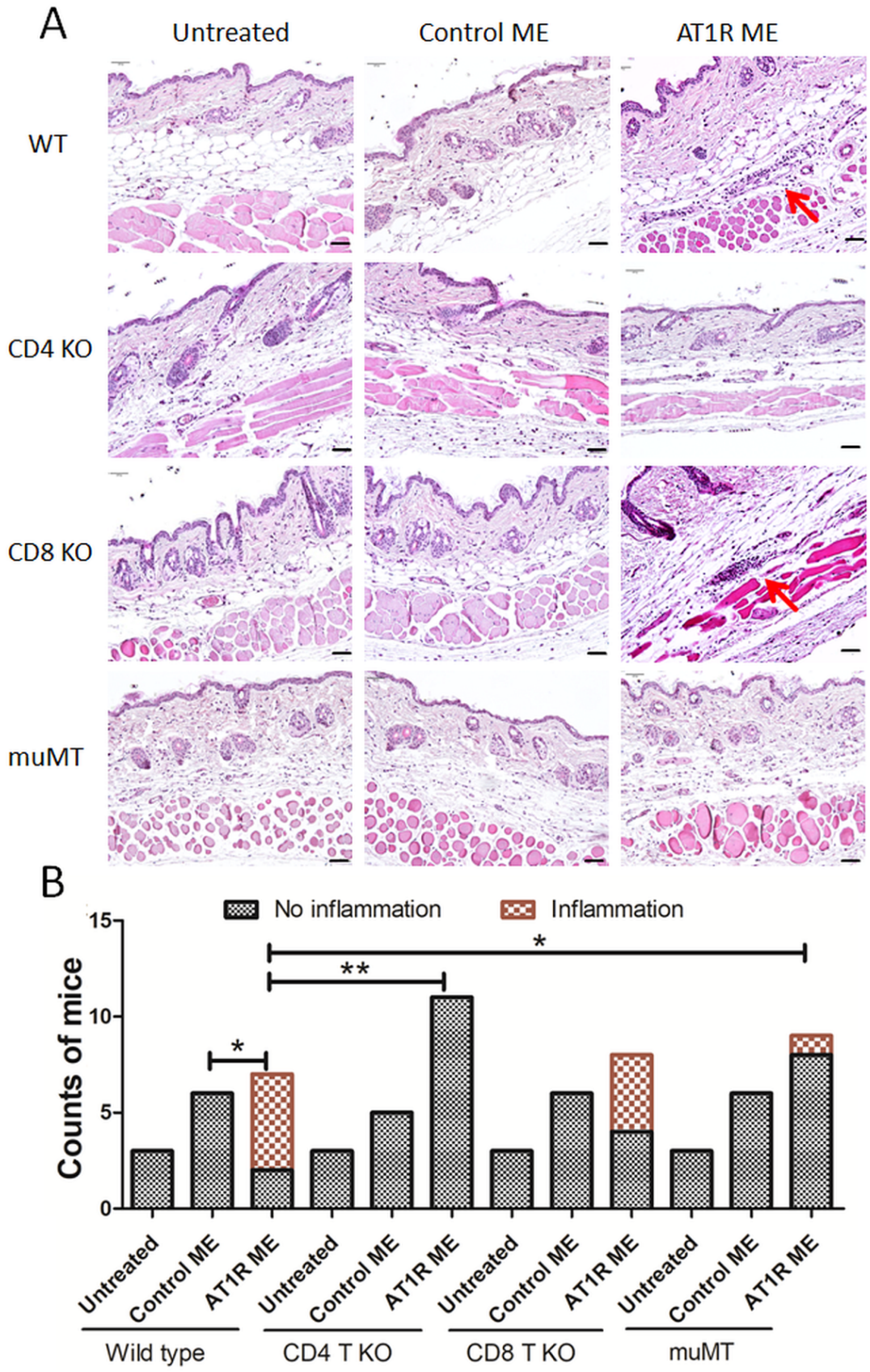

Figure 9. CD4$^+$ T cells and B cells are required for AT1R-induced skin inflammation in mice. Wild type (WT), CD4$^+$ T cell deficient (CD4 KO), CD8$^+$ T cell deficient (CD8 KO) and B cell deficient

(muMT) mice were immunized with control ME or AT1R ME. **A.** Representative micrographs of H&E stained skin paraffin sections of untreated, control ME-immunized or AT1R ME-immunized mice. Red arrows indicate inflammatory cell infiltration around blood vessels. Scale bar = 50 μm. **B.** Incidence of skin inflammation in untreated, control ME-immunized or AT1R ME-immunized mice. Mice with and without skin inflammation are indicated in black and orange colors, respectively. P values were calculated by using Fisher's test. *, $p<0.05$, **, $p<0.01$.

3.1.5. $CD4^+$ T cells and B cells are indispensable for the development of the skin fibrosis

Skin fibrosis is the most common hallmark pathological change in patients with SSc, and it is also a key feature of the AT1R-induced mouse model. Consequently, the development of skin fibrosis in WT, $CD4^+$ T cell deficient, $CD8^+$ T cell deficient and B cell deficient mice was investigated. Skin fibrosis was quantified by using two objective assessments. By microscopical determination of the thickness of the collagen layer of mouse skin after Masson's Trichrome stain and by quantitative measurement of the collagen content using a Sircol collagen detection kit. Both skin thickness and collagen content were significantly increased in the AT1R ME-immunized wild type mice as compared to untreated and control ME-immunized wild type mice (Figure 10), which confirms that C57BL/6J mice develop skin fibrosis after immunization with AT1R ME. In the $CD8^+$ T cell deficient mice, AT1R ME-immunization induced a significantly elevated skin thickness and collagen content compared to control ME-immunization (Figure 10), indicating that AT1R-induced skin fibrosis is not affected by deficiency of $CD8^+$ T cells. However, the AT1R-induced skin fibrosis was not observed in $CD4^+$ T cell deficient mice and B cell deficient mice (Figure 10).

Taken together, AT1R ME-immunization induced immunological and histopathological features of SSc such as production of anti-AT1R IgG, pulmonary inflammation, skin inflammation and fibrosis in wild type and $CD8^+$ T cell deficient mice. However, such SSc-like features were not induced in $CD4^+$ T cell or B cell deficient mice, suggesting that both $CD4^+$ T cells and B cells are indispensable for the development of disease in the AT1R-induced mouse model for SSc.

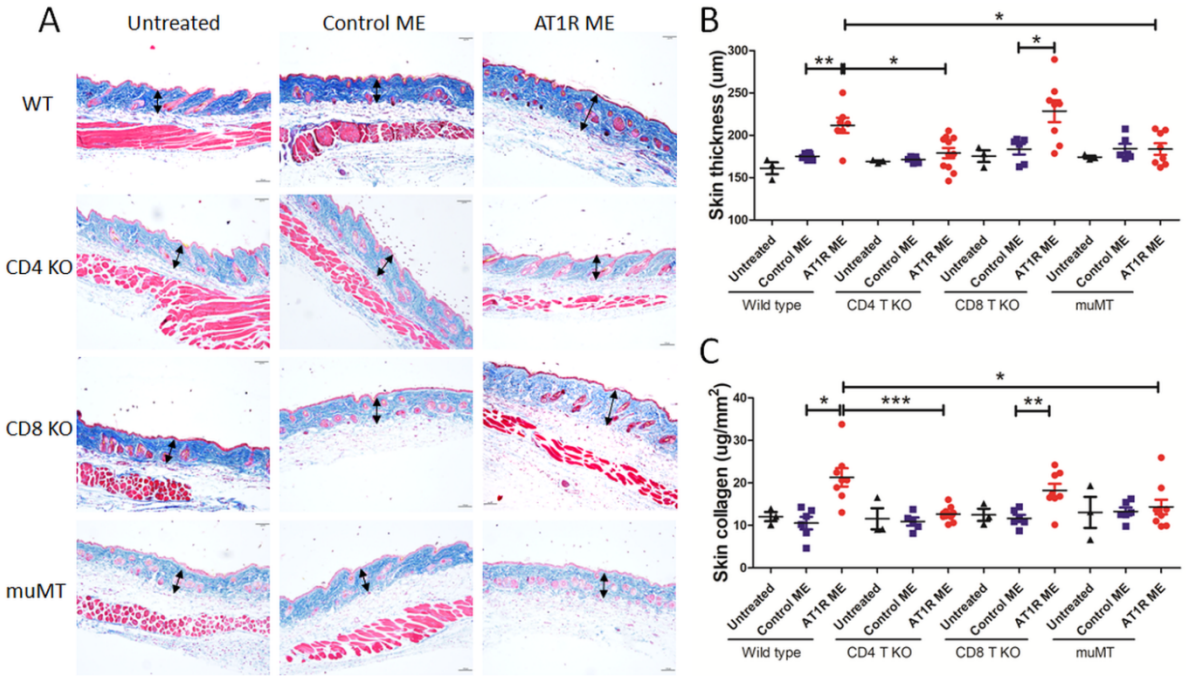

Figure 10. CD4$^+$ T cells and B cells are required for AT1R-induced skin fibrosis in mice. Wild type (WT), CD4$^+$ T cell deficient (CD4 KO), CD8$^+$ T cell deficient (CD8 KO) and B cell deficient (muMT) mice were immunized with control ME or AT1R ME. **A.** Representative micrographs of Masson's Trichrome stained skin paraffin sections from untreated, control ME-immunized or AT1R ME-immunized mice. Double-headed arrows indicate the collagen layer of the skin. Scale bar = 100 µm. **B**. Quantitative analysis of skin thickness. Thickness of the skin was defined as the thickness of the collagen layer stained in blue from the Masson's Trichrome staining. **C.** Quantitative analysis of collagen content was determined using Sircol collagen detection kit and expressed as µg per mm^2 of the skin. Statistical analysis was performed using Mann Whitney U test or Student t test depending on the normality of data. *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$.

3.2. Role of complement C3 in the active mouse model

As the center player of the humoral immune response, the main function of B cells is to produce antibodies, which is in most cases depended on the help of CD4$^+$ T cells [168]. Given that B cells and CD4$^+$ T cells are indispensable for the AT1R-induced mouse model of SSc, autoantibodies against AT1R high likely play an essential role in the pathogenesis of the disease. Autoantibodies are capable to induce pathology via multiple pathways, which generally can be categorized into 2 groups, Fc-dependent and Fc-independent pathways. The Fc-dependent pathways include stimulating immune cells via Fc receptor and activating complement system via immune complex, whereas Fc-independent pathway indicate autoantibodies induce pathology by a direct agonizing effect on the receptor after binding, namely, functional

autoantibodies. I hypothesized that anti-AT1R IgG are functional autoantibodies whose Fab portion cause abnormal activation of AT1R and subsequent pathology. To exclude the Fc-dependent pathways, the role of complement activation in the AT1R-induced mouse model for SSc was determined by immunizing mice deficient in complement component 3 (C3) which is the key molecule for complement activation [169].

3.2.1. C3 deficiency does not affect the production of anti-AT1R autoantibodies

To evaluate the role of C3, the production of autoantibodies against AT1R in the blood of C3 deficient and wild type control mice was measured. As shown in Figure 11, immunization with AT1R ME induced the production of anti-AT1R IgG in both wild type and C3 deficient mice, and the antibodies titer in the two groups were comparable, while immunization with control ME did not. Therefore, this finding suggests that C3 deficiency has no effect on the production of anti-AT1R autoantibodies.

