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Investigation of the role of T and B cells in a novel humanized mouse model for systemic sclerosis

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Zusammenfassung

Hintergrund und Zielsetzung: Die Systemische Sklerose (SSc) ist eine komplexe autoimmune Bindegewebserkrankung mit unklarer Pathogenese. Vor kurzem wurde in unserer Arbeitsgruppe ein neuartiges humanisiertes Mausmodell für SSc etabliert, bei dem mononukleäre periphere Blutzellen (PBMC) aus SSc-Patienten in *Rag2*$^{-/-}$*Il2R*$^{-/-}$ immundefiziente Mäuse übertragen werden. Mäuse, die PBMC von SSc-Patienten erhalten, entwickeln systemische Entzündungssymptome in Lunge, Niere und Leber, welche in dieser Form auch in der SSc auftreten. In der vorliegenden Studie sollte die Rolle der menschlichen T- und B-Zellen in der Pathogenese der experimentellen SSc im humanisierten Mausmodell untersucht werden.

Methoden: PBMC wurden aus 11, in der Rheumatologischen Klinik der Universität Lübeck behandelten, SSc-Patienten isoliert. Humane $CD3^+$ T- oder $CD19^+$ B-Zellen wurden mit Hilfe magnetischer Zellsortierung (MACS) aus den PBMC entfernt. Um eine Erkrankung bei Mäusen zu induzieren, wurden 2×10^7 PBMC vor bzw. nach T- oder B-Zelldepletion mittels intraperitonealer (i.p.) Injektion in die *Rag2*$^{-/-}$*Il2R*$^{-/-}$ Mäuse übertragen. Die Empfängermäuse wurden 12 Wochen nach dem Transfer abgetötet und Blut, Milz, Lunge, Niere, Herz, Leber, Muskel, Ösophagus, Darm sowie Haut für weitere Untersuchungen gesammelt. Menschliche T- und B-Zellen wurden vor dem Transfer und in der 6. und 12. Woche nach dem Transfer mittels Durchflusszytometrie (FACS) bestimmt. Weiterhin wurden die Serumspiegel des humanen Gesamt-IgGs, sowie die Spiegel spezifischer Antikörper gegen Angiotensin-II-Typ-1-Rezeptors (AT1R) und Endothelin-1-Typ-A-Rezeptors (ETAR) in den Empfängermäusen mittels ELISA gemessen. Die Histopathologie der Haut und der inneren Organe wurde in Schnitten nach Hämatoxylin- und Eosin-(H&E-)Färbung untersucht. Definierte Zellpopulationen in den erkrankten Organen wurden durch immunhistochemische Verfahren bestimmt.

Ergebnisse: Sowohl T-Zellen als auch B-Zellen konnten durch Depletion erfolgreich aus den PBMC der Patienten entfernt werden. Dabei zeigte sich, dass Mäuse, welche Gesamt-PBMC aus SSc-Patienten erhalten hatten, eine gegenüber Mäusen mit T- oder-B-Zell depletierten PBMC deutlich erhöhte Sterblichkeitsrate aufwiesen. Dieser Effekt setze sich in der Entwicklung des Krankheitsbildes der experimentellen SSc fort. Während Mäuse nach Tranfer von Gesamt-PBMC massive Enzündungszeichen in Lunge, Niere und Leber entwickelten, waren Tiere nach Übertragung von T- oder B-Zell-depletierten PBMC weitgehend vor einer Erkrankung geschützt. Weitergehende Untersuchungen zeigten, dass die Mäuse nach Erhalt der Gesamt-PBMC gegenüber der Kontrollgruppe höhere Zahlen an humanen Leukozyten im peripheren Blut und signifikante Mengen an humanem IgG und anti-AT1R und anti-ETAR-Autoantikörpern aufwiesen. Darüber hinaus bildeten Mäuse, denen, Gesamt-PBMC transferiert wurden, eine strukturierte Architektur der Milz aus, die durch eine Pulpa alba mit funktionellen Keimzententren charakterisiert war. Diese Strukturierung war weder in unbehandelten *Rag2*$^{-/-}$*Il2R*$^{-/-}$ Mäusen noch in Tieren nach Übertragung

von T- oder B-Zell-depletierten PBMC sichtbar. Die IHC-Analyse zeigte, dass die Pulpa alba von Mäusen nach Transfer von PBMC aus SSc-Patienten überwiegend aus humanen $CD20^+$ B-Zellen und $CD4^+$ T-Zellen sowie einer geringen Menge an humanen $CD8^+$ T-Zellen und $CD138^+$ Plasmazellen gebildet wird.

Schlussfolgerungen: Diese Studie zeigt, dass sowohl T- als auch B-Zellen eine essentielle Rolle in der Pathogenese der experimentellen SSc spielen. Die Identifikation von humanen Lymphozyten als Treiber der Erkrankung im verwendeten Tiermodell liefert einen starken Hinweis darauf, dass ein vergleichbarer Mechanismus in der SSc im Menschen existieren könnte.

Summary

Background and objective: Systemic sclerosis (SSc) is a complex autoimmune connective tissue disease with an unclear pathogenesis. Recently, a novel humanized mouse model for SSc was established in our group by transferring peripheral blood mononuclear cells (PBMC) from SSc patients to *Rag2*$^{-/-}$*Il2R*$^{-/-}$ immunodeficient mice. Mice receiving PBMC from SSc patients but not from healthy controls develop systemic inflammation in lung, kidney and liver, partially resembling SSc. In the current study, I aimed to investigate the role of human T and B cells in this humanized mouse model for SSc.

Methods: PBMC were isolated from 11 SSc-patients which were under treatment at the Department of Rheumatology, University of Lübeck. Human CD3$^+$ T or CD19$^+$ B cells were depleted from PBMC using a magnetic activated cell sorter (MACS). To induce disease in mice, 2 × 10^7 PBMC before or after T- or B-cell depletion were transferred into *Rag2*$^{-/-}$*/Il2R*$^{-/-}$ mice via intraperitoneal (i.p.) injection. The recipient mice were sacrificed at week 12 after the transfer, and blood, spleen, lung, kidney, heart, liver, muscle, esphogus, intestines as well as skin were collected for further evaluation. Human T and B cells were determined using flow cytometry (FACS) analysis before the transfer and at the 6th and 12th week after the transfer. Serum levels of total human IgG, anti-angiotensin-II type1 receptor (AT1R) IgG and anti-endothelin-1 type A receptor (ETAR) IgG in recipient mice were determined by ELISA. Histopathology of the skin and inner organs were evaluated by hematoxylin and eosin (H&E) staining. Moreover, histology and cellularity of murine spleens were determined by H&E and immunohistochemstry (IHC) staining, respectively.

Results: Both T cells and B cells were efficiently depleted from patients' PBMC. After transfer, mice received whole PBMC from SSc patients showed a significantly higher mortality rate than mice received T or B cell-depleted PBMC. At week 12 after cells transfer, mice engrafted with whole PBMC showed higher levels of human leukocytes in peripheral blood than mice received corresponding T or B cell-depleted PBMC. Strikingly, mice transferred with whole PBMC from SSc patients developed systemic inflammation in the lung, kidney and liver, while such inflammation was not observed in mice transferred with corresponding T or B cell-depleted PBMC. During the course of the experiment, considerable amount of human IgG and anti-AT1R and anti-ETAR autoantibodies were detectable in mice after transfer of whole PBMC but not T or B cell-depleted PBMC. Moreover, mice transferred with whole PBMC developed a typical white pulp in their spleens containing functional germinal centers (GCs) which was absent in untreated *Rag2*$^{-/-}$*/Il2R*$^{-/-}$ immunodeficient mice, or did not contain functional GC seen after engraftment with T or B cell-depleted PBMC. IHC analysis demonstrated that the splenic white pulps in mice transferred with PBMC from SSc patients consisted of predominantly human CD20$^+$ B cells and CD4$^+$ T cells, as well as small amount of human CD8$^+$ T cells and CD138$^+$ plasma cells.

Conclusions: This study demonstrates that both T and B cells are indispensable for the development of the disease of experiment SSc. The identification of human lymphocytes as drivers of the disease in the animal model used here argues strongly for the presence of a corresponding mechanism in SSc in humans.

Abbreviation

SSc, systemic sclerosis; PBMC, peripheral blood mononuclear cells; MACS, magnetic activated cell sorter; FACS, flow cytometric analysis; AT1R, angiotensin-II type1 receptor; ETAR, endothelin type A receptor; H&E, hematoxylin and eosin; IHC, immunohistochemstry; GCs, germinal centers; ACR, American College of Rheumatology; EULAR, European League Against Rheumatism; ACA, anti-centromere-specific antibodies; ATA, anti-DNA topoisomerase I antibodies; GIT, gastrointestinal tract; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; ACE, angiotensin-converting enzyme; SCR, scleroderma renal crisis; PAH, pulmonary arterial hypertension; NOR, nucleolus organizing regions; MMP, matrix metalloproteinases; TGF, transforming growth factor; Treg, regulatory T cells; AHR, arylhydrocarbon receptor; HLA, human leukocyte antigen; *BANK1*, B cell scaffold protein with ankyrin repeats 1; INF. interferon; *IRF4*, interferon regulatory factor 4; *STAT4*, signal transducer and activator of transcription 4; *TNFAIP3*, tumor necrosis factor alpha-induced protein 3; *IRAK1*, interleukin-1 receptor-associated kinase 1; *TERT*, telomerase reverse transcriptase; *ATP8B4*, ATPase phospholipid transporting 8B4; *DNASE1L3*, deoxyribonuclease 1 like 3; *ATG5*, Autophagy Related 5; PBC, primary biliary cirrhosis; SLE, systemic lupus erythematosus; MG, myasthenia gravis; PV, pemphigus vulgaris; T1DM, type 1 diabetes mellitus; SS, Sjogren's syndrome; RA, rheumatoid arthritis; BP, bullous pemphigoid; PF, pemphigus foliaceus; AS, ankylosing spondylitis; RTX, rituximab; ARRIVE, Animal Research: Reporting of In Vivo Experiments; FCS, fetal cow serum; BSA, bovine serum albumin; i.p., intraperitoneally; RBC, red blood cell; RT, room temperature; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; KO, knock out; AMA, anti-mitochondrial antibody; Tfh, follicular helper T; TCR, T cell receptor; MHC II, major histocompatibility complex; ICOS, inducible costimulator; ICOS-L, inducible costimulator ligand.

1. Introduction

1.1 Systemic sclerosis

Systemic sclerosis (SSc), also known as scleroderma, is a systemic autoimmune connective tissue disease which is characterized by autoimmunity, inflammation, vasculopathy and fibrosis (1). The prevalence and incidence of SSc vary considerably among different ethnic populations (1). For example, high prevalence (276–443 per million) and incidence (14–24 per million per year) have been reported in populations of European ancestry, whereas Asian populations are associated with low prevalence (<150 per million) and incidence (<11 per million per year) (2-6). Like many other autoimmune diseases, SSc is featured by gender difference, with female-to-male ratios ranging from 3:1 to 14:1 among geographical populations(3, 5).

1.1.1 Clinical features in SSc

In 1980, a preliminary classification criteria for SSc was proposed by American College of Rheumatology (ACR) (7). However, the 1980 ACR classification criteria for SSc lacks adequate sensitivity for early SSc and limited cutaneous SSc. In 2013, the ACR and European League Against Rheumatism (EULAR) jointly proposed a new criteria for the classification of SSc in which both clinical manifestations and immunological parameters are incorporated to enable an improved and earlier diagnosis (8). The diagnostic parameters of the 2013 ACR/EULAR criteria are as follows: 1) skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints, 2) finger tip lesions, 3) telangiectasia, 4) abnormal nailfold capillaries, 5) pulmonary arterial hypertension and/or interstitial lung disease, 6) Raynaud's phenomenon, and 7) SSc-related autoantibodies including anti-centromere-specific antibodies (ACA) and anti-DNA topoisomerase I antibodies (ATA) and anti-RNA polymerase III antibodies, with maximal scores of 9, 3, 2, 2, 2, 3 and 3, respectively. Patients with a total score of 9 or more could be definitely diagnosed as SSc (8).

Majority of patients with SSc suffer from skin manifestations, such as skin fibrosis, Raynaud's phenomenon and ischemic ulcers (9). Besides skin involvement, many inner organs including the lung, kidney, heart, and gastrointestinal tract (GIT) are often affected in SSc (10). Generally, SSc is classified into two main subtypes based on the extend of skin involvement: 1) limited cutaneous SSc (lcSSc) in which skin sclerosis is restricted to the hands and, to a lesser extent, to the face and neck, and 2) diffuse cutaneous SSc (dcSSc) in which patients develop extensive skin sclerosis (11). In addition to the 2 major subsets, there are 2 infrequent subtypes of SSc, SSc *sine* scleroderma in which skin is not involved, and SSc overlap syndrome in which patients develop at least two connective tissue diseases at the same time (12).

Compared to other autoimmune disorders, SSc is featured by a poor prognosis, with a 10-year survival rate of 60-70% (13). The SSc-related death is often a consequence of involvement of inner organs (2, 4, 6). Prior to the use of angiotensin-converting enzyme (ACE) inhibitors in 1980's, scleroderma renal crisis (SCR) was the leading cause of the SSc-related death (14, 15). SRC occurs in approximately 5% of SSc patients, most commonly in those with dcSSc and early-stage (<4 years) disease (1, 14). Generally, the development of SRC starts with the deposition of mucopolysaccharides in the vascular intima, renal fibrosis and thickening of glomerular basement membrane and proteinuria, which consequently leads to the malignant hypertension and oligo-anuric acute renal failure (16). Currently, the leading causes of SSc-related death are the lung manifestations, including pulmonary fibrosis and pulmonary arterial hypertension (PAH) (1, 17, 18).

In addition to the kidney and lung, many other inner organs can be affected in SSc. For example, GIT involvement occurs in approximately 90% of patients with SSc. The most common SSc-associated sympotom in GIT is gastroesophageal reflux disease owing to the impairment of esophageal motility and the incompetence of the lower oesophageal sphincter (1, 19). Additionally, the oral cavity, stomach and intestines as well as the liver and pancreas were also commonly affected in SSc (19, 20). With regard to cardiac involvement, myocardial damage, conduction system fibrosis, coronary artery vasospasm and structural alterations and pericardial damage have been observed in patients with SSc (21). Consequently, SSc patients develop a variety of cardiac complications such as systolic or diastolic dysfunction, impaired ventricular filling, arrhythmias, and congestive heart failure (21-23). Besides, a notable proportion of patients with SSc also develop musculoskeletal complications such as artheropathy and myositis, which may lead to extremity dysfunction and disability (1, 24).

