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Generation and characterization of antibodies directed against angiotensin II receptor type 1 (AT1R)

Dissertation

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- Sektion Medizin-

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Zusammenfassung

Hintergrund: Systemische Sklerose (SSc) ist eine chronische Bindegewebserkrankung, die durch Autoimmunität, Vaskulopathie und Fibrose gekennzeichnet ist. Befunde klinischer Studien haben gezeigt, dass Autoantikörper gegen den Angiotensin II Typ 1 Rezeptor (AT1R) eine Rolle bei der Pathogenese von SSc spielen könnten. Erst kürzlich wurde in unserer Gruppe ein neuartiges Mausmodell für SSc etabliert, in welchem Mäuse mit humanem AT1R (hAT1R) immunisiert wurden. Dieses Modell liefert starke Hinweise darauf, dass Autoantikörper gegen hAT1R in der Pathogenese der ein zentrale Rolle spielen. Ziel dieser Studie war es daher, Autoantikörper gegen AT1R zu generieren und diese zu charakterisieren.

Methoden: Polyklonale Antikörper gegen hAT1R wurden aus Seren hAT1R-immunisierter Mäuse mit Hilfe der Affinitätschromatographie über Protein G oder A aufgereinigt. Monoklonale Antikörper gegen hAT1R wurden aus hAT1R-immunisierten Mäusen mit konventioneller Hybridomtechnik erzeugt. Die funktionale Aktivität von anti-hAT1R-Antikörpern wurde über ihre Kapazität zur Bindung an Epithelzellen und zur Aktivierung von Kardiomyozyten und Monozyten bestimmt.

Ergebnisse: Es wurden polyklonale Antiseren gegen hAT1R sowie drei verschiedene monoklonale Anti-hAT1R-Antikörper in Mäusen generiert welche zuvor mit dem respektiven Antigen immunisiert wurden. Sowohl poly- als auch monoklonale Antikörper banden an die Oberfläche von Epithelzellen. Im Vergleich zu entsprechenden Kontrollen erhöhten polyklonale Antikörper gegen hAT1R die Kontraktionsrate von Kardiomyozyten und förderten die Interleukin-8-Produktion in Monozyten. Beide Effekte konnten durch Losartan, einem AT1R-spezifischen Inhibitor, blockiert werden. Zwei der drei monoklonalen IgGs gegen hAT1R erhöhten ebenfalls die Kontraktionsrate der Rattenkardiomyozyten und vermittelten die Sektretion von Interleukin 8 aus menschlichen Monozyten. Diese Funktionen konnten zumindest teilweise durch Losartan blockiert werden.

Schlussfolgerungen: Sowohl polyklonale als auch monoklonale Antikörper gegen AT1R stellen funktionelle Autoantikörper mit einer agonistischen Wirkung auf AT1R dar. Meine Ergebnisse unterstützen die Hypothese, dass Autoantikörper gegen AT1R pathogen sein könnten und damit sehr wahrscheinlich zur Pathogenese der SSc beitragen.

Summary

Background: Systemic sclerosis (SSc) is a chronic connective tissue disease which is characterized by autoimmunity, vasculopathy and fibrosis. Evidences from clinical studies have shown that autoantibodies against angiotensin II type 1 receptor (AT1R) may play a role in the pathogenesis of SSc. Very recently, a novel mouse model for SSc has been established in our group by immunizing mice with human AT1R (hAT1R). This model provides strong evidence that autoantibodies against hAT1R are pathogenic in the process of SSc. In this study, I aimed to generate and characterize autoantibodies against AT1R.

Methods: Anti-hAT1R polyclonal IgG and three different anti-hAT1R monoclonal IgGs were generated from mice immunized with human AT1R. Monoclonal antibodies against hAT1R were generated from hAT1R-immunized mice using conventional hybridoma technique. Functions of anti-hAT1R antibodies were evaluated by their capacity to bind to hAT1R on the cell membrane of epithelia and to activate cardiomyocytes and monocytes.

Results: Anti-hAT1R polyclonal IgG and three different anti-hAT1R monoclonal IgGs were generated from mice immunized with human AT1R. Both types of antibodies against AT1R were able to bind to the membrane of epithelial cells. As compared to corresponding controls, polyclonal antibodies against hAT1R increased the cell beating rate of cardiomyocytes and promoted interleukin 8 production in monocytes. Both effects could be blocked by Losartan, an AT1R specific inhibitor. Two out of 3 monoclonal IgGs against hAT1R induced an increased beating rate of rat cardiomyocytes and increased the production of interleukin 8 from human monocytes compared to corresponding isotype controls. These functions were at partially sensitive to treatment with Losartan.

Conclusions: Both polyclonal and monoclonal antibodies against AT1R represent functional autoantibodies with an agonistic effect on AT1R. My results support the hypothesis that autoantibodies against AT1R might be pathogenic and contribute to the development of SSc.

1. Introduction

1.1. Autoimmune diseases and Systemic sclerosis (SSc)

1.1.1. Autoimmune diseases

Autoimmune diseases represent a group of disorders characterized by an attack of the immune system to its host [1]. Under physiological condition, our immune system does not react to host structures, which is named as immunological self-tolerance [2]. However, self-tolerance can be broken, resulting in the generation of autoreactive T cells and autoantibodies [3][4]. Consequently, such autoreactive T cells and autoantibodies can mediate inflammation and/or damage in tissues or organs of the host, resulting in autoimmune disease. So far, approximately 80 different autoimmune diseases have been described [5], which affect 5-10% of the world population [6]. Autoimmune diseases can be categorized into two groups, systemic autoimmune diseases which affect multiple organs such as rheumatoid arthritis (RA), systemic sclerosis (SSc) and systemic lupus erythematosus (SLE), and organ-specific autoimmune diseases, including multiple sclerosis (MS), autoimmune thyroid disease, Addison's disease and type 1 diabetes (T1D) [5].

Both genetic and environmental factors contribute to the development of autoimmune diseases. Hundreds of genetic loci have been identified to be associated with various autoimmune disorders [7]. For example, human leukocyte antigen (HLA) loci are associated with numerous autoimmune diseases[8], suggesting an important role of antigen presentation in the development of those disorders . In addition to HLA genes, plenty of genetic variations within genes involved in multiple biological processes have be identified as susceptibility variants for autoimmune diseases[9]. Environmental factors, such as infection, microbiota, chemicals and sex hormones contribute significantly to the development of autoimmune disorders. For example, parvovirus and Epstein-Barr virus (EBV) infections are highly related to rheumatoid arthritis (RA) and autoimmune thyroid disease, while a Hepatitis-A-Virus (HAV) infection is associated with T1D and autoimmune hepatitis (AIH) [10][11][12][13][14]. Moreover, it has long been known that some drugs can cause autoimmune disease-like symptoms. For example, DNA methylation inhibitors such as procainamide and hydralazine can cause a SLE-like disease[15]. In addition, for most autoimmune diseases there is a clear sex difference in prevalence, suggesting a role of sex hormones in the disease pathogenesis [16][17].

1.1.2. Systemic sclerosis (SSc)

SSc is a systemic autoimmune disease which affects multiple organs. SSc is characterized by three hallmarks, autoimmunity, vasculopathy and fibrosis [18]. During the past decades, several diagnostic criteria have been used for the diagnosis of SSc. In 1980, a preliminary criteria for the

classification of SSc was proposed by American College of Rheumatology (ACR) [19]. Another set of criteria was proposed by Nadashkevich et. al in 2004, in which laboratory parameters were included in the diagnosis of SSc [20]. The most recent classification criteria for SSc were proposed in 2013 by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) [21]. The ACR/EULAR classification consists of 8 clinical and immunological parameters each of which is given a maximal score. These diagnostic parameters are 1) skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints, 2) skin thickening of the fingers, 3) fingertip lesions, 4) telangiectasia, 5) abnormal nailfold capillaries, 6) pulmonary arterial hypertension and/or interstitial lung disease, 7) Raynaud's phenomenon, and 8) SSc-related autoantibodies (anticentromere (ACA), anti–topoisomerase I (ATA), anti–RNA polymerase III), with maximal scores of 9, 4, 3, 2, 2, 2, 3 and 3 respectively. Patients with a total score of more than 9 are classified as definitely positive for SSc.

The prevalence and incidence of SSc vary among populations, with a prevalence of 7- 489 cases per million population and an incidence of 0.6-122 new cases per million population per year [22]. Like many other rheumatoid diseases, SSc occurs more prominent in females [23], where number of female patients are 3-4 times as high as compared to males [24]. Although relatively rare, SSc is featured by a high mortality, with an average life expectancy 16-34 years less than age- and sex-matched healthy controls [25].

Although the exact aetiology and pathogenesis of SSc has not been fully understood, it is believed that both genetic and environmental factors contribute to the development of the disease. So far, many susceptibility genes have been identified for SSc, including a couple of genes within HLA loci [26] and approximately 20 non-HLA genes such as Interferon regulatory factor 5 (*IRF5*), Interleukin-12 (*IL12*), Signal transducer and activator of transcription 4 (*STAT4*), and Cluster of Differentiation 247 (*CD247*) [27] . With regard to environmental factors, it has been shown that exposure to chemicals such as silica, trichloroethylene, and bleomycin, are highly related to SSc initiation [28][29]. Beside chemicals, viral and bacterial infections have been suggested to be associated with SSc [30][31]. For example, Radiac and colleagues reported that *helicobacter pylori* (*H. pylori*)-positive SSc patients showed higher severity score compared to *H. pylori*-negative ones, suggesting that *H. pylori* infection correlates with disease severity of SSc [31].

Like other rheumatoid diseases, SSc shows a great heterogeneity in clinical manifestations among tissues/organs [32]. This heterogeneity is presented as not only the various patterns of organ involvement but also the difference in the types of manifestation in a single organ, e.g. pulmonary fibrosis and pulmonary arterial hypertension (PAH) in the lung.

Most SSc patients express skin manifestations, including fibrosis, nailfold capillary abnormality, Raynaud's phenomenon and digital ulcers. Among these, Raynaud's phenomenon and nailfold capillary abnormality are two early features commonly seen in patients, followed by the development of digital ulcers and fibrosis. According to the status of the skin involvement, SSc can be classified into three forms, limited cutaneous SSc (lcSSc) in which patients only have skin manifestation of distal extremities, with or without face and upper neck involvement, diffuse cutaneous SSc (dcSSc) where skin manifestations extent from distal extremities to proximal limbs and the trunk, and SSc without skin involvement [27].

All three forms of SSc can be associated with further damage to internal organs such as lung, heart, kidney, and the gastrointestinal tract (GIT). Lung involvement is very prominent in patients with SSc. The most common clinical symptoms in the lung of SSc patients are exertional dyspnea and dry cough [33]. Pulmonary interstitial fibrosis and PAH are two main complications, which represent also the two leading causes of death in SSc [34]. With regard to cardiac manifestations, myocardial damage, conduction system fibrosis, coronary artery vasospasm and structural alterations, as well as pericardial damage are the main pathologies. Consequently, patients with SSc show systolic or diastolic dysfunction, impaired ventricular filling, arrhythmias, and congestive heart failure [35][36]. Renal manifestations in SSc are prominently caused by the vascular abnormality. The most severe renal manifestation of SSc is the scleroderma renal crisis (SRC), which was the leading cause of death before angiotensin-converting-enzyme (AEC) inhibitors were used for treatment [37][38]. In addition to SRC, further renal manifestations, such as mucopolysaccharides deposition in vascular intima, renal fibrosis and thickening of glomerular basement membrane and proteinuria are observed [39]. Gastrointestinal tract involvement is the most common visceral lesion of SSc [40]. All parts of the digestive tract can be affected, with esophageal involvement being the most common one. Clinically, SSc patients often suffer from gastrointestinal symptoms include dysphagia, odynophagia, heartburn and regurgitation, vomiting, epigastric pain, abdominal bloating, and chronic intestinal pseudo-obstruction [41][42][43]. An overview of multiple organ involvement in SSc is depicted in Figure 1 [44].

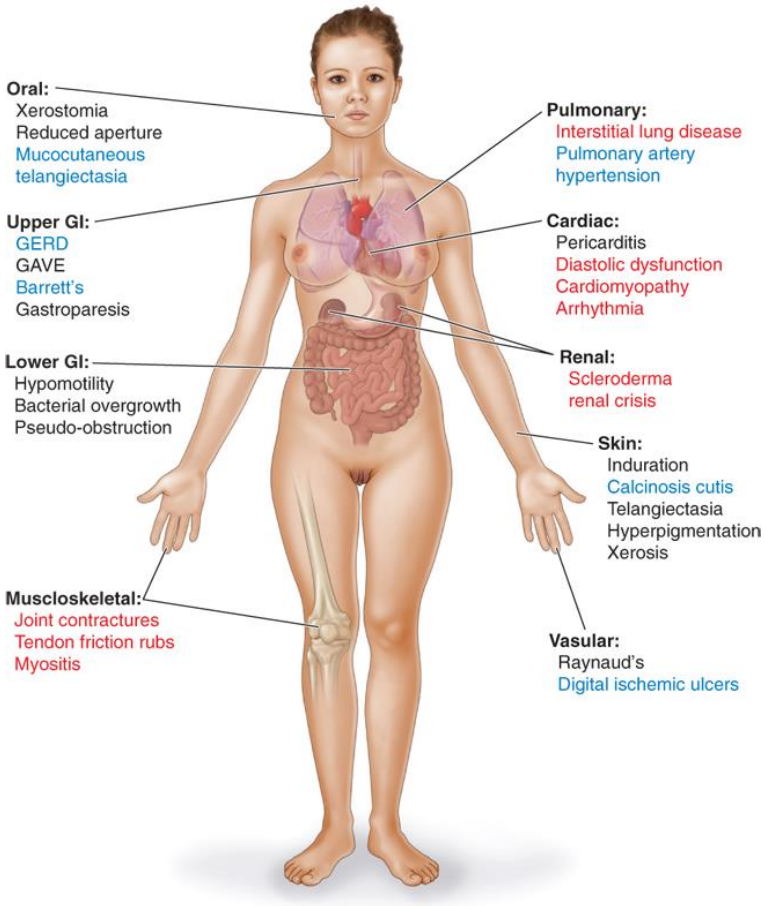

Figure 1. Multi-organ involvement in systemic sclerosis (according to [44])**.** Prominent complications more common in diffuse cutaneous SSc are shown in red; complications more common in limited cutaneous SSc in blue; symptoms occurring in both forms are shown in black.

Internal organ manifestations of SSc do not only reduce the life quality of patients, but represent the major causes of the death in patients with SSc [38][45].

1.2. G-protein-coupled receptors (GPCRs) and angiotensin II type 1-receptor (AT1R)

1.2.1. G-protein-coupled receptors (GPCRs)

G protein coupled receptors (GPCRs), also known as seven transmembrane-domain receptors, are the largest protein superfamily located on the cell membrane. As integral membrane proteins, GPCRs consist of an extracellular N-terminal domain, 3 extracellular loops (ECL), 3 intracellular loops (ICL), and an intracellular C-terminal domain.

In humans, GPCRs comprise more than 1,000 receptors encoded by more than 800 genes [46], which make them the largest family of receptors [47]. Furthermore, GPCRs are broadly expressed on almost all cell types and tissues [48]. Thousands of natural components with diverse chemical properties can bind to GPCRs as ligands, ranging from photons, small organic molecules to biomacromolecules such as proteins [49].

GPCRs exert principally their biological function by coupling to GTP binding proteins (G proteins) which are heterotrimeric proteins composed of 3 subunits, α, β and γ [50]. Once an agonistic ligand binds to the a GPCR, the receptor will undergo conformational change and be activated. Subsequently, the receptor activates the attached G-protein by causing the exchange of GTP for GDP on the Gα subunit. As a consequence of the activation, G proteins dissociate into an alpha (Gα) and a beta-gamma (Gβγ) subunit. The dissociated Gα and Gβγ subunits can act on their target proteins which further lead to the generation of second messengers such as cAMP, cGMP, IP3, and the activation of further downstream intracellular signaling pathways.

By regulating various biological processes, GPCRs play important roles in the activation, function and homeostasis of numerous type of cells, which makes them essential regulators in human body [51]. Due to their important roles in both physiological and pathological conditions, GPCRs are also a favorite therapeutic target for various human diseases [52].

1.2.2. Renin-angiotensin system (RAS)

The renin-angiotensin system (RAS) is a hormone system that regulates blood pressure and fluid balance in the body [53]. It consists of a series of peptide hormones and corresponding regulating enzymes. The RAS plays a key role in the regulation of cardiac, renal, and vascular physiology, and its activation is essentially involved in many pathologic conditions such as hypertension, heart failure, and renal disease [54]. Generally, RAS can be divided into two categories, circulating and tissue RAS, also called classical and local RAS, respectively [55].

Angiotensinogen, renin, angiotensin I (Ang I), ACE, Ang II, and mineralocorticoid (mainly aldosterone) are main components of the RAS. Angiotensinogen, a polypeptide secreted by liver is the basal substrate of the circulating RAS system. Circulating angiotensinogen can be cleaved into Ang I. Angiotensin I can be further converted to the active peptide, Ang II, by angiotensin converting enzyme (ACE) in the lungs [56][57][58][59][60]. In the local RAS, Ang II is produced by local ACE or non-ACE enzymes in tissues such as heart, central nervous system (CNS), kidney, pancreas, eyes and adrenal gland [61][62][63][64]. The local RAS can regulate tissue function more directly and more efficiently by paracrine and autocrine hormones.