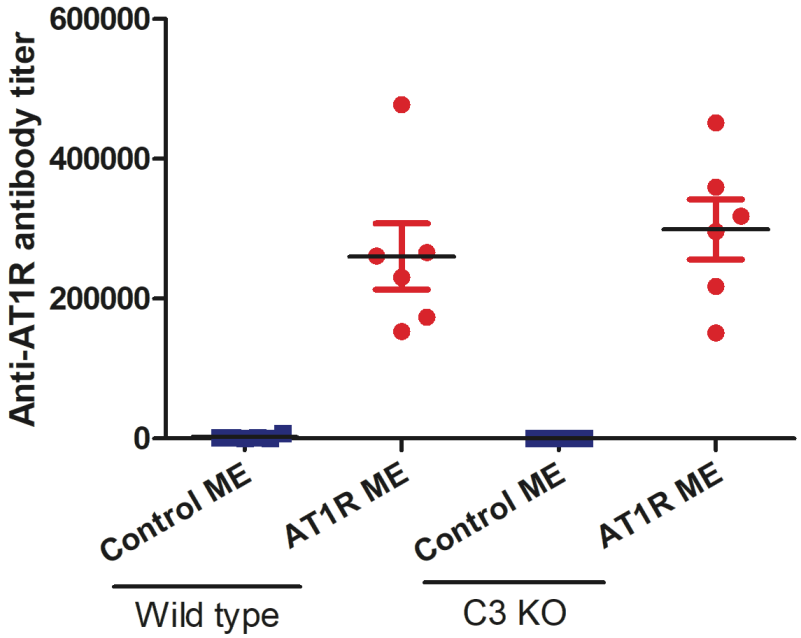

Figure 11. Anti AT1R autoantibodies in the blood of wild type and C3 deficient mice. Wild type and C3 deficient (C3 KO) mice were immunized with control ME or AT1R ME. Levels of anti-AT1R IgG were determined in sera by ELISA. Statistical analysis was performed using Mann Whitney U test or Student t test depending on the normality of data. **, $p<0.01$, ***, $p<0.001$.

3.2.2. C3 deficiency does not affect the production of pro-inflammatory cytokines

Next, the effect of deficiency of C3 on cytokine production in mice was investigated. Serum levels of IL-1α, IL-4, TNF-α and IFN-γ in wild type and C3 deficient mice were determined as described in section 3.1.2 above. Consistently, wild type mice immunization with AT1R ME showed significantly higher levels of IL-1α, IL-4, TNF-α and IFN-γ than mice immunized with control ME (Figure 12). Notably, no significant difference was observed in serum levels of any of the four cytokines between AT1R ME-immunized wild type and C3 deficient mice (Figure 12). This finding suggests that C3 is not required for the dysregulation of cytokines in this mouse model.

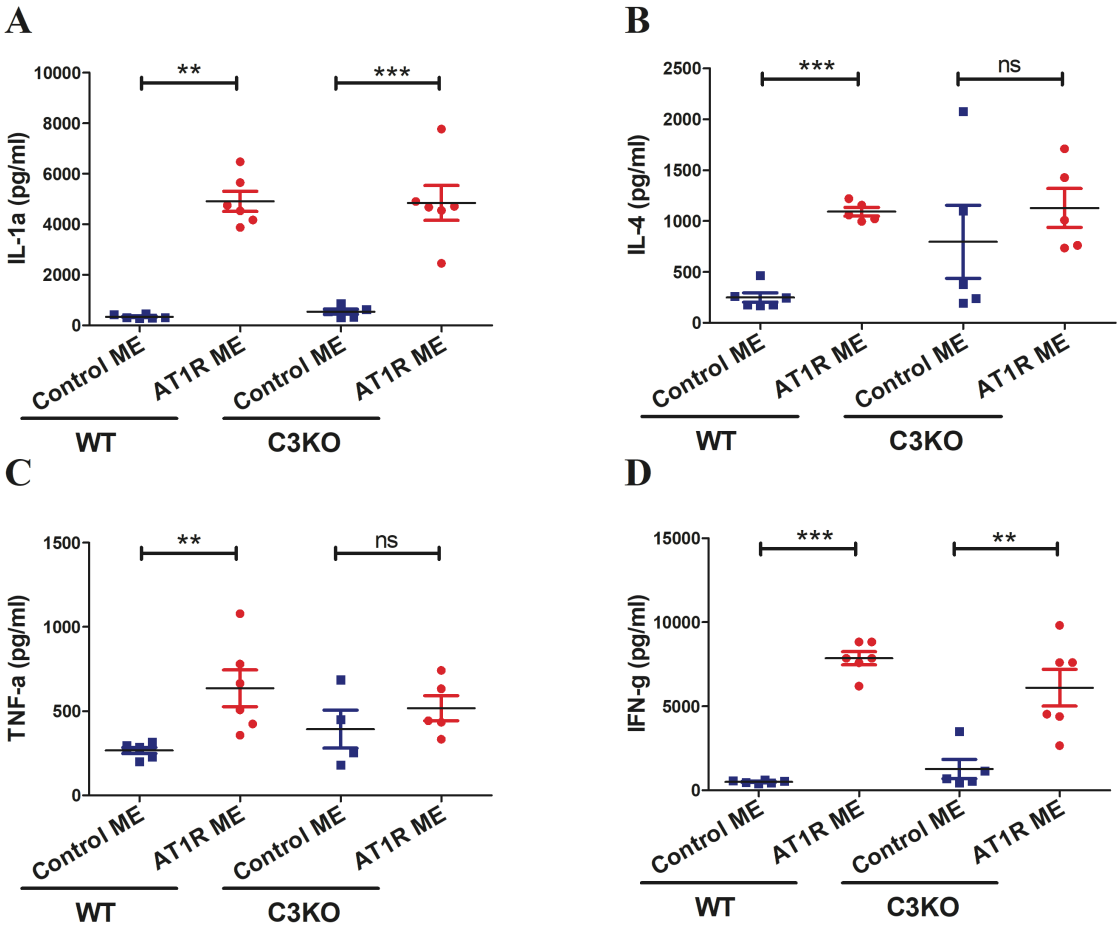

Figure 12. Serum levels of cytokines in wild type and C3 deficient mice. Wild type and C3 deficient (C3 KO) mice were immunized with control ME or AT1R ME. Serum levels of IL-1α (**A**), IL-4 (**B**), TNF-α (**C**) and IFN-γ (**D**) were detected in sera of mice using ELISA kit purchased from PeproTech. Statistical analysis was performed using Mann Whitney U test or Student t test depending on the normality of data. ns, not significant, **, $p<0.01$, ***, $p<0.001$.

3.2.3. C3 deficiency promotes pulmonary inflammation

Lung inflammation in mice was determined on HE stained paraffin sections as described in section 3.1.3 above. Compared with corresponding control mice immunized with control ME, both AT1R ME-immunized wild type and C3 deficient mice developed more severe lung inflammation. When compared with wild type controls, C3 deficient mice developed more severe inflammation in the lung after the immunization with AT1R (Figure 13). This finding not only suggests that complement C3 is not required for development of lung inflammation, but also suggests an unexpected protective role of C3 in the development of AT1R-induced pulmonary inflammation.

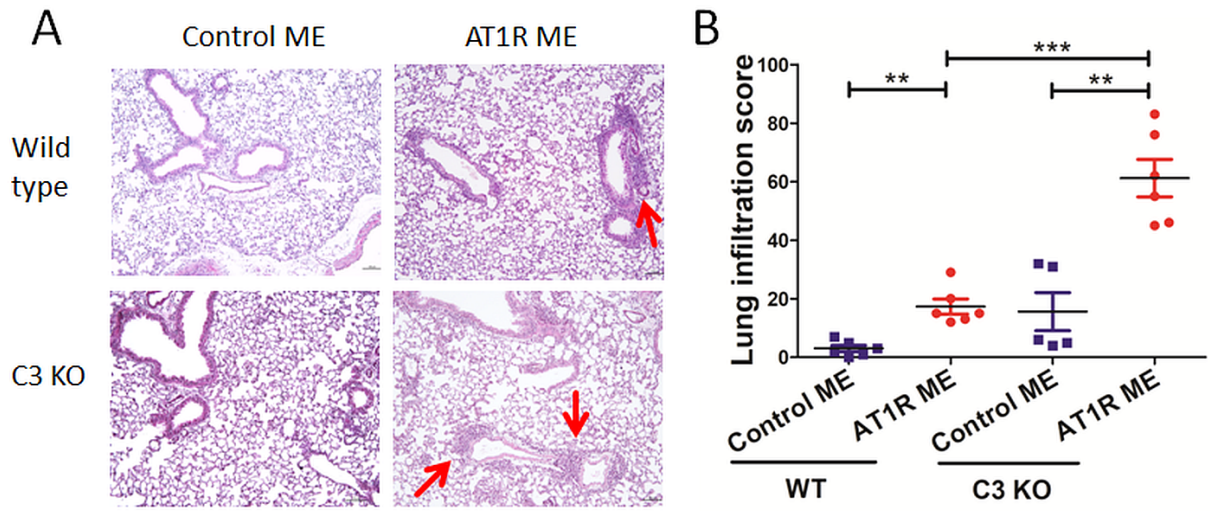

Figure 13. Development of pulmonary inflammation in wild type and C3 deficient mice. Wild type and C3-deficient (C3 KO) mice were immunized with control ME or AT1R ME. **A.** Representative micrographs of H&E stained lung sections of control ME- or AT1R ME-immunized mice. Red arrows indicate inflammatory cell infiltration in the lung. Scale bar = 100 µm. **B.** Quantitative analysis of pulmonary inflammation in mice. Severity of pulmonary inflammation was quantified by the size and number of infiltrates in the lung and scored in a double-blinded fashion. Statistical analysis was performed using Mann Whitney U test or Student t test depending on the normality of data. **, $p<0.01$, ***, $p<0.001$.

3.2.4. C3-deficiency does not affect the development of skin inflammation

The development of inflammation in the skin of wild type and C3 deficient mice was assessed by H&E staining. Four out of 6 wild type mice immunized with AT1R developed perivascular infiltration in the skin, while none of 6 wild type mice immunized with control ME did (Figure

14). In the C3 deficient mice, 5 out of 6 mice immunized with AT1R ME, but none out of 5 mice immunized with control ME developed skin inflammation (Figure 14). No significant difference in the incidence of skin inflammation between AT1R ME-immunized wild type and C3 deficient mice, suggesting that C3 is not required for the development of skin inflammation.

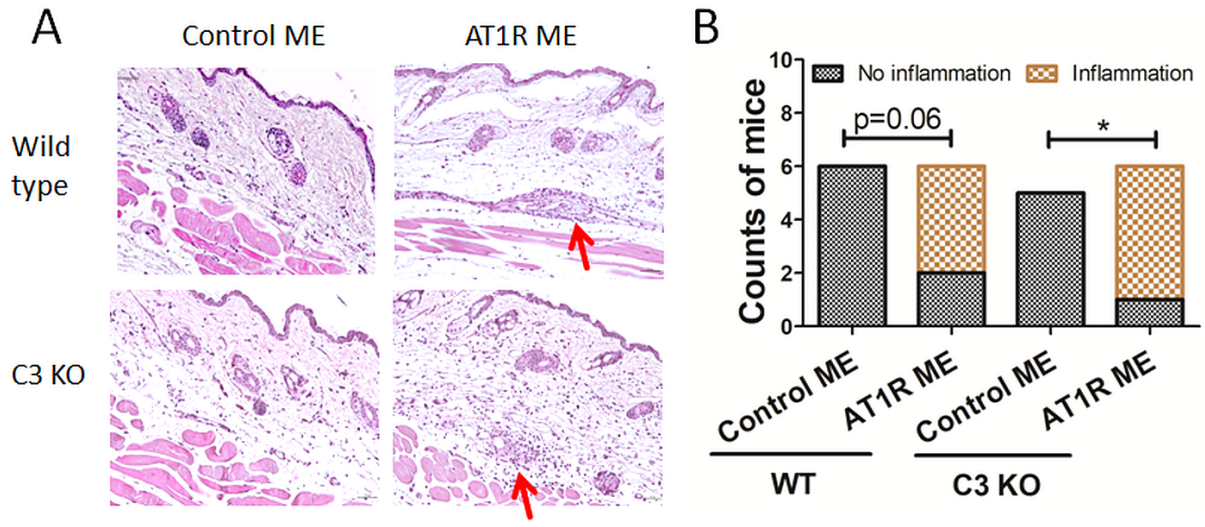

Figure 14. Development of skin inflammation in wild type and C3 deficient mice. Wild type and C3-deficient (C3 KO) mice were immunized with control ME or AT1R ME. **A.** Representative micrographs of H&E stained skin sections of control ME- or AT1R ME-immunized mice. Red arrows indicate inflammatory cell infiltration around blood vessels. Scale bar = 50 µm. **B.** Incidence of skin inflammation in mice. The numbers of mice with and without skin inflammation are indicated by black and orange colors, respectively. P values were calculated by Fisher's test. *, $p<0.05$.