1.1.2 Immunological features of SSc

SSc is associated with hyper-γ-globulinemia and the presence of various autoantibodies (25). Among SSc-associated autoantibodies, ACA, ATA and anti-RNA polymerase III antibodies are currently used as diagnostic markers for the disease (8, 26). Usually, ATA and anti-RNA polymerase III antibodies are strongly associated with dcSSc and a poor prognosis, while ACA are associated with lcSSc (26). Besides the three autoantibodies, anti-Th/To antibodies and autoantibodies against nucleolus organizing regions (NOR) of 90-kDa antigen (anti-NOR-90 antibodies) have also been demonstrated to be associated with SSc (27-29). Notably, high levels of anti-Scl70 antibodies are associated with an increased extend of cutaneous involvement (30), and the former anti-Th/To antibodies are associated with lcSSc and pulmonary hypertension (28). Although the abovementioned autoantibodies are associated with SSc, none of them have been

shown to be pathogenic.

Recently, some further autoantibodies have been shown to be associated with SSc and/or its clinical manifestations. These autoantibodies which are specified as “functional” if its direct interaction with an identified target antigen leads to a molecular pathway activation or inhibition that can be replicated in an experimental setting, are suggested to contribute to the development of SSc (31). These autoantibodies are: 1) autoantibodies against endothelial cells which are able to induce endothelial activation and apoptosis (32-34), 2) anti-angiotensin-II type1 receptor (AT1R) and anti-endothelin type A receptor (ETAR) which are capable to stimulate the corresponding receptor and induce the production of pro-inflammatory and pro-fibrotic cytokines (35-38); 3) anti-fibroblast antibodies which are able to trigger pro-adhesive and pro-inflammatory phenotypic changes in fibroblasts (39-42); 4) anti-matrix metalloproteinases (MMP)-1 and -3 antibodies which are capable to prevent the degradation of excess collagen in the extracellular matrix (43, 44); and 5) anti-fibrillin 1 antibodies which are able to induce fibroblast activation and the release of sequestered transforming growth factor (TGF)-β from fibrillin-1-containing microfibrils into the extracellular matrix (45-47). However, the pathogenicity of those functional autoantibodies needs to be verified in future studies.

Apart from autoantibodies, SSc is also featured by inflammatory infiltration in multiple organs. Histological studies indicate that such infiltrates are present at very early stages, preceding the onset of fibrosis (48, 49). These cellular infiltrates are composed mostly of T cells, which are dominated by $CD4^+$ T cells associated with a small amount of $CD8^+$ T cells (49, 50). It has been proposed that $CD4^+$ T cell subsets such as Th1, Th17, Th22, Th9 play a role in the inflammatory phase of SSc and Th2 cells contribute to tissue fibrosis (51-56). In addition, abnormailties of regulatory T cells (Treg) have also been observed in SSc, where the proportions of functionally impaired Treg cells and profibrotic cytokine-producing Tregs are increased in SSc patients as compared to healthy subjects (57-59).

1.1.3 Risk factors for SSc

As many other autoimmune diseases, SSc is a consequence of the interaction between enviromental risk factors and genetic predisposition.

1.1.3.1 Role of environmental factors in SSc

Recently, increasing evidences have demonstrated that environmental and occupational factors contribute to the development of SSc, such as silica dust, solvents, chemicals, drugs, infectious agents and air pollution (60-62). Numerous infectious agents including Parvovirus B19,

Cytomegalovirus, Epstein-Barr virus, Retroviruses have been proposed as possible triggering factors for SSc (63). Since there is considerable homology between viruses and autoantigens, molecular mimicry may play a role in initiating autoimmune responses in SSc (64). Apart from infectious microorganisms, biological substances might also contribute to the development of SSc. For example, air pollutants could not only bind to the arylhydrocarbon receptor (AHR) to regulate Th17 and Treg cells, but also induce epigenetic changes to contribute to the development of autoimmune diseases (65). Another example is silica, a substance which is able to induce pulmonary inflammation and fibrosis (66). A epidemiological study showed that the risk of SSc appears to be markedly associated with high cumulative exposure of silica dust (67). In addition, some drugs such as bleomycin or paclitaxel are also associated with the development of SSc (68). Finally, organic solvents, including different compounds, i.e. white spirit, aromatic, aliphatic-chain, chlorinated solvents, ketones and welding fumes, have been reported to be associated with SSc (67, 69, 70). These environmental factors potentially affect immune-inflammatory responses and/or fibrotic responses by directly acting on cellular signaling pathways (65, 71) and/or through inducing epigenetic changes (72-74).

1.1.3.2 Role of genetic factors in SSc

It has been estimated that the relative risk of developing SSc is 1.6% in families with a history of SSc and 0.026% in general population, suggesting a role of genetic risk factors in the development of the disease(75, 76). Dissecting the genetic basis of SSc is a valuable strategy to explore the pathogenesis of the disease. So far, many susceptibility genes of SSc have been identified, suggesting that they are involved in the development of the disease (74, 77-80).

Among these susceptibility genes, human leukocyte antigen (HLA) loci show the strongest association with SSc. Many HLA class II genes such as *HLA-DQA1*, *HLA-DQB1*, *HLA-DPB1* and *HLA-DRB1* have been shown to be associated with the susceptibility to SSc (74, 79, 81). In 1981, Gladman et al. reported that *HLA-DR5* which consists *HLA-DRB1*11* and *HLA-DRB1*12* is associated with and increased risk for SSc in Caucasian (82), for the first time demonstrating an association between HLA class II genes and SSc. Further studies refined the association and showed that *HLA-DRB1*11*, in particulary *HLA-DRB1*1104* is strongly associated with the increased risk for SSc(83, 84). In addition to Caucasian, the strong association of *HLA-DRB1*11* or *HLA-DRB1*1104* with SSc has been observed in Greece, Hispanic, Iranian, Turkish and African populations (83, 85-89). Besides, *DQA1*0501* and *DQB1*0301* which form a haplotype with *HLA-DRB1*11:04* have been also suggested to be associated with SSc (83, 90, 91). Notably, the strong association of *HLA-DRB1*11* has not been observed in East Asian populations most likely due to the lack of the *HLA-DRB1*11:04* allele (92-94). Furthermore, the SSc-associated *HLA* alleles vary

among different East Asian population. In East Asian populations, *DRB1*1502* has been reported to be strongly associated with SSc in both Japanese and Korean populations (95, 96). In addition, two *DRB1*1502*-linked alleles (*DQB1*0601*, *DPB1*09:01*) as well as *DPB1*0301* have been reported to be associated with increased risk for SSc in different East Asian populations (92, 93, 95). Taken together, SSc is mainly associated with HLA class II gene, suggesting that negative selection and activation of autoreactive $CD4^+$ T cells are involved in the development of SSc.

Many non-HLA genes have been identified to be associated with susceptibility to SSc. According to their functions, these susceptibility genes can be classified into 7 categories: 1) T cell-related genes such as *CD2* (97), *IL12A* (98) and *IL12RB1* (99); 2) B cell-related gene such as B cell scaffold protein with ankyrin repeats 1 (*BANK1*) (100, 101); 3) Interferon (INF) signaling associated genes such as interferon regulatory factor 4 (*IRF4*) (102), *IRF5* (103-105) and signal transducer and activator of transcription 4 (*STAT4*) (103, 105, 106); 4) NF-kappa B signaling-related genes such as tumor necrosis factor alpha-induced protein 3 (*TNFAIP3*) (107) and interleukin-1 receptor-associated kinase 1 (*IRAK1*) (105); 5) Genes encoding enzymes including telomerase reverse transcriptase (*TERT*) (100), ATPase phospholipid transporting 8B4 (*ATP8B4*) (108), and deoxyribonuclease 1 like 3 (*DNASE1L3*) (98, 109); 6) Autophagy-related genes such as Autophagy Related 5 (*ATG5*) (98) and 7) extracellular matrix components related genes: *COL4A3* (100), *COL4A4* (100), *COL5A2* (100), *COL22A1* (100), *COL13A1* (100) and *CTGF* (105), suggesting that those biological components are involved in the pathogenesis of SSc.

In addition to genetic factors, epigenetic modifications including post-translational modifications, DNA-methylation, microRNAs, and long non-coding RNAs have been recently suggested to contribute to the development of SSc (74).

Taken together, the pathogenesis of SSc is a consequence of the interaction between genetic and/or epigenetic predispositions and enviromental risk factors. However, the precise pathomechanisms of the disease are still largely unknown.

1.2 Humanized mouse models for SSc

Animal models, particularly mouse models, are powerful research tools for exploring the pathogenesis of the human diseases (110). However, there are still considerable differences between humans and mice, which results in a pathophysiological gap between human diseases and mouse models. One strategy to bridge the gap is to use humanized mouse models.

1.2.1 Humanized mouse model for autoimmune diseases

In general, there are three main strategies for the generation of humanized mouse models, namely transfer of human immune cells, transfer of human target tissues, and humanization via genetic modification (110-113).

In 1983, Bosma *et al*. reported on a severe combined immunodeficiency mutation in a mouse strain termed C.B.-17 scid/scid (hereafter SCID) which is caused by a lack of functional T cells and B cells (114). The discovery of the SCID mice provides an ideal donor for grafting of xenogenetic tissue or cells, opening the field of establishing humanized mouse models via the transfer of human tissues/cells into mice. In 1989, Krams et al. reported the first humanized mouse model for autoimmune disease by transferring PBMC from patients with primary biliary cirrhosis (PBC) into SCID mouse (115). After the adoptive transfer, the recipient mice generated human autoantibodies against mitochondria and evolved biliary lesions after the transfer resembling PBC (115). Since then, several humanized mouse models have been generated by the transfer of PBMC from patients with various autoimmune disease, such as systemic lupus erythematosus (SLE) (116, 117), myasthenia gravis (MG) (118), pemphigus vulgaris (PV) (119), type 1 diabetes mellitus (T1DM) (120), and Sjogren's syndrome (SS) (121). The PBMC transfer-induced humanized model provides a powerful research tool to investigate the pathogenicity of human immune cells.

Beside the transfer of immune cells, transfer of target tissues was also used to induce humanized mouse models. In 1994, Geiler et al. reported a humanized mouse model for rheumatoid arthritis (RA) generated by transferring normal human cartilage together with rheumatoid synovial tissue or fibroblasts into SCID mice (122). Since this humanized mouse model is featured by human cartilage destruction, a major characteristic of RA, it has been applied to investigate the mechanism underlying the tissue damage of RA (123). Thereafter, transfer of human tissue, together with or without human autoantibodies or immune cells, has been used to induce humanized mouse models for other autoimmune diseases including bullous pemphigoid (BP) (124), pemphigus foliaceus (PF) (125) and T1DM (126).

Another important strategy to establish humanized mouse model is to generate genetically humanized mice (110). One type of this genetically humanized model is mice expressing strong susceptibility gene associated with a human autoimmune disease. For example, *HLA-B27* is a strong risk allele of ankylosing spondylitis (AS). The *HLA-B27* transgenic mice lacking α2-microgloblin developed spontaneous inflammatory arthritis (127), make it a humanized mouse model for AS. Another type of genetically humanized mouse model is mice bearing human antigen. For instance, Nishie et al (128) generated a genetically humanized mouse model for BP by

injecting human autoantibodies into *COL17*-knockout mice bearing human *COL17A* gene.

1.2.2 Humanized mouse model for SSc

So far, more than ten different mouse models for SSc have been established (129, 130), among which only one is humanized. In the latter model, three-dimensional bioengineered skin containing human keratinocytes and fibroblasts isolated from skin of SSc patients or healthy donors is generated *ex vivo* and then grafted onto the backs of SCID mice (131). After grafting the regenerated human skin from SSc patients exhibits typical scleroderma phenotypes in recipient mice, while the corresponding control skin does not (131). Although this mouse model provides a tool to investigate the pathogenetic events in skin, it does not allow studying the role of immune cells in the disease pathogenesis. Very recently, our laboratory generated a novel humanized mouse model for SSc by transfer of PMBC from patients into *Rag2*$^{-/-}$*/Il2R*$^{-/-}$ immune deficient mice lacking T, B and NK cells. By the 12th weeks after the transfer, mice received PMBC from SSc patients developed systemic inflammation in inner organs such as the lung, kidney, while no such effects were seen in mice after transfer of PMBC from healthy donors (**Figure 1**). Interestingly, mice transferred with PBMC from SSc patients who were treated with Rituximab (RTX, anti-CD20 monoclonal antibodies) showed no inflammation in any organs **(Figure 1)**. Moreover, mice which received PBMC from SSc patients generated higher levels of human total IgG as well as anti-AT1R and anti-ETAR antibodies. Although mice after transfer of PBMC from healthy controls also generated comparable amount of human total IgG, they produced significantly lower amount of anti-AT1R and anti-ETAR antibodies. In addition, transfer of PBMC from RTX-treated patients did not result in any immunological or histopathological symptoms.

This novel humanized mouse model for SSc, may be suitable to explore the role of immune cells in the pathogenesis of the disease. Additionally, this preliminary result also indicates a role of B cells in the development of the disease. However, this assumption needs to be verified in a more detailed study.