1.2.3. Angiotensin II and angiotensin II type 1 receptor (AT1R)

Angiotensin II, an octapeptide, is a crucial mediator of the RAS. Ang II mediates intense contraction of systemic arterioles and promotes vasopressin and oxytocin release from the pituitary gland. Furthermore, it also increases the secretion of aldosterone from adrenal cortex, thus promotes the reabsorption of water and sodium in renal tubulars and increases sympathetic nervous excitements. The abovementioned features make Ang II currently the most effective substance that elevates the blood pressure.

Angiotensin II exert its biological effect mainly via two major subtype of membrane receptors in mammalians: angiotensin II type 1 receptor (AT1R) and angiotensin II type 2 receptor (AT2R) [65]. Additional 2 subtypes of Ang II receptors, AT3R and AT4R, have also been reported in 1992, but their functional role appears unclear.

AT1R and AT2R were first identified and pharmacologically characterized in 1989 [66]. In human, AT1R is broadly and ubiquitously expressed in various organs and tissues, and this wide distribution among tissues is consistent with its function of regulation of the blood pressure, electrolyte and fluid balance [67]. In contrast to human, rodents such as rats and mice carry two subtypes of AT1R , namely AT1AR and AT1BR [68]. AT1AR and AT1BR show approximately 95% sequence homology at the protein level [68]. AT1AR has a similar tissue distribution pattern to human AT1R, while AT1BR predominantly express in anterior pituitary gland and adrenal zona glomerulosa in murine [69].

In myocardium and blood vessel walls, binding of Ang II to AT1R leads to the activation of mitogen-activated protein kinase (MAPK) and the activation of transforming growth factor-β (TGF-β) which in turn activate Smad2/3 [70][71][72]. In addition, the AT1R activation can also upregulate NAD(P)H oxidase (NOX), and thus lead to an increased reactive oxygen species formation [73]. Activated MAPK and increased reactive oxygen species (ROS), together with phosphorylated Smad2/3 can increase the expression of matrix metalloproteinase (MMP), TGF-β, connective tissue growth factor (CTGF), and plasminogen activator inhibitor 1 (PAI-1) genes [74][75][76] which possess pro-proliferation, pro-inflammatory and pro-fibrotic effects and consequently lead to endothelial dysfunction, inflammation and fibrosis, respectively [76][77]. By contrast, activation of AT2R leads to an opposite effect as that of activation of AT1R under majority of circumstances [78][79][80].

In the renal system, AT1R expresses broadly on glomerular mesangial cells, afferent arterioles and

efferent arterioles, proximal and distal tubules, while AT2R expresses on vasculature and tubules, especially on renal proximal tubule cells [81]. Ang II affects renal functions mainly via AT1R on vascular and tubular elements. For example, Ang II-AT1R binding can induce renal arteriolar vasoconstriction, including constriction of arcuate arteries, interlobular arteries, afferent and efferent arterioles, as well as the vasa recta, maintaining vascular tone of the kidney [82][83]. Regarding the binding of Ang II-AT2R, it has been shown to be able to influence on the renal secretion of sodium, a process also named natriuresis [84][85].

During immune responses, Ang II shows proinflammatory properties, mainly via activating AT1R. Ang II enhances the production of inflammation-related molecules, such as proinflammatory cytokines, chemokines and cell adhesion molecules. It has been reported that Ang II-AT1R binding facilitates the production of many proinflammatory cytokines including IL-6, IL-17 and tumor necrosis factor α (TNF-α) [86][87][88]. Ang II also promotes the production of many chemokines such as interleukin 8 (IL-8, CXCL8) which is chemotactic for neutrophils, and monocyte chemoattractant protein type 1 (MCP-1, CCL2) which recruits macrophage into the vascular intima [86] [88]. Beside promoting the production of cytokines and chemokines, Ang II also increases the expression of adhesion molecules in both arterioles and venules, including E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1) and intercellular cell adhesion molecule-1 (ICAM-1) [89][90]. In addition, Ang II can stimulate the production of prostaglandins and vascular endothelial cell growth factor (VEGF) in human vascular smooth muscle cells, thus increase vascular permeability and initiate inflammatory responses [91]. Moreover, Ang II can exert its proinflammatory properties by direct activation of immune cells. For example, Ang II can act on monocytes and enhance their differentiation into macrophage-like cells [92]. Another target is nature killer (NK) cells which express both AT1R and AT2R. Ang II is able to trigger the calcium signaling, chemotaxis, and cell proliferation. Furthermore, NK cells can produce and deliver Ang II to inflammatory sites, thus forming a positive feedback loop and thus amplifying the inflammation [93]. Dendritic cells (DC) and T cells have also been shown to be regulated by Ang II, where the migration, differentiation and antigen presenting of DC [94] and the proliferation, activation, cytokine production and differentiation of T cells [95][96] are promoted by Ang II.

1.3. Functional autoantibodies (AABs) and anti-AT1R AAB

1.3.1. Functional autoantibodies

Autoantibodies are antibodies that targeting antigens from the organism itself, also called autoantigens. Autoantigens consist of a variety of molecules that could be the target of autoantibodies, including nucleic acid, carbohydrate, lipids and proteins. Among autoantibodies,

antinuclear antibodies (ANAs), a group of antibodies against multiple nuclear constituents, are most common ones and have been found in many autoimmune diseases [97].

The presence of autoantibodies is one of the hallmarks of many autoimmune diseases. According to their respective pathogenic principles, autoantibodies can be categorized into two groups. The first group are classical autoantibodies which induce disease pathology mainly via Fc part by activation of the complement system and/or FcR on immune cells. A typical example for this pathway are anti-COL7 antibodies in epidermolysis bullosa acquisita [98]. The second group consists of functional antibodies which act as agonistic or antagonistic ligands of the targeting receptors [99]. As a consequence, binding of functional autoantibodies to receptors leads to the activation or inactivation of the receptor. A function example typical for such autoantibodies are anti-N-methyl-D-aspartic acid receptor (NMDAR) antibodies which act as antagonist of the NMDAR and thus mediate autoimmune encephalitis [100].

1.3.2. Anti-GPCR autoantibodies

As abovementioned, GPCRs belong to the largest family of cell surface receptors. After binding to GPCRs, functional autoantibodies can exert agonistic, inhibitory or synergistic effects on the receptors [99]. In this way, anti-GPCR autoantibodies can directly affect the receptor-mediated cell signaling cascade, which leads to physiological or pathological changes. The first functional autoantibodies against GPCR were discovered in the 1970s in sera of patients with Grave's disease [101]. The thyrotropin (TSH) receptor, a GPCR expressed on thyroid cells, plays an essential role in thyroid physiology and pathology. Autoantibodies against the TSH receptor bind to the receptor as an agonistic ligand, leading to the typical hyperthyroidism, the key feature in Graves' disease [102].

Regarding the binding site of autoantibodies against GPCRs, it has been suggested that stimulatory or agonistic autoantibodies bind to epitopes on either the 1st or the 2nd ECL, whereas, inhibitory or blocking autoantibodies recognize the 3rd ECL of the receptors [103]. After binding, autoantibodies against GPCRs might regulate receptor function via multiple mechanisms. First of all, the two antigen-binding Fab portions of anti-GPCR antibodies can mediate receptor dimerization via crosslinking, and thus activate the receptor. Second, the stimulatory effect of anti-GPCR antibodies can also be transduced via inhibiting of GPCR desensitization and internalization, thus leading to constant receptor-mediated signaling. Third, binding of autoantibodies against GPCRs can competitively inhibit the binding of physiological ligand of the receptors, mediating an antagonistic effect. Fourth, antagonistic effect of autoantibodies against GPCRs can also be mediated via receptor desensitization and internalization, which renders GPCRs unresponsive to their

physiological ligands [104]. Finally, autoantibodies against GPCR with synergistic effect show no effect by themselves, but the presence of those autoantibodies can amplify the effect of the physiological ligands of GPCRs [99].

Functional autoantibodies against GPCR also play an important role in pathological conditions. Besides aforementioned anti-TSH receptor antibodies, several other autoantibodies against GPCRs have been suggested to be associated with multiple human diseases.

β1 and β2 adrenergic receptors (AR) are GPCRs which recognize catecholamines, a type of neurotransmitters. It is not surprising that autoantibodies against β1-AR have been found in several heart-related diseases, including cardiomyopathy[105][106][107], arrhythmias [108] and myocarditis [109]. Autoantibodies against β2-AR are associated with Chagas' patients[106], allergic asthma [110], complex regional pain syndrome [111], Alzheimer's disease [112] and open angle glaucoma [113]. Autoantibodies against α1-AR, another member of adrenergic receptors[114], show up in diverse types of hypertension [115][116][117] and increased serum levels of autoantibodies against α1-AR have been observed in patients with Alzheimer's disease or type II diabetes mellitus [112] [118].

1.3.3. Functional autoantibodies against GPCRs in systemic sclerosis

SSc is featured by a panel of autoantibodies, including aforementioned ACA, ATA and anti-RNA polymerase III which are used as diagnostic markers for the disease[21]. Apart from these three classic autoantibodies, some other autoantibodies have been reported to be associated with SSc.

Anti-endothelial cell antibodies (AECA) are autoantibodies that bind and react to endothelial cells (EC). AECA were first reported in 1970s by Lindqvist and Osterland [119]. In patients with SSc, the prevalence of AECA is approximately 40%, and those autoantibodies are directly linked to vascular injury and endothelial cell damage[120] [121]. In line with this clinical evidence, *in vitro* findings have shown that AECA positive sera or purified IgG could bind to endothelial cells and cause endothelial cell damage and dysfunction [138][123][124]. Furthermore, *in vivo* transfer of AECA-positive sera from a humanized SSc model in UCD-200 chickens induce endothelial cell apoptosis in healthy chicken embryos [125]. Since endothelial cell damage and endothelial dysfunction is considered as an initial event in the pathogenesis of SSc, it is conceivable that AECA contribute to this process.

Autoantibodies against another cell type, fibroblast, have been also detected in SSc. In 2002, Chizzolini et al. reported that autoantibodies recognizing lung fibroblasts were found in 58% SSc

patient, with a higher frequency in dcSSc than in lcSSc [126]. Furthermore, IgG isolated from anti-fibroblast antibody positive SSc sera enhance the production of IL-6, IL-1α and IL-1β from fibroblast, while IgG isolated from control sera could not, suggesting a proinflammatory role of these autoantibodies [126].

However, it needs to be pointed out that the specific autoantigens of both AECA and anti-fibroblast antibodies are undefined. Therefore, it is not clear whether they are classic or functional autoantibodies. In addition to these two autoantibodies, several defined functional autoantibodies have been reported to be associated with SSc.

1.3.3.1. Anti-platelet-derived growth factor receptor antibodies

Platelet-derived growth factor receptor (PDGFR) is a surface tyrosine kinase receptor for members of the PDGF family. In 2006, Baroni et al. for the first time reported that autoantibodies against PDGFR with an agonistic effect were specifically detected in patients with SSc [127]. According to this study, IgG isolated from SSc patient sera could recognize native PDGFR and induce ROS production in mouse-embryo cell line specifically via PDGFR and tyrosine phosphorylation, while IgG from healthy control could not [127]. Furthermore, anti-PDGFR antibody positive SSc IgG could 1) increase ROS accumulation; 2) increase α–smooth-muscle actin (α-SMA) and collagen type I synthesis; 3) induce myofibroblast conversion from fibroblasts [127] [128] . Yamakage et al. showed that TGF-β could upregulate the expression of PDGFR and the sensitivity to PDGF stimulation in SSc fibroblasts [129]. Finally, besides their role on fibroblasts, stimulatory autoantibodies against PDGFR from SSc are able to induce the proliferation and migration of pulmonary vascular smooth muscle cells [130]. Taken together, previous studies have demonstrated that autoantibodies against PDGFR with an agnostic effect can be detected in SSc patients and those autoantibodies have the potential to mediate a fibrotic process.

1.3.3.2. Anti-endothelin1 type A receptor antibodies

Endothelin-1 type A receptor (ETAR) is a GPCR for endothelin, a peptide that play a role in potent and long-lasting vasoconstriction. In 2011, Riemekasten et al. reported that autoantibodies against ETAR were found in more than 80% patients with SSc, which is significantly higher than in healthy control and other autoimmune diseases including RA and primary Sjögren's syndrome (pSS) [131]. Furthermore, this study has demonstrated that high levels of autoantibodies against ETAR are associated with several disease manifestations, such as digital ulcers, lung fibrosis, PAH and disease mortality [131], suggesting that they are involved in the pathogenesis of SSc. This notion is supported by *in vitro* studies which show that anti-ETAR antibodies are able to exert effect on multiple type of cells [132][133]. For example, epithelial cells respond to anti-ETAR antibodies by

extracellular signal-regulated kinase 1/2 (ERK1/2) phosphorylation, Ca^{2+} accumulation, as well as an increased production of TGF-β, IL-8, IL-6 and CCL2 [132]. Autoantibodies against ETAR can also affect fibroblasts by increasing the synthesis of type I collagen in a dose-dependent manner [132]. Moreover, immune cells are also target of autoantibodies against ETAR, which is well exemplified by the observation that autoantibodies against ETAR are able to promote the migration of T cells and neutrophils. In addition, autoantibodies against ETAR can stimulate the secretion of IL-8 and chemokine (C-C motif) ligand 18 (CCL18) from peripheral blood mononuclear cells (PBMCs) [133]. In summary, functional autoantibodies against ETAR are present in patients with SSc and might contribute to the pathogenesis of the disease.

1.3.3.3. Anti-angiotensin II type 1 receptor (AT1R) antibodies

Anti-AT1R antibodies were found first in preeclamptic patients by Wallukat et al. in 1999 [134]. According to their study, autoantibodies against AT1R are present in 90% preeclamptic patients, but not in healthy control individuals. Moreover, IgG isolated from sera of preeclamptic patients stimulated AT1R on neonatal rat cardiomyocytes *in vitro*, while IgG isolated from healthy controls did not [134]. Notably, the agonistic effect of IgG from preeclamptic patients on rat cardiomyocytes could be inhibited by Losartan, suggesting the presence of functional autoantibodies against AT1R [134]. Subsequently, functional autoantibodies against AT1R were found some other diseases, including SSc [135][136] [131].

Interestingly, although AT1R and ETAR are two different GPCRs in terms of sequence similarity, ligand recognition and function, level of anti-AT1R antibodies are highly correlated with levels of anti-ETAR antibodies in SSc patients [131]. Similar to anti-ETAR antibodies, anti-AT1R antibodies are detected in the majority of patients with SSc and associated with an increased risk of multiple disease manifestations, including scleroderma renal crisis, lung fibrosis, PAH and disease mortality [131]. *In vitro,* autoantibodies against AT1R can promote the migration of neutrophils and endothelial cells, and increase the synthesis of collagen in fibroblast [132]. In addition, autoantibodies against AT1R also show stimulatory effect on PBMC in the secretion of IL-8 and CCL18 [133]. Ludwig et al. summarized the *in vitro* mechanisms of anti-AT1R and anti-ETAR autoantibodies-induced pathology in SSc, as shown in Figure 2 [98].

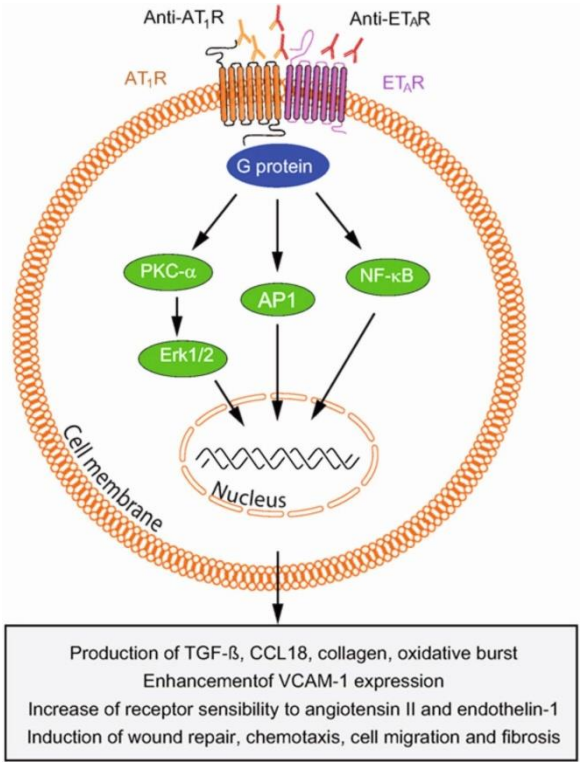

Figure 2. Summarized *in vitro* pathological mechanisms induced by anti-AT1R and anti-ETAR autoantibodies in systemic sclerosis (according to [98]**).** Abbreviations: PKC-α, protein kinase C-α; NF-κB, nuclear factor-κB; AP-1, activator protein 1.

Taken together, clinical and *in vitro* evidence suggest that autoantibodies against AT1R and ETAR are functional autoantibodies and might be pathogenic in SSc. This notion is further supported by an *in vivo* experiment, where Kill and colleagues showed that a transfer of IgG isolated from anti-AT1R and anti-ETAR antibody positive sera from SSc patient induced neutrophil accumulation in lungs of recipient mice [132]. However, the pathogenic role of these autoantibodies needs to be further verified *in vivo* using animal models, e.g. via active immunization.