3.2.5. C3-deficiency does not affect the development of skin fibrosis

Finally, we evaluated the development of skin fibrosis in wild type mice and C3-deficient mice. In wild type mice, both skin thickness and collagen content were significantly increased in the AT1R ME-immunized mice compared to control ME-immunized mice, confirming the AT1R-induced skin fibrosis. Similarly, AT1R-induced skin fibrosis was also observed in C3-deficient mice (Figure 15). Furthermore, both skin thickness and collagen content were not significantly different between wild type and C3-deficient mice immunized with AT1R ME (Figure 15). This result suggests that C3 is dispensable for the development of skin fibrosis.

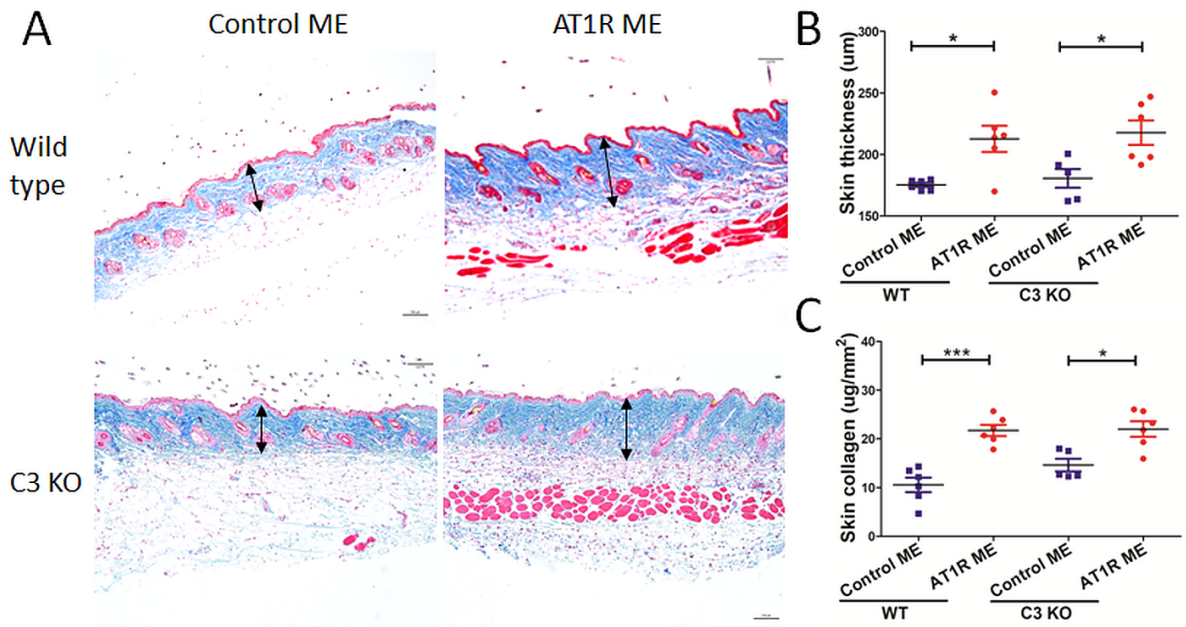

Figure 15. Development of skin fibrosis in wild type and C3 deficient mice. Wild type and C3-deficient (C3 KO) mice were immunized with control ME or AT1R ME. A. Representative micrographs of Masson's Trichrome stained skin sections of control ME- or AT1R ME-immunized mice. Double-headed arrows indicate the collagen layer of the skin. Scale bar = 100 µm. **B**. Quantitative analysis of skin thickness. Thickness of the skin was defined as the thickness of the collagen layer stained in blue from the Masson's Trichrome staining. **C.** Quantitative analysis of collagen content Collagen content was determined using Sircol collagen detection kit and expressed as µg per mm^2 of the skin. Statistical analysis was performed using Mann Whitney U test or Student t test depending on the normality of data. *, $p<0.05$, ***, $p<0.001$.

Collectively, deficiency of complement C3 did not prevent disease development in the AT1R-induced mouse model for SSc. These findings not only suggest that C3 is dispensable in the disease pathogenesis, but also support the hypothesis that anti-AT1R autoantibodies mediate disease pathology in a Fc-independent manner.

3.3. Anti-AT1R monoclonal antibodies are pathogenic

Previously, our group generated three monoclonal antibodies against AT1R from mice immunized with AT1R ME. One of the three monoclonal antibodies, namely mAb5.2a, has been shown to be a functional autoantibody with agonistic effect to AT1R (unpublished data) *in vitro*. To evaluate the potential pathogenicity of anti-AT1R autoantibodies, mAb5.2a

monoclonal antibody was transferred into the mice via repetitive injection into mouse ear (Figure 16).

Antibody	Mouse strain	Antibody amount	Antibody volume
mAb 5.2a	C57Bl/6J	100ug	50ul
Mouse IgG2a isotype control	C57Bl/6J	100ug	50ul

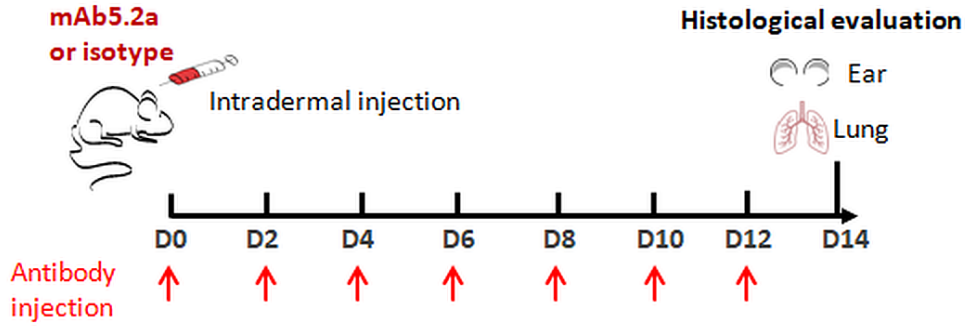

Figure 16. Experimental design of the antibody transfer. A. Monoclonal antibodies and mice used in this experiment. B. Schematic overview of the experimental setup. Briefly, 100 µg mAb5.2a or mouse IgG2a isotype antibodies in 50 µl PBS solution were transferred to each ear via intradermal injection on day 0, 2, 4, 6, 8, 10 and 12. Two weeks after the first injection, mice were sacrificed and the lung and ears were collected for further evaluation.

3.3.1. Transfer of mAb5.2a promotes inflammation in ear skin

Two weeks after the first injection of the antibodies, histological alterations of murine ears were determined on H&E stained paraffin sections. Murine ears treated with mAb5.2a showed a strong infiltration of inflammatory cells, whereas only very mild skin inflammation was observed in mice which received antibodies of the corresponding IgG isotype control (Figure 17A). Quantitative analysis demonstrated that the difference in severity of ear inflammation between the two groups was highly significant ($P<0.001$) (Figure 17B), suggesting that mAb5.2a is able to promote local inflammation in the ear.

To explore whether local injection of mAb5.2a could mediate pathological changes also in peripheral tissues, lung sections of mice were assessed after H&E staining. Interestingly, mild pulmonary inflammation was detected in 5 out of 11 mAb5.2a treated mice. By contrast, none of 14 mice treated with IgG isotype control developed inflammation in the lung (Figure 17 C

and D). The significant difference in the incidence of pulmonary inflammation (P<0.01) suggests that local injection of mAb5.2a is able to induce pathological changes in peripheral internal organs.

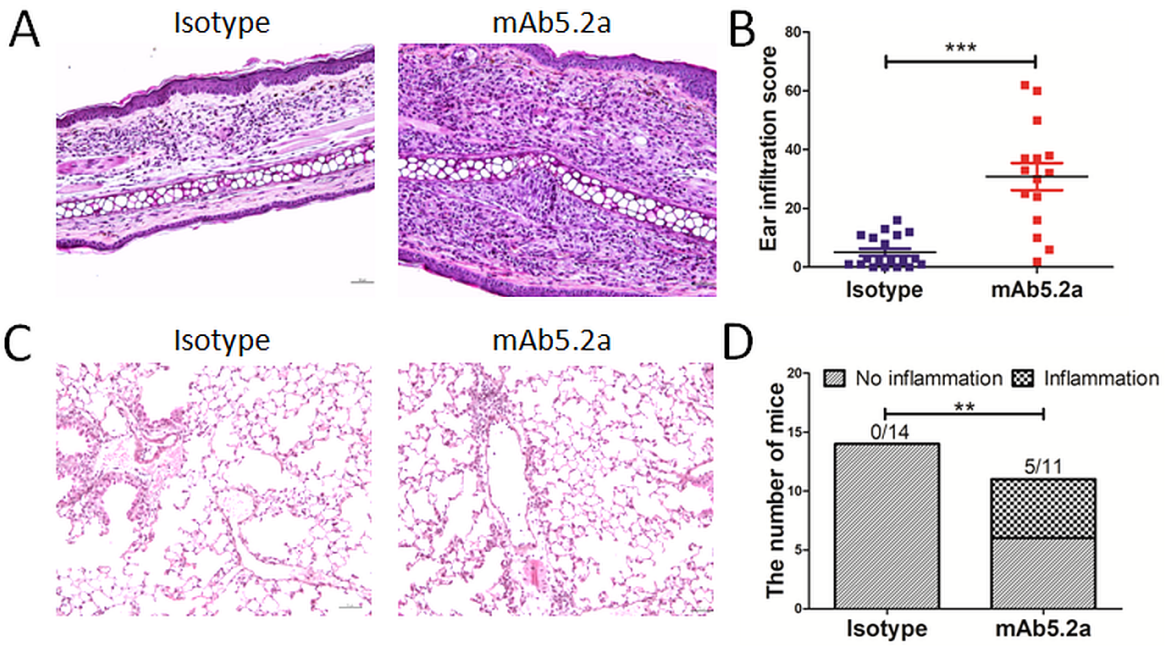

Figure 17. Development of inflammation in the ear and lung of mice after transfer of anti-AT1R monoclonal antibodies (mAb5.2a). A. Representative micrographs of H&E stained ear skin sections of mice treated with monoclonal anti-AT1R antibodies (mAb5.2a, n=15, right) or isotype IgG antibodies (IgG2a, n=18, left). Scale bar=50 µm. **B**. Quantified analysis of the severity of inflammation in ear skin. Severity of skin inflammation was quantified by scoring the sizes and numbers of infiltrates in a double-blinded fashion. P value was calculated by Mann Whitney test. ***, p<0.001. **C.** Representative micrographs of H&E stained lung sections of mice treated with monoclonal anti-AT1R antibodies (mAb5.2a, n=11, right) or isotype control antibodies (IgG2a, n=14, left). Scale bar=50 µm. **D**. Incidence of lung inflammation in mice treated with anti-AT1R antibodies (mAb5.2a) or isotype control antibodies. Statistical analysis was performed using Fisher's test. **, p<0.01.