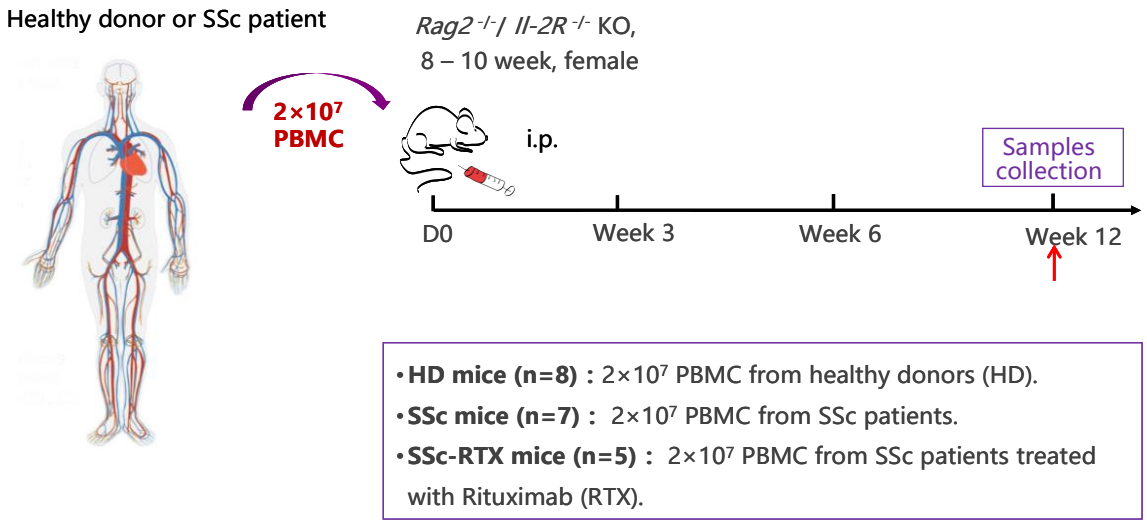

Figure 1. Experimental SSc in a humanized mouse model. Human PBMC are isolated from SSc-patients or healthy controls and transferred to *Rag2*$^{-/-}$/*Il2R*$^{-/-}$ mice. By the 12th weeks after the transfer, mice received PMBC from SSc patients developed SSc-like syndromes, while no such effects were seen in mice received PMBC from healthy donors or Rituximab-treated SSc patients; SSc, systemic sclerosis; HD, healthy donor; PBMC, peripheral blood mononuclear cells; KO, knock out; i.p., intraperitoneally.

1.3 Aim of the study

As described above, a humanized mouse model for SSc was successfully generated in our group by transferring PBMC from patients. However, the pathomechanisms as well as the disease-driving cells in this model are currently unknown. Based on clinical observations and on preliminary findings derived from this model, I hypothesize that both human T and B cells play an indispensable role in the development of experimental SSc.

To prove this hypothesis, the effect of B or T cell depletion from PBMC of SSc patients prior to their transfer into immunodeficient mice was analyzed in terms of disease development, mortality, and architecture of lymphatic organs in the recipient animals.

2. Materials and Methods

2.1 Materials

2.1.1 Patients enrollment

In total, 11 SSc patients were enrolled for this study in the Department of Rheumatology, University of Lübeck, Lübeck, Germany. Of them, 2 patients were recruited in December 2017, 1 patient was recruited in January 2018, 3 patients were recruited in October 2018, and 5 patients were recruited in November 2018. All patients fulfilled the 2013 ACR/EULAR criteria for SSc (8). Patients gave an informed written consent to this study, and the study was approved by the Institutional Review Board of the University of Lübeck (**Number: 16-199, Date: 14/11/2016**).

2.1.2 Mice

Female B10;B6-*Rag2*^{tm1Fwa} *Il2rg*^{tm1Wjl} **(***Rag2*$^{-/-}$*Il2R*$^{-/-}$**)** mice were purchased from Taconic Biosciences, Inc., USA. These mice were housed under specific pathogen-free conditions at the animal facilities of the Research Center Borstel. All animal studies were in compliance with the (ARRIVE) Animal Research: Reporting of In Vivo Experiments guidelines and were carried out according to European Union Directive 2010/63/EU.

All animal studies were approved by the Animal Research Ethics Board of the Ministry of Energy Change, Agriculture, Environment, Nature, and Digitalization, Kiel, Germany (**Number: V 241 – 50577/2017 (108-8/17), Date: 05/10/2017**).

2.1.3 Equipment and consumables

Name	Manufacturer
Absorbance reader	Tecan Trading AG, Switzerland
Analytical balance	Sartorius research GmbH, Germany
autoMACS® Pro Separator	Miltenyi Biotec, Germany
Balance	Kern&Sohn GmbH, Germany
4°C, -20°C, -80°C refrigerator	Thermo Fisher, USA
Biosafety cabinet	Heraeus, Germany
Fast protein liquid chromatography	Pharmacia Biotech, Sweden
Microplate Reader	Tecan life science, Switzerland
Super-centrifuge	Hettich, Germany
Thermostat water bath	Julabo, Germany
Syringes (1ml, 20ml, 50ml)	Merck, Germany

Cassette for paraffin embedding	VWR International GmbH, Germany
Cells counter	Schärfe system GmbH, Germany
Centrifuge	Hettich lab technology, Germany
Cytocentrifuge	Thermo Fisher scientific, USA
Electronic pressure pot	Instant pot company, Germnay
Fluid aspiration system	Vacuubrand GmbH, Germany
Heating plate	Heidolph instrument, Germany
Heating stage	Medite GmbH, Germany
Image acquisition system	Nikon Corporation, Japan
Luer syringe	Beckton Dickinson, USA
Micro-emulsifying needle	Merck KGaA, Germany
Microscope	Olympus corporation, Japan
Microtome	Leica biosystems nussloch, Germany
Microtome blade	Feather safety razor Co., Ltd, Japan
Mini centrifuge	Carl Roth GmbH, Germany
Molds for cryo-embedding	Sakura Finetek Europe, Netherlands
Multi-channel pipettes	Sartorius research GmbH, Germany
Needles	Beckton Dickinson, USA
Orbital shaker	Gesellschaft für Labortechnik mbH, Germany
PAP pen	Kisker Biotech GmbH & Co. KG, Germany
Paraffin embedding workstation	Thermo Fisher scientific, USA
PH meter	Knick Elektronische Messgeräte GmbH, Germany
Pipette controller	Brand GmbH + CO KG, Germany
Scissors and forceps	Karl Hammacher GmbH, Germany
Serum collection tubes	Beckton Dickinson, USA
Single channel pipettes	Brand GmbH + CO KG, Germany
Slides	R. Langenbrinck GmbH, Germany
Spectrophotometer	Thermo Fisher scientific, USA
Spin tissue processor	Thermo Fisher scientific, USA
Syringes (1ml, 20ml, 50ml)	Merck, Germany
Thermocycler	Bio-Rad Laboratories GmbH, USA
Pipette tips (10µl, 100µl, 1000µl)	Sarstedt, AG&Co, Germany

Ultra-low freezer	Thermo Fisher scientific, USA
Vortex mixer	Scienfific industries, inc, USA
Water bath	Gesellschaft für Labortechnik mbH, Germany
Filters (0.2µm, 0.45µm)	Merck, Germany
SepMate-50 tube	Stemcell technologies incoporation, Germany
Tubes (0.5 ml, 1.5 ml, 2.0 ml)	Sarstedt, Germany
Tubes (15 ml, 50 ml)	Sarstedt, Germany
Cell culture plates (6-well, 24-well, 48-well, 96-well, flat-bottom)	Corning, USA
AT1R ELISA Screening plates	CellTrend, Germany
ETAR ELISA Screening plates	CellTrend, Germany
Fusion plates (24-well, flat-bottom)	Greiner Bio-One, Germany
MaxisorpTMSurface ELISA plates (96-well, round-bottom)	Thermo Fisher, USA
Microtiter plates (96-well, round-bottom)	Greiner Bio-One, Germany

2.1.4 Chemical reagents

Name	**Company**	**Catalogue number**
Pancoll human	PanBiotech, Aidenbach, Germany	P04-60500
1 mol/L HCL	Merck KGaA, Germany	1.09057.9010
1 mol/L NaOH	Merck KGaA, Germany	1.09137.9010
30% H_2O_2 solution	Carl Roth, Germany	9681.4
4% Formalin	Carl Roth GmbH, + Co. KG, Germany	P087.1
Bovine serum albumin	PAA laboratories GmbH, Germany	K41-001
Citric acid	Merck KGaA, Germany	1.00244.1000
EDTA	Merck KGaA, Germany	1.08418.0250
Tris base	Biomol, Germany	CDX-T0244
Eosin G	Carl Roth GmbH, + Co. KG, Germany	3137.2

Ethanol	Sigma Aldrich chemie GmbH, Germany	32205
Fetal bovine serum	PAN biotech, Germany	P100703
Glycine	Carl Roth, Germany	3790.3
Hematoxylin Gill II	Carl Roth GmbH, + Co. KG, Germany	T864.2
KCl	Merck KGaA, Germany	1.04936.0500
KH_2PO_4	Merck KGaA, Germany	A434173
Mounting medium	Merck KGaA, Germany	1.07961.0500
Na_2HPO_4	Merck KGaA, Germany	1.06580.1000
NaCl	Merck KGaA, Germany	1.06404.5000
$NaCO_3$	Merck KGaA, Germany	1.06392.1000
NH_4Cl	Merck Chemicals, Germany	1011450500
Paraffin	Sigma-aldrich international GmbH, Germany	327204
RPMI 1640 medium	Biochrom GmbH, Germany	FG1415
TMB solution	eBioscience incorporation	00-4201-56
Trypan blue	Chroma-gesellschaft schmidt&Co, Germany	11660
Tween-20	Sigma-Aldrich, USA	087K0137

2.1.5 Buffers and solutions

Name	**Recipe**
3% H_2O_2 solution	30 % H_2O_2 solution: 1 ml PBS: 9 ml
Antigen retrieval buffer: Citrate acid (PH=6.0)	Tri-sodium citrate: 2.94 g Milli Q water: 1000 ml Mix, adjust pH to 6.0 with 1 mol/L HCl
Antigen retrieval buffer: Tris-EDTA (PH=9.0)	Tris base 1.21g EDTA:0.37g Distilled water: 1000m Mix to dissolve. Adjust pH to 9.0 with 1mol/L HCL
ELISA coating buffer	$NaHCO_3$: 8.4g

	Na2CO3:3.56
	H2O: 1L, adjust pH to 9.5
Blocking solution	BSA: 1g
	PBS: 100 ml
PBS Buffer	NaCl: 8 g
	KCl: 0.2 g
	Na_2HPO_4: 1.15 g
	KH_2PO_4: 0.2 g
	Milli Q water: 1 L, adjust pH to 7.2 with HCL
ELISA wash buffer (PBST)	0.5mlTween-20
	1L PBS, PH=7.4
ELISA diluted buffer	1L PBS
	0.5ml Tween-20
	1g BSA
Red blood cells lysis buffer	NH_4Cl: 8.34 g
	EDTA: 0.037 g
	$NaCO_3$: 1.0 g
	Milli Q water: 1000 ml
	Adjust pH to 7.2--7.4
Stop solution	Fetal bovine serum: 20 ml
(for stopping lysis of red blood cells)	PBS: 1000 ml

2.1.6 Antibodies

Name	**Company**	**Catalogue number**
APC mouse anti human CD8	Biolegend incorporation, USA	344722
BV421 mouse anti human CD3	Biolegend incorporation, USA	300434
BV650 mouse anti human CD4	Biolegend incorporation, USA	300536
FITC-mouse-anti-human CD45	Biolegend incorporation, USA	368508
Percp/cy5.5 mouse anti human CD20	Biolegend incorporation, USA	302326

anti-mouse IgG1-BV421	BD Biosciences	400158
anti-mouse IgG1-BV650	BD Biosciences	400164
anti-mouse IgG1-APC	BD Biosciences	400122
anti-mouse IgG2b-Percp/cy5.5	BD Biosciences	400338
anti-mouse IgG1-FITC	BD Biosciences	400110
Mouse anti human CD20 (IgG2a)	Dako, USA	M0755
Rabbit anti human CD4	Abcam, UK	ab133616
Mouse anti human CD8 (IgG1)	Biolegend, USA	372902
Mouse anti human CD138 (IgG1)	Biolegend, USA.	356505
Mouse IgG1	Biolegend, USA.	400124
Mouse IgG2a	Biolegend, USA.	401504
Rabbit IgG	Lubeck University provide	-
Mouse anti human IgG Fc	Southern biotech	9040-01
Human IgG (stardard protein)	Sigma, USA	I8640
Biotin-conjugated goat anti-rabbit IgG	Jackson ImmunoResearch Laboraties. Inc.	111-065-144
Biotin-conjugated goat anti-mouse IgG	Jackson ImmunoResearch Laboraties. Inc., USA,	115-066-062
HRP conjugated donkey anti-human IgG (H+L)	Jackson ImmunoResearch,	709-035-149

2.1.7 Kits

Name	**Company**	**Catalogue number**
ABC kit	Vector laboratory, USA	PK-6100
Avidin - Biotin blocking kit	Vector laboratory, USA	SP-2001
DAB kit	Vector laboratory, USA	SK-4100
Human CD3 microbeads	Miltenyibiotec	5171011505
Human CD19 microbeads	Miltenyibiotec	5171025006

2.2 Methods

2.2.1 Isolation of human PBMC

To prepare PBMC, 45 ml of peripheral blood from each patient with SSc was collected and placed into a 50 ml tube containing heparin. Blood samples were diluted with an equal volume of PBS containing 2% of fetal cow serum (FCS), then transferred to 50 ml Sepmate-50 tubes containing density gradient medium Pancoll (PanBiotech, Aidenbach, Germany). After 10 min of centrifugation at 1200 g at room temperature, the top layer of cells (PBMC) were collected into new 50 ml tubes. The collected PBMC were washed with PBS containing 2% FCS three times. Finally, the number of cells were counted, and then resuspended in RPMI1640 medium (Biochrom GmbH, Germany) at a concentration of 1×10^8 cells/ml. Cell viability was assessed by trypan blue exclusion test.

2.2.2 Depletion of human T or B cells from PBMC

To deplete human T and/or B cells from PBMC, 2×10^7 PBMC isolated from patients with SSc were resuspended in 160 µL MACS buffer (PBS with 0.5% bovine serum albumin (BSA)). Subsequently, 40 µL human CD3 microbeads (Miltenyibiotec, Order No.:130-050-101) or human CD19 microbeads (Miltenyibiotec, Order no.: 130-050-301) were added to the cell suspension, gently mixed and then incubated for 15 min at 4°C. After 10 min of centrifugation at 1200 g at 4°C, cell pellets were resuspended up to 1.25×10^8 cells in 500 µL MACS buffer. Next, cell samples were placed in the sample position of the AutoMACS, automatically diluted with running buffer and flowed through the Column. For the removal of CD3 positive T cells or CD19 positive B cells, the AutoMACS program 'DEPLETE' was assigned using the Separation menu. Consequently, the magnetically labeled T or B cells were retained in the AutoMACS Column, while non-labeled cells passed through the column and were collected. After the T- or B-cell depletion, PBMC were centrifuged, washed and resuspened in RPMI 1640 medium. For the corresponding whole PBMC control samples, cells without incubation with microbeads went though the AutoMACS program.