1.4. Induced autoimmunity to AT1R in mice

Animal models are invaluable research tools for the investigation of human autoimmune diseases. Beside their usage in the exploration of pathogenic mechanisms and in the search for novel therapeutics, animal models can also help to explore the role of autoantigens in diseases [137]. For example, induction of autoimmune disease-like symptoms in animals by immunization with a potential autoantigen is an important criterion for identifying a disease-relevant autoantigen[138]. Consequently, immunizing animals with a GPCR could be a powerful strategy to determine the role of autoimmunity against the receptor *in vivo*.

However, induction of functional antibodies recognizing conformational epitopes of cell surface-expressed receptors is challenging since the proteins retain their physiological structure only in the context of the cell membrane. Immunization with peptides or recombinant proteins will largely induce antibodies to linear epitopes or conformational epitopes not present in the native molecule [139]. So far, genetic immunization of mice with cDNA of the target protein is the only strategy to circumvent this problem. An example for this is that the application of cDNA encoding the human thyrotropin receptor leads to the formation of functional antibodies against the receptor and a mild disease phenotype [140].

Very recently, our group has developed a novel strategy of immunization to induce functional autoantibodies against cell surface-expressed receptors. We immunized mice with membrane extracts from cells overexpressing human AT1R (hAT1R) emulsified in CFA. The immunization induced a strong immune response to hAT1R. Furthermore, the immunized mice developed disease manifestations resembling cardinal features of SSc, including skin perivascular infiltration, skin fibrosis and pulmonary inflammation (unpublished data). Immunizing mice with AT1R provides not only provide a novel mouse model for SSc, but also demonstrated that autoimmunity to AT1R is a pathogenic event, supporting a role of autoantibodies against AT1R in the pathogenesis of SSc. However, the function of autoantibodies against AT1R in this new mouse model need to be further characterized and investigated.

1.5. Aim of this study

As aforementioned, both clinical and experimental evidence indicate that autoimmunity to AT1R is involved in the development of experimental SSc and SSc patients. Based on this evidence, I hypothesize that autoantibodies against AT1R are functional and thus contribute to the development of SSc.

In this study I evaluated the function of autoantibodies against hAT1R. To reach this goal, the function of polyclonal antibodies (pAbs) against hAT1R isolated from hAT1R-immunized mice was analyzed. To specifically evaluate the function of defined anti-hAT1R antibodies, monoclonal antibodies (mAbs) directed to this receptor were generated via hybridoma technology [141] [142] [143] and characterized.

2. Materials and Methods

2.1. Materials

All materials are summarized in the below three tables which list consumable materials, chemicals and reagents, and equipment, respectively.

2.1.1. Consumable materials

Products	**Manufacturer**
0.2µm, 0.45µm filters	Merck, Germany
0.5 ml, 1.5 ml, 2.0 ml tubes	Sarstedt, Germany
15 ml, 50 ml tubes	Sarstedt, Germany
Cell culture plates (6-well, 24-well, 48-well, 96-well, flat-bottom)	Corning, USA
AT1R 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
Membrane Extract ELISA Screening plates	CellTrend, Germany
Microtiter plates (96-well, round-bottom)	Greiner Bio-One, Germany
Syringes (1ml, 20ml, 50ml)	Merck, Germany
T25, T75 cell culture flasks	Sarstedt, Germany

2.1.2. Chemicals and reagents

Products	**Manufacturer**
Aqua dest	B. Braun, Germany
BIOCHIP HEp-2 cells Slides	Euroimmun, Germany
BM Condimed H1 Hybridoma cloning Supplement(10X)	Roche, Germany
Bovine IgG standard	RCB, Germany
BSA, low endotoxin	PAN-Biochtech, Germany
CHO cell membrane extracts	CellTrend, Germany
Ciprofloxacin hydrochlorid	Fluka Biochemica, Germany
ColorpHast pH test strips	Merck, Germany
Complete Freund adjuvant	Sigma-Aldrich, USA

DIL SPE.	CellTrend, Germany
DMEM	Biochrom AG, Germany
Dylight 649 goat anti-mouse IgG antibody	Biolegend, USA
ELISA coating buffer	Thermo Fisher, USA
Fetal calf serum	PAN-Biochtech, Germany
Fluorodeoxyuridine	Sigma-Aldrich, USA
Glycine HCl buffer	Sigma-Aldrich, USA
Goat anti-Bovine IgG Ab-HRP	Life technologies, USA
Goat anti-Mouse IgG Ab-HRP	Jackson immunoreaearch, USA
Gold mounting agent with DAPI (with DAPI)	Thermo Fisher, USA
HAT supplement (50x)	Gibco, USA
HCl	Sigma-Aldrich, USA
Hemocytometer	Thermo Fisher, USA
HEPES	Merck, Germany
HiTrap Protein G Affinity column	GE Healthcare, USA
HT supplement (50x)	Gibco, USA
Human AT1R	CellTrend, Germany
Human IL-8 ELISA kit	Biolegend, USA
Incomplete Freund adjuvant	Sigma-Aldrich, USA
IsoStrip Mouse Monoclonal Antibody Isotyping Kit	Roche, Germany
LEAF IgG 2a isotype ctrl	Biolegend, USA
LEAF IgG 2b isotype ctrl	Biolegend, USA
L-Glutamin	PAA, Germany
Losartan Potassium	Sigma-Aldrich, USA
Lyophilized Protein A	GE, USA
Mouse IgG standard	RCB, Germany
NA/LE IgG 1 isotype ctrl	BD Bioscience, USA
NaOH	Sigma-Aldrich, USA
PEG 1500	Roche, Germany
Penicillin/Streptomycin-solution	PAN-Biochtech, Germany
Phosphate Buffer solution (Na2HPO4)	Merck, Germany
Red blood cell lysis buffer	Sigma-Aldrich, USA
Roti®Histofix 4% (paraformaldehyde, 4%)	Roth, Germany
Roti® Histokit II	Roth, Germany
Roti®-Safe GelStain	Roth, Germany

Roticlear	Roth, Germany
RPMI 1640 with HEPES	Gibco, USA
SM20-I medium	Merck, Germany
Sodium citrate	Sigma-Aldrich, USA
Sulfric acid (1 M H2SO4)	Merck, Germany
Tetramethylbenzidine (TMB)	Thermo Fisher, USA
Tris-HCl buffer	Sigma-Aldrich, USA
Triton X-100	Sigma-Aldrich, USA
Trypan blue	Chroma-gesellschaft schmidt&Co, Germany
Tween-20	Sigma-Aldrich, USA
Ultrafree Centrifugal Filter Device	Merck, Germany
Ultra-low IgG fetal calf serum	PAN, Germany
Ultrapure water	Merck, Germany
Valsartan	Sigma-Aldrich, USA

2.1.3. Equipment

Equipment	**Manufacturer**
4°C, -20°C, -80°C refrigerator	Thermo Fisher, USA
Biosafety cabinet	Heraeus, Germany
Carbon dioxide cell incubator	Thermo Fisher, USA
Confocal fluorescence microscope SP5	Leica, Germany
Fast protein liquid chromatography	Pharmacia Biotech, Sweden
Microplate Reader	Tecan life science, Switzerland
MiniPerm classic Bio-reactor	Sarstedt, Germany
MiniPerm Nutrition Module	Sarstedt, Germany
MiniPerm Production Module	Sarstedt, Germany
Nanodrop 1000 spectrophotometer	Thermo Fisher, USA
Phase contrast microscope	ZEISS, Germany
Super-centrifuge	Hettich, Germany
Thermostat water bath	Julabo, Germany
Universal Turning Device	Sarstedt, Germany

2.2. Methods

2.2.1. Induction of anti-hAT1R polyclonal antibodies in mice

Eight-nine weeks old female C57BL/6J mice were immunized with 0.2 mg membrane extracts isolated from CHO cells overexpressing hAT1R in 50 µl PBS emulsified with equal volume of complete Freund adjuvant (CFA) via subcutaneous injection to the footpad at day 0. Three weeks after the primary immunization, mice were boosted with 0.2 mg hAT1R in 50 µl PBS, emulsified with 50 µl incomplete Freund adjuvant (IFA). In the control group, mice were treated in the same procedure with same amount of control membrane extracts isolated from normal CHO cells (ME). Nine weeks after the first immunization, all mice were sacrificed and blood samples were collected for IgG isolation. All animal studies were approved by the Animal Research Ethics Board of the Ministry of Energy Change, Agriculture, Environment, Nature, and Digitalization, Kiel, Germany (Az V 241 – 47120/2017 (104-8/17), from October 5th, 2017).

2.2.2. Isolation of anti-hAT1R polyclonal antibodies

After standing without anticoagulants for 30 min at room temperature, blood samples collected from mice immunized with hAT1R or ME control were centrifuged at 6000g for 10 mins. Then, supernatants were collected as serum sample. Sera from untreated healthy female C57BL/6J mice were also collected using the same procedure. To isolate IgG, serum samples were first diluted with equal amount of PBS and mixed at 4 °C for 1 hour. The well mixed samples were filtered by passing through 0.45µm filter and then applied onto HiTrap protein G Affinity column. Subsequently, the sera-loaded columns were washed with PBS and eluted with 0.1M glycine HCl buffer (pH 2.7). Finally, the eluted IgG fractions were neutralized with 1 M Tris-HCl buffer (PH 9.0) to a neutral PH value, and the IgG concentrations were determined photometrically at a wavelength of 280 nm using Nanodrop 1000 spectrophotometer.

2.2.3. Enzyme-linked immunosorbent assay (ELISA)

Ninety-six well microtiter plates pre-coated with membrane extracts isolated from CHO cells overexpressing hAT1R or membrane extracts isolated from normal CHO cells were used for ELISA to detect antibodies against hAT1R or control ME in sera or cell culture supernatant. Diluted sera or cell culture supernatants were added onto plate and incubated for 2h at 4 °C. The microtiter plates were washed by PBS-Tween for 3 times and incubated with goat anti-mouse IgG-HRP antibody (1:5000 dilution) for 1 hour at room temperature. Bound antibodies were visualized by colorimetric reaction to 0.1 ml TMB added to each well as the substrate of peroxidase, and the

TMB-peroxidase reaction was terminated by 1 M H_2SO_4. Finally, optical density (OD) values were determined at 450/620nm by Microplate Reader [131] .

2.2.4. Generation of anti-AT1R monoclonal antibodies

2.2.4.1. Immunization of mice

Eight weeks old female C57BL/6J mice were immunized with 0.2 mg hAT1R membrane extracts in 50 µl PBS emulsified with 50 µl complete Freund adjuvant (CFA) via subcutaneous injection to the footpads. Two weeks later, animals were boosted with 0.2 mg hAT1R membrane extract in 50 µl PBS emulsified with 50 µl incomplete Freund adjuvant (IFA). At day 34 after the first immunization, mice were boosted again with 0.1 mg hAT1R membrane extract in 100 µl PBS. At day 36 after the first immunization, mice were sacrificed. Serum, spleen and two draining lymph nodes were collected for further experiments.

2.2.4.2. Fusion of myeloma cells with mouse lymphocytes

The immortal mouse myeloma cell line (Ag8.653) was cultured in DMEM supplemented with 1% L-glutamine, 1% Penicillin/Streptomycin-solution (PEST) and 10% fetal calf serum at 37°C in 5% CO_2. Spleen and draining lymph nodes from the immunized mouse were dissociated in PBS and filtered by 70 µm cell strainer to prepare a single cell suspension. After lysis of red blood cells (RBC) by incubation in RBC lysis buffer, the single cell suspension was washed twice and then suspended in RPMI 1640 medium. Subsequently, cell fusion between mouse lymphocytes and myeloma cells (Ag8. 653) was conducted in the presence of polyethylene glycol (PEG). Briefly, the suspended myeloma cells were added to the lymphocytes (ratio of 1:1). Cells were centrifuged at 300 g for 10 min and the supernatant was discarded. The cell pellet (2 x 10^8 cells) was then warmed at the 37 °C in water bath, and 1ml pre-warmed PEG 1500 was added to the pellet. Cells were gently rotated for 1 min to facilitate the fusion. In the next step, 5 ml warm RPMI 1640 medium was stepwise added to the suspension over a time period of 5 min to dilute the PEG, followed by addition of further 40 ml warm RPMI 1640 medium. Finally, cells were centrifuged at 300 g for 10 min, and cells were used for further culture [141] [143].

2.2.4.3. Hybridoma culture

After cell fusion, cells were counted by the hemocytometer and resuspended in HAT selection medium in which only the myeloma-B hybrid cells can grow [144]. (DMEM supplemented with 1% L-glutamine, 1x HAT solution and 10% fetal calf serum) at a concentration of 5 x 10^5 / ml. Cells were seeded into fusion plates with 1 ml cell suspension/well and cultured at 37°C in 10% CO_2 [145].

2.2.4.4. Hybridoma screening

After primary hybridoma clusters became visible macroscopically, hybridoma screening was conducted to single out anti-AT1R specific monoclonal antibodies-secreting hybridomas. Briefly, supernatants collected from fusion wells were tested by hAT1R ELISA. Unspecific binding was determined by the reaction of supernatants to plates covered with control ME. Samples with high OD450 values (>0.5) on hAT1R ELISA plate and low OD 450 values (<0.1) on control ME ELISA plate were chosen for further selections.

2.2.4.5. Hybridoma subcloning

Cells from cultures with high reactivity to hAT1R but low to ME controls were chosen for subcloning. Each hybridoma cluster in promising fusion wells was picked out by 20µl pipette tip and seeded into a separate single well in 96-well plates and culture was continued in 200 µl HAT selection medium. When subclones proliferated to large cell clusters, supernatants were collected and retested by ELISA.

2.2.4.6. Limiting dilution

Hybridoma clones chosen from subcloning were resuspended in culture medium and counted by a hemocytometer. Then cells were diluted and cultured at concentrations of 10, 5, 2, 1, 0.5 cells/ well, in 200 µl HAT selection medium. When hybridoma cell proliferation was observed, respective supernatants were collected and tested by ELISA. Hybridoma clones positive for anti-AT1R IgG but negative for anti-ME IgG derived from wells with the lowest cell concentration was selected for next limiting dilution. Limiting dilutions were repeated 5 to 7 rounds until all wells containing hybridoma clusters were positive for anti-hAT1R antibodies [146] [145].

During the limiting dilution, hybridoma culture medium was slowly changed from HT medium (DMEM supplemented with 1% L-glutamine, 1x HT solution and 10% fetal calf serum) to normal DMEM medium (DMEM supplemented with 1% L-glutamine and 10% fetal calf serum).

2.2.4.7. Determination of mAbs subclass and light-chain composition

Subclass and light-chain composition of monoclonal antibodies were analyzed by IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche) according to the manufacturer's instruction. Briefly, culture supernatants from hybridoma cell lines were diluted 1:10 in 1% BSA and well mixed. Then one isotyping strip was put into the detection tube which contains 150 µl diluted culture supernatants, and incubated for 10 min until the color changed to blue at the respective position on the strip indicating the different subclass and light-chain of the corresponding monoclonal antibody.

2.2.4.8. mAbs large-scale production

A bioreactor (MiniPerm classic Bio-reactor) was used for the large-scale production of monoclonal antibodies. Hybridomas were suspended at the concentration around $1x10^6$/ml in 38 ml fresh culture medium (DMEM supplemented with 1% L-glutamine and 10% fetal calf serum) and

cultured in a Production Module of the reactor. The Nutrition Module connected to the Production Module was filled with 350 ml DMEM medium (DMEM supplemented with 1% L-glutamine and 5% fetal calf serum). Hybridomas were cultured at 37°C in 10% CO_2 with low speed persistent rotation on a Universal Turning Device.

Culture medium in the Nutrition Module was renewed every second day. Hybridoma cells were counted every day. When the cell concentration surmounting 5 $x10^6$/ml were reached, cell suspensions were taken out by a sterile syringe and centrifuged at 300 g for 10 min, then 700 g for 25min and culture supernatants were collected for further purification of the antibodies. [147]

2.2.4.9. Purification of antibodies

The column for monoclonal antibody purification were prepared according to the manufacturer's instruction. 1.5g lyophilized Protein A powder was dissolved in 50 ml Aqua dest (ultrapure) and equilibrated for 15 min at room temperature. Then the bead suspension was centrifuged at 300 g for 3 min and washed with 50ml distilled water for 3 times. The pellet was suspended in 50ml PBS and transferred into a glass column and stored at 4°C until use.

To purify monoclonal antibodies, culture supernatants were diluted with an equal amount of PBS and subsequently filtered through a 0.45µm filter. Diluted samples were then applied to the Protein A column connected to a fast protein liquid chromatography (FPLC) system. The loaded column was washed with PBS and eluted with Na-citrate buffer (pH 3-5) and eluted fractions were collected. The concentration of the eluted IgG in each fraction was determined photometrically at λ 280nm using a Nanodrop 1000 spectrophotometer.

To concentrate monoclonal antibodies, the eluted IgG fractions were applied to the Centrifugal Filter Device (0.1 µm pore size) and centrifuged at 5000 g until the required concentrate volume of IgG fractions was achieved. Finally, the concentrated antibody solutions were neutralized to pH 7.0 by NaOH or HCl.