3.3.2. Composition of the inflammatory infiltrates in the ear and lung of mice injected with mAb5.2a

To determine the cell types in inflammatory infiltrates in the ear and lung of mice injected with mAb5.2a, immunohistochemistry staining on the corresponding sections were performed with antibodies recognizing T cells, B cells and neutrophils. As shown in Figure 18, ear tissue treated

with mAb5.2a showed infiltrations dominated by neutrophils, and to a much lesser extent the presence of B cells and T cells. By contrast, in the lung of mice injected with mAb5.2a, infiltrates consisted predominantly of T cells, while neutrophils and B cells were only rarely observed (Figure 18).

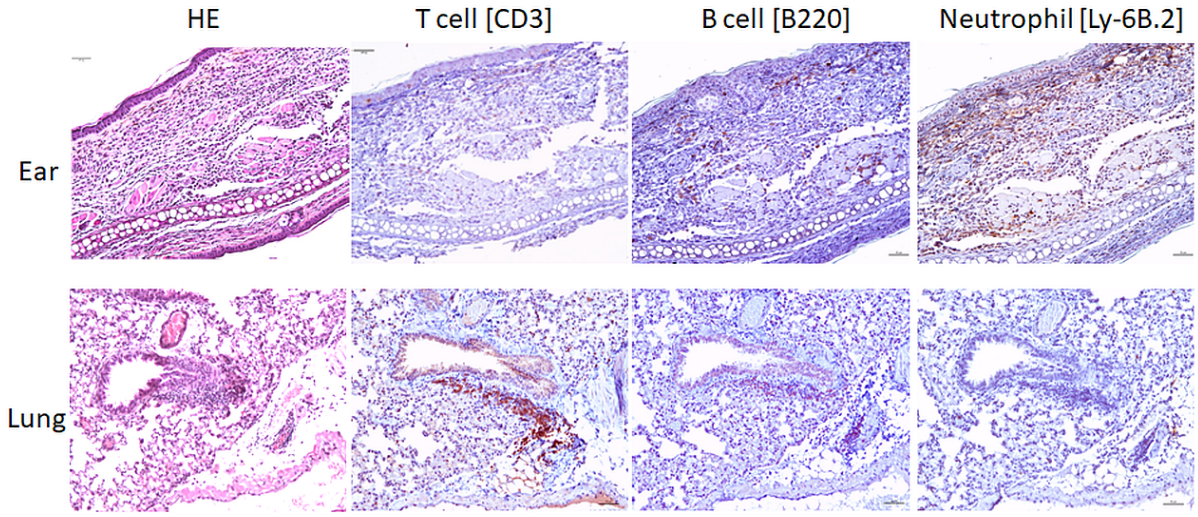

Figure 18. Cellularity of the inflammatory infiltrates in ear and lung of mice treated with monoclonal anti-AT1R antibodies. T cells, B cells and neutrophils were detected by immunohistochemistry (IHC) staining of ear (top) and lung (bottom) sections using antibodies against CD3 (T cells), B220 (B cells) and Ly-6B.2 (neutrophils), respectively. Representative micrographs are shown. Scale bar = 50 µm.

3.3.3. mAb5.2a induced pathology is depend on the expression of AT1R

To determine whether mAb5.2a-induced pathology is dependent on the specific binding of the monoclonal antibody to murine AT1R, mAb5.2a antibody was transferred into AT1R knockout mice which are deficient in both AT1a and AT1b receptors. As shown in Figure 19, administration of mAb5.2a, but not IgG isotype control, induced strong inflammation in ear skin of wild type littermate control mice, which is consistent with previous findings in the section 3.3.1. By contrast, no significant difference was observed in skin inflammation between mAb5.2a-treated and IgG isotype control-treated AT1R deficient mice. Moreover, injection of mAb5.2a induced significantly more severe inflammation in wild type mice than in AT1R deficient mice (Figure 19). Therefore, this finding demonstrated that the expression of AT1R is required for mAb5.2a induced skin inflammation.

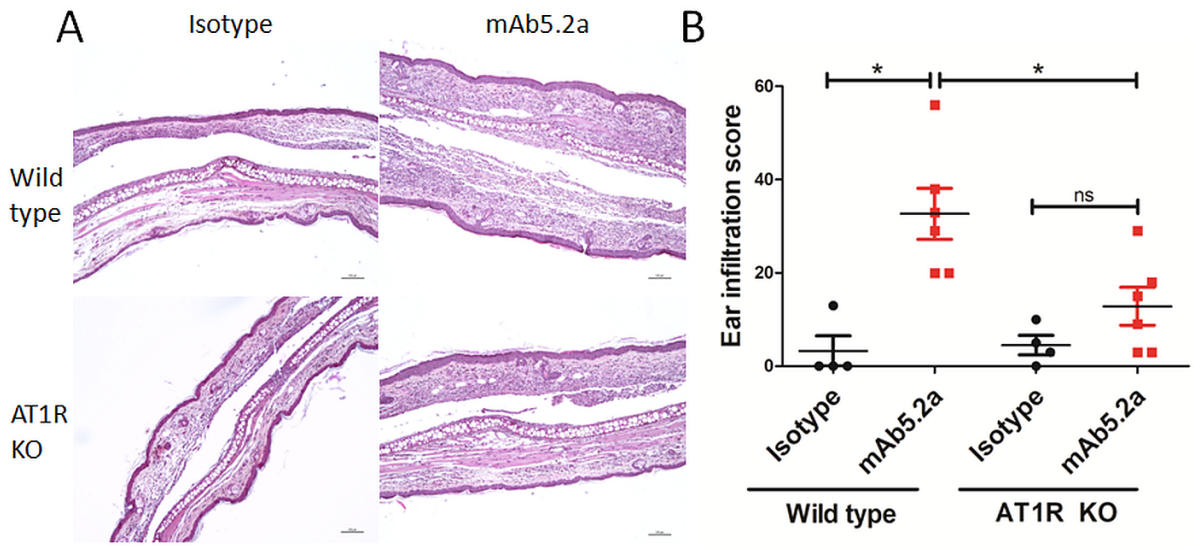

Figure 19. Development of mAb5.2-induced ear inflammation in mice. AT1R deficient mice (AT1R KO) and their littermate controls (Wild type) were injected with mAb5.2a or murine IgG2a isotype control. A. Representative micrographs of H&E stained ear sections from wild type and AT1R deficient mice. Scale bar =100 µm. B. Quantified analysis of the severity of inflammation in ear skin. Severity of inflammation was defined as ear infiltration score and calculated as described in the legend of Figure 16. Statistical analysis was performed using Mann Whitney U test or Student's t test depending on the normal distribution of data. ns, not significant *, $p<0.05$.

Taken together, passive transfer of monoclonal antibodies against AT1R via i.d. injection to mouse ear could not only induce strong local infiltration in the skin but also mediate inflammation in the lung, suggesting that functional autoantibodies against AT1R are pathogenic. Moreover, the anti-AT1R monoclonal antibody-induced inflammation is dependent on the binding of autoantibodies to the specific antigen.

3.4. Fibroblasts are major cells bound by anti-AT1R monoclonal antibodies in the skin.

To explore the mechanism underlying the mAb5.2a induced inflammation, the type of resident cells in the skin bound by mAb5.2a was determined. For this purpose, cryosections of murine ear from untreated mice were prepared, stained with mAb5.2a or mouse IgG2a isotype control, and binding was visualized by Dylight649-conjugated secondary antibodies in the confocal microscope. As shown in Figure 20, dermal resident cells were able to bind with mAb5.2a and cellular binding with mAb5.2a occurred predominately on cells located in the dermis layer of the skin.

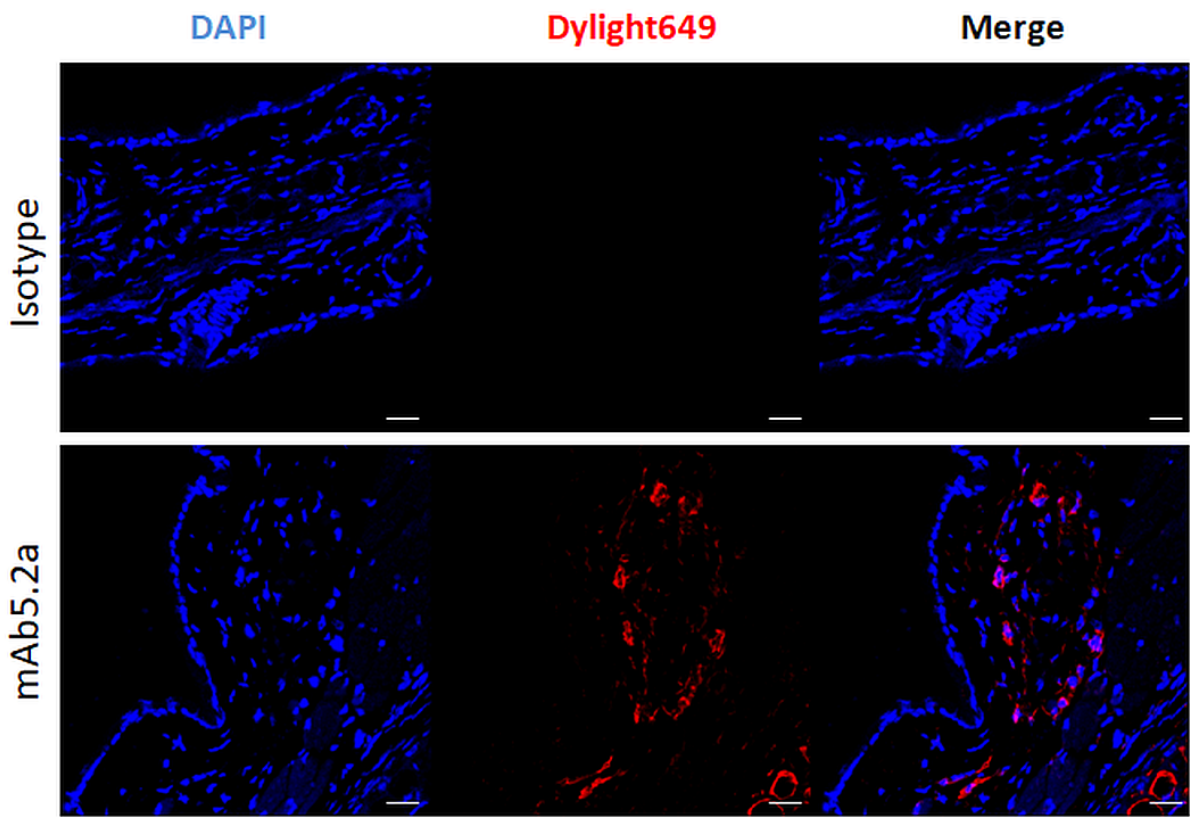

Figure 20. Binding of mAb5.2a with resident cells in mouse ear. Cryosections of murine ear from untreated mice were prepared, stained with either monoclonal anti-AT1R antibodies (mAb5.2a, bottom panel) or murine IgG isotype control (IgG2a, up panel), and then visualized using Dylight649-labeled goat anti-mouse IgG secondary antibody (red). Stained sections were counterstained with DAPI (blue). Representative micrographs of IF of ear cryosections from untreated mice are shown. Scale bar = 50 μm.