2.2.3 Adoptive transfer of PBMC

Rag2$^{-/-}$*Il2R*$^{-/-}$ mice, eight to ten-week-old, were used for the experiments. Then, 2×10^7 PBMC with or without depletion of T or B cells in 100 µl RPMI 1640 medium were injected intraperitoneally (i.p.) into each recipient mouse. Mice which received whole PBMC, T-cell-depleted PBMC and/or B-cell-depleted PBMC from a patient were regarded as a paired group for comparison.

2.2.4 Isolation of leukocytes from murine blood

At the 6th week and 12th week after PBMC transfer, 100 µl of blood from each mouse was collected in a 1.5ml tube containing 20 µl heparin solution (Braun, 5.000 I.E./ml). Subsequently, 1 ml cold PBS was added to the blood samples, centrifuged for 10 min (400g, 4°C) to separate the plasma.

Then, 1 ml of red blood cell (RBC) lysis buffer was added to resuspend the cell pellet, and the cell suspension was transferred to tube containing another 4 ml of RBC lysis buffer to lyse erythrocytes. After 3 min of incubation at room temperature (RT), 15 ml stop solution (0.1% BSA solution or 2% FCS in PBS) was added into the tube to stop the cell lysis. After centrifugation at 300g at 4 °C for 10 min, cell pellets were suspended and washed twice with cold PBS. Finally, numbers of cells were counted, and cells were suspended in PBS for further by flow cytometry (FCAS) analysis.

2.2.5 Preparation of murine serum samples

Murine serum samples were prepared from peripheral blood collected from mice. At 12^{th} week, blood samples were collected from the hearts after the mice were sacrificed with CO_2. Peripheral blood samples were stored at room temperature for at least 30 min, then centrifuged at 6000 g for 10 min, and supernatants were collected stored at -80°C for further analysis.

2.2.6 Flow cytometry (FACS)

To evaluate the cellularity of human PBMC and murine peripheral blood cells, 1×10^6 cells from each sample were used for FACS. Briefly, cells were added to a FACS tube containing 2 ml of PBS without Ca^{++} and Mg^{++}, and then centrifuged at 300 g at 4°C for 5 min. Cell pellet was suspended in 50 µl PBS without Ca^{++} and Mg^{++} containing human IgG (61 µg/ml, Sigma) and live/dead stain kit (Invitrogen, L23105, 1:1000 diluted), incubated for 20 minutes at 4°C to block the Fc receptors, and further stained with cellular markers by adding and incubating for 20 minutes at 4 °C with 50 µl antibody mixture which was composed of the following fluorescent chromes-conjugated antibodies: anti-human CD3-BV421 (BD Biosciences, clone UCHT1), anti-human CD4-BV650 (BD Biosciences, clone 2RPA-T4), anti-human CD8-APC (BD Biosciences, clone SK1), anti-human CD20-Percp/cy5.5 (BD Biosciences, clone 2H7) and anti-human CD45-FITC (BD Biosciences, clone 2D1). For control staining, cells were stained with a mix of corresponding isotypes including anti-mouse IgG1-BV421 (BD Biosciences, clone MOPC-21), anti-mouse IgG1-BV650 (BD Biosciences, clone MOPC-21), anti-mouse IgG1-APC (BD Biosciences, clone MOPC-21), anti-mouse IgG2b-Percp/cy5.5 (BD Biosciences, clone MPC-11), anti-mouse IgG1-FITC (BD Biosciences, clone MOPC-21). After the incubation, cells were washed twice with 2 ml FACS buffer, centrifuged at 400 g at 4°C for 5 min, resuspended in 200 µl FACS buffer, and finally fixed by adding 100 µl of 4% paraformaldehyde solution. The fixed samples were measured by using LSR II flow cytometer (BD, USA) within three days, and the data were analyzed using FACS Express software (De Novo Software, USA, version 5).

2.2.7 Enzyme-linked immunosorbent assay (ELISA)

Levels of human total IgG, human anti-AT1R and anti-ETAR antibodies were determined by using Enzyme-linked immunosorbent assay (ELISA).

A quantitative ELISA was used to determine human total IgG in murine sera. Briefly, a 96-well ELISA plate (Corning Incorporated, Corning, NY14831, USA) was coated with 100 µl mouse anti-human IgG Fc (Southern Biotech, 9040-01) (5 µg/ml, diluted in coating buffer (0.15 M Carbonate-Bicarbonate, pH 9.6)) overnight at 4 degree. The plate was washed with washing buffer (PBS with 0.05% Tween 20, pH 7.4), incubated with 200 µl blocking solution (PBS containing 1% bovine serum albumin (BSA)) at 37°C for 1 hour. Then 100 µl mouse serum samples prepared in log dilution (diluted from 1:500 to 1:500.000 in blocking solution) or diluted standard samples (Human IgG, Sigma, I8640) were added and incubated with the plate for 1 hour at RT. The standards were diluted with blocking solution from 12.2 mg/ml to 20 ng/ml, and further to 10 ng/ml, 5 ng/ml, 2.5 ng/ml, 1.25 ng/ml, 0.625 ng/ml, 0.3125 ng/ml and 0 ng/ml. After washing, 100 µl HRP-conjugated donkey anti-human IgG (H+L) (Jackson ImmunoResearch, 709-035-149, 1:50.000 diluted with blocking solution) was added and incubated at 37°C for 1 hour. Binding of the secondary antibodies was visualized by addition of 100 µl TMB substrate solution, and the reaction was stopped by adding 100 µl of 1 M H_2SO_4. OD value was measured at 450 nm in a microtiter plate reader (Tecan life science, Switzerland). Standard curves were generated, and levels of human IgG in murine serum samples were calculated in relation to the corresponding standards run in parallel.

Human IgG against AT1R or ETAR in mouse serum samples were detected by using commercial ELISA Kit (Celltrend, Germany) according to the provided protocol, with slight modification. Briefly, plates coated with membrane extract from CHO cells overexpressing human AT1R or ETAR (Celltrend, Germany) were incubated with serum samples (1:100 diluted in sample diluent). After incubation with HRP-conjugated donkey anti-human IgG (H+L) (Jackson ImmunoResearch, 1:25000 dilution with sample diluent) for 1 h at RT, the signal was visualized by TMB. OD values were measured at 450 nm on a microreader (Tecan life science, Switzerland).

2.2.8 Histology

Murine organs including the skin, lung, liver, kidney, muscle, esophagus, intestines, muscle and spleens were collected, fixed in 4% paraformaldehyde for 24 h. After dehydration and paraffinization, the samples were embedded in paraffin, and then sectioned at thickness of 4-µm for histological analysis. In the below table the detailed procedure of the dehydration and paraffinization is summarized.

Dehydration and paraffinization

Step	Reagent	Incubation
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	absolute ethanol	1 hour
7	absolute ethanol	1 hour
8	absolute ethanol	1 hour
9	xylene	1 hour
10	xylene	1 hour
11	paraffin	1 hour and 30 min
12	paraffin	1 hour and 30 min

For histological evaluation, paraffin sections were stained with hematoxylin and eosin (H&E). The protocol of H&E staining is summarized in the table below. Images were taken by using bright-field microscopy (Olympus, DS-Ri) and analyzed using NIS-Elements analysis system. The severity of inflammation was defined as the percentage of area with inflammation (affected area/total area ×100%).

HE staining

Step	Reagent	Incubation
Deparaffinization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Re-hydration	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 hematoxylin solution (No.2)	20 min
Bluing	running tap water	10 min
Staining	Eosin (1%, acidic) counterstain	3 min
	Tap water	10 seconds
De-hydration	Ethanol 70%	10 seconds
	Ethanol 96% I	10 seconds
	Ethanol 96% II	10 seconds
	Absolute ethanol I	10 seconds
	Absolute ethanol II	3 min
Clearing	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Mounting	Entellan (Merck)	

2.2.9 Immunohistochemistry (IHC) staining

Immunohistochemistry staining (IHC) was performed on paraffin sections of mouse spleen sections to detect human $CD4^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells and and $CD138^+$ plasma cells. Briefly, slides were subjected to 10 mM citrate buffer (PH = 6.0) at 117°C for 4 minutes to retrieve human CD4 and CD20 antigens, or incubated in Tris-EDTA buffer (10 mM Tris, 0.5 mM EDTA, pH = 9) at 117°C for 10 minutes to retrieve human CD8 and CD138 antigens. After antigen retrieval, endogenous peroxidase, endogenous biotin and unspecific binding were blocked with 3% H_2O_2, biotin blocking solution (Vector Laboratories, Inc. Burlingame, CA94010, USA) and 5% BSA (Sigma, St. Louis., MO, USA, CAS9048-46-8) solution, respectively. Then, sections were incubated overnight at 4 °C with primary antibodies against human CD4 (Abcam, UK, Cat. No.: ab133616, Clone: EPR6855, 1:100 dilution), human CD20 (Dako, Santa Clara, CA, USA, Cat. No.: M0755. Clone: L26, 1:100 dilution), human CD8 (Biolegend, San Diego, CA, USA. Cat.No.:372902. Clone: C8/144B, 1:100 dilution) or human CD138 (Biolegend, San Diego, CA, USA. Cat.No.356505. Clone: M15, 1:100 dilution). Meanwhile, corresponding isotype control antibodies including normal rabbit IgG (provided by the Department of Dermatology, Univeristy of Lübeck, 1:100 dilution), murine IgG1 (Biolegend, San Diego, CA, USA. Cat.No.:400124. Clone: Mopl-21, 1:100 dilution) and murine IgG2a (Biolegend, San Diego, CA, USA. Cat.No.: 401504.

Clone: MG2a53, 1:100 dilution) were used. After the incubation with primary antibodies or isotype controls, slided were incubated with corresponding biotin-conjugated secondary antibodies including goat anti-rabbit IgG (Jackson ImmunoResearch Laboraties. Inc., Baltimore, USA, catalog number: 111-065-144, 1:600 dilution) for primary antibodies against CD4 staining, goat anti-mouse IgG (Jackson ImmunoResearch Laboraties. Inc., Baltimore, USA, catalog number: 115-066-062, 1:1000 dilution) for primary antibodies against CD20, CD8, and CD138 at RT for 50 min, followed by incubation with ABC reagents (Vector Laboratories, Inc. Burlingame, CA, USA, PK-6100) for 30 min at RT. Subsequently, the slides were incubated with 3,3-diaminobenzadine (DAB substrate kit, Vector Laboratories, Inc. Burlingame, CA, USA, Cat. No.: SK-4100.) as a chromagen to visualized the immunoreactivity, and then counterstained with hematoxylin for 5 min. Images were taken by using bright-field microscopy (Olympus, Japan). In the table below the detailed procedure of the IHC staining is summarized.

Immunohistochemistry

Step	Reagent	Incubation
Deparaffinization	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Re-hydration	Absolute ethanol I 100%	5 min
	Absolute ethanol II 100%	5 min
	Ethanol 96%	5 min
	Ethanol 70%	5 min
	Ethanol 40%	5 min
	Deionized water	5 min
Antigen retrieval	10Mm citrate buffer (PH＝6.0)	117°C , 4min (50 min in antigen reitrieval pot)
	Tris-EDTA buffer (pH= 9)	117°C , 10min (50 min in antigen reitrieval pot)
Cool down	10Mm citrate buffer (PH＝6.0)	20 min

Blocking endogenous peroxidase	3% H_2O_2 solution	15 min
	PBS washing	5 min, three times
Blocking endogenous biotin	Avidin solution	15 min
	PBS washing	5 min
	Biotin solution	15 min
	PBS washing	5 min, three times
Blocking unspecific binding	5% BSA	50 min
Incubation of primary antibodies	Primary antibodies diluted in PBS	4°C , overnight
	PBS washing	5 min, three times
Incubation of biotinylated secondary antibodies	Biotinylated secondary antibodies diluted in PBS	50 min, RT
	PBS washing	5 min, three times
Preparation of avidin and biotinylated HRP complex (ABC solution)	100 ul of avidin solution (Reagent A) and 100 µl of biotinylated HRP solution (Reagent B) in 5 ml of PBS	30 min, RT
Incubation of ABC solution	ABC solution	30 min, RT
	PBS washing	5 min, three times
Incubation of DAB solution	84 ul of buffer stock solution, 100 µl of DAB reagent and 80 µl of H_2O_2 in 5 ml of Deionized water	2 min, RT
	Tap water	5 min, three times

Counterstaining	Gill's hematoxylin solution (No.2)	1 min
Bluing	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
Clearing	Xylene I	5 min
	Xylene II	5 min
	Xylene III	5 min
Mounting	Entellan (Merck)	

2.2.10 Statistical Analysis

Data were analyzed using GraphPad5 Prism software (La Jolla, California, U.S.A.). Quantitative data with normal distribution are presented as mean ± standard deviation (mean ± SD), while data without normal distribution are expressed as medians (min, max). To assess the significance of differences of quantitative data, the paired t test was applied when values were under normal distribution, while Wilcoxon matched pairs test was performed when values were not normally distributed. Differences in rates of mouse death were analysed by using Fisher's exact test. The cumulative survival curve was analysed by using Kaplan–Meier analysis. A p value < 0.05 was considered statistically significant.

3. Results

3.1 Experimental setup of the study

In order to investigate the role of human T and B cells in the pathogenesis of the humanized SSc mouse model, the current study was designed as follows: First of all, PBMC were isolated from 11 patients with SSc. T or B cells were depleted from PBMC using MACS separators with CD3 and anti-CD19-conjugated microbeads, respectively. Then 2×10^7 PBMC, with or without depletion of T or B cells were transferred into *Rag2*$^{-/-}$*/Il2R*$^{-/-}$ mice via i.p. injection. Twelve weeks after the transfer, mice were sacrificed and blood and tissue samples were collected for further evaluation. **Figure 2** shows an overview of the experimental scheme of this study.