2.2.4.10. Determination of bovine IgG

A competition ELISA was used to determine bovine IgG in purified mouse monoclonal antibody solutions. Microtiter plates were precoated with 100 µl bovine IgG solution (400ng/ml) overnight at 4°C, and then blocked with 1% BSA in PBS at 37°C for 2 h. Then diluted monoclonal antibody solutions or bovine IgG served as standard samples were preincubated with equal volume of 1: 2000 diluted goat anti-bovine IgG-HRP antibody in microtiter plates for 30 min at 37°C. 100 µl preincubated samples were added into the microtiter plates precoated with Bovine IgG, and incubated for 1 h at 37 °C. The microtiter plates were washed by PBS-Tween for 3 times. Bound antibodies were visualized by colorimetric reaction to 0.1 ml TMB added to each well as the substrate of peroxidase, and the TMB-peroxidase reaction was terminated by 1 M H_2SO_4. Finally,

optical density (OD) values were recorded at 450/620nm by Microplate Reader and the concentration of bovine IgG was determined according to the standard curve of bovine IgG run in parallel.

2.2.5. Immunofluorescence staining

Binding of polyclonal antibodies against hAT1R isolated from mouse sera and monoclonal antibodies against hAT1R purified from hybridoma culture supernatants to human epithelial type 2 (HEp-2) cells were determined using indirect immunofluorescence staining. BIOCHIP slides precoated with HEp-2 cells were incubated with antibodies (20µg/ml) for 30 min at room temperature (RT). Then the slides were rinsed by PBS-Tween, incubated with Dylight 649 labeled goat anti-mouse IgG antibody for 30 min to detect the binding of pAb, and mounted with Coverslips using Gold mounting agent with DAPI. Finally, fluorescent signals were determined using confocal fluorescence microscope.

2.2.6. Cardiomyocytes beating assay

The assay for functional activities of antibodies on cardiomyocytes was performed in cooperation with BerlinCures, Berlin, Germany. Cardiomyocytes prepared from hearts of neonatal rats were cultured as monolayer for 4 to 10 days at 37°c in SM20-I medium supplemented with 10% heat inactivated neonatal calf serum and 2 µM fluorodeoxyuridine. To determine the effect of anti-AT1R antibodies on cardiomyocytes, cultured cardiomyocytes were treated with anti-AT1R antibodies and corresponding controls. The beating rate of the cells were measured at a heated stage (37°C) of an inverted microscope and the basal beating rate was determined at 6 marked fields on the bottom of the culture flask for 15 sec. One hour after the treatment of the autoantibodies, the beating rate of cardiomyocytes was measured again. The effects of the autoantibodies were expressed as “increase of number of beats / 15 sec”.[148]

2.2.7. IL-8 release assay

Human monocyte cell line (THP1) were cultured at 37°C in RPMI 1640 supplemented with HEPES and 10% Ultra low IgG- fetal calf serum. To determine the effect of anti-AT1R antibodies on monocytes, THP1 cells were seeded in 48 well plates at the concentration of $8.3x10^6$/ml and anti-AT1R IgG or corresponding control IgG was added and cells were incubated for 24 hours. To

determine the specificity of the effect, in some samples 1 μM Losartan potassium was added 5 hours before the antibody treatment. Cell culture supernatants were collected 24 hours after antibody stimulation and amounts of IL-8 produced were detected by ELISA[149].

IL-8 concentrations in the culture supernatants were determined by a Human IL-8 ELISA kit according to the manufacturer's instruction. Briefly, supernatant samples were incubated in ELISA plates pre-coated with anti-human IL-8 capture antibody for 2 hours at room temperature, washed with PBS-Tween for 3 times, incubated with anti-human IL-8 detection antibody for 1 hour, and further incubated with Avidin-HRP for 30min, Subsequently, bound antibodies were visualized by colorimetric reaction to 0.1 ml TMB added to each well as the substrate of peroxidase, and the TMB-peroxidase reaction was terminated by 1 M H_2SO_4. Finally, optical density (OD) values were recorded at 450/620nm by Microplate Reader and the concentration of IL-8 was determined according to the standard curve of IL-8 run in parallel.[133]

2.2.8. Statistical analysis

All data are expressed as the mean ± SD. For quantitative data in normal distribution, statistical analysis was performed with unpaired Student’s t test or one-way ANOVA test. For quantitative data that did not follow Gaussian distribution, Mann-Whitney U test was applied. Fisher`s test was used to assess the significance of qualitative data. P values less than 0.05 were considered as statistically significant.

3. Results

3.1. Generation and Characterization of polyclonal antibodies against hAT1R

3.1.1. Generation of anti-hAT1R polyclonal antibodies

Nine weeks after the first immunization with hAT1R or control ME, mouse sera were collected for the isolation of pAbs. In total, 1.3 ml and 1.5 ml sera were collected from hAT1R-immunized mice and control ME-immunized mice, respectively. In addition, 0.4 ml sera from untreated control C57BL/6J mice were also collected (Table 1). Total IgG from serum samples was isolated by protein G-Sepharose column. Finally, fractions of 8.72 mg, 14.98 mg, and 0.29 mg IgGs were isolated from hAT1R-immunized, control ME-immunized, and untreated mice, respectively. Purified IgG fractions were adjusted to 1.0 mg/ml in PBS, sterilized by passing through 0.22 μm filters, and stored at -80 °C for further use.

Table 1. Generation of anti-hAT1R and anti-ME polyclonal antibodies.

Group of mice	Volume of mouse sera (ml)	pAb amount (mg)
Unimmunized	0.4	0.29
control ME immunized	1.5	14.98
hAT1R immunized	1.3	8.72

3.1.2. Binding of anti-hAT1R polyclonal antibodies to epithelial cells

In a first step binding of polyclonal IgG isolated from hAT1R-immunized mice to AT1R-expressing human epithelial cells (HEp-2 cell line) was determined by immunofluorescence staining. Polyclonal IgG isolated from hAT1R-immunized mice bound to the membrane of HEp-2 cells, while polyclonal IgG from control ME-immunized mice or from unimmunized mice did not (Figure 3), suggesting that anti-hAT1R IgG are able to bind the receptor on the membrane.

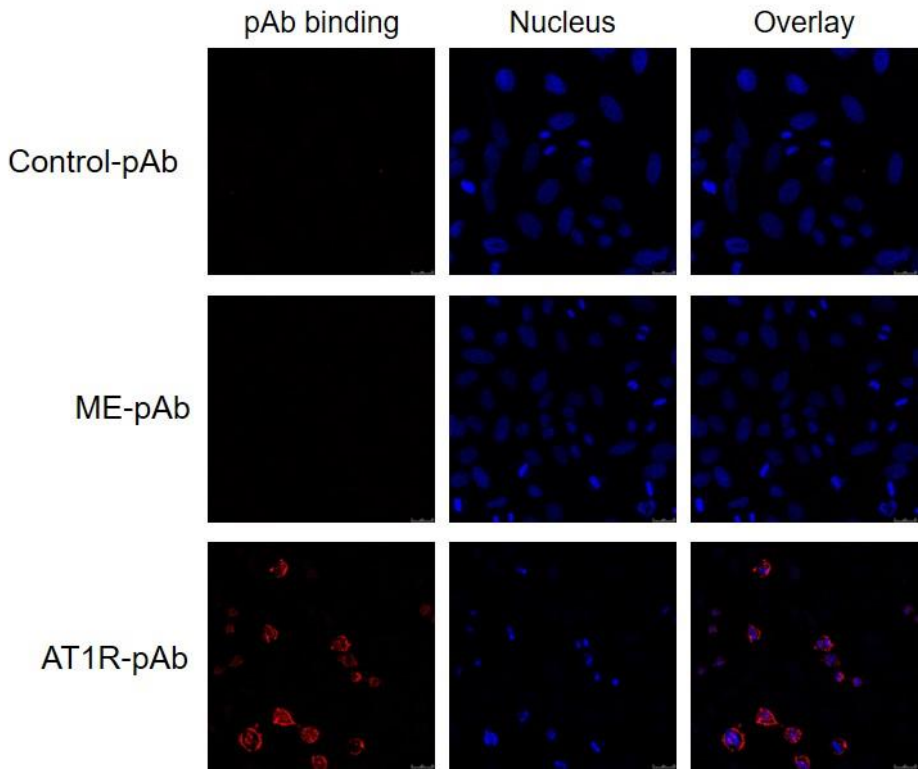

Figure 3. Binding of anti-hAT1R polyclonal antibodies (pAb) to human epithelial type 2 cells (HEp-2). HEp-2 cells on BIOCHIP slides were incubated with 20µg/ml polyclonal IgG from untreated control mice (control pAb), control ME-immnuized mice (ME-pAb) or hAT1R-immunized mice (AT1R pAb) for 30 min at RT. The binding of mouse IgG to the cell membrane was detected by using immunofluorescent staining with Dylight 649 labeled goat anti-mouse IgG antibody. Red signal indicate bound IgG, while blue signal (DAPI) indicate nuclear DNA.

3.1.3. Anti-hAT1R polyclonal antibodies activate cardiomyocytes

Given that anti-hAT1R polyclonal IgG are able to bind to the receptor, in the next step the capacity of the IgG acting as a ligand of AT1R was investigated. It has been shown that AT1R is expressed on cardiomyocytes and the activation of AT1R leads to an increased cell beating rate [99][150][151]. In the first step, the capacity of anti-hAT1R polyclonal IgG to affect the beating rate of rat cardiomyocytes was determined. As compared to the control, polyclonal IgG from hAT1R-immunized mice significantly increased cell beating rate by 4 times/15 sec., while polyclonal IgG from control ME-immunized mice showed no effect (Figure 4A). To verify that the stimulatory effect of the anti-hAT1R polyclonal IgG is mediated specifically via AT1R activation, cardiomyocytes were stimulated by anti-hAT1R polyclonal IgG in presence or absence of Losartan, a specific inhibitor of AT1R. As shown in Figure 4B, the stimulatory effect of anti-hAT1R polyclonal IgG was completely blocked by Losartan, suggesting that the stimulatory effect of the antibodies is mediated by activation of AT1R.

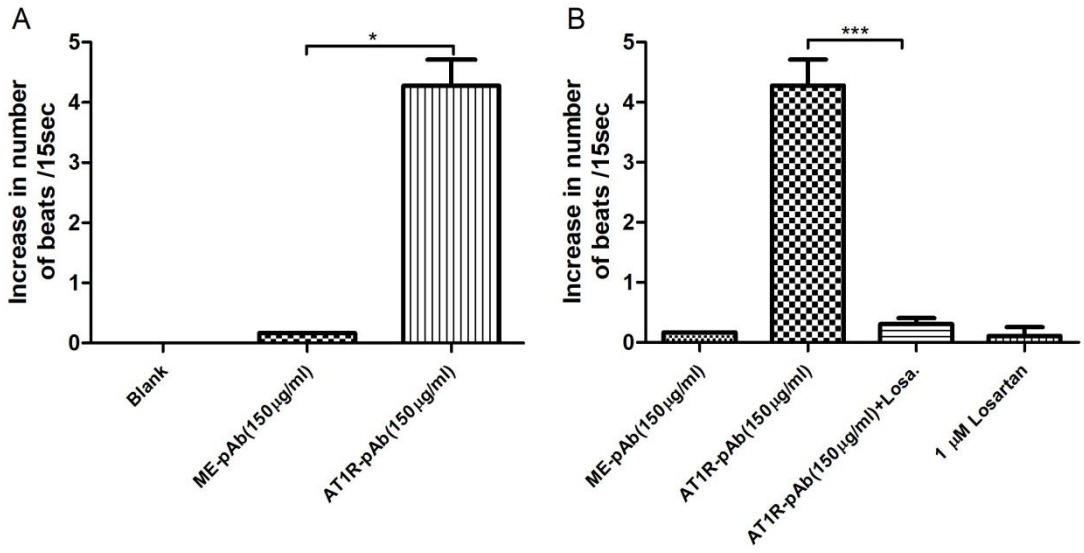

Figure 4. Effect of anti-hAT1R IgG polyclonal IgG on cardiomyocytes. Rat cardiomyocytes were cultured with polyclonal IgGs in presence or absence of 1 μM Losartan, and cell beating rate was measured as the readout. **(A)** Effect of anti-hAT1R polyclonal IgG (AT1R-pAb) and anti-control ME polyclonal IgG (ME-pAb) on the beating rate of cardiomyocyte. **(B)** Losartan blocked the stimulatory effect of anti-hAT1R polyclonal IgG on the beating rate. Result are presented as mean ± SD (n=4), and statistical analysis was performed using unpaired t test (* = $p<0.05$, *** = $p<0.001$.)

3.1.4. Anti-hAT1R polyclonal antibodies promote IL-8 release from monocytes

To confirm that anti-hAT1R polyclonal IgG are able to activate AT1R, human monocytic THP-1 cells were stimulated with the different IgG fractions described in 3.1.1. As compared to cells treated with anti-ME polyclonal IgG and control polyclonal IgG, cells treated with anti-hAT1R polyclonal IgG produced significant higher level of IL-8, suggesting a stimulatory role of anti-hAT1R polyclonal IgG on monocytes (Figure 5A). Furthermore, the promoting effect of anti-hAT1R polyclonal IgG on IL-8 production from monocytes could be blocked by Losartan, demonstrating that the effect is due to the action of functional anti-hAT1R IgG on AT1R (Figure 5B).

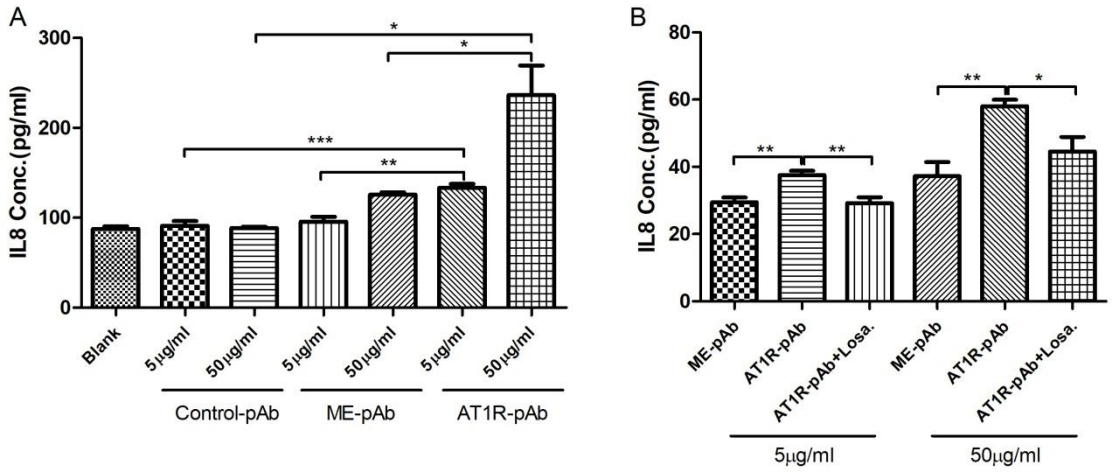

Figure 5. Agonistic effect of anti-hAT1R polyclonal IgG on human monocytes. Human monocytic THP-1 cells were cultured with IgGs at the concentrations indicated in presence or absence of 1 μM Losartan for 24 hours. The concentration of IL-8 in the supernatant was determined by ELISA. **(A)** Effect of anti-hAT1R polyclonal IgG (AT1R-pAb), anti-control ME polyclonal IgG (ME-pAb) and control IgG (control pAb) on monocytes. **(B)** Losartan blocked the stimulatory effect of anti-hAT1R polyclonal IgG on IL-8 production from monocytes. Data are presented as mean ± SD (n=4), and statistical analysis was performed using unpaired t test (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.)

Taken together, polyclonal IgG isolated from hAT1R-immunized mice bind to membrane receptors, activate rat cardiomyocytes as well as human monocytes in an AT1R-dependent manner.

3.2. Generation and characterization of monoclonal antibodies against hAT1R

The use of polyclonal antibodies derived from mouse sera for functional studies has several disadvantages. On one hand, antibodies specifically recognizing AT1R represent only a small percentage of the total IgG isolated from the sera. On the other hand, polyclonal IgG against an antigen are composed of IgGs recognizing different epitopes of the antigen and are most likely a mixture of stimulatory and non-stimulatory antibodies [152] [153]. The low purity and high heterogeneity of those polyclonal IgGs make it difficult to apply them to the investigation of antigen specific IgG. For these reasons, generation of monoclonal antibodies specifically targeting single epitope of AT1R and the characterization of such mAbs were conducted.

3.2.1. Generation of hybridoma clones

Hybridoma technique was used to generate anti-AT1R mAbs. At day 36 after the first immunization with membrane extracts isolated from CHO cells overexpressing hAT1R, serum

sample from the immunized mouse was collected for antibody detection. Antibodies against hAT1R were detected by ELISA. The hAT1R-immunized mouse developed high levels of IgG against hAT1R, while the serum did not show any reactivity to control ME (Figure 6), suggesting antigens present in the control extract did not induce a relevant immune response.