To define the cell types which bind to mAb5.2a, single cells from ear and skin of untreated mice were prepared. The isolated primary murine skin cells were subsequently stained with mAb5.2a in combination with cell-specific markers for identifying fibroblasts, macrophages and dendritic cells by flow cytometry (Figure 21A). To remove the cell clumps or duplets and dead cells, single cells with viability were isolated by using the strategy depicted in Figure 21B and used for subsequent cytometric analysis. To determine the proportion of different cell types in the murine skin cells binding with mAb5.2a, mAb5.2a positive binding cells were firstly gated out from single viable cells. Due to the conjugated fluorescence of dendritic cell marker CD11c (FITC) was overlapped with mAb5.2a (Alexa488), two flow cytometric panels were designed: one panel was used for fibroblast and macrophage (Figure 21C, upper panel), and the other was used for dendritic cells (Figure 21C, bottom panel). As shown in Figure 21C,

mAb5.2a positive cells contained fibroblasts as the major population, which accounted for approximately 60% of all mAb5.2a positive cells referring to the quadrants of flow cytometric analysis. By contrast, macrophages and dendritic cells accounted for only 2.7% and 2% of all mAb5.2a positive cells by referring to quadrants, respectively.

Moreover, to investigate the proportion of fibroblast in the ear and skin of mice binding with mAb5.2a, a different flow cytometric gating strategy compared to the strategy used in Figure 21C was used, in which fibroblasts were firstly gated out as $CD45^-$ $CD140a^+$ cells from single viable cells. Subsequently, the binding of mAb5.2a to the fibroblasts as well as the binding of IgG2a isotype control to fibroblasts were examined. As shown in Figure 21D, compared to the IgG2a isotype control, the flow cytometric histogram plots showed a single peak and higher level of signal intensity of mAb5.2a, which suggested that the whole cell population of fibroblasts binds with mAb5.2a.

To further confirm the binding of mAb5.2a to the cell surface of murine fibroblasts, binding of mAb5.2a with L929 mouse fibroblast cell line was determined by using ibidi slides which allowed the fibroblasts to grow the bottom surface of ibidi slides and thus the living fibroblasts can directly be used for immunofluorescence staining without detaching from the bottom surface. As shown in Figure 22, obvious surface binding of L929 cells with mAb5.2a was observed and no binding with IgG2a isotype control was observed, further confirming the binding activity of mAb5.2a to the cell surface of fibroblasts.

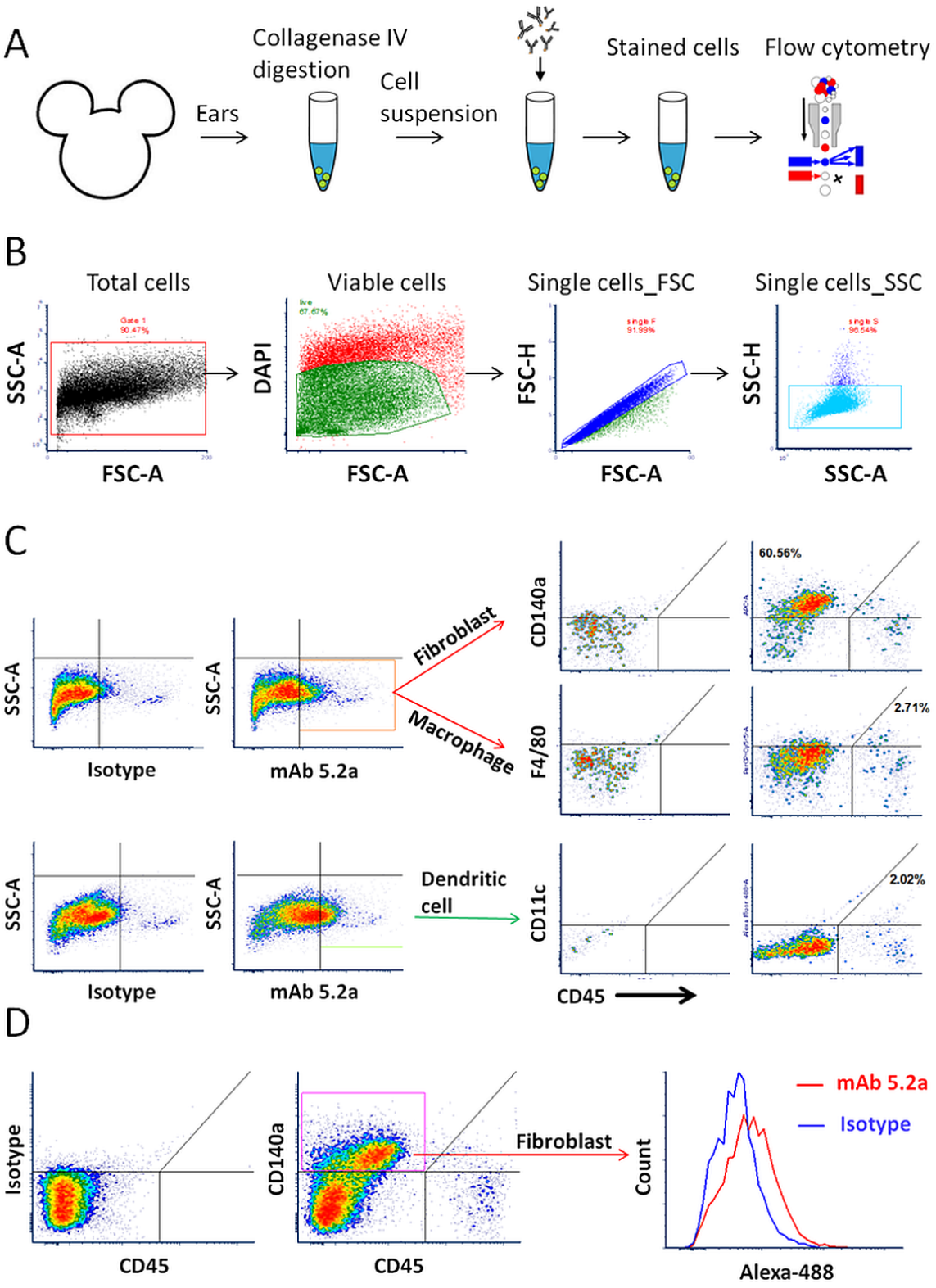

Figure 21. Binding of mAb5.2a to cells in ear and skin of mice. Cells isolated from ear and skin of untreated mice were stained with mAb5.2a or murine IgG2a isotype control in combination with cell-specific surface markers for fibroblasts ($CD45^-$ $CD140a^+$), macrophages ($CD45^+$ $F4/80^+$) and dendritic cells ($CD45^+$ $CD11c^+$). **A.** Schematic workflow of the experiment to isolate and stain cells from ear and skin of mice. **B.** Flow cytometric gating strategy for identifying single and viable cells from total cells isolated from murine ear and skin. Viable cells (green) were firstly gated from total cell population

(black) and subsequently used for gating single cells in the forward scatter (blue). Single cells in the forward scatter were further gated by the side scatter to isolate single cells (cyan) for subsequent gating. Representative flow cytometric color dot plots were presented. FSC, forward scatter; SSC, side scatter; FSC-A, area of forward scatter; SSC-A, area of side scatter; FSC-H, height of forward scatter; SSC-H, height of side scatter. **C**. Flow cytometric gating strategy for identifying different subsets of cells in the mAb5.2a positive cell population using cell specific surface makers for fibroblasts, macrophages and dendritic cells. Flow cytometric density plots were presented. **D**. Flow cytometric gating strategy for determining the proportion of mAb5.2a binding cells in the fibroblast population. Flow cytometric density plots (left) and histogram plot (right) were presented.

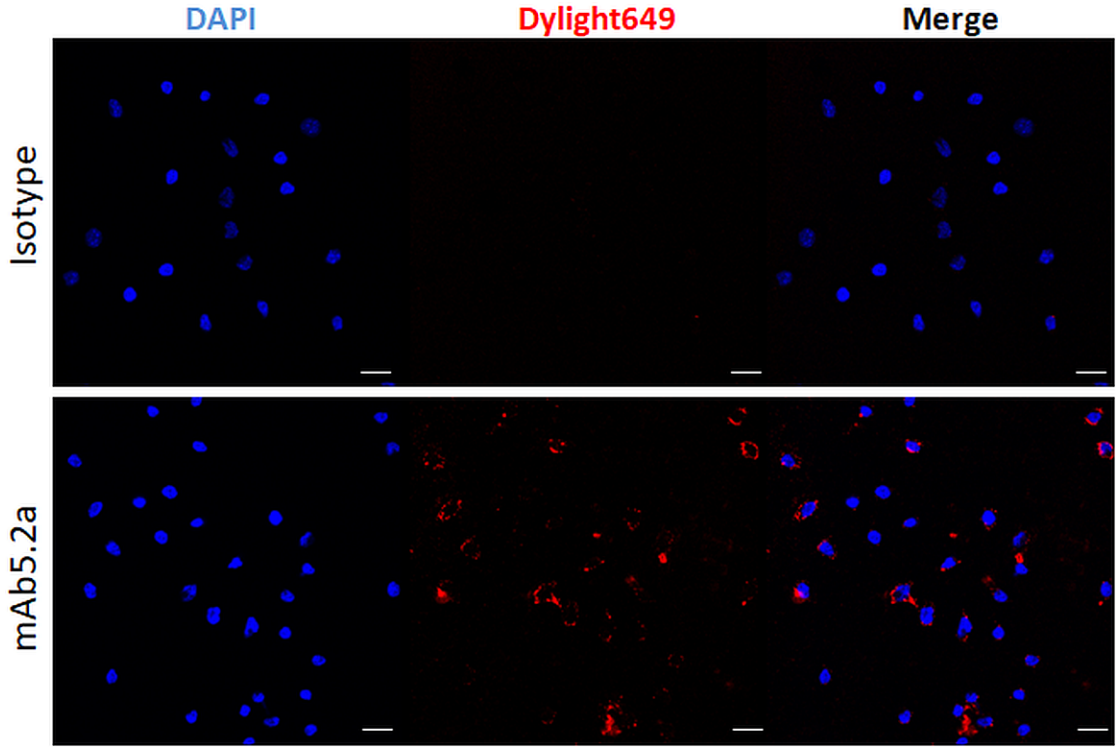

Figure 22. Binding of monoclonal mAb5.2a to the surface of mouse fibroblast cell line L929. Living L929 cells were stained with either anti-AT1R monoclonal antibodies (mAb5.2a, bottom panel) or murine IgG isotype control (IgG2a, up panel), and visualized by Dylight649-labeled goat anti-mouse IgG secondary antibody (red). Stained sections were counterstained with DAPI (blue). Representative IF micrographs are shown. Scale bar = 50 μm.

3.5 Binding complex of mAb5.2a with AT1R

The results of this study provide evidence that functional autoantibodies against AT1R play a central role in the pathogenesis of experimental SSc. In order to understand the mode of action of these autoantibodies, it is essential to elucidate the molecular mechanism of the interaction

between receptor and antibody. In a first approach, computer-based models were used to predict the structure of antibody-receptor binding (Figure 23A). For this purpose, cDNA of heavy chain and light chain of mAb5.2a were sequenced in cooperation with Dr. Antje Müller (University of Lübeck) and Primer 5.0 was utilized to translated the cDNA sequence into protein sequence of mAb5.2a, while the protein sequence of AT1R was retrieved from Uniprot database (Figure 23B).