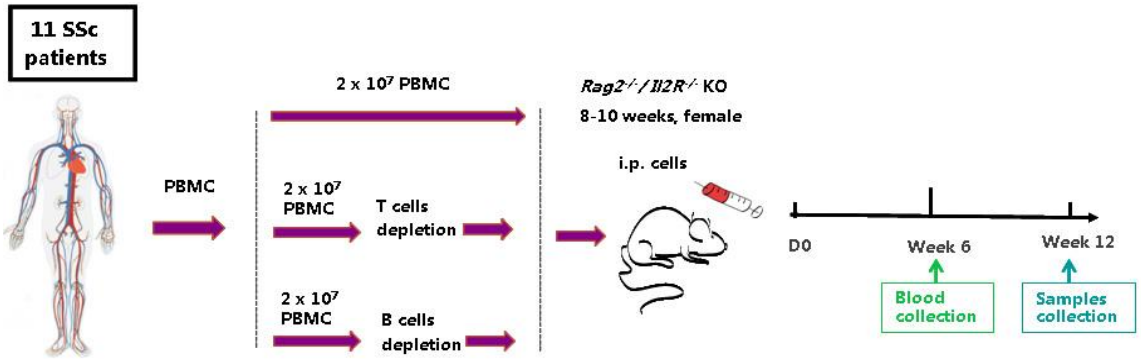

Figure 2. An overview of the experimental scheme. PBMC, peripheral blood mononuclear cells; KO, knock out; i.p., intraperitoneally.

3.2 Demographic and clinical features of patients with SSc

In total, 11 SSc patients (3 males and 8 females) with 9 lcSSc patients and 2 dcSSc were enrolled in this study. Demographic and clinical characteristics of these patients are summarized in Table 1**.** The mean age and median disease duration of SSc patients were 57 years and 5.1 years, respectively. Of the 11 patients, 9, 4, 4, 3, 2, 2, 2, 1, 1 and 1 were positive for ANA, anti-Scl70 antibodies, anti-AT1R antibodies, anti-ETAR antibodies, ACA, anti-CENP-B antibodies, AMA-M2, anti-RNA-polymerase III antibodies, anti-Th/To antibodies and anti-NOR-90 antibodies, respectively. All 11 patients developed Raynaud syndrome, and part of them showed complications in the lung, kidney, heart and gastrointestinal tract (Table 1). Table 1 also summarizes the treatment information of those patients.

Table 1. Demographic and clinical features of patients with SSc

	SSc (n = 11)
Age (years, mean ± SD)	57.0 ± 13.8
Gender (female, n, %)	8 (72.7)
Disease duration (years, median(min,max))	5.1 (1-16)
SSc classification (n, %)	
lcSSC	9(81.8)
dcSSc	2(18.2)
Antibodies positivity (n, %)	
ANA	9 (81.8)
Anit-Scl70 antibodies	4 (36.4)
ACA	2 (18.2)
CENP-B	2 (18.2)
AMA-M2	2(18.2)
Anti-Th/To antibodies	1 (9.1)
anti-NOR-90 antibodies	1(9.1)
Anti- RNA-polymeraseIII antibodies	1(9.1)
Anti-AT1R antibodies	4 (36.4)
Anti-ETAR antibodies	3 (27.3)
Symptoms of signs (n, %)	
Raynaud-syndrome	11 (100)
Fatigue	3 (27.3)
Lung fibrosis	4 (36.4)
PHA	3 (27.3)
Nephrology involvement	4 (36.4)
Cardiac involvement	3 (27.3)
Calcinosis, ulcera	4 (36.4)
Abnormal nailfold capillaries	4 (36.4)
Arthralgia	1 (9.1)

Sicca-symptom	3 (27.3)
Gastroesophageal reflux	3 (27.3)
Treatment (n, %)	
Prednisolon	2 (18.2)
Mycofenolat-Mofetil	1 (9.1)
Ciclosporin	1 (9.1)
Hydroxychloroquin	4(36.4)
Arcoxia	1 (9.1)
Bosentan	2(18.2)
Metoprolol	1 (9.1)
Ramipril	3 (27.3)
Nitrendipin	1 (9.1)
Vitamin D	1 (9.1)
Ilomedin Infusion intravenous	8 (72.7)

SSc, systemic sclerosis; lcSSc, limited cutaneous SSc; dcSSc, diffuse cutaneous SSc; ANA, anti-nuclear antibodies; ACA, anti-centromere-specific antibodies;AMA, anti-mitochondrial antibody; anti-NOR-90, anti-a 90-kDa component of the nucleolus-organizing region of chromatin. AT1R, angiotensin-II type1 receptor; ETAR, endothelin-1 type A receptor; PHA, pulmonary arterial hypertension.

3.3 Depletion of T- or B- cells from human PBMC

PBMC were isolated from patients with SSc, with a cell viability of more than 90% which was indicated by trypan blue exclusion test. After the depletion of T or B cells, the depletion efficiency was evaluated by using FACS analysis. As shown in **Figure 3**, T cell depletion resulted in a dramatic and significant decrease in human $CD3^+$ T cells in PBMC (**Figure 3A**). Further evaluation of two major subtypes of T cells demonstrated that $CD4^+$ T cells were almost completely depleted in all samples (**Figure 3B**), while $CD8^+$ T cells could only partially be removed (**Figure 3C**). Ablation of B cells was highly efficient, and a complete deficiency of $CD20^+$ B cells was observed in all samples (**Figure 3D**).

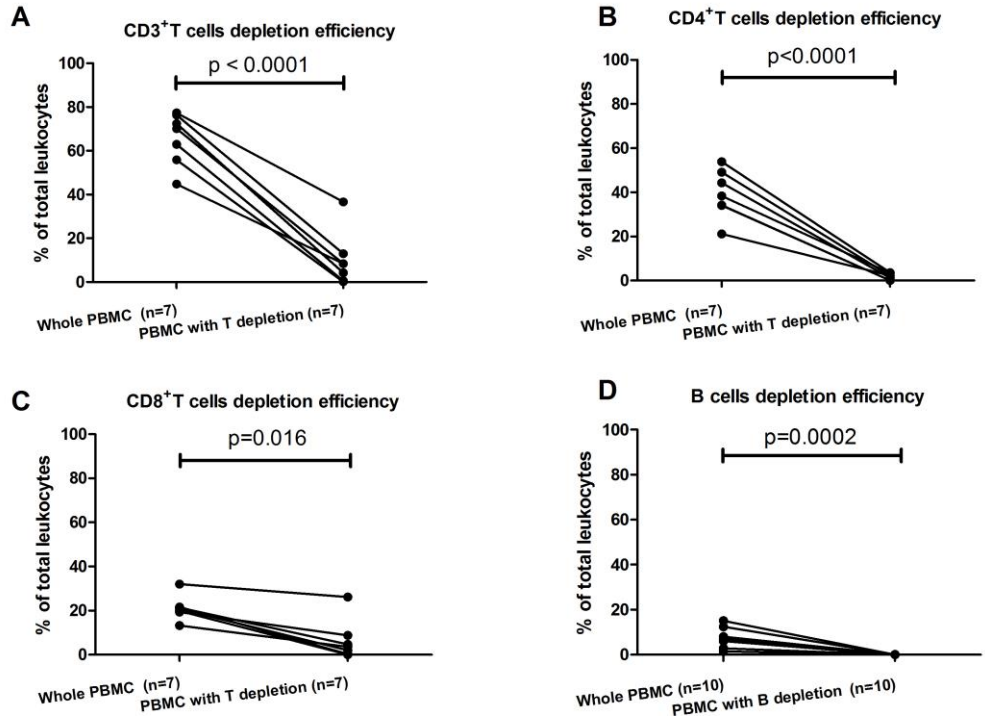

Figure 3. Efficiency of depletion of human T or B cells from PBMC. Levels of $CD3^+$ T cells **(A)**, $CD4^+$ T cells **(B)**, $CD8^+$ T cells **(C)**, and $CD20^+$ B cells **(D)** were determined by FACS analysis and presented as % of total human leukocytes in PBMC. Statistical significance of comparison in A, B and D was determined using the paired t test, and statistical significance of comparison in C was determined by Wilcoxon match pairs test.

Eighteen mice were transferred with whole PBMC (n=6), T-cell-depleted PBMC (n=6) or B-cell-depleted PBMC (n=6) from 6 patients with SSc. Two mice were transferred with whole PBMC (n=1) or T-cell-depleted PBMC (n=1) from one SSc patient. Eight mice were transferred with whole PBMC (n=4) or B-cell-depleted PBMC (n=4) from 4 SSc patients. Taken together, this experiment consisted of 7 paired groups of whole PBMC vs T-cell-depleted PBMC and 10 paired groups of whole PBMC vs B-cell-depleted PBMC.

3.4 PBMC transfer-induced mortality in mice

Six mice transferred with whole PBMC from patients with SSc died or had to be sacrificed due to the severe disease before the end of the experiment, while no mice transferred with T or B cell depleted PBMC from SSc patients died or had to be sacrificed. Kaplan–Meier analysis showed significant difference in cumulative survival between whole PBMC-transferred group and T cell depleted PBMC-transferred group as well as between PBMC-transferred group and B cell depleted

PBMC-transferred group **(Figure 4A).** Moreover, whole PBMC-transferred mice had significantly higher death rates than mice transferred with T- or B-cell-depleted PBMC **(Figure 4B)**.

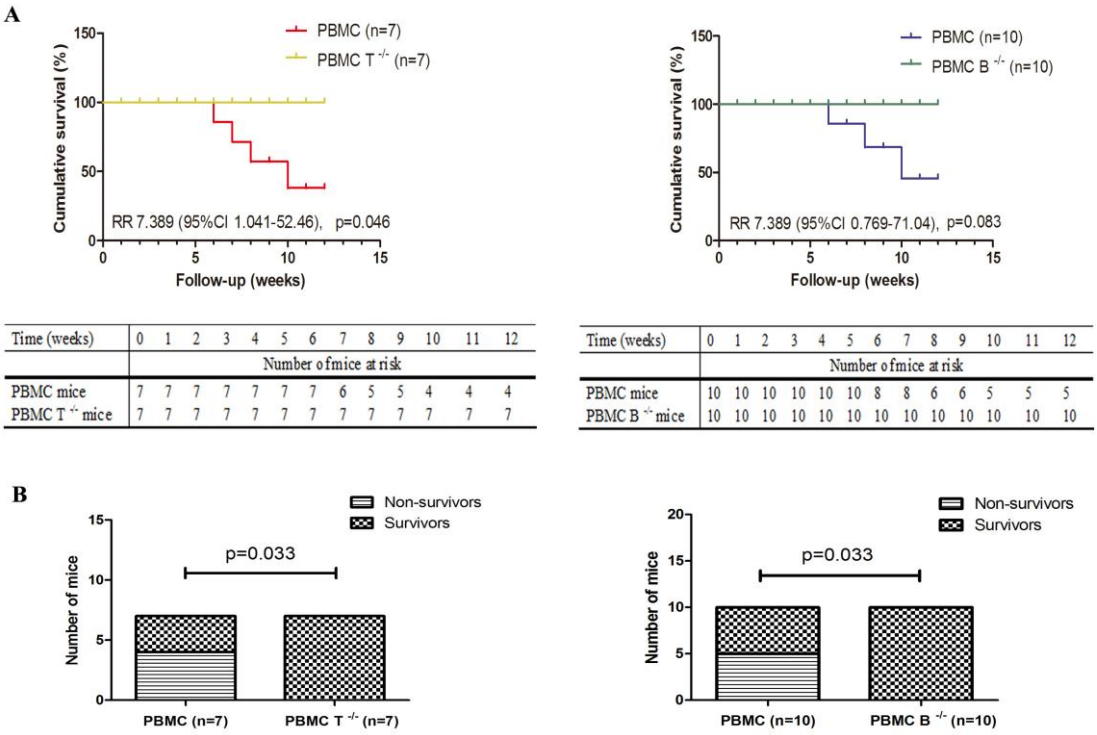

Figure 4. Mortality in mice after engraftment with patient-derived PBMC. (A) Kaplan–Meier survival curve illustrating the survival difference between whole PBMC-transferred mice and mice transferred with T- or B-cell-depleted PBMC. Numbers of mice are indicated on the curves, and the time axis is right-censored at 12 weeks. **(B)** Mice transferred with whole PBMC showed significantly higher death rates compared to mice transferred with T- or B-cell depleted PBMC. Comparison of survival curves (**A**) and death rates (**B**) between whole PBMC-transferred mice and mice transferred with T- or B-cell-depleted PBMC were determined using the Log-rank (Mantel-Cox) test and Fisher's exact test, respectively. P values reflect comparisons between mice transferred with whole PBMC and mice transferred with T- or B-cell-depleted PBMC. PBMC, mice transferred with whole PBMC from SSc patients, PBMC $T^{-/-}$, mice transferred with T-cell depleted PBMC from SSc patients, PBMC $B^{-/-}$, mice transferred with B-cell depleted PBMC from SSc patients.

3.5 Adoptively transferred human PBMC in murine peripheral blood

To evaluate whether the transferred human PBMC survive in recipient mice, we detected human $CD45^+$ leukocytes, $CD3^+$ T cells, $CD4^+$ T, $CD8^+$ T and $CD20^+$ B cells in mouse blood at the 6^{th} and 12^{th} week after transfer by FACS analysis.

By the 6^{th} week after transfer, human leukocytes could be detected in peripheral blood of recipient mice in all three groups, suggesting that the transferred human PBMC survived in the recipient mice. As expected, mice transferred with whole PBMC showed significant higher levels of human $CD45^+$ leukocytes, $CD3^+$ T cells and $CD4^+$ T than mice which had received T cell-depleted PBMC

(**Figure 5A**). However, no significant difference was observed with regard to levels of human cells between mice transferred with whole PBMC and mice received B-cell depleted PBMC (**Figure 5B**).