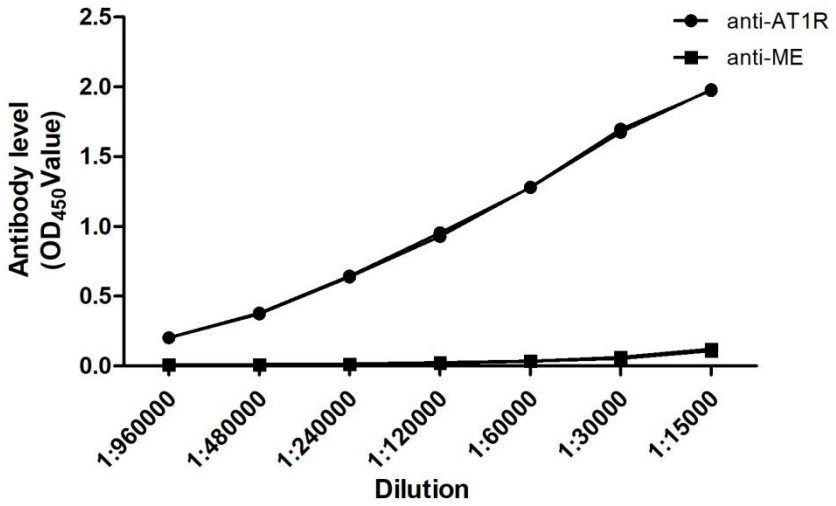

Figure 6. Antibody production in a hAT1R-immunized mouse. ELISA plates coated with hAT1R or control ME were used for antibody detection. A serum sample of the hAT1R-immunized mouse was diluted and incubated with hAT1R or control ME coated ELISA plates for 2h at 4 °C, then goat anti-mouse IgG-HRP was used as the 2nd antibody for a 1h incubation at RT with agitation. Binding of the secondary antibody was visualized by a colorimetric reaction to TMB and expressed as optical density (OD).

Mice immunized with hAT1R were used as starting material for the collection of lymphocytes from the mouse and the generation of hybridoma cells. Spleen and two inguinal lymph nodes of the mouse were collected for the preparation of single cell suspension. In total, 76.1 x 10^6 lymphocytes were isolated from the hAT1R-immunized mouse. In the next step, equal amounts of mouse myeloma cells (Ag8.653) were fused with the isolated lymphocytes in the presence of PEG. After incubation, 75 x 10^6 fused hybrid cells were further cultured with HAT selection medium in 150 fusion wells.

Ten days after the fusion, hybridoma clusters were observed in most wells. Supernatants from each fusion well were then collected for detecting immune reactivity against hAT1R and control ME by ELISA. Cells derived from supernatants with high reactivity to hAT1R and low reactivity to control ME were selected as desired candidates. An overview of the reactivities of supernatants from all 150 fusion wells to hAT1R and control ME is depicted in Figure 7. Most supernatants showed a similar reactivity to hAT1R and control ME. Only 5 out of 150 fusion wells exerted high

anti-hAT1R bindings but low interaction with control ME. These five fusion wells were well no. 2.10, 3.24, 5.2, 6.7, and 7.6.

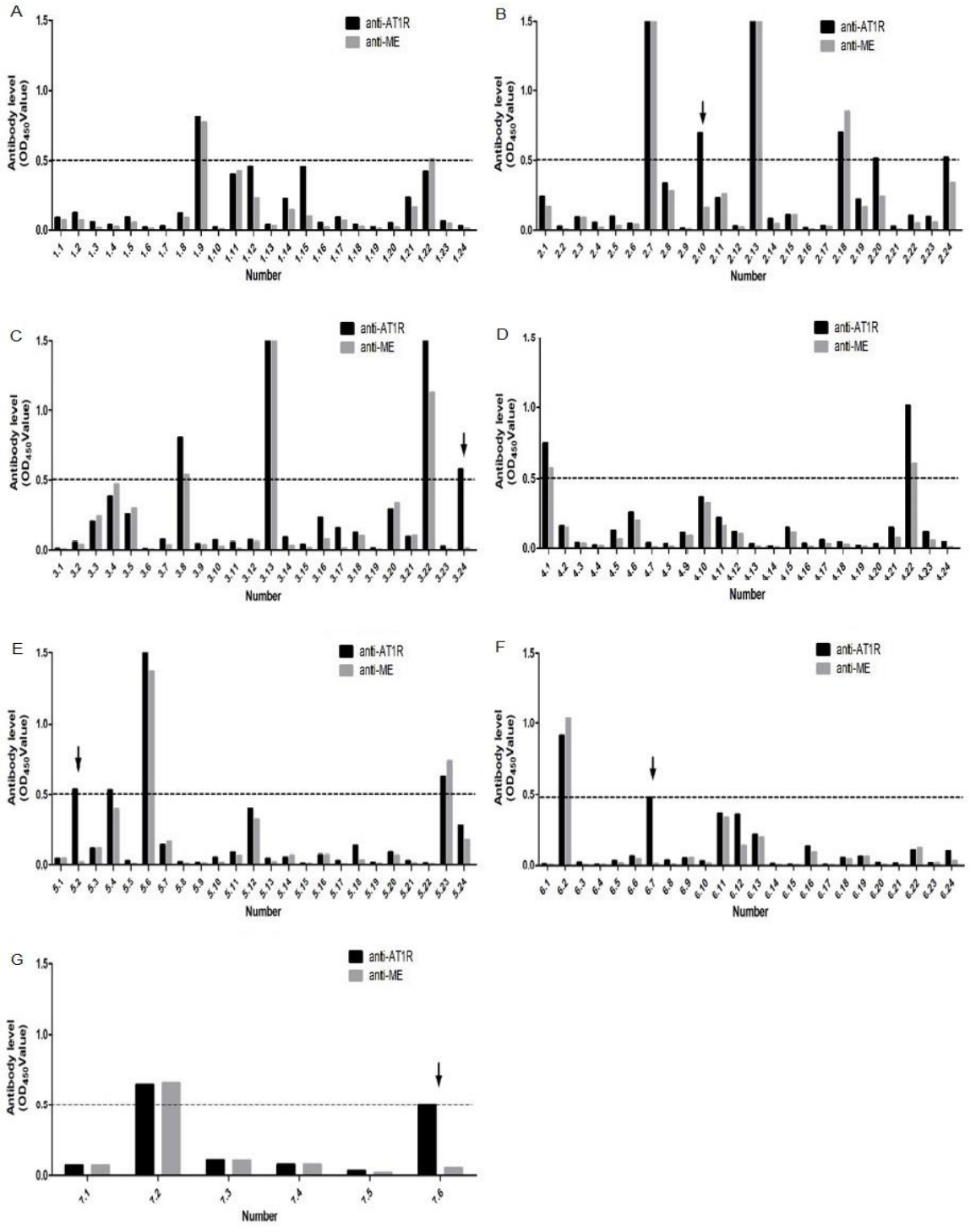

Figure 7. IgG reactivity to hAT1R and control ME in supernatants derived from 150 fusion wells. Hybridoma culture supernatants from 150 fusion wells on 7 plates (plate 1 to 7 (A to G)) were determined for their IgG reactivities to hAT1R (anti-AT1R) and control ME (anti-ME) by ELISA. The X-axis indicates the code of fusion wells, while the Y-axis presents the IgG reactivities to hAT1R and control ME. Arrows show desired fusion wells with high reactivities to hAT1R but low reactivities to control ME.

Since each of the five candidate wells contained multiple hybridoma clusters, hybridoma sub-cloning was further performed. In total, 49 hybridoma clusters were picked out from the 5 fusion wells and cultured on 96-well plates. Eight days after the culture, the 49 hybridoma clones proliferated and formed large clusters. Supernatants from each of 49 wells were then collected to determine their IgG reactivity to hAT1R and control ME by ELISA. As shown in Figure 8, IgG reactivities to hAT1R among 49 hybridoma clones varied considerably, ranging from low or no reactivity to high reactivity. It should be mentioned that most supernatants showed higher IgG reactivities to hAT1R than to control ME. Finally, 4 of hybridoma clones which showed high anti-hAT1R IgG levels but low anti-ME IgG levels were selected for the next step.

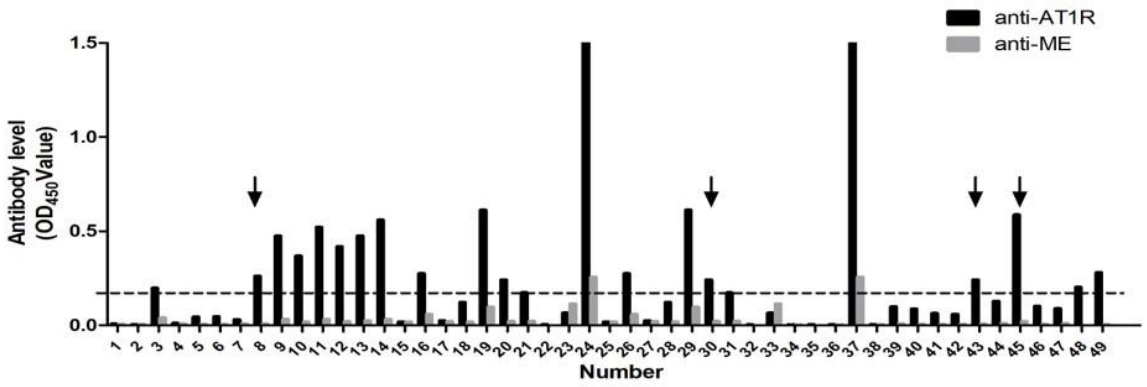

Figure 8. IgG reactivities to hAT1R and control ME in supernatants of 49 hybridoma clones. Culture supernatants of 49 hybridoma clones were determined for their IgG reactivities to hAT1R(anti-AT1R) and control ME (anti-ME) by ELISA. The X-axis indicates the No. of hybridoma clones, while the Y-axis presents the IgG reactivities to hAT1R and control ME. Arrows show desired fusion wells with high reactivities to hAT1R but low reactivities to control ME.

The 4 selected hybridoma clones, 2.10.13, 5.2.6, 6.7, and 7.6.2 were subjected to limiting dilutions to obtain wells seeded with single cells on 96-well cell culture plates. Ten-to-fifteen days after the culture, supernatants from wells with hybridoma clusters were tested for IgG reactivities to hAT1R. As shown in Figure 9, all wells of 5.2.6 hybridoma clone were positive for anti-hAT1R IgG, while a very small portion of wells of the other three clones were anti-hAT1R IgG positive. In detail, all 128 tested wells of the 5.2.6 clone were positive for IgG against hAT1R, while 25 out of 256 tested wells (9.77%) of the 2.10.13 clone, 1 out of 43 test wells (2.33%) of the 6.7 clone, and 1 out of 12 tested wells (8.33%) of the 7.6.2 clone were positive for IgG against hAT1R (Table 2). From those anti-hAT1R IgG positive wells, one well from each hybridoma clones were selected for the 2nd round limiting dilution.

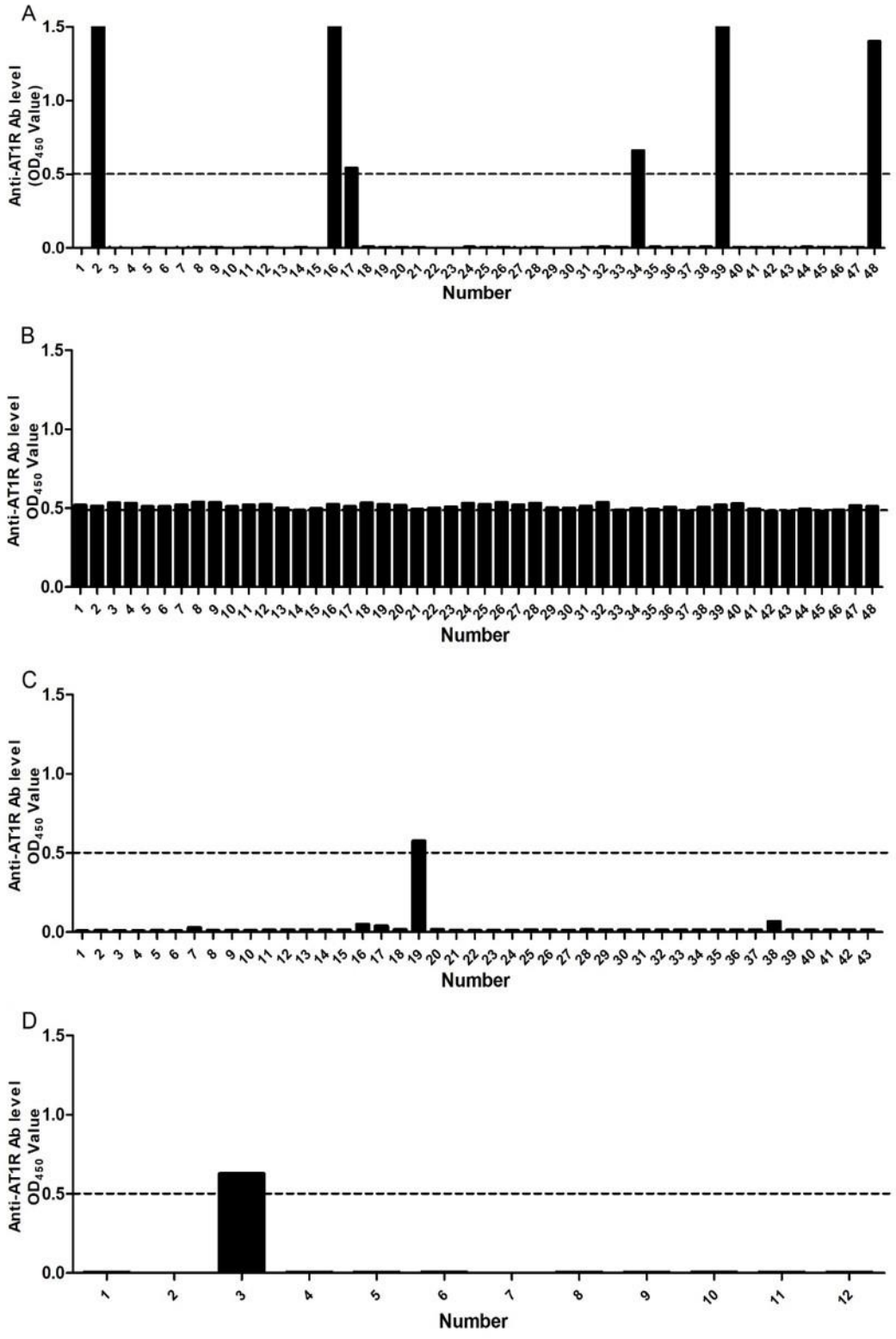

Figure 9. IgG reactivities to hAT1R in supernatants of hybridoma cells in the 1st round of Limiting dilution. Culture supernatants from wells of 2.10.13 **(A)**, 5.2.6 **(B)**, 6.7 **(C)** and 7.6.2 **(D)** hybridoma cells were tested for their IgG reactivities to hAT1R. Representative samples are shown. The X-axis indicates the No. of wells, while the Y-axis presents the IgG reactivities to hAT1R.

Table 2. Summary of the hybridoma clones secreting anti-hAT1R antibodies from the 1st round of limiting dilution.

Hybridoma clone	Number of wells tested	Number of positive wells	Positive rate (%)
2.10.13	256	25	9.77
5.2.6	128	128	100
6.7	43	1	2.33
7.6.2	12	1	8.33

The four wells 2.10.13.58, 5.2.6.8, 6.7.51 and 7.6.2.65 were subjected to the 2nd limiting dilution. Fifteen days later, supernatants from wells with hybridoma clusters were collected and tested for IgG reactivities to hAT1R using ELISA. Table 3 summarizes results of all four hybridoma clones, and representative results are showed in Figure 10. For the 2.10.13.5 clone, 93 out of 143 tested wells (65.03%) were anti-hAT1R IgG positive, in which the positive rate was considerably increased. In contrast to a 100% positive rate of anti-AT1R IgG in the first round limiting dilution, the 5.2.6.8 clone only showed a positive rate of 71.63% (53 out of 74 tested wells). The positive rate of anti-AT1R IgG in the 7.6.2.65 clone also considerably increased to 73.49% (122 out of 166 tested wells). Unfortunately, none of 150 tested wells of the 6.751 clone was anti-AT1R IgG positive, which led to the loss of this hybridoma clone. Finally, one well from each of 2.10.13.58, 5.2.6.8 and 7.6.2.65 clones were chosen for the 3rd round limiting dilution.

Table 3. Summary of the hybridoma clones secreting anti-hAT1R antibodies from the 2nd round of limiting dilution.