After the sequences of mAb5.2a and AT1R were obtained, the structures of them were predicted by using suitable web-based servers. To model the structure of Fv portion of anti-AT1R antibody mAb5.2a, SAbPred, a web based server designed by Oxford Protein Informatics Group (OPIG) for antibody structural modeling [164], was utilized. This webserver provides structural modeling for Fv portion of antibody and recognition of complementary-determining regions (CDRs) with high accuracy. Amino acid sequence of mAb5.2a was submitted into SAbPred server and default settings were used for structure modeling and recognition of CDRs. Predicted structure of Fv of anti-AT1R antibody was downloaded and visualized by using PyMol open source molecular visualization system, which was presented in Figure 23C. SWISS-MODEL server developed by Swiss Institute of Bioinformatics [166] was utilized to model the structure of AT1R receptor based on protein structure homology-modeling algorithms. Protein sequence of human AT1R obtained from Uniprot database was submitted to SWISS-MODEL server, predicted structure model of AT1R was downloaded and visualized by using PyMol, which was showed in Figure 23D.

Subsequently, to model the structure of binding complex of antibody and receptor, conformational structure of Fv portion of mAb5.2a and AT1R receptor were submitted to ZDOCK server which has been widely used for antibody-antigen docking [167]. Simulated structure of the binding complex was downloaded from ZDOCK server and further edited and colored in PyMol open source molecular visualization system. As shown in Figure 23E, mAb5.2a binds well with the extracellular domain of AT1R. Moreover, analysis of the polar contact and hydrogen bonds at the interface between mAb5.2a and AT1R demonstrated that mAb5.2a binds to N terminal, the second and third extracellular loop (ECL) of AT1R, and both the light chain and heavy chain of mAb5.2a are involved in the interaction (Figure 23F).

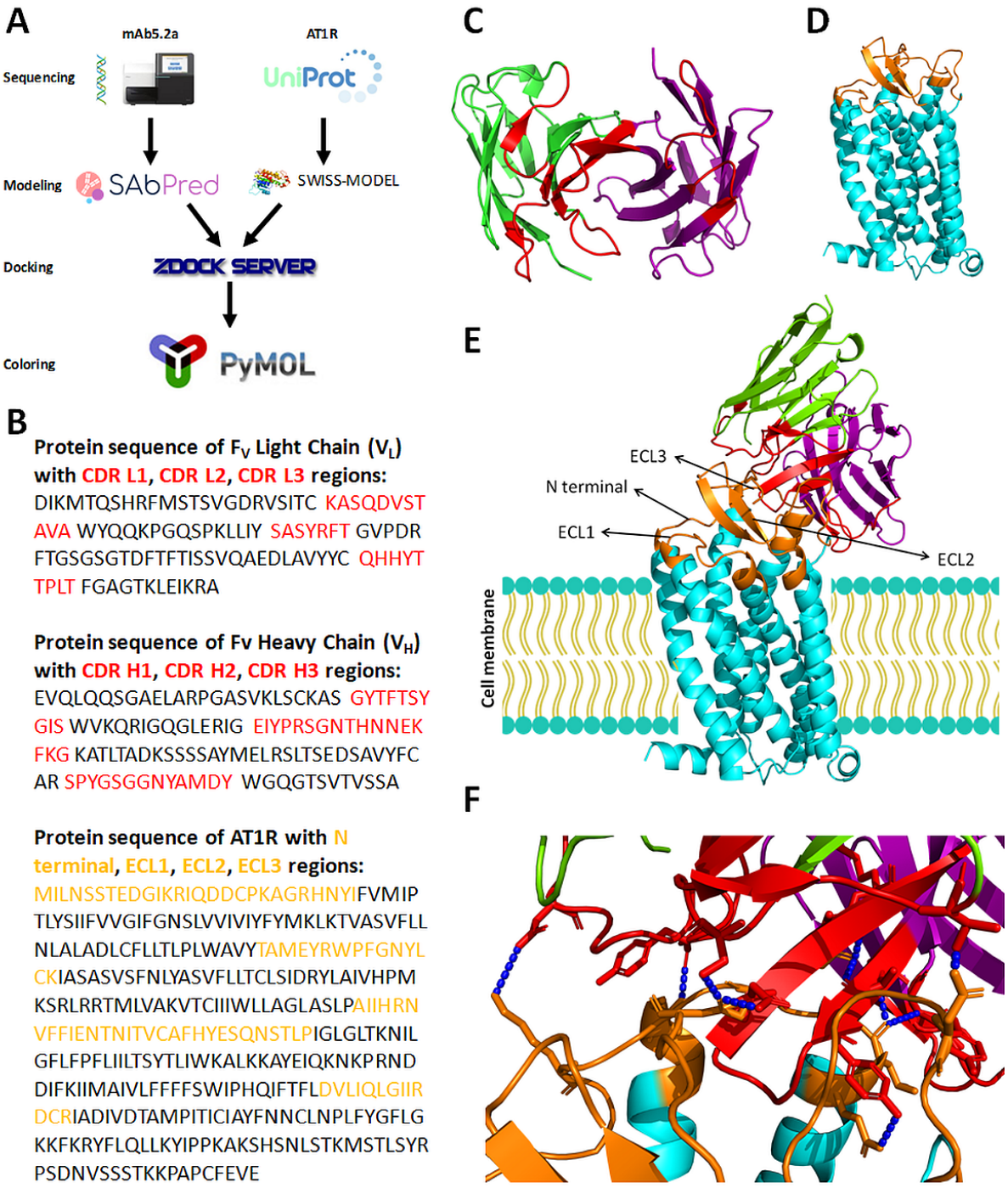

Figure 23. Computational model of the binding complex of monoclonal anti-AT1R antibody with AT1R. A. Workflow diagram for predicting the structure of binding complex of anti-AT1R antibody with AT1R. **B**. Protein sequences of light chain and heavy chain of variable fragment of monoclonal anti-AT1R antibodies **(**mAb5.2a) with the complementary-determining regions (red) obtained by sequencing and protein sequence of AT1R retrieved from uniprot database (protein ID, P30556) with extracellular regions (yellow). **C.** Structure of the variable fragment (Fv) of mAb5.2a predicted by using SAbPred server. Purple, heavy chain of Fv fragment of mAb5.2a; green, light chain of Fv fragment of mAb5.2a; red, CDRs of heavy and light chain of mAb5.2a. **D.** Structure of the AT1R predicted by using

SWISS-MODEL server. Cyan, AT1R; yellow, extracellular loops (ECLs) of AT1R. **E.** Simulated structure of complex of variable fragment (Fv) of mAb5.2a and human AT1R on the cell membrane. Cyan, AT1R; yellow, ECLs of AT1R; purple, heavy chain of Fv fragment of mAb5.2a; green, light chain of Fv fragment of mAb5.2a; red, CDRs of heavy and light chain of mAb5.2a. **F.** Polar contact and hydrogen bonds (blue dot bar) at the interface between the N terminus and extracellular loops (ECLs) of human AT1R (yellow) and complementary-determining regions (CDRs) (red) of mAb5.2a.

4. Discussion

SSc is an autoimmune-mediated complex connective tissue disorder with unclear pathogenesis. It is widely believed that autoantibodies play an important role in the pathogenesis of SSc, though the underlying mechanisms are largely unknown [170]. Generally, autoantibodies are able to induce pathological changes through multitude of pathways which differ considerably among autoimmune diseases [171].

In this study, I investigated the pathogenicity of autoantibodies against AT1R by immunizing mice with membrane extract of cells overexpressing human AT1R and by transferring functional antibodies against AT1R into mice. These studies demonstrated that 1) Both $CD4^+$ T cell deficient mice and or B cell deficient mice are resistant to the AT1R-induced SSc-like disease, whereas $CD8^+$ T cell deficient mice are as susceptible as wild type mice, suggesting that $CD4^+$ T cells and B cells play an essential role in the development of disease; 2) C3 deficient mice are susceptible to AT1R-induced SSc-like disease, suggesting that complement activation is not indispensable for the disease pathogenesis; 3) local transfer of mAb5.2a into the ear of mice induces local inflammation in the ear as well as inflammation in the lung, demonstrating that functional monoclonal autoantibodies against AT1R are pathogenic in mice; 4) in the murine skin, fibroblasts are the main target cells of functional monoclonal antibodies against AT1R. Taken together, these results demonstrate that autoantibodies against AT1R are able to induce pathological changes in mice, implicating a pathogenic role of anti-AT1R autoantibodies in SSc.

Mouse models of SSc

Mouse models of systemic sclerosis are powerful tools for investigating the pathogenesis of the disease [172]. Until now, more than 20 different mouse models of SSc have been generated, which belong largely to two groups: induced mouse model such as the bleomycin induced mouse model and spontaneous mouse model like Fli-1 deficient mouse model [172]. To study the role of the immune system in SSc, a perfect model would be an immunization-induced mouse model. However, although many studies have reproduced the dysregulation of immune response and autoimmunity in the mouse model, most of these SSc mouse model are not generated by induction of an autoimmune response and thus hardly be used to study the role of autoimmunity in pathogenesis of SSc.

Recently, a mouse model of SSc was established in our group by immunizing C57BL/6J mice with AT1R membrane extract. These mice developed autoantibodies against AT1R followed by inflammation and skin fibrosis, which provide us an ideal tool to study the role of autoimmunity in the pathogenesis. Beside our immunization induced mouse model, in 2011, Yoshizaki and his colleagues showed that immunization of DNA topoisomerase I with Freund's complete adjuvant is able to induce skin and lung fibrosis, and immune dysregulation including increased frequency of Th2 cells and elevated production of IL-6 and TGF-β [173]. They also showed that elevated production of IL-6 is important as loss of IL-6 attenuates skin and lung fibrosis and abnormal B cell activation which correlates with skin and lung fibrosis [173]. In addition to immunization of DNA topoisomerase I, immunization of type V collagen can induce skin and lung fibrosis and autoimmunity as well [174]. These studies supported our finding that dysregulated immune response is a pathogenic driver in the development of SSc like disease.

Studies on the pathogenesis of other induced or spontaneous mouse models of SSc also revealed the important role of immune dysregulation and autoimmunity in the disease development [172][175]. Bleomycin induced mouse model is widely used to study the pathogenesis of tissue fibrosis in systemic sclerosis, which reproduced several key features of SSc including skin and lung fibrosis, immune dysregulation and autoimmunity [175]. In a further study it has been shown that depletion of T cells was able to ameliorate the tissue fibrosis of bleomycin induced mouse model [176]. Beside this, deficiency of CD19, a positive regulator of B cell activation, suppressed the development of skin and lung fibrosis, immune deregulation and autoimmunity in the bleomycin induced mouse model [177]. In the recent established Fli-1 knockout mouse model of SSc, the development of tissue fibrosis was found to be autoreactive T cells dependent [178]. All these findings were in line with our finding that CD4 T cells and B cells are important for the development of SSc like diseases.

Contribution of lymphocytes to the pathogenesis of AT1R-induced mouse model for SSc

Since $CD4^+$ T cells, $CD8^+$ T cells and B cells are present in the affected tissues of SSc and they are suspected to contribute to the development of the disease [86], [88], [89], [98], the role of those three types of lymphocytes in the AT1R-induced mouse model for SSc was investigated. Interestingly, $CD8^+$ T cell-deficient mice were as susceptible to the disease as wild type control mice, while both $CD4^+$ T cell-deficient mice and B cell-deficient mice were resistant. These results directly support an essential role of $CD4^+$ T cells and B cells, but not $CD8^+$ T cells in the development of AT1R-induced SSc-like disease.