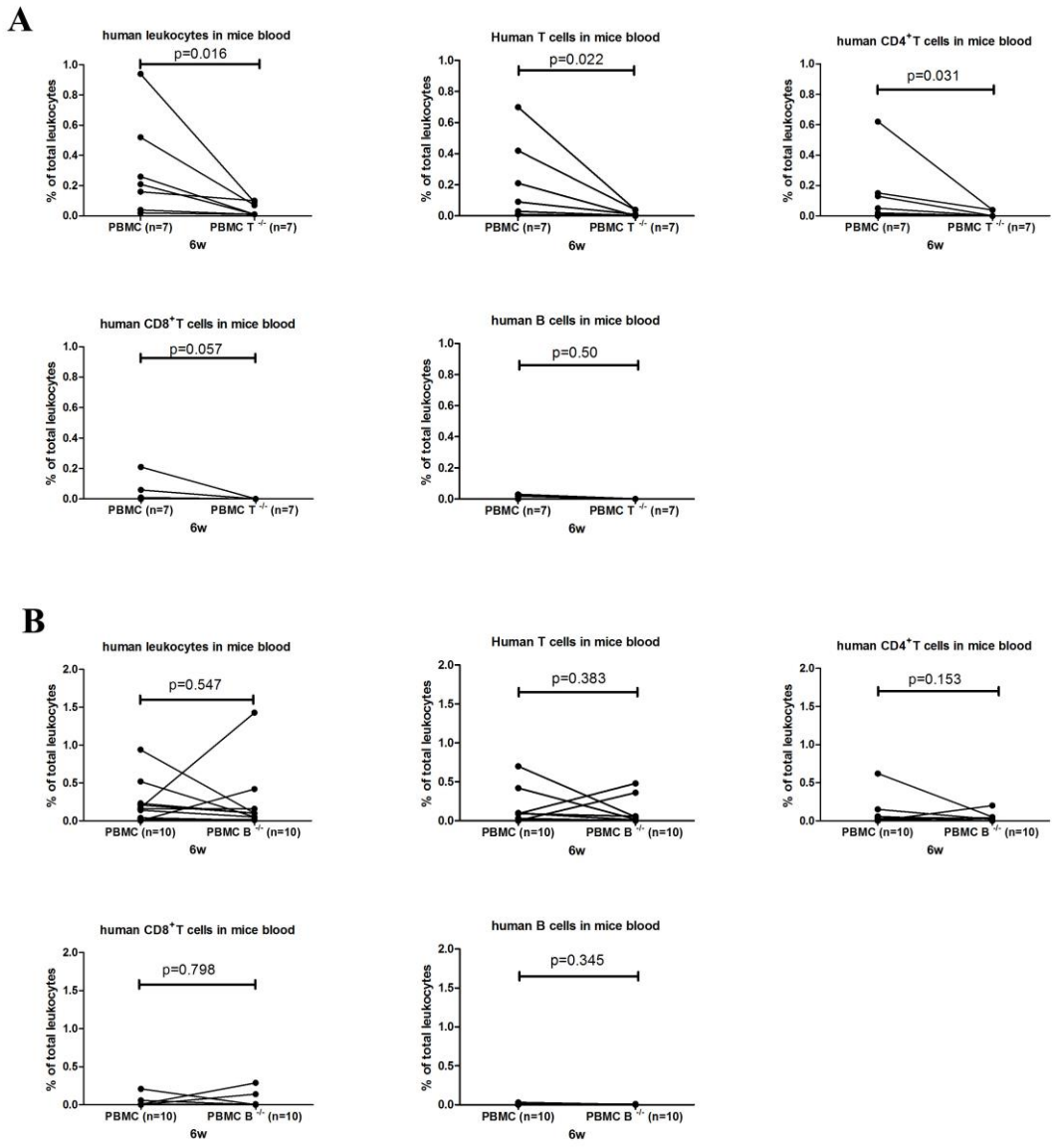

Figure 5. Human leukocytes in peripheral blood of recipient mice at week 6 after transfer. (A) Comparison between mice transferred with whole PBMC (n=7) and mice transferred with T-cell depleted PBMC (n=7) in levels of human $CD45^+$ leukocytes, $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells and $CD20^+$ B cells. (**B**) Comparison between mice transferred with whole PBMC (n=10) and mice transferred with B-cell depleted PBMC (n=10) in levels of human leukocytes, $CD3^+$T cells, $CD4^+$T cells, $CD8^+$T cells and $CD20^+$B cells. P values reflect comparisons between mice transferred with whole PBMC and mice transferred with T- or B-cell depleted PBMC. Statistical significance was determined using the Wilcoxon matched pairs test.

At week 12 after transfer, a comparison between groups was performed only with a limited number of paired samples because several mice transferred with whole PBMC died or had to be sacrificed

before the end of the experiment. As shown in **Figure 6A**, a tendency of increased levels of human CD45$^+$ leukocytes, CD3$^+$ T cells, CD4$^+$ T cells, CD8$^+$ T cells and CD20$^+$ B cells in the peripheral blood was observed in mice transferred with whole PBMC compared to mice transferred with T-cell depleted PBMC, which was, however, not significant. Similar results were observed in the comparison between mice transferred with whole PBMC and mice transferred with B-cell depleted PBMC (**Figure 6 B**).

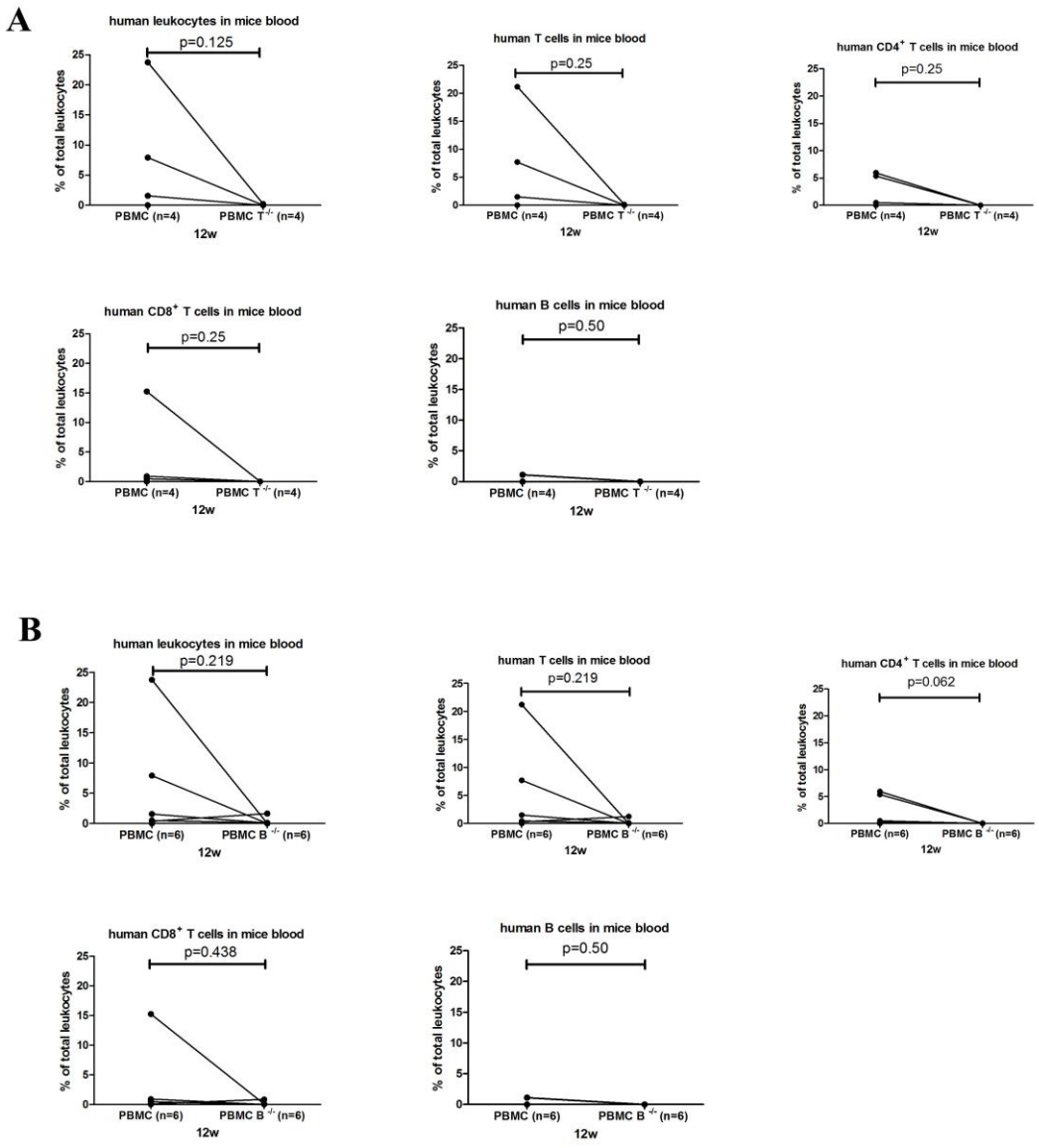

Figure 6. Human leukocytes in peripheral blood of recipient mice at week 12 after transfer. (A) Comparison between mice transferred with whole PBMC (n=4) and mice transferred with T-cell depleted PBMC (n=4) in levels of human leukocytes, CD3$^+$ T cells, CD4$^+$T cells, CD8$^+$T cells and CD20$^+$ B cells. (**B**) Comparison between mice transferred with whole PBMC (n=6) and mice transferred with B-cell depleted PBMC (n=6) in levels of human CD45$^+$ leukocytes, CD3$^+$T cells, CD4$^+$T cells, CD8$^+$T cells and CD20$^+$ B cells. P values reflect comparisons between mice transferred with whole PBMC and mice transferred with T- or B-cell depleted PBMC. Statistical significance was determined using the Wilcoxon match pairs test.

3.6 Architecture and cellularity of spleens after transfer of human PBMC

Next, we determined whether the transferred human PBMC were present in the spleens of recipient mice. As expected, spleens of untreated *Rag2*$^{-/-}$*Il2R*$^{-/-}$ immunodeficient mice were small and did not show normal white pulp which consists of functional germinal center **(Figure 7A)**. However, spleens of *Rag2*$^{-/-}$*Il2R*$^{-/-}$ mice transferred with whole PBMC from SSc patients were large and characterized by well-structured white pulps (**Figure 7B**). However, such well-structured white pulp was not observed in spleen of mice (0 out of 7) transferred with T-cell depleted PBMC (**Figure 7C**) and rarely observed in spleen of mice (2 out of 10) transferred with B-cell depleted PBMC (**Figure 7D**).

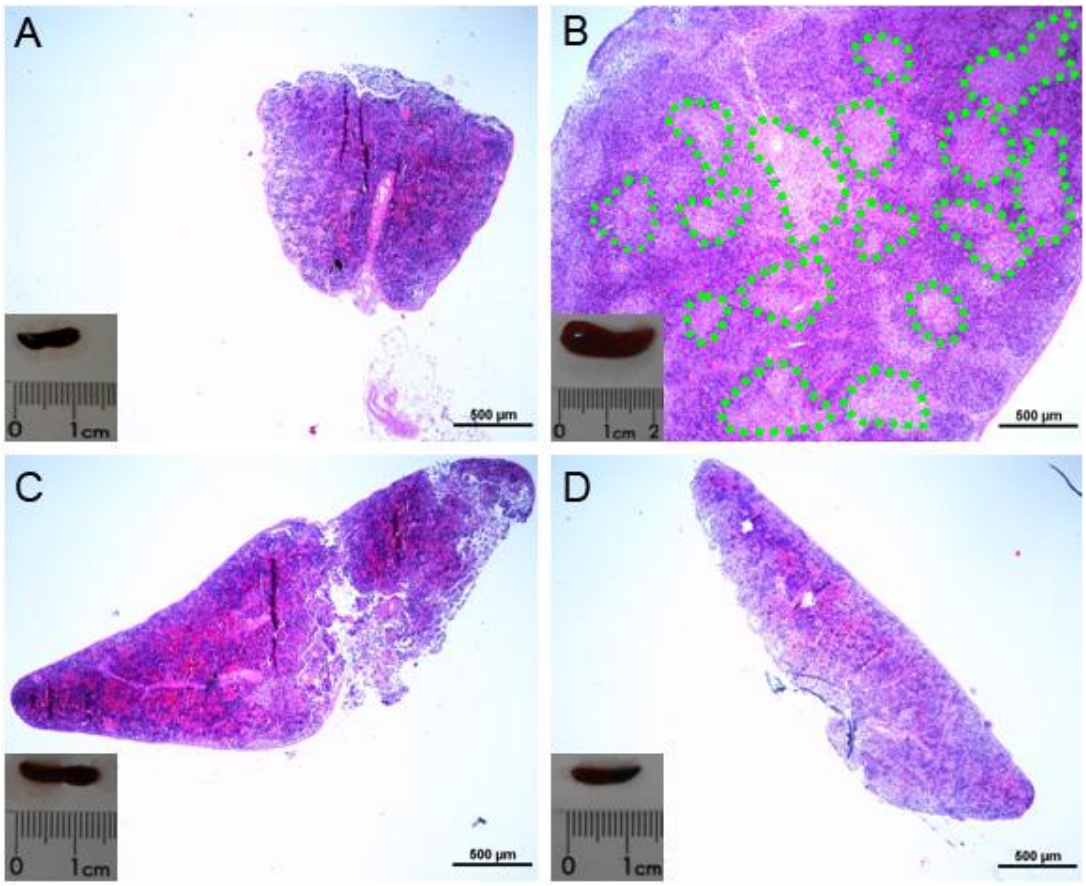

Figure 7. Morphology of the splenic white pulp in recipient mice. Representative pictures of H&E-stained spleen sections of untreated *Rag2*$^{-/-}$*Il2R*$^{-/-}$ mice (**A**), *Rag2*$^{-/-}$*Il2R*$^{-/-}$ mice transferred with whole PBMC from SSc patients (**B**), *Rag2*$^{-/-}$*Il2R*$^{-/-}$ mice transferred with T-cell depleted PBMC (**C**) and *Rag2*$^{-/-}$*Il2R*$^{-/-}$ mice transferred with B-cell depleted PBMC (**D**). The dotted green line indicates the well-structured white pulp. Bars = 500 µm.

Next, the distribution of human lymphocytes in murine spleen was investigated using IHC staining. As shown in **Figure 8**, the well-structured splenic white pulps in mice transferred with whole PBMC from SSc patients consisted predominantly of human $CD20^+$ B cells and $CD4^+$ T cells as well as small amount of human $CD8^+$ T cells and $CD138^+$ plasma cells (**Figure 8**). However, very few $CD4^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells or $CD138^+$ plasma cells could be detected in spleens of all the mice transferred with T-cell depleted PBMC (**Figure 8**). With regard to mice transferred with B-cell depleted PBMC, small amount of $CD4^+$ T cells and $CD8^+$ T cells, but neither $CD20^+$ B cells nor $CD138^+$ plasma cells were observed in spleen without white pulp (**Figure 8**), and many T cells but not B cell or plasma cells could be detected in murine spleen with white pulp-like stuctures (data not shown).