Hybridoma clone	Number of wells tested	Number of positive wells	Positive rate (%)
2.10.13.58	143	93	65.03
5.2.6.8	74	53	71.62
6.7.51	150	0	0
7.6.2.65	166	122	73.49

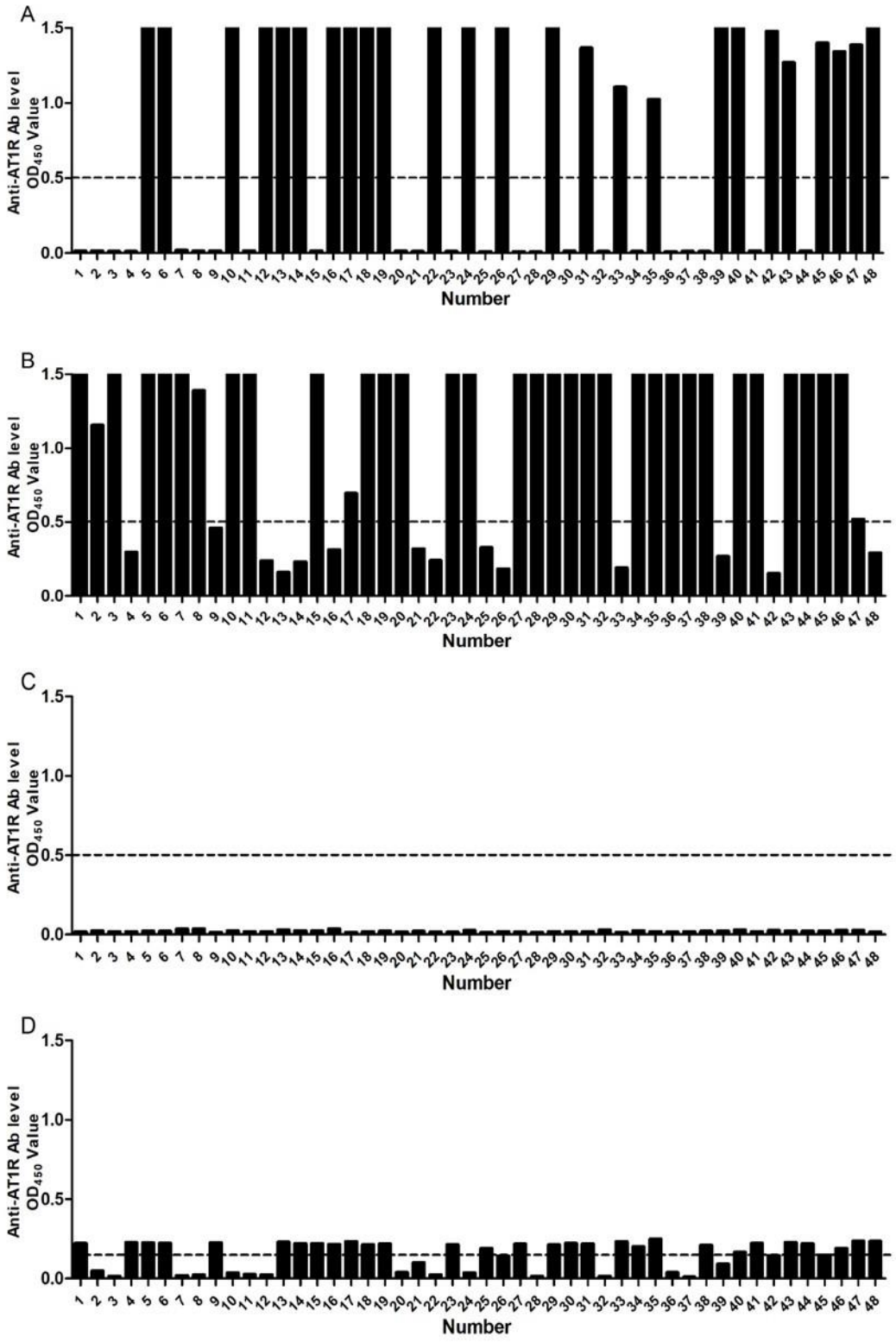

Figure 10. IgG reactivities to hAT1R in supernatants of hybridoma cells in the 2nd round of limiting dilution. Culture supernatants from wells of 2.10.13.58(**A**), 5.2.6.8(**B**), 6.7.51(**C**) and 7.6.2.65 (**D**) hybridoma cells were tested for their IgG reactivities to hAT1R. Representative samples are shown. The X-axis indicates the No. of wells, while the Y-axis presents the IgG reactivities to hAT1R.

The three selected wells, 2.10.13.58.19; 5.2.6.8.12; 7.6.2.65.1, were subjected to the 3rd limiting dilution. Fifteen days after the culture, supernatants from wells with hybridoma clusters were tested for IgG reactivities to hAT1R. As shown in Figure 11 and Table 4, all test wells of 2.10.13.58.19 and 7.6.2.65.1 clones were anti-hAT1R IgG positive, and such positive rate in the 5.2.6.8.12 clone was 79.66% (47 out of 59 tested wells). Again, one well from each clone were selected for the 4th round limiting dilution.

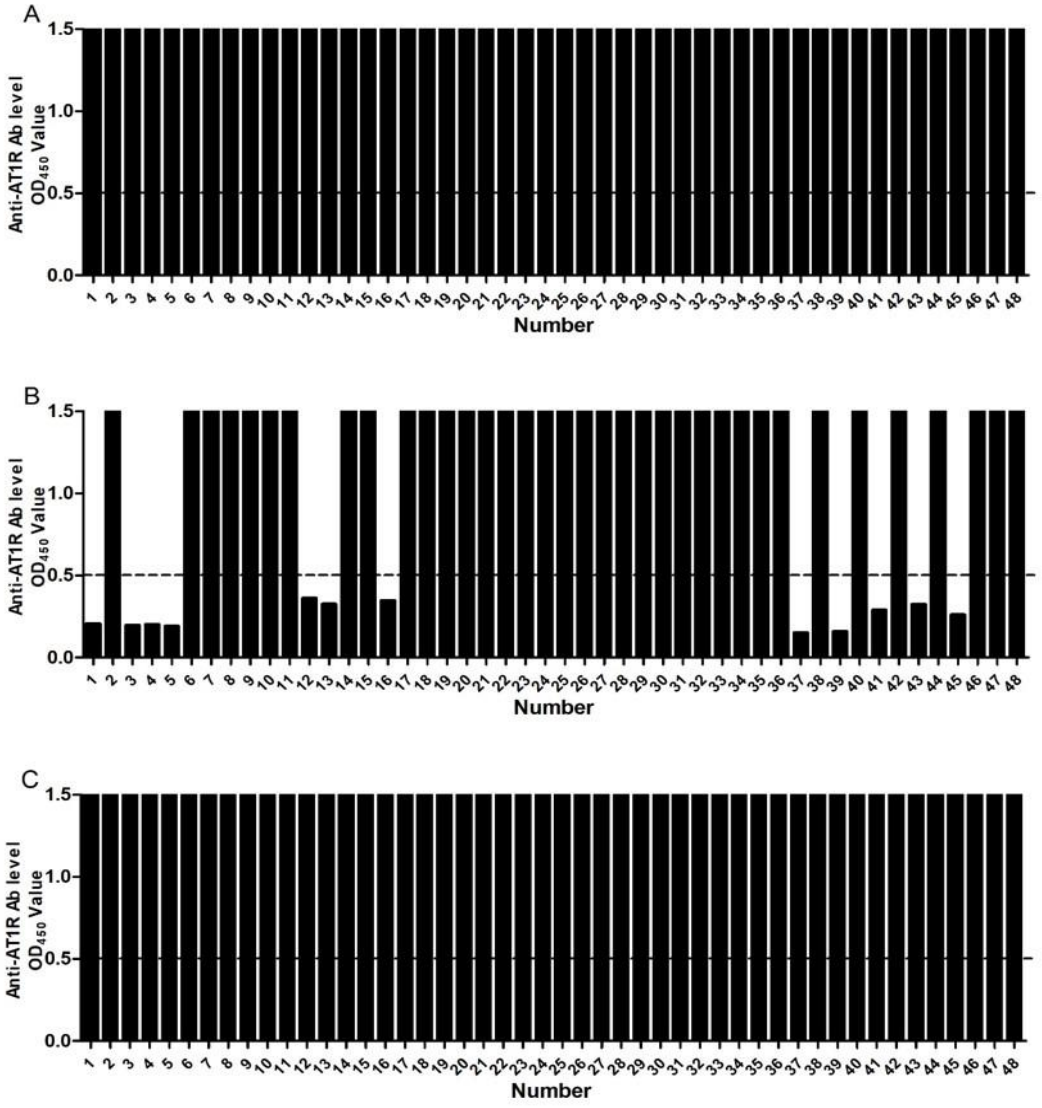

Figure 11. IgG reactivities to AT1R in supernatants of hybridoma cells in the 3rd round of limiting dilution. Culture supernatants from wells of 2.10.13.58.19(**A**), 5.2.6.8.12(**B**) and 7.6.2.65.1 (**C**) hybridoma cells were tested for their IgG reactivities to hAT1R. Representative samples are shown. The X-axis indicates the No. of wells, while the Y-axis presents the IgG reactivities to hAT1R.

Table 4. Summary of the hybridoma clones secreting anti-hAT1R antibodies from the 3rd round of limiting dilution

Hybridoma clone	Number of LD wells in ELISA screening	Number of positive wells	Positive rate (%)
2.10.13.58.19	128	128	100
5.2.6.8.12	59	47	79.66
7.6.2.65.1	172	172	100

The three wells, 2.10.13.58.19.6; 5.2.6.8.12.2; 7.6.2.65.1.12 were subjected to the 4th round of limiting dilution. Fifteen days after the culture, supernatants from wells with hybridoma clusters were tested for anti-hAT1R IgG. As shown in Table 5 and Figure 12, in consistent with the results of the 3rd round limited dilution, all tested wells of 2.10.13.58.19.6.1 (132/132) and 7.6.2.65.1.12.1 (78/78) were anti-hAT1R IgG positive. For the 5.2.6.8.12.2.5 clone, all 314 tested wells were anti-hAT1R positive, reaching a positive rate of 100%. To further purify the three hybridoma clones, we performed the 5th round of limiting dilution.

Table 5. Summary of the hybridoma clones secreting anti-hAT1R antibodies from the 4th round of limiting dilution

Hybridoma clone	Number of LD wells in ELISA screening	Number of positive wells	Positive rate (%)
2.10.13.58.19.6	132	132	100
5.2.6.8.12.2	314	314	100
7.6.2.65.1.12	78	78	100

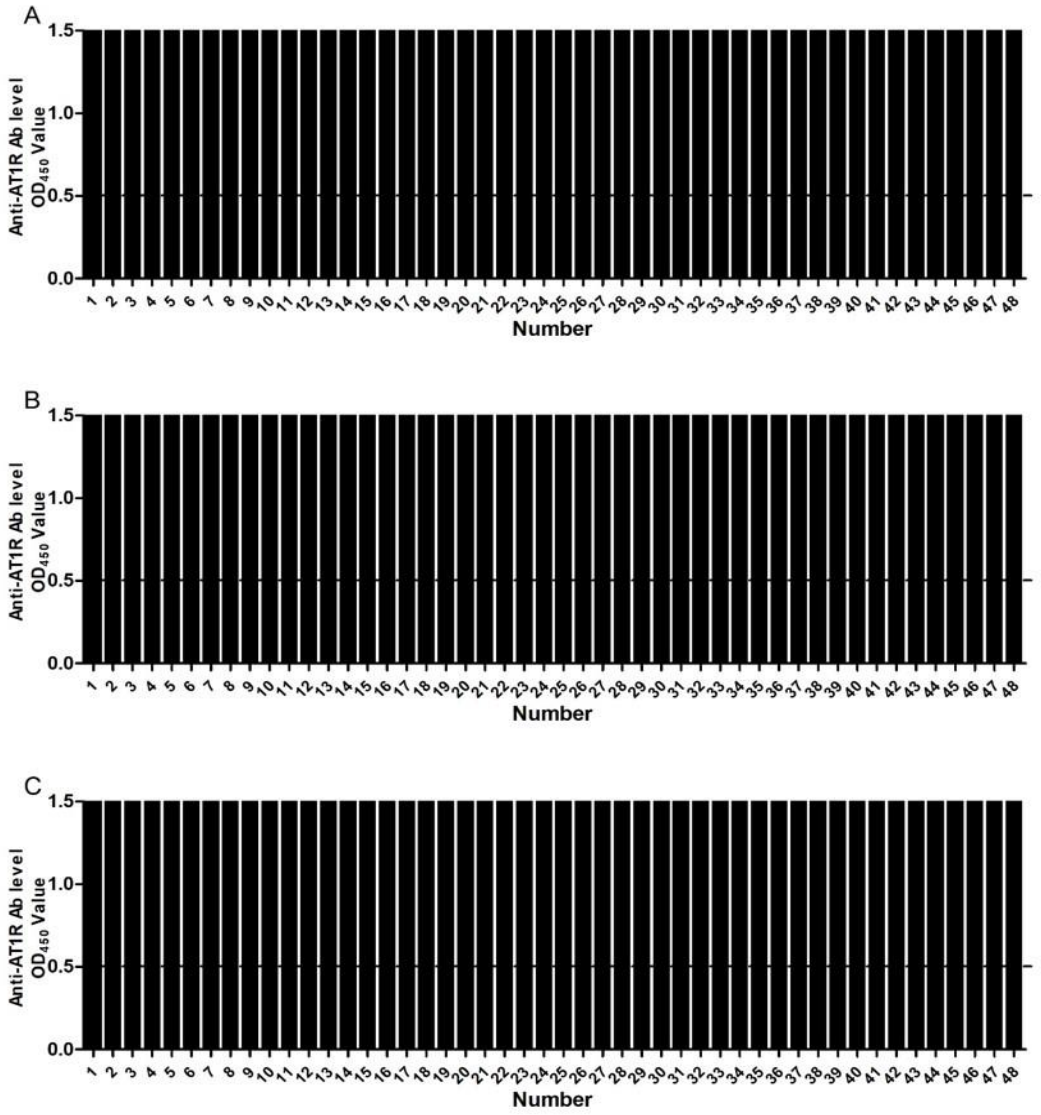

Figure 12. IgG reactivities to hAT1R in supernatants of hybridoma cells in the 4th round of Limiting dilution. Culture supernatants from wells of 2.10.13.58.19.6(**A**), 5.2.6.8.12.2(**B**) and 7.6.2.65.1.12 (**C**) hybridoma cells were tested for their IgG reactivities to hAT1R. Representative samples are shown. The X-axis indicates the No. of wells, while the Y-axis presents the IgG reactivities to hAT1R.

The three wells, 2.10.13.58.19.6.1, 5.2.6.8.12.2.5 and 7.6.2.65.1.12.1. were subjected to the 5th round of limiting dilution. As expected, all test wells of all three hybridoma clones were anti-hAT1R positive (Figure 13 and Table 6). Since anti-AT1R IgG positive rates of the three hybrid clones were 100% for at least 2 rounds of limiting dilution (4th and 5th round), no additional round of limiting dilution was performed.

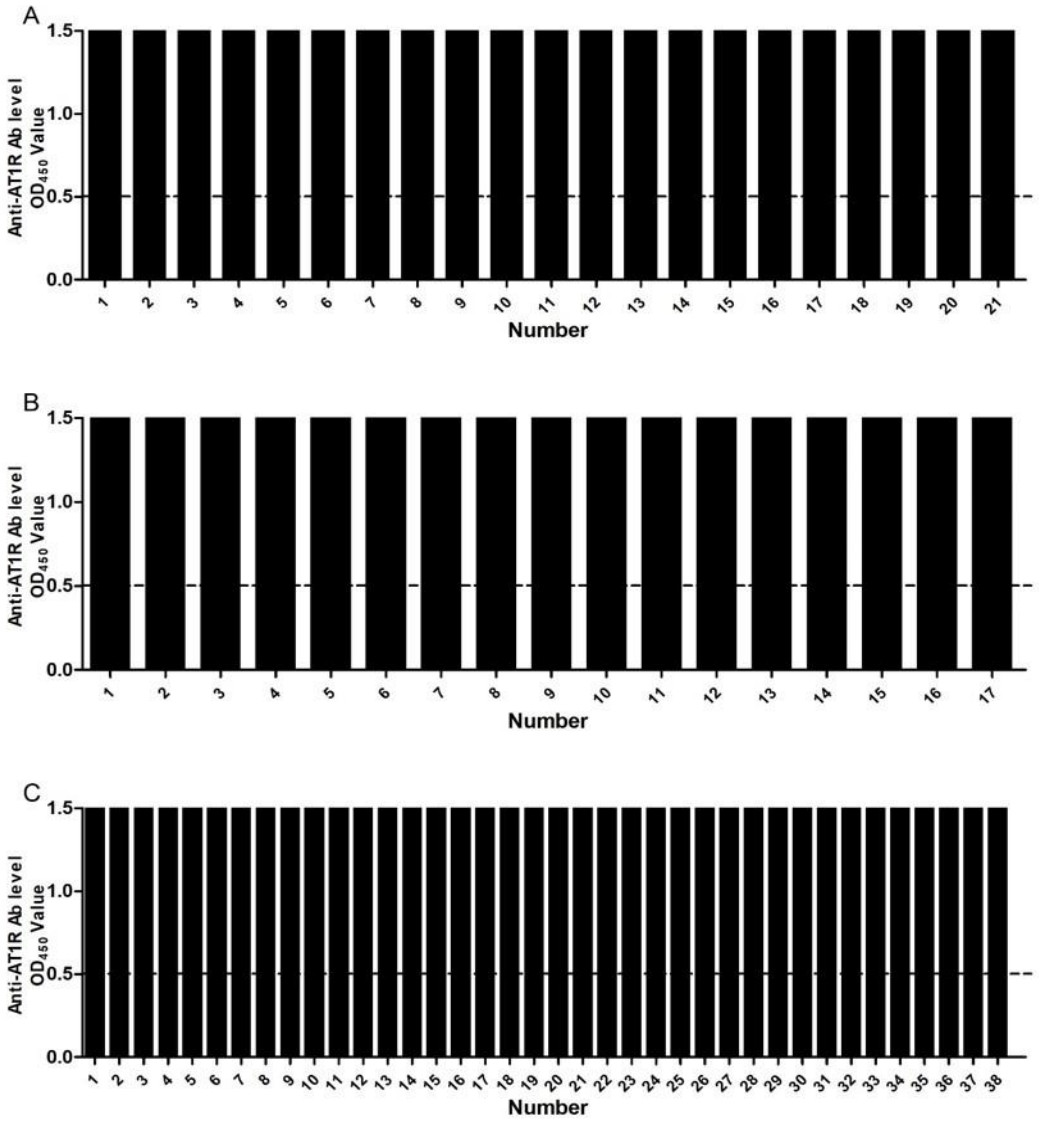

Figure 13. IgG reactivities to hAT1R in supernatants of hybridoma cells in the 5th round of limiting dilution. Culture supernatants from wells of 2.10.13.58.19.6**(A)**, 5.2.6.8.12.2**(B)** and 7.6.2.65.1.12**(C)** hybridoma cells were tested for their IgG reactivities to hAT1R. Representative samples are shown. The X-axis indicates the No. of wells, while the Y-axis presents the IgG reactivities to hAT1R.