Neither $CD4^+$ T cell deficient mice nor B cell deficient mice produced any detectable levels of anti-AT1R IgG after the immunization with AT1R, demonstrating that autoantibodies against AT1R are produced in a T cell-dependent manner. Based on these findings, together with the observation of lacking disease pathology in $CD4^+$ T cell deficient-mice and B cell-deficient mice, we speculate that the disease in this novel mouse model for SSc is mainly mediated by anti-AT1R autoantibodies. This hypothesis is further supported by the fact that functional antibodies against AT1R are able to induce inflammation in the skin and lung of mice.

T cells are the dominant inflammatory cells in the infiltrate in affected tissues and organs in SSc, especially in early stages of the disease [88], [89]. This is consistent with our previous data showing that T cells are the main cells in the inflammation of skin and lung. Peripheral blood mononuclear cells (PBMC) from SSc patients show an increased capacity of transendothelial migration compared with PBMC from healthy controls [179], and this increase is due to the migration of $CD3^+$ T cells and mainly to a larger proportion of $CD4^+$ T cells [179]. In line with this notion, inflammatory infiltrates in the skin and lung of SSc patients are featured by T cells, and $CD4^+$ T cells predominate over $CD8^+$ T cells, resulting a predominance of $CD4^+$ T cells and an increased CD4/CD8 ratio [61]. Beside this, it has been shown that SSc patients have more activated $CD4^+$ T cells in the peripheral blood than healthy subjects [180]. These results suggest that, in contrast to $CD8^+$ T cells, $CD4^+$ T cells in SSc patients are prone to transvascular migration and might play important role in the pathogenesis of SSc. This notion is supported by the data of this study which demonstrates that a lack of $CD4^+$ T cells prevented the development of SSc like disease in the mouse model while lack of $CD8^+$ T cells did not.

$CD4^+$ T cells could contribute to the development of experimental SSc through several mechanisms. Since this study shows that anti-AT1R autoantibodies are pathogenic in mice, helping B cells to produce autoantibodies should be a major contribution of $CD4^+$ T cells to the disease pathogenesis. Moreover, antigen specific autoreactive $CD4^+$ T cells might also directly interact with endothelial cells of the blood vessel. This notion is supported by previous studies showing that T cells are able to bind to and to injure endothelial cells and thus promote perivascular inflammation [59], [60].

In addition to these two mechanisms, activated $CD4^+$ T cells could also contribute to the disease development via the production of cytokines in an autoantibody-independent manner [168], [181]. This idea is supported by my observation that pro-inflammatory cytokines IL-1a, IL-4 and TNF-a were significantly increased in the serum of AT1R immunized wild type mice as compared to control ME immunized mice. These results are in line with findings observed in

the patients with SSc. It has been revealed that pro-inflammatory cytokines, especially Th2 cytokines including IL-4, IL-6 and IL-13 are elevated in peripheral blood and affected tissues of patients and involved in the pathogenesis of the disease [64]. Th2 cytokines such as IL-4 and IL-13 are capable in promoting the proliferation of fibroblasts and collagen production *in vitro* [182], [183]. Findings in animal models, which genetic deletion of IL-13 attenuates fibrosis in mice whereas overexpression of IL-13 leads to tissue fibrosis support this view [184]. An oligonucleotide microarray study revealed that two Th2 polarization-associated genes, interleukin 2 receptor subunit beta (*IL2RB*) and GATA binding protein 3 (*GATA3*), are significantly upregulated in the peripheral blood cells of SSc patients [185]. These data indicate that SSc is a disease associated with a Th2-type autoimmune response [186], and that Th2 polarization may have crucial impact on its pathogenesis [2], [88], [94]. Although SSc is predominately a Th2-cytokine-driven disease, elevated levels of Th1 cytokines such as IFN-γ have been observed in SSc patients compared to healthy controls [187], which was confirmed by our mouse model too.

The above-mentioned mechanisms underlying $CD4^+$ T-cells' contribution are best exemplified in the development of pulmonary inflammation in the AT1R-induced mouse model of SSc. Although both $CD4^+$ T cell-deficient mice and B cell-deficient mice are resistant to the AT1R-induced pulmonary inflammation, the two mouse strains showed a slight difference in disease manifestations in the lung. As expected, untouched $CD4^+$ T cell-deficient and B cell-deficient mice showed no evidence of pulmonary inflammation. However, in contrast to control ME- or AT1R ME-immunized $CD4^+$ T cell deficient mice which did not develop any pulmonary inflammation, both control ME- and AT1R ME-immunized B cell deficient mice develop a mild and comparable inflammation in the lung. These findings suggest that the pulmonary inflammation in the AT1R-induced mouse model of SSc is composed of two parts, one is mediated by specific immune responses to AT1R and the other is caused by immune responses to components of control ME or the adjuvant used. The former is dependent on $CD4^+$ T cells and B cells, and thus most likely associated with the generation of autoantibodies. The latter is dependent on $CD4^+$ T cells but not B cells, and thus likely mediated by cytokines released by $CD4^+$ T cells.

The B cell-deficient mice (muMT) were generated by Kitamura et al through disrupting the gene encoding mu chain in the precursor cells of B cells (pre-B cells). Consequently, the development of B cells is arrested at the stage of pre-B cell and mature B cells are absent in the muMT mice [188], [189]. However, subsequent studies showed that the lack of mu chain is not

a mandatory requirement of B cell development and B1 cells which are able to secrete unspecific IgG and IgE are present in the muMT mice [188], [190]. Therefore, the leakage of B1 cells in muMT mice needs to be considered when the findings with the B cell deficient mice are interpreted.

This study demonstrated that B cells play an important role in development of systemic inflammation in the AT1R ME-induced mouse model for SSc. One the one hand, as mentioned above, B cell deficient mice are resistant to AT1R ME-immunization induced pulmonary inflammation. On the other hand, B cell deficiency also greatly inhibits the development of perivascular infiltrates in the skin.

The essential role of B cells is not limited to lung inflammation. While wild-type mice developed fibrosis in the skin after immunization with AT1R ME, no differences were observed in skin thickness or skin collagen contents between AT1R ME-immunized and control ME-immunized B cell deficient mice. These findings suggest that B cells are key player in the development of skin fibrosis in the AT1R-induced mouse model of SSc. My results are in line with findings from a previous study in which Hasegawa and colleagues reported that depletion of B cell significantly suppressed the skin fibrosis in the tight-skin ($Tsk^{/+}$) mouse model of SSc [191]. Beside evidence from experimental models, clinical studies also suggest a role of B cells in the development of skin fibrosis in SSc. For example, in patients with SSc, increased numbers of B cells have been observed in the affected skin and the number of B cells is positively correlated with the progression and disease severity of SSc [192]. In addition, B cell-depleting monoclonal antibodies, e.g. Rituximab, have been reported to be effective in improving skin fibrosis in SSc patients, further supporting an important role of B cells in the development of dermal fibrosis [193].

There could be multitude of mechanisms underlying the contribution of B cells to the SSc-like disease in mice. Given that the main function of B cells is to produce antibodies and this study showed that autoantibodies against AT1R are pathogenic in mice, generation of autoantibodies should be an important mechanism underlying the B cell contribution in the novel mouse model. However, beside producing autoantibodies, B cells could also contribute to inflammation and fibrosis via other pathways including antigen presentation, formation of tertiary lymphoid organs (TLOs), and release of pro-inflammatory cytokines [194], [195]. B cells express MHC II and costimulatory molecules and are able to present antigen, leading to the activation and differentiation of $CD4^+$ T cells and thus contribute to the disease manifestation. For example, it has been reported that MHC II and costimulatory molecules (i.e., CD40, CD80 and CD86)

are upregulated on the donor B cells in a mouse model of chronic graft-versus-host disease (GVHD), which augments the clonal expansion and differentiation of donor T cells and finally promotes the development of skin inflammation and fibrosis [196]. TLOs are aggregates of lymphocytes and stromal cells in an organized structure which occur outside of the secondary lymphoid organs [197]. Formation of TLOs has been widely observed in affected organs in many systemic autoimmune diseases, such as synovial tissue in RA, kidneys in SLE and salivary glands in pSS. TLOs support the affinity maturation, clonal selection and differentiation of autoreactive B cells and thus contribute to the disease manifestation of disease [198]. In the AT1R-induced mouse model for SSc, TLOs-like aggregate of T lymphocytes and B lymphocytes have been observed in the lung of affected mice, suggesting a potential role of TLOs-like structure in the disease pathogenesis (unpublished data). Finally, infiltrated B cell might be involved in the pathogenesis via secretion of pro-inflammatory and pro-fibrotic cytokines like IL-4 and IL-6 [195], [199]. This notion is supported by the data from the current study that levels of IL-4 and IL-6 are elevated in the SSc mouse model and deficiency of B cells completely inhibits the production of IL-4 and IL-6 as well as disease manifestations in the mouse model. Further evidence is brought by another animal model of SSc, in which B cells isolated from the bleomycin induced mouse model of SSc produced high levels of profibrotic cytokines after stimulation with BAFF [199]. In addition, in vitro co-culture experiments suggest that IL-6 and TGF-β secreted by B cells are able to stimulate the differentiation of fibroblast into myofibroblasts [104], which is supported by data from the SSc patients. It has been shown that B cells from SSc patients are capable of secreting significant higher level of IL-6 than B cells from healthy controls after stimulation with BAFF [104]. In addition, SSc patients treated with Rituximab show decreased levels of IL-6 which is correlated with improvement of skin fibrosis [193], suggesting a B-cell derived IL-6 plays a role in the pathogenesis of SSc.

Role of the complement activation in the AT1R-induced mouse model for SSc

The complement system is a fundamental part of innate immune system and plays an essential role in several physiological activities, including host defense against pathogens, bridging the innate and adaptive immune response and assisting the disposal of immune complex [200]. The system is composed of more than 50 plasma proteins, including complement C1 to C9 [201]. It can be activated through three distinctive pathways, namely classical, alternative and lectin pathways. For all three complement activation pathways, the conversion of C3 to C3b is

necessary for full activation of the complement system [201]. Therefore, complement C3 deficient mice are an ideal tool for the investigation of the role of complement system in experimental models of diseases.

As one of main effector mechanisms of antibody-mediated immunity, complement activation has been demonstrated to be involved in the pathogenesis of multiple autoimmune diseases, including SLE, bullous pemphigoid, pemphigus valguris and epidermolysis bullosa acquista (EBA) [201]. For instance, EBA is an autoimmune blistering disease caused by autoantibodies against collagen type VII (COL7), a major component of anchoring fibrils of the dermal-epidermal adhesion [202]. Affected skin biopsy from patients with EBA is featured by complement deposition at the dermal-epidermal junction, and it has been shown that complement activation is an important step in the development of experimental EBA in mice [202].

Since my results suggest an essential role of anti-AT1R autoantibodies, I determined whether complement activation is required for the development of disease in this mouse model by investigating C3 deficient mice. As shown here, AT1R-immunized C3-deficient mice generated anti-AT1R autoantibodies and developed inflammation in the lung and skin as well as skin fibrosis. Therefore, complement system is a dispensable factor for the disease development in this mouse model. This notion is supported by evidence from studies showing that levels of complement components in sera of patients with SSc are not correlated with disease manifestations and activity, including skin fibrosis and pulmonary hypertension [203], [204]. Moreover, as the autoantibodies against AT1R are agonizing antibodies which have been confirmed in vitro experiments (unpublished data), therefore the autoantibodies against AT1R induced pathological changes does not require the complement system.

Surprisingly, in this AT1R-induced mouse model for SSc, C3 deficient mice developed significantly more severe inflammation in the lung than wild type control mice, suggesting an unexpected anti-inflammatory role of complement C3. Interestingly, no such differences were seen in the skin where inflammation and fibrosis reached similar scores in wild type and knockout mice. This suggests that the anti-inflammatory effect of complement C3 is lung specific.