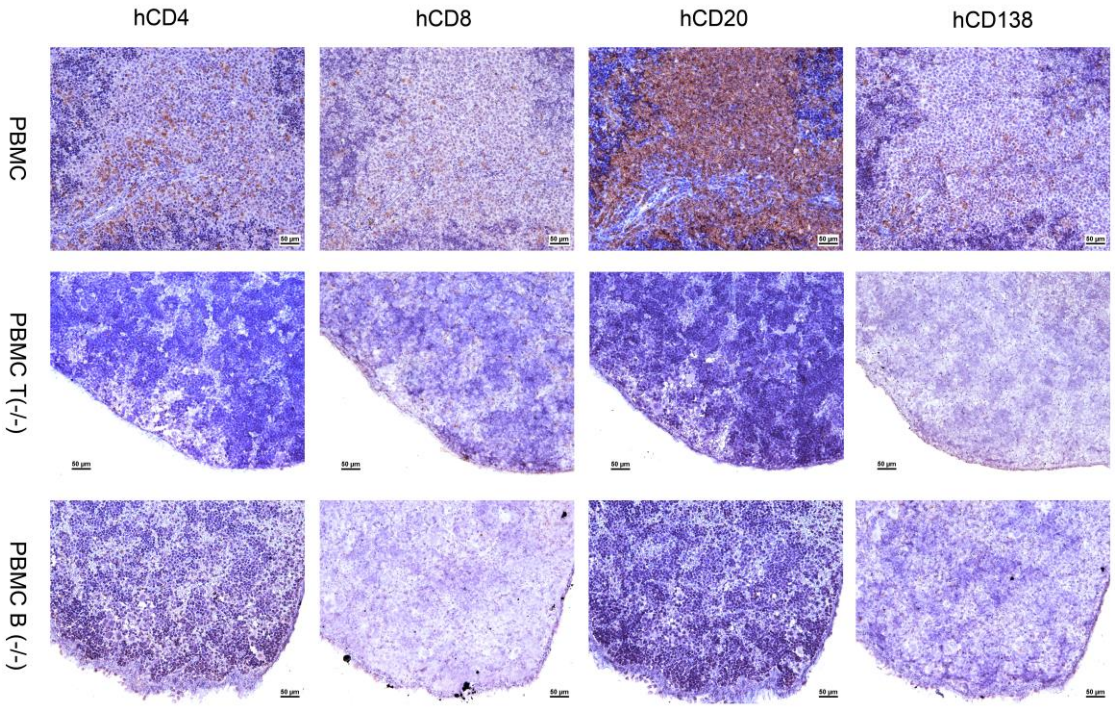

Figure 8. Human lymphocytes in the spleens of recipient mice. Representative pictures of IHC-stained spleen section for detecting human $CD4^+$ T, $CD8^+$ T, $CD20^+$ B and $CD138^+$ plasma cells. PBMC, mice transferred with whole PBMC from SSc patients, PBMC T(-/-), mice transferred with T-cell depleted PBMC from SSc patients, PBMC B(-/-), mice transferred with B-cell depleted PBMC from SSc patients. Bars=50 μm.

3.7 Production of human IgG and autoantibodies

Given the presence of human lymphocytes in spleens of recipient mice, I next investigated whether these lymphocytes were functional by determining human antibodies in murine sera. Since the 6 mice died or had to be sacrificed before the end of the experiment, serum samples of 5 of them

were collected when they died, and serum sample from one mouse was not available. Sera from all other mice were prepared at week 12 after the transfer.

First, levels of human total IgG were determined in sera of mice. As shown in the **Figure 9 A, B**, mice transferred with whole PBMC from SSc patients generated considerable amount of human IgG. When compared to mice transferred with whole PBMC, both mice transferred with T-cell depleted PBMC ($p = 0.031$) and mice transferred with B-cell depleted PBMC ($p=0.004$) produced dramatically and significantly less amount of human IgG.

In the following step, levels of two SSc-related human autoantibodies, anti-AT1R and anti-ETAR IgG, were analyzed in mice sera. Mice transferred with whole PBMC produced both SSc-related autoantibodies. By contrast, neither mice transferred with T-cell depleted PBMC nor mice transferred with B-cell depleted PBMC produced anti-AT1R or anti-ETAR autoantibodies **(Figure 9 C, D, E, F)**.

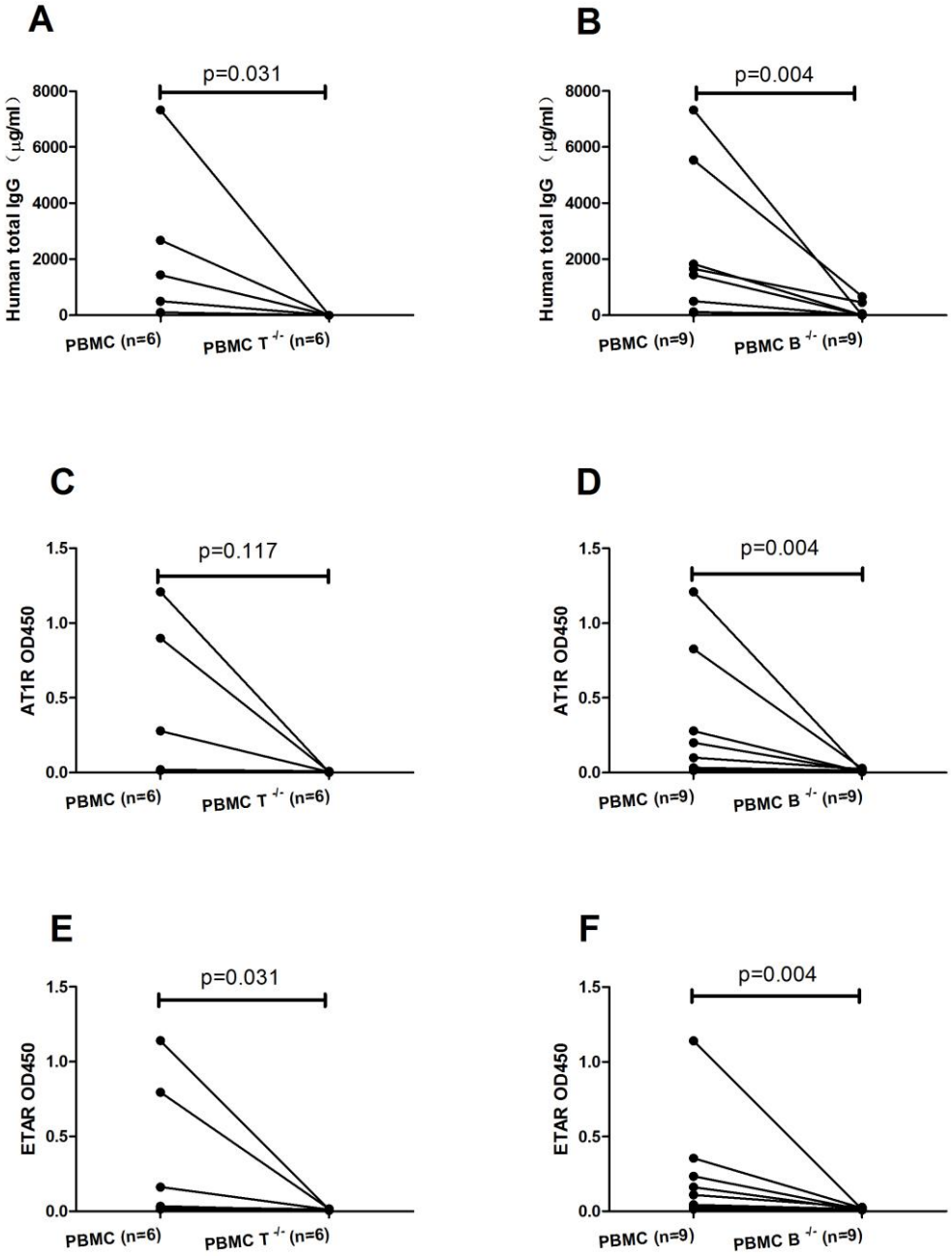

Figure 9. Production of human antibodies in recipient mice. (A, B) Serum levels of human total IgG were significantly higher in the mice transferred with whole PBMC from SSc patients than those in the mice transferred with T-cell depleted (n=6) or B-cell depleted (n=9) PBMC. Comparison of serum levels of anti-AT1R IgG **(C, D)** and anti-ETAR IgG **(E, F)** between mice transferred with whole PBMC from SSc patients and mice transferred with T-cell depleted (n=6) or B-cell depleted (n=9) PBMC. *P* values reflect comparisons between mice transferred with whole PBMC and mice transferred with T- or B-cell depleted PBMC. Statistical significance of comparison in A, B, D, E, F was determined using Wilcoxon matched pairs test, and statistical significance of comparison in C was determined by pair t test.

3.8 Tissue inflammation in recipient mice after transfer of human PBMC

Finally, to assess the effect of human T and B cells in disease manifestation, the histopathology of mice tissues including the lung, kidney, liver, heart, muscle, intestines, esophagus and skin were evaluated. Although 6 mice received whole PBMC from SSc patients died or were sacrificed before the end of experiment, tissues of all mice were collected and analyzed.

Mice transferred with whole PBMC from SSc patients showed inflammation in the lung, kidney and liver (**Figure 10**), but not in heart, muscle, intestines, esophagus or skin (data not shown). However, little or no inflammation in the lung, kidney and liver was observed in mice transferred with T cell-depleted PBMC or B cell-depleted PBMC (**Figure 10**). Therefore, these results suggest that both T and B cells are required for the disease manifestation in this humanized mouse model for SSc.

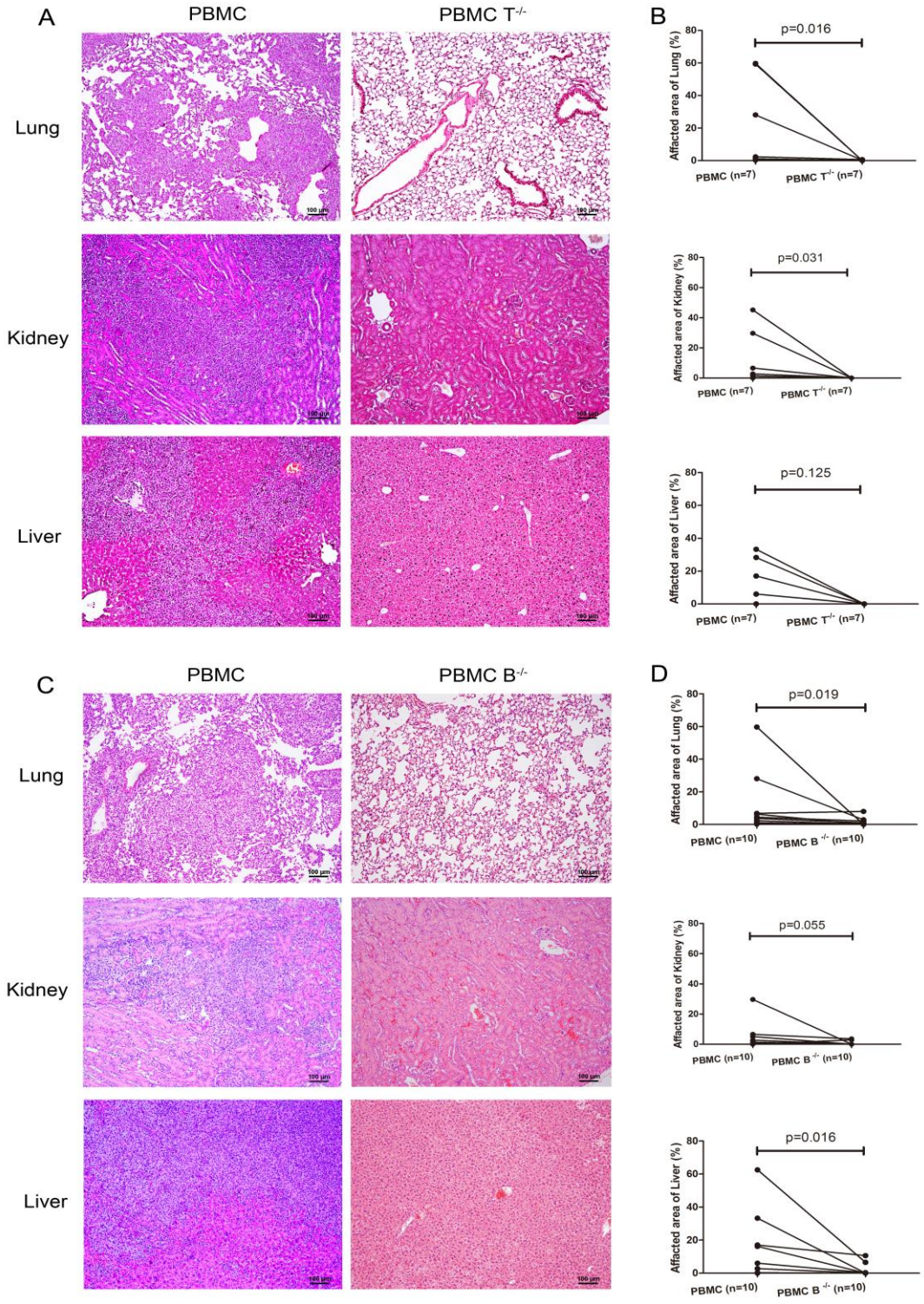

Figure 10. Histopathology of the lung, kidney and liver of recipient mice. (A) Representative micrographs of different tissues, **(B)** Quantitative analysis of inflammation in the lung, kidney and liver of mice transferred with PBMC or T-cell depleted PBMC from SSc patients. **(C)** Representative micrographs of different tissues, **(D)** Quantitative analysis of inflammation in the lung, kidney and liver of mice transferred with PBMC or B-cell depleted PBMC from SSc patients. *p* values reflect comparisons between mice transferred with PBMC and mice transferred with T- or B-cell depleted PBMC. Bars=100 μm. Statistical significance was determined by Wilcoxon matched pairs test.

4. Discussion

In the present study, I investigated the role of human T and B cells in a humanized mouse model of SSc in which disease is induced by adoptive transfer of patient-derived PBMC into *Rag2*$^{-/-}$*Il2R*$^{-/-}$ mice. According to my results, transfer of whole PBMC from SSc patients could i) restore the well-structured splenic white pulps in immunodeficient recipient mice, ii) induce production of human IgG and SSc-related autoantibodies, and iii) produced disease symptoms and increase mortality in the animals. However, when those PBMC were depleted from T or B cells before transfer, recipient mice showed neither immunological nor clinical features of SSc-like disease. Therefore, this study for the first time provides direct evidence that both human T and B cells play a crucial role in the pathogenesis of experimental SSc in a model closely resembling the disease in human.