Table 6. Summary of the hybridoma clones secreting anti-hAT1R antibodies from the 5th round of limiting dilution

Hybridoma clone	Number of LD wells in ELISA screening	Number of positive wells	Positive rate (%)
2.10.13.58.19.6.1	21	21	100
5.2.6.8.12.2.5	17	17	100
7.6.2.65.1.12.1	38	38	100

3.2.2. Subclass and light chain composition of 3 anti-hAT1R monoclonal antibodies

After the limiting dilution, 3 anti-hAT1R IgG producing hybridoma clones (2.10.13.58.19.6.1, 5.2.6.8.12.2.5 and 7.6.2.65.1.12.1) were generated. To determine subclasses and light-chain compositions of the 3 monoclonal antibodies, we tested culture supernatants from the hybridoma clones by using Isotyping Kit.

As summarized in Table 7, the subclasses of 3 monoclonal antibodies, 2.10.13.58.19.6.1, 5.2.6.8.12.2.5 and 7.6.2.65.1.12.1 were IgG 2b, IgG 2a and IgG 1, respectively. In addition, the light chains of the 3 monoclonal antibodies were all κ. Hereafter, the three hybridoma clones and their corresponding monoclonal antibodies were renamed as 2.2b, 5.2a and 7.1.

Table 7. Subclasses and light chain composition of three anti-hAT1R monoclonal antibodies.

Hybridoma	Subclass	Light-chain	New name
2.10.13.58.19.6.1	IgG 2b	κ	2.2b
5.2.6.8.12.2.5	IgG 2a	κ	5.2a
7.6.2.65.1.12.1	IgG 1	κ	7.1

3.2.3. Large scale production of 3 anti-hAT1R monoclonal antibodies

To produce amounts of monoclonal antibodies sufficient for their further characterization, the 3 hybridomas were cultured consecutively in miniPERM bioreactors. Several hundred ml of cell culture supernatants from the miniPERM production module were collected for monoclonal antibody purification using protein A-Sepharose column. Finally, 50 mg mAb 2.2b, 25 mg 5.2a, and 100 mg 7.1 mAbs were purified for further tests.

3.2.4. Bovine IgG% in anti-hAT1R monoclonal antibodies

During monoclonal antibodies generation, cell culture medium was supplemented with 10% fetal calf serum containing bovine IgG. To ensure that the purified murine monoclonal antibodies were not contaminated with bovine IgG, concentrations of bovine IgGs in the purified murine monoclonal antibodies were determined by competitive ELISA. As shown in Table 8, less than 0.5% bovine IgG were detected in all 3 monoclonal antibodies, suggesting that the three monoclonal antibody preparation reached a purity sufficient for all further approaches.

Table 8. Bovine IgG in prurified murine anti-AT1R monoclonal antibodies.

Monoclonal antibodies	Bovine IgG percentage (%)
2.2b	0.02
5.2a	0.12
7.1	0.37

3.2.5. Cross reactivity of anti-hAT1R monoclonal antibodies to ETAR

AT1R and ETAR display significant sequence identities on the protein level which may result also in cross reacting antibodies. This idea may be supported by the observation, that in SSc patients an autoimmune response to AT1R is always associated with a response to ETAR. Next, whether the three anti-hAT1R monoclonal IgGs react to hETAR were determined. mAb 2.2b showed strong reactivity to both hAT1R and hETAR, while mAb 5.2a and mAb 7.1 react specifically to hAT1R, but not to hETAR (Figure 14).

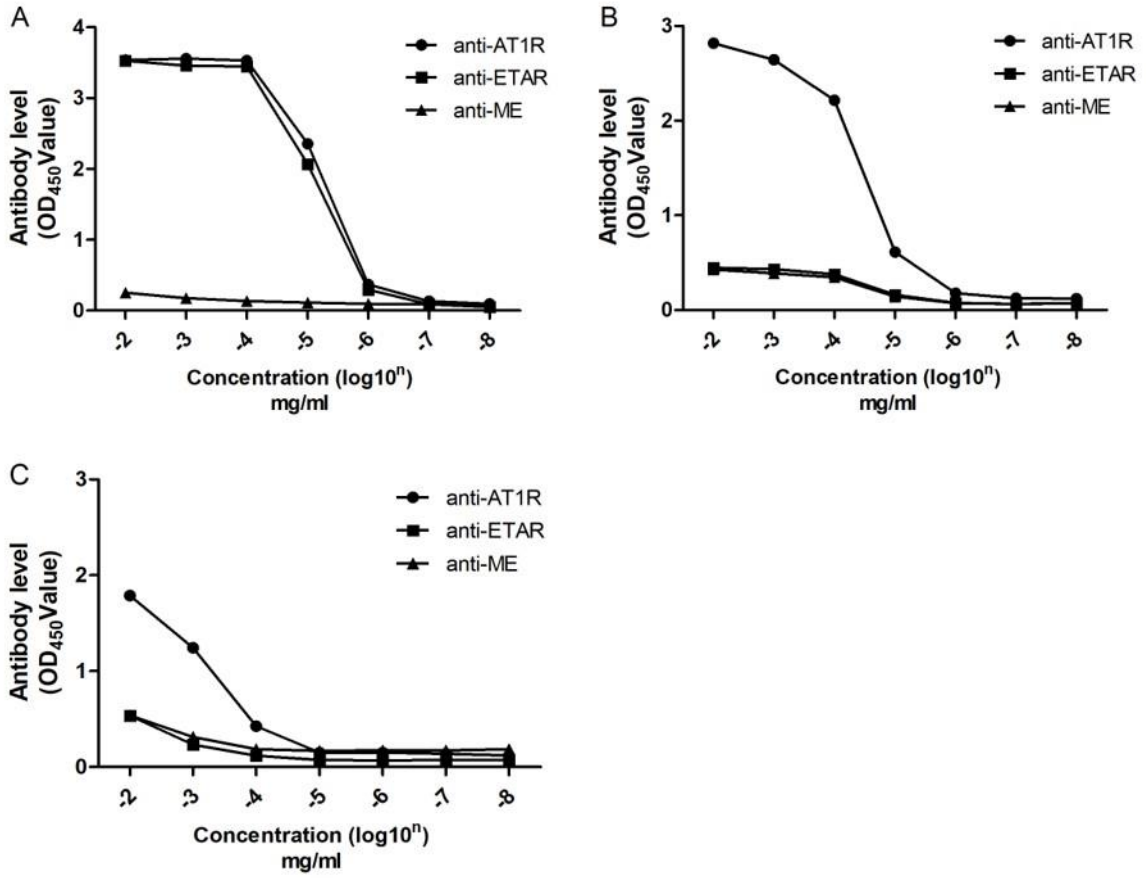

Figure 14. Cross reactivities of autoantibodies against hAT1R to hETAR. ELISA plates pre-coated with hAT1R, hETAR or control ME were used for antibody detection. mAb 2.2b **(A)**, mAb 5.2a **(B)** and mAb 7.1 **(C)** samples were diluted into different concentrations as indicated and exposed to the different antigens for

2h at 4 °C, then goat anti-mouse IgG-HRP was used as the 2nd antibody for a 1h incubation at RT with agitation. Binding of the secondary antibody was visualized by a colorimetric reaction to TMB and expressed as optical density (OD).

3.2.6. Binding of anti-hAT1R monoclonal antibodies to epithelial cells

In the next step, the three anti-hAT1R monoclonal antibodies were tested for their ability to bind to human epithelial cells (HEp-2) which express the receptor on the surface. Using indirect immunofluorescence staining, all of 3 anti-hAT1R monoclonal antibodies (mAb 2.2b, mAb 5.2a and mAb7.1) bound to cell membrane of HEp-2 cells, while their corresponding isotype controls did not (Figure 15).

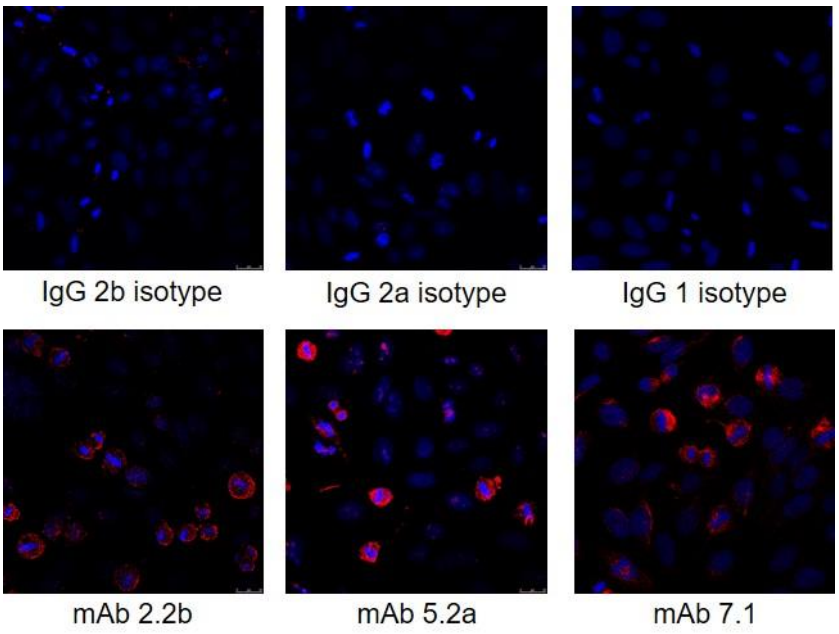

Figure 15. Binding of anti-hAT1R monoclonal antibodies to human epithelial type 2 cells (HEp-2). HEp-2 cells on BIOCHIP slides were incubated with 20µg/ml mAb or isotype control for 30 min at room temperature. The binding of mAb was detected using immunofluorescent staining with Dylight 649 labeled goat anti-mouse IgG antibody. Red signal indicates the bound monoclonal antibodies, while blue signal (DAPI) stands for nucleus.

3.2.7. Effect of anti-hAT1R monoclonal antibodies on cardiomyocytes

Given that all three monoclonal antibodies could bind to cell membrane, their ability to stimulate the receptor on cardiomyocytes were further investigated. Rat cardiomyocytes express a variety of G-protein coupled receptors on cell surface and activating some receptors can exert chronotropic effects on these cells. Among them, AT1R activation promotes positive, while ETAR activation leads to negative chronotropic effects on cardiomyocytes, respectively [99][148]. Monoclonal

antibody 5.2a increased the beating frequency of cardiomyocytes compared to the corresponding isotype controls ($p<0.001$) and the stimulatory effect was completely blocked by Losartan (Figure 16A), suggesting the effect is due to its action on AT1R. As demonstrated in 3.3.5, 2.2b is a bispecific mAb that binds to AT1R and ETAR. Monoclonal antibody 2.2b increased the beating frequency of cardiomyocytes compared to the corresponding isotype controls ($p<0.05$) (Figure 16B). However, 2.2b decreased the beating frequency of cardiomyocytes in presence of Losartan, and showed no effect on the beating frequency of cardiomyocytes in presence of both Losartan and BQ123 (Figure 16B), indicating a bispecific activation of AT1R and ETAR by 2.2b. The other anti-hAT1R monoclonal antibody, 7.1, decreased cardiomyocytes beating frequency. Unexpectedly, this decrease could be inhibited by adding of BQ123, the antagonist of ETAR (Figure 16C).

To verify the bispecific effect of monoclonal antibody 2.2b, the cells were incubated with BQ123 prior to the stimulation with mAb. Under this experimental setting, 2.2b increased the beating frequency of cardiomyocytes and the stimulatory effect was completely blocked by Losartan, suggesting the effect is due to its action on AT1R. (Figure 17A). When cells were preincubated with Losartan prior to the stimulation with mAb, 2.2b decreased the beating frequency and the inhibitory effect was completely blocked by BQ123, suggesting this effect is due to the activation on ETAR (Figure 17B).

Therefore, all three anti-hAT1R mAbs showed a specific function on cardiomyocytes, where 5.2a mAb was agonistic to AT1R, 2.2b mAb was agonistic to both AT1R and ETAR, and 7.1 mAb was agonistic to ETAR.

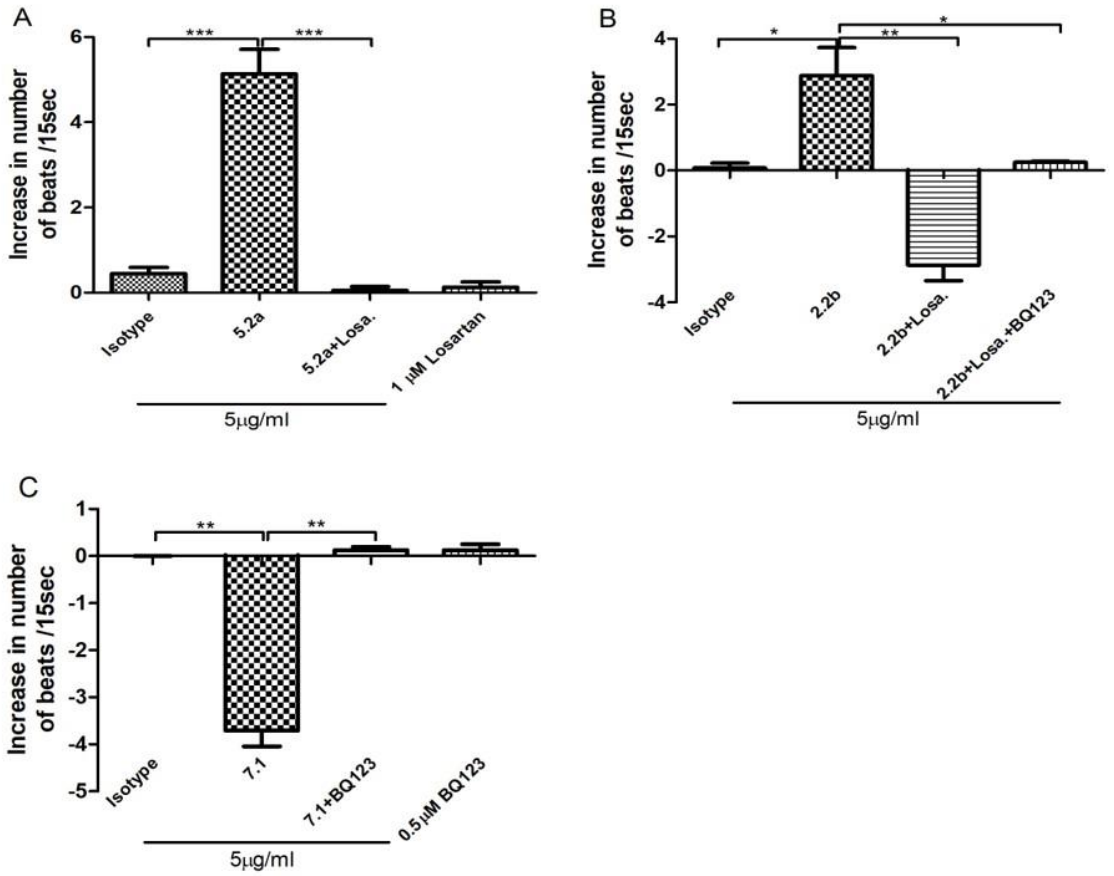

Figure 16. Effect of three anti-hAT1R monoclonal antibodies on rat cardiomyocytes. Cardiomyocytes were stimulated with anti-hAT1R mAbs 5.2a **(A)**, 2.2b **(B)**, and 7.1 **(C)**, or antibodies of the corresponding isotype in presence or absence of Losartan (1 μM), BQ123 (0.5μM) or a combination of Losartan and BQ123. Effect on **c**ell beating rate is presented as increase in number of beats/15 second which is shown on Y-axis. Data are presented as mean ± SD (n=6), and statistical analysis was performed using unpaired t test (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.)