Two possible mechanisms may contribute to the unexpected anti-inflammatory role of complement C3. Complement activation is involved in the clearance of immune complex, apoptotic bodies, dying cells and debris which is important for maintaining immune homeostasis [200]. Thus, complement C3 deficiency could impair the function of the clearance,

leading to dysregulation of immune responses and consequent inflammation [205]. Furthermore, it has been shown that airway epithelial cells are capable to internalize complement C3 from exogenous sources, and internalized complement C3 protects airway epithelial cells from stress-induced death (e.g. from oxidative stress) by influencing pathways which are independent of programming cell death [206]. Therefore, the lack of complement C3 could promote the cell death of airway epithelial cells, which might be a trigger of pulmonary inflammation [206].

Mechanism underlying the pathogenicity of monoclonal antibody against AT1R

Over the past decade, studies have accumulated more and more evidences regarding the pathogenic role of functional autoantibodies against AT1R in SSc. This includes elevated anti-AT1R IgG in sera of patients, strong association between levels of autoantibodies against AT1R with disease severity and mortality, *in vitro* demonstration of agonistic effects of anti-AT1R IgG, and experimental pathogenicity of IgG isolated from patients with high levels of anti-AT1R autoantibodies [160], [162], [163]. However, a direct proof of the pathogenic effect of anti-AT1R autoantibodies was still missing. In this study, I could show that transfer of functional monoclonal autoantibodies against AT1R directly induce inflammation in the skin and lung in naïve mice. These results for the first time provide evidences that autoantibodies against AT1R are indeed pathogenic in mice.

Moreover, deficiency in AT1R expression prevented mice from mAb5.2a-induced inflammation, confirming that anti-AT1R monoclonal antibodies promote disease pathology via binding to AT1R. AT1R is expressed by a broad variety of cells, including keratinocytes, fibroblasts, endothelial cells and immune cells in the skin [147], [207]. By analysis of single cells isolated from murine ears, this study shows that fibroblasts are the dominant cells binding mAb5.2a in the skin, suggesting that dermal fibroblasts are main target cells of monoclonal antibodies against AT1R. In addition, immunofluorescence staining of skin samples showed that endothelial cells also bind mAb5.2a, providing another type of target cell of functional antibodies against AT1R.

The accumulative prevalence of autoantibodies mediated autoimmune diseases is over 2.5% up to date [171]. Pathological mechanisms of autoantibody-induced pathology considerably differ among diseases [131], [171]. Therefore, understanding of the precise pathomechanisms of autoantibody-mediated pathologies would strongly benefit the development of novel treatment

of autoimmune diseases [171], [208]. According to a comprehensive review from Ludwig et al., the autoantibody-induced pathology can be categorized into seven groups based on the underlying mechanism of action: 1) mimicking receptor stimulation; 2) blocking of neural transmission, by receptor blockade; 3) induction of altered signaling; 4) triggering uncontrolled microthrombosis; 5) uncontrolled neutrophil activation; 6) cell lysis; 7) induction of inflammation at the site of autoantibody binding [171]. Generally, these seven pathways can be grouped into two major pathways, Fc-dependent and Fc-independent pathways [171]. According to our hypothesis that autoantibodies against AT1R mediate pathology through an Fc-independent pathway. Specifically, functional autoantibodies bind to a receptor as an agonist and mediate a sustained activation, which eventually induces inflammation and other pathological changes. This hypothesis is supported by the finding that complement activation is dispensable for the AT1R-induced SSc-like disease. In addition, it is also supported by previous findings showing IgG containing higher level of AT1R autoantibodies purified from SSc patients are capable in stimulating the secretion of inflammatory cytokines and chemokines such as IL-8 and CCL18 [161].

By contrast to physiological ligands bind the GPCRs on the cell membrane in the hydrophobic pocket site, the functional autoantibodies most likely bind the extracellular domains of GPCRs [136]. Moreover, understanding the interaction of autoantibody against AT1R with AT1R at the molecular level could not only let us know how the antibody against AT1R bind and activate the receptor AT1R, but also provide important information for rational design of effective therapeutics to block the pathological activation of AT1R. The classical way to describe the interaction between the antibody and AT1R is by determining the three-dimension structure of the antibody-AT1R complex by X-ray crystallography, which is an often time-consuming and laborious process with high failure rate [209]. However, due to the significant advances in computational biology for structural prediction of antibody-antigen complexes, computational docking provides us a rapid and affordable alternative route to obtain the structural information of such complexes [209], especially for GPCR like AT1R which tertiary and quaternary structure is strongly affected upon purification [210].

By virtue of computational docking, the interaction of antibody against AT1R and AT1R was determined. Predicted model of antibody-AT1R complex showed that the N terminal, the second and third extracellular loops of AT1R are involved in the interaction, which suggest the extracellular domains are important for the binding of AT1R autoantibodies. This finding is supported by the experimental results that agonizing autoantibodies against AT1R recognize

the second extracellular loop of AT1R receptor [211]. Beside this, other functional autoantibodies against GPCR such as anti-beta1-adrenergic receptor autoantibodies and anti-muscarinic type 3 receptor which have been shown recognize the extracellular domains of GPCR [136]. Immunization of rabbits with peptides belong to the second extracellular loop of the beta1-adrenergic receptor induced production of autoantibodies against beta1-adrenergic receptor and dilated right and left heart ventricles, which can be prevented when the rabbits were treated with antagonists of the beta1-adrenergic receptor, suggesting the pathogenic effects of autoantibodies against the second extracellular loop of the receptor [212], [213]. Altogether, the binding of antibody mAb5.2a against AT1R to the extracellular domains of AT1R could play an essential role in the pathogenic effects of mAb5.2a.

Interestingly, local injection of monoclonal antibody against AT1R into skin did not only induce skin inflammation, but also caused inflammation in the lung. Most likely, small amounts of injected monoclonal autoantibodies are transported to the lung via blood circulation and subsequently stimulate the lung tissue cells resulting in a peripheral inflammation. Although monoclonal autoantibodies against AT1R induce inflammation in both skin and the lung, there are remarkable differences in the composition of the inflammatory infiltrates between the two organs. Analysis of affected tissues by immunohistochemistry showed that the infiltrates in skin are composed predominantly of neutrophils, while pulmonary infiltrates consist mainly of lymphocytes, especially T cells. There might be two possible explanations for this phenomenon. First, the difference could due to different target cells affected by the antibodies in either organ. As shown here, the predominant cells that express AT1R in the skin are fibroblasts, and those fibroblasts are also the major target cells of the injected monoclonal antibodies against AT1R. By contrast, when the locally injected circulating monoclonal antibodies reach the lung via circulation, they most likely bind to endothelial cells which express AT1R as shown here and by others [214]. Therefore, the interaction of functional anti-AT1R autoantibodies with different target cells might lead to the secretion of cell-type specific chemotactic factors which attract different groups of immune cells. However, this hypothesis needs to be verified in future studies. A second explanation could be that the strong increase of neutrophils in the locally affected tissue may be explained by the rather high concentration of anti-AT1R antibodies near the injections site presumably leading to a massive release of neutrophil-recruiting chemokines. By contrast, concentration of anti-AT1R antibodies in the lung can be expected as significantly lower resulting in a release of lymphocytes-attracting chemokines.

It is also interesting to notice that cellular infiltrates in the skin induced by AT1R immunization differ significantly from those induced by AT1R-directed monoclonal antibodies. While immunization resulted in infiltrates consisting of B and T cells, administration of antibodies promoted a local infiltration of neutrophils at the injection site and the accumulation of T cells in the periphery of the lung. One possible explanation could be, as mentioned above, the presence of high concentration of anti-AT1R antibodies in the ear of mice which may lead to a release of neutrophil-attracting chemokines. Moreover, a reason for the lack of B cells in the lungs of antibody-transferred mice could be the fact that in such passive model an activation of the adaptive immune system including the expansion of B cells is lacking. Within this view, attraction of T cells into the affected tissue could be one essential function of AT1R-autoantibodies in promoting inflammation.

5. Conclusion

In this study, the role of $CD4^+$ T cells, $CD8^+$ T cells and B cells in a mouse model of SSc induced by immunization with AT1R membrane extract was investigated. Moreover, the pathogenicity of autoantibodies against AT1R derived from this model was evaluated *in vivo*. I could show that $CD4^+$ T cells and B cells play an indispensable role in the pathogenesis of the AT1R-induced mouse model for SSc, suggesting the important role of autoantibodies in the development of disease in the mouse model. Deficiency of complement C3 did not prevent the development of AT1R-induced SSc-like disease, demonstrating that the complement system does not drive the pathology in this model and providing indirect evidence that the pathogenic function of AT1R autoantibodies maybe linked to their capacity to directly activate AT1R. Furthermore, transfer of monoclonal anti-AT1R antibodies induced inflammation in the skin and lung of naïve mice, confirming the pathogenicity of autoantibodies against AT1R which is most likely mediated by fibroblast as the major cell population in binding these antibodies in murine skin. In summary, these results provide for the first time direct evidence that functional autoantibodies against AT1R are pathogenic in mice, substantiating the idea that these autoantibodies represent one major pathogenic driver in human SSc.

6. Outlook

My results suggest that autoantibodies against AT1R act as a ligand of AT1R and consequently mediate pathology in the mice. However, this notion needs to be validated in future studies. To provide further evidence for receptor-activation as pathogenic principle of the antibodies, it is of significance to prepare F(ab')2 fragment of them and determine their pathogenicity in mice. Beside this, further exploration of the mechanism underlying the anti-AT1R autoantibody-induced pathology will help us to understand the pathogenesis of human SSc. Thus, in future studies it will be important to clarify the question which cells represent the responder to the autoantibodies against AT1R. Moreover, to deeper understand the mechanisms of the disease, it will be essential to delineate the inflammatory cascade triggered by anti-AT1R autoantibodies. Finally, given a pathogenic role of anti-AT1R autoantibodies, it is highly interesting to evaluate whether autoantibodies against AT1R and cells relevant for their production could serve as potential therapeutic target for the treatment of SSc in humans.

7. References

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Publications

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Conferences

Conference	Location and date	Contribution
The European League Against Rheumatism Congress	Madrid, 2019	Oral presentation ("OP0188 Pathogenicity of Functional Autoantibodies against AT1R in a Mouse Model of Systemic Sclerosis")
GRK1727 Autumn Retreat	Bad Segeberg, 2019	Oral presentation ("Pathomechanisms in experimental systemic sclerosis")
GRK1727 Spring Retreat	Lübeck, 2019	Oral presentation ("Pathomechanisms in experimental systemic sclerosis")
NDI3-New Developments in Immunology, Inflammation and Infection	Borstel, 2019	Organization
BBRS Retreat	Ammersbek, 2019	Oral presentation ("Pathomechanisms in experimental systemic sclerosis")
NDI3-New Developments in Immunology, Inflammation and Infection	Borstel, 2018	Poster ("Pathomechanisms of experimental systemic sclerosis")
GRK1727 Autumn Retreat	Crivitz, 2018	Oral presentation ("Pathomechanisms in experimental systemic sclerosis")
GRK1727 Spring Retreat	Lübeck, 2018	Oral presentation ("Pathomechanisms in experimental systemic sclerosis")
NDI3-New Developments in Immunology, Inflammation and Infection	Borstel, 2017	Poster ("Pathomechanisms of experimental systemic sclerosis")
GRK1727 Autumn Retreat	Bad Segeberg, 2017	Oral presentation ("Pathomechanisms in experimental systemic sclerosis")

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