4.1 Role of T cells in experimental SSc

In the current study, mice transferred with PBMC from SSc patients developed functional splenic germinal centers (GCs) and systemic inflammation in the lung, kidney and liver, while mice transferred with T cell-depleted PBMC from SSc patients did not. These results show that human T cells play an indispensable role in the pathogenesis of this novel humanized mouse model.

In patients with SSc, inflammatory cells in affected tissues consist mostly of T cells, where $CD4^+$ T cells dominate while $CD8^+$ T cells are less prominent (49, 50). This phenomenon has also been observed in the humanized mouse model used in the current study, where human T cells including both $CD4^+$ and $CD8^+$ T cells are present in affected murine organs such as the lung, kidney and liver.

After being activated, $CD4^+$ T cells differentiate into distinct subtypes, including Th1, Th2, Th17, Treg and follicular helper T (Tfh) cells, and each subtype plays different roles in adaptive immune responses of SSc (132-136). Several studies have suggested that the imbalance of Th1/Th2 responses contributes to the pathogenesis of SSc (135, 137, 138). For example, Boin et al (138) reported that Th2 cells are more frequent than Th1 cells in peripheral blood of SSc patients than in controls, and the Th1/Th2 ratio is correlated with lung forced vital capacity in SSc with active interstitial lung diseases (138). Moreover, Th17/Treg imbalance has also been demonstrated to be associated with SSc pathogenesis (53, 132). A substantial amount of studies have suggested that Th17 cells and their cytokines such as IL-17A, IL-21, and IL-22, are associated with the development of prominent features of SSc (52, 139-142). By contrast, a decreased level of Treg cells has been observed in patients with SSc, particularly in patients with late-stage disease (143). However, a decreased level of Treg cells is associated with an increased level of Th17 cells in peripheral blood of SSc patients (144), leading to an increased ratio Th17/Treg. As a consequence,

the Th17/Treg cells imbalance further promotes Th17 cell-mediated inflammatory processes (132).

In addition to $CD4^+$ T cells, $CD8^+$ T cells have also been suggested to be involved in pathogenesis of SSc in several studies (145-150). Recently, Li et al (148) showed that circulating and skin-resident $CD8^+CD28^-$ T cells are increased in both peripheral blood and affected skin of SSc in the early stage of the disease. The $CD8^+CD28^-$ T cells in SSc patients exhibited an effector memory phenotype, a strong cytolytic activity and a high capacity of producing the profibrotic cytokine IL-13 (148). Furthermore, SSc patient-derived $CD8^+$ T cells are able to secrete type 2 cytokines such as IL-4, IL-5, and IL-9 as well as pro-inflammatory cytokines including IFNγ, TNFα and IL-17 (146, 147). Therefore, the pro-inflammatory, pro-fibrotic and cytotoxic features of $CD8^+$ T cells enable them to contribute to the development of SSc.

4.2 Role of B cells in experimental SSc

This study shows that, apart from T cells, B cells are also indispensable for the disease development in the humanized mouse model of SSc. These findings are in line with clinical observations, in which an association between B cell abnormalities and the development and progression of SSc has been shown (25, 151, 152). Moreover, in this model the substantial improvement of SSc-patients after B-cell depletion can be reproduced (153-156).

Interestingly, recipient mice transferred with whole PBMC from SSc patients generated not only human IgG but also human autoantibodies against AT1R and ETAR. Previously, Riemekasten and colleagues have demonstrated that serum levels of anti-AT1R and anti-ETAR antibodies are significantly higher in patients with SSc than healthy subjects and patients with other autoimmune diseases (32-34). Meanwhile, those two autoantibodies have been shown to be associated with disease manifestations of SSc, such as pulmonary fibrosis, PAH and mortality (32-34). Moreover, *in vitro* studies have revealed that anti-AT1R and anti-ETAR antibodies are functional autoantibodies with agonistic effects on their receptor (35-38). For example, both anti-AT1R and anti-ETAR IgG can activate endothelial cells and induce the secretion of pro-inflammatory and pro-fibrotic cytokines (35-38). Therefore, anti-AT1R and anti-ETAR IgG are SSc-associated autoantibodies and potential contributors to the pathogenesis of the disease. In this humanized mouse model, recipient mice transferred with whole PBMC from SSc patients produce significantly higher levels of anti-AT1R and anti-ETAR IgG than those transferred with PBMC from healthy subjects (unpublished data), supporting the note that these two autoantibodies are SSc-related. Moreover, transfer of B-cell-depleted or T-cell-depleted PBMC from SSc patients did not induce the production of anti-AT1R or anti-ETAR IgG in recipient mice, suggesting that the

two autoantibodies are produced in a T-cell-dependent manner. Although recent results of our group strongly argue for a pathogenic role of these antibodies, their function in this model remains to be clarified.

Although not investigated here, it is conceivable that mice transferred with PBMC from SSc patients could generate beside anti-AT1R and ETAR some other autoantibodies which might contribute to the development of disease. Autoantibodies can contribute to the pathogenesis of autoimmune disease manifestation through multiple mechanisms, including mimicking receptor stimulation, blocking the function of antigen, induction of altered signaling, triggering uncontrolled microthrombosis, cell lysis, neutrophil activation, and induction of inflammation (157). Concerning the role of autoantibodies in SSc, some possible effects have been suggested such as 1) acting as ligand and inducing the production of pro-inflammatory and pro-fibrotic cytokines (35-38), 2) triggering pro-adhesive and pro-inflammatory phenotypic changes in fibroblasts (39-42); 3) preventing the degradation of excess collagen in the extracellular matrix (43, 44); and 4) activating fibroblast (45-47).

In addition to autoantibodies production, B cells are able to contribute to the disease development by releasing cytokines and acting as antigen presenting cells. For example, B cells from SSc mouse model or SSc patients produce considerable levels of IL-6 which subsequently induce Th2-dominant immune responses (158-161). Moreover, B cells could act as APCs and thus influence the function of T cells (160-162).

4.3 Interaction between T and B cells

In this study, only mice transferred with whole PBMC developed SSc-like disease, while mice transferred with T- or B-cell depleted PBMC did not. This suggests an essential role of interaction between T and B cells in disease pathogenesis. The importance of the cooperation between T and B cells has been demonstrated in several other animal models for SSc too (163-165). For example, Phelps et al. have shown that only the simultaneous injection of both T and B cells from tight skin mice into C57BL/6 Pa/Pa mice could induce tight skin-related phenotypes such as dermis thickening, enhanced collagen gene transcription, inflammatory infiltration of the skin, and production of autoantibodies (163). Another study performed by Hasegawa et al. reported that anti-DNA topoisomerase I antibody (ATA) secretion could only be detected when B cells retrieved from ATA-positive SSc patients are cocultured with T cells. Noteworthy, B cells did not produce ATA when cultured with pokeweed mitogen and recombinant topoisomerase I alone (166). In addition, Komura et al (164) have demonstrated that the communication between B and T cells is dependent

on CD40/CD40-L signaling in tight-skin mice. Finally, Tanaka et al (165) reported that the ICOS/ICOS-L axis was particularly important in the cooperation between B and T cells in bleomycin (BLM) induced mouse model of SSc. These findings from animal models suggest that an interaction between T and B cells is required for the development of SSc.

Germinal centers (GCs) are specialized compartments in secondary lymphoid organs, and they are vital for the development of effective adaptive immune responses (167-170). Both B and T cells are fundamental components of GCs (170). In the current study, immune deficient mice transferred with whole PBMC from SSc patients developed splenic white pulp which contains functional GCs, while mice transferred with T cell depleted PBMC did not. In addition, although 2 out of 10 mice transferred with human B-cell-depleted PBMC formed splenic white pulp-like stuctures which are composed of human T cells, these two mice did not produce autoantibodies and showed no evidence of histopathology, suggesting that these white pulp-like structures contain no functional GCs. These results indicate that both T and B cells are indispensable for the formation of functional GCs. Mechanistically, GC formation is dependent on chemokines related to lymphocyte homing, e.g. chemokine (C-X-C motif) ligand 13 (CXCL13). Tfh cells, a subtype of $CD4^+$ T cells, produce CXCL13 and thus contribute to homing of B and T cells into primary follicles as well as GC formation in secondary lymphoid tissues (171).

GCs are also the main location where T and B cells interact with each other. In GCs, Tfh cells provide key signals to antigen-primed B cells to undergo proliferation, isotype switching and somatic hypermutations, leading to the generation of long-living plasma cells and memory B cells during immune responses (167, 172-174). Previous studies have demonstrated that the interaction between T and B cells is dependent on T cell receptor (TCR)/major histocompatibility complex (MHC II), CD28/D80-CD86, CD40-L/CD40 and inducible costimulator (ICOS)/ inducible costimulator ligand (ICOS-L) signaling in SSc (166, 175-177). Besides $CD4^+$ T cells, $CD8^+$ T cells are also involved in GCs formation and B cells maturation (178, 179). In a humanized mouse model of rheumatoid arthritis induced by adoptive transfer of human synovium and PBMC, GC function in the rheumatoid lesion is dependent on $CD8^+$ T cells, which is indicated by the fact that depletion of $CD8^+$ T cells disrupts synovial GCs and decrease the antibody production of antibodies (180).

In the current study, mice transferred with T-depleted PMBC not only were lacking of human T cells but also showed limited number of B cells in blood and spleen. This finding suggests that the proliferation and differentiation of B cells are essentially dependent on T cells in blood and spleen. Meanwhile, 8 of 10 mice transferred with B-depleted PMBC showed limited numbers of T cells,

while 2 mice showed considerable number of T cells in blood and slpeen, suggesting that the B cells are also involved in but not indispensable for the proliferation of T cells in blood and spleen. A possible reason for this is that T cell activation and proliferation is also dependent on other professional APCs such as dendritic cells and macrophages.

4.4 Clinical relevance of the humanized mouse model for SSc

In this model, mice develop immunological and histopathological features mimicking the human SSc after transfer of patient cells, providing a platform to study the pathogenesis of the disease. Notably, recipient mice transferred with PBMC from different SSc patients showed considerable differences in their disease phenotype with regard to autoantibody production, histopathology of inner organs and disease-related mortality. Most likely, the heterogeneity in disease manifestation among recipient mice reflects the differences of PBMC derived from individual donors. Therefore, this model could be further developed to generate individualized, patient-specific models of the disease. Although this approach has to be further validated and explored in the future, the idea of a personalized optimization of treatment regimes on a model instead of a patient has become a very attractive concept.

The current study suggests that both human T and B cells are indispensable for the development of SSc. This finding indicates that T cells, B cells and molecules involved in the interaction between T and B cells, such as TCR/MHC II, CD28/D80-CD86, CD40-L/CD40 and ICOS/ICOS-L might be targets of the treatment. So far, RTX, a chimeric monoclonal anti-CD20 B-cell-depleting antibody, has been approved for clinical use and demonstrated to be an efficient medicine for SSc patients (153-156). Thus, T-cell-targeted therapies or those targeting on interaction between T and B cells could be novel candidate treatments of the disease.

4.5 Limitations of this study

Two limitations in this study need to be mentioned here. The first limitation is that all patients were treated with drugs, and the treatment varied considerably among patients. The heterogeneity in treatment might affect the disease manifestations in recipient mice. The second one is that the humanized model used in this study only mimics part of features of the disease. SSc is featured by autoimmunity, systemic inflammation, fibrosis and vasculopathy. In this humanized mouse model, recipient mice are featured by autoimmunity and systemic inflammation but develop neither fibrosis nor vasculopathy. Therefore, this model is not suitable for investigating the pathogenesis of fibrosis and vasculopathy. One possible reason for the lack of tissue fibrosis and vasculopathy is

that both disease manifestations are results of the interaction between human autoimmune cells and target tissues. Accordingly, a transfer of both PBMC from SSc patients and target tissue, e.g. human skin from healthy subjects into immunodeficient mice might partially overcome this limitation. This strategy has been applied in animal model of another autoimmune disease- alopecia areata (181).

5 Conclusion and perspectives

5.1 Conclusion

In this study, we have shown that both T and B cells essentially contribute to the development of immunological and pathological features in the humanized mouse model, including GCs formation, production of autoantibodies, systemic inflammation and disease mortality. Furthermore, this study also provides evidence that human T and B cells depend on each other for their survival, function and proliferation in mice. In conclusion, the current study has demonstrated that both human T and B cells are required for the development of disease in the humanized mouse model for SSc.

5.2 Perspectives

Although this study represents a potential individualized model for SSc which is helpful for personalized medicine, it needs to be further optimized and improved. On the one hand, transfer of PBMC from patients prior to any treatment might induce disease manifestations reflecting human disease better. On the other hand, induction of fibrosis and vasculopathy in mice will be an ideal humanized mouse model for SSc.

Furthermore, the mechanisms underlying the role T and B cells in this humanized mouse model should be explored in the future. Many interesting questions in the aspect need to be addressed, e.g. which subtypes of T and B cells contribute to the disease development, whether autoantibodies are important for the disease pathogenesis, and, if yes, which specificity do these autoantibodies have. Addressing these questions will greatly help to understand this model as well as SSc.

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1. **Yaqing Shu**[#], Wei Qiu[#], Junfeng Zheng, Xiaobo Sun, Junping Yin, Xiaoli Yang, Xiaoyang Yue, Chen Chen, Zhihui Deng, Shasha Li, Yu Yang, Fuhua Peng, Zhengqi Lu, Xueqiang Hu, **Frank Petersen, Xinhua Yu.** HLA class II allele *DRB1*16:02* is associated with anti-NMDAR encephalitis. *Journal of Neurology, Neurosurgery, and Psychiatry* 2019;90:652-658 ([#]co-first authors)

2. **Yaqing Shu**, Youming Long, Shisi Wang, Wanming Hu, Jian Zhou, Huiming Xu, Chen Chen, Yangmei Ou, Zhengqi Lu, Alexander Y. Lau , **Xinhua Yu**, Allan G. Kermode & Wei Qiu. Brain histopathological study and prognosis in MOG antibody-associated demyelinating pseudotumor. *Annals of Clinical and Translational Neurology* 2019; 6:392-396

3. **Yaqing Shu**, Yue Xu, Chen Chen, Jing Li, Rui Li, Haotian Wu, Xueqiang Hu, Zhengqi Lu, **Xinhua Yu**, Wei Qiu. Serum Bilirubin and Albumin in Anti-N-Methyl-D-Aspartate Receptor Encephalitis. *Neuroimmunomodulation* 2018;25:206-214