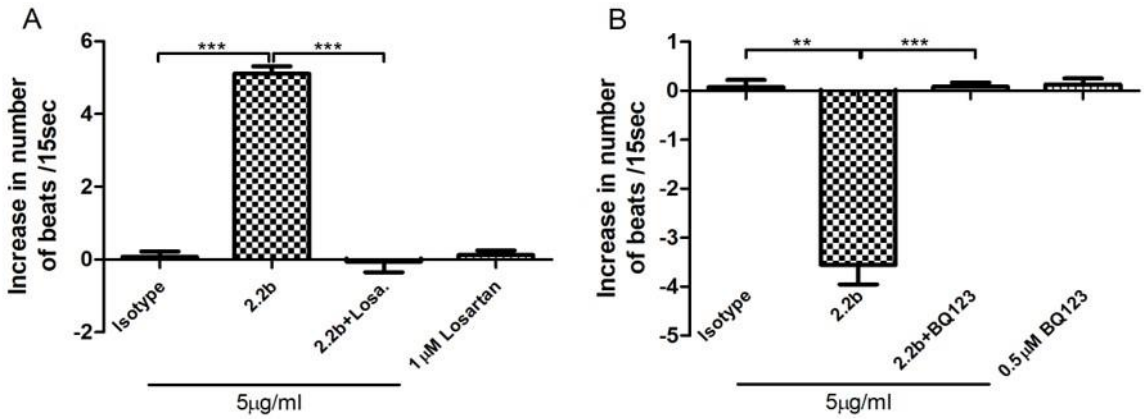

Figure 17. Effect of anti-hAT1R monoclonal antibody 2.2b on rat cardiomyocytes. Cardiomyocytes were pretreated with BQ123 (0.5μM) **(A)**, or Losartan (1 μM) **(B)**, and then stimulated with mAb 2.2b in presence/absence of Losartan or BQ123. Effect on **c**ell beating rate is presented as increase in number of beats/15 second which is shown on Y-axis. Data are presented as mean ± SD (n=6), and statistical analysis was performed using unpaired t test (** = $p<0.01$, *** = $p<0.001$.)

3.2.8. Effect of anti-AT1R monoclonal antibodies on monocytes

To confirm functional roles of the three anti-AT1R monoclonal antibodies, their effect on human monocytes was further investigated. Cells of the human monocytic cell line THP-1 were stimulated with anti-AT1R mAbs in presence or absence of Losartan, and IL-8 in supernatants was determined as a readout. As compared to isotype controls, mAb 2.2b significantly increased the IL-8 production from monocytes, and the increase could be partially blocked by Losartan, suggesting an agnostic effect of mAb 2.2b (Figure 18A). At high concentrations (50 μg/ml), mAb 5.2a also significantly increased the IL-8 production from monocytes compared to isotype control (Figure 18B). The stimulatory effect of mAb 5.2a could not be blocked by either Losartan or another AT1R specific antagonist, Valsartan (Figure 18B, C), but was partially and significantly blocked by a combination of both inhibitors (Figure 18D). mAb 7.1 showed no effect on IL-8 secretion from monocytes exceeding that observed in isotype controls (Figure 18E).

Taken together, these results show that all three anti-hAT1R monoclonal antibodies are able to bind to AT1R expressed on cell membranes as wells to immobilized receptor protein *in vitro*. However, the three mAbs show different biological effect on the receptors, where 5.2a shows an agonistic effect on AT1R, 2.2b shows an agonistic effect on both AT1R and ETAR, and 7.1 shows no effect on AT1R but an agonistic effect on ETAR.

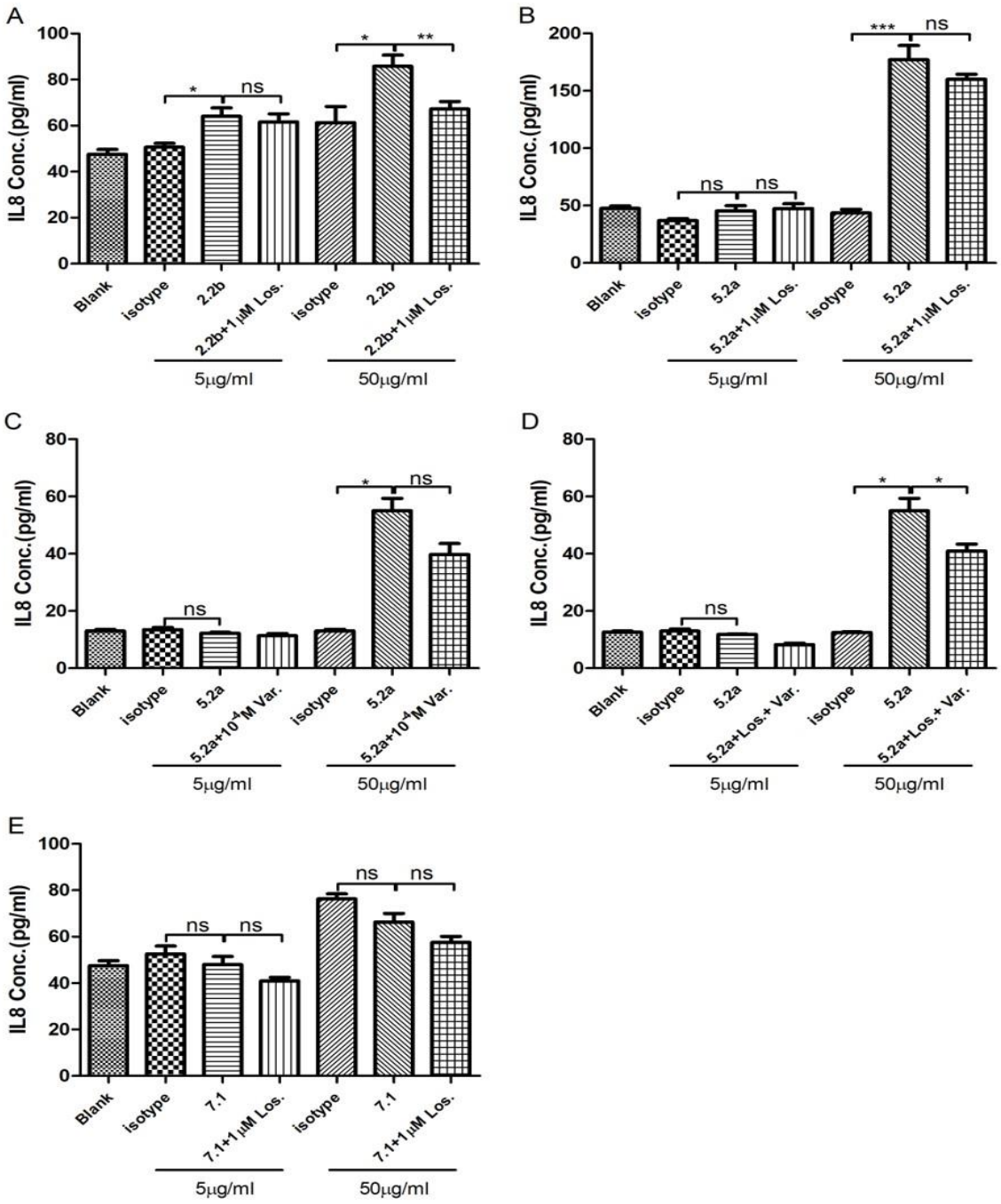

Figure 18. Effect of anti-hAT1R monoclonal antibodies on human monocytes. Human monocytic cell line THP-1 were stimulated with anti-hAT1R mAbs 2.2b (**A**), 5.2a (**B, C, D**), and 7.1 (**E**), or their corresponding isotype controls in presence or absence of Losartan (1 μM), Valsartan (10^{-4} M) or the combination of Losartan (10^{-5} M) and Valsartan (10^{-4} M) for 24 hours. The concentration of IL-8 in the supernatant was determined by ELISA. Data are presented as mean ± SD (n=4), and statistical analysis was performed using unpaired t test (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.)

4. Discussion

In the present study, anti-hAT1R pAbs and three anti-hAT1R mAbs were generated from hAT1R-immunized mice and characterized. By using *in vitro* assays such as ELISA, cell-based binding assay, and function of monocytes and cardiomyocytes, those anti-hAT1R pAbs and mAbs were characterized. Results show that both anti-hAT1R pAbs and three anti-hAT1R mAbs could bind to native AT1R on cell membrane. *In vitro* functional assays showed that anti-hAT1R pAbs and 2 of anti-AT1R mAbs are functional autoantibodies with an agonistic effect to AT1R. Therefore, this study for the first time demonstrates that immunization of mice with hAT1R results in the expression of functional autoantibodies to the antigen and supports a role of autoantibodies against AT1R in the pathogenesis of SSc.

4.1. Functional autoantibodies against AT1R

AT1R is expressed on many cell types, including epithelial cells, and the activation of AT1R plays an important role in numerous physiological processes[154] [155]. Human epithelial type 2 (HEp-2) cells are widely used for the laboratorial detection of autoantibodies in many autoimmune diseases [156]. The current study demonstrates that anti-hAT1R pAbs isolated from hAT1R-immunized mice can bind to AT1R on cell membrane of HEp-2, suggesting it might act as ligand of the receptor.

It has been reported that Ang II, the natural ligand of AT1R, modulates the function of cardiomyocytes by activating AT1R and consequently increasing the cell beating frequency [99]. In this study, *in vitro* experiments demonstrated that anti-hAT1R pAbs increased the beating rate of cardiomyocytes. Furthermore, anti-hAT1R pAbs-induced increase of beating rate could be blocked by Losartan, suggesting that such effect is due to AT1R activation. Therefore, these data suggest that anti-hAT1R pAbs are functional antibodies and act as agonists to AT1R on cardiomyocytes. This notion is further supported by the observed effect of anti-hAT1R pAbs on monocytes. AT1R is known to be highly related to the production of SSc-relevant cytokines. IL-8, a proinflammatory and profibrotic chemokine mainly produced by monocytes and endothelial cells, is found elevated in sera and in BALF of patients with SSc and SSc-ILD respectively [157][158]. Using an *in vitro* experimental system, Gunther et al. have shown that human PBMC stimulated with autoantibodies against AT1R derived from SSc patients produce increased levels of IL-8 and CCL18 and such increase can be inhibited by Losartan [133], suggesting a proinflammatory and profibrotic feature of autoantibodies against AT1R. In the present study, anti-hAT1R pAbs derived from hAT1R-immunized mice increased the IL-8 production from monocytes, and the increase could be partially

blocked by Losartan, supporting the proinflammatory effect of anti-hAT1R pAbs to AT1R.

Beside polyclonal IgG against hAT1R, three monoclonal IgGs generated in this study have also been demonstrated to be functional autoantibodies. In the current study, three murine monoclonal IgGs against hAT1R were generated. The antibodies termed 2.2b, 5.2a, and 7.1, belong to IgG2b, IgG2a and IgG1 subclasses, respectively. All of three monoclonal IgGs were able to bind to the AT1 receptor on human epithelial type 2 cells, suggesting that they recognize naive structure of AT1R. Notably, the three anti-hAT1R monoclonal IgGs showed distinct reactivity to ETAR and exerted different biological functions on the AT1 receptor.

As mentioned above, AT1 and ETA receptors are expressed on the membrane of rat cardiomyocytes. Activation of AT1R exerts positive chronotropic effect on the cells, while activation of ETAR exerts an opposite effect [99][148]. The current study demonstrated that 2.2b could activate both AT1R and ETAR on cardiomyocytes, and the activation could be blocked by antagonist of the two receptors, respectively. Furthermore, the agonistic effect of 2.2b on AT1R has also been demonstrated with THP-1 cells, where 2.2b simulation increased the IL-8 production from THP-1 cells and such increase could be blocked by Losartan. Therefore, 2,2b are bispecific mAb to AT1R and ETAR, in terms of both binding and activation.

Monoclonal IgG 5.2a binds specifically to AT1R, but not to ETAR. Furthermore, 5.2a mAb could activate AT1R on both cardiomyocytes and THP-1 cells. Notably, Losartan alone could block the effect of 5.2a mAb on cardiomyocytes but not that on THP-1 cells. Moreover, a combination of Losartan and Valsartan could at least partially block the effect of 5.2a on IL-8 production. Losartan is one of the angiotensin II receptor blockers (ARBs) that shows a selective antagonism to AT1R but not AT2R. It has been shown that different ARBs have different binding kinetics to the receptor [159]. Among ARBs, the competitive antagonist Losartan have relatively lower affinity to AT1R compared with candesartan and irbesartan which show insurmountable antagonism to the receptor [160]. Valsartan, another competitive antagonist of AT1R, shows a higher binding affinity and blockage effect to AT1R than Losartan. Different ARBs also differ pharmacologically in terms of their blocking mechanism, bioavailability, metabolism and the respective duration of the blocking effect. Such pharmacological differences may explain the inhibitory effect induced by a combination of Losartan and Valsartan during 5.2a stimulation of monocytes. Pharmacologically different ARBs, Losartan and Valsartan exert their blocking effect to AT1R via different mechanism. Thus, a combined application of both antagonists showed a combined efficiency and higher competitiveness to AT1R compared with single antagonist application.

Although both 2.2b and 5.2a mAbs exert an agonistic effect on AT1R, they differ in their sensitivity to the antagonist, suggesting a major difference between the two AT1R-activating mAbs. There are two possible reasons for their heterogeneous reactivity to Losartan. The first one is that the two monoclonal IgGs show different binding affinity to the receptor. Due to a high binding affinity of 5.2a, it is difficult to completely block the agonistic effect of 5.2a with Losartan or Valsartan. Another possible reason for the difference is that the two agonistic monoclonal IgGs recognizing different epitopes which can be bound by Losartan with different affinity.

Clone 7.1 binds specifically to AT1R, but not to ETAR. However, the further characterization revealed that mAb 7.1 could not activate AT1R on monocytes or cardiomyocytes. Unexpectedly, this antibody showed a negative chronotropic effect on cardiomyocytes, and this effect could be totally blocked by the ETAR antagonist, BQ123. These results suggest that 7.1 mAb bind to AT1R but show an agonistic effect on ETAR. Since this antibody did not exert any immunoreactivity to ETAR, the agonistic effect is most likely to be exerted in an indirect manner. The mechanism underlying this effect remains to be further explored.

Therefore, although all three monoclonal IgGs are directed against AT1R, they are characterized by individual features with regard to immunoreactivity and functionality. This phenomenon has been also observed by Itai et al. when they characterizing100 anti-human epidermal growth factor receptor 2 (HER2) monoclonal antibodies. Among the 100 mAbs, many of them show binding activity to HER2 in flow cytometry analysis and immunohistochemical analysis, while only 3 mAbs react to the antigen in Western blot examination [161]. Such heterogeneity may due to the difference between various *in vitro* testing methods for monoclonal antibodies characterization.

Taken together, the three anti-hAT1R monoclonal IgGs are of different subclasses, most likely recognize different epitopes and thus possess different functions. This finding is in line with the notion that polyclonal antibodies against a certain antigen are of great heterogeneity in both structure and function [153][162].

According to my results, both polyclonal and monoclonal IgGs against hAT1R generated from immunized mice react to both human and rat AT1R. One possible explanation is that the immunized mice do produce anti-human AT1R specific antibodies but such antibodies show cross reactivity between human and rat AT1R due to the similarities in protein sequence and the structure.

Collectively, the current study has demonstrated that both anti-hAT1R pAbs and mAbs could be functional antibodies with an agnostic effect. The agonistic effect of anti-hAT1R autoantibodies is in consistence with previous findings that Ang II is able to modulates the function of multiple cells, including monocytes [163], macrophages [92], NK cells [93], Dendritic (DC) cells [94], T cells [96] and cardiomyocytes[99].

4.2. Role of autoantibodies against AT1R in the mouse model of SSc

The functional characterization of anti-hAT1R pAbs and mAbs also shed some lights on the disease pathogenesis of the hAT1R-induced mouse model of SSc. In this model, hAT1R immunization induce multiple SSc-like phenotypes, including lung inflammation, skin inflammation and fibrosis (data not published). This hAT1R-induced mouse model demonstrates that autoimmunity to AT1R is a pathogenic event. However, it is not clear which type of autoimmunity, cellular or humoral one, play a key role in the disease development of the mouse model.

The evidence that anti-hAT1R pAbs and mAb are functional and agonistic support a key role of humoral autoimmunity in the pathogenesis. It has been shown that continuously administration of Ang II subcutaneously is able to induce skin inflammation and fibrosis [164]. In addition, transgenic mice overexpressing endothelin I also develop a spontaneous SSc-like disease [165]. These previous findings suggest that activation of AT1R or ETAR might be a pathogenic event in the pathogenesis of SSc. Therefore, anti-AT1R IgGs with agonistic effect might also be pathogenic and thus involved in the disease development in the SSc-like disease in this mouse model. This notion can be verified in two ways in the future study. The first is to investigate the the hAT1R-induced SSc-like disease in B cell deficient mice to determine whether humoral autoimmunity to hAT1R is required for the disease development. The second is to transfer anti-hAT1R antibodies into healthy mice, which allows to determine whether anti-hAT1R antibodies are sufficient for the induction of SSc-like disease phenotypes.

In addition, two out of the three anti-hAT1R monoclonal IgGs show an agonistic effect to AT1R and one of them even exerts bispecific effect on both AT1R and ETAR, making them of special interest. Since transfer of autoantibodies is a commonly used strategy of modeling human autoimmune disease[137], transfer of these two anti-hAT1R monoclonal IgGs into mice might lead to a new mouse model for SSc which allow us to further explore the role of autoantibodies against AT1R in the pathogenesis of SSc.

4.3. Role of autoantibodies against AT1R in the pathogenesis of SSc

The current study also provides some evidence for our understanding the pathogenesis of SSc. The agonistic effect of anti-AT1R pAbs supports a pathogenic role in the development of human diseases. Previously, Riemekasten et al. have shown that levels of anti-AT1R antibodies are significantly increased in patients with SSc compared with healthy controls and patients with other autoimmune diseases [131]. Moreover, the presence of anti-AT1R antibodies is associated with disease manifestations, including lung fibrosis, PAH and mortality [131]. These clinical evidences suggest that autoantibodies against AT1R might play a pathogenic role in the development of SSc. This notion is partially supported by some *in vitro* findings showing that IgG isolated from SSc patient with high level of anti-AT1R antibodies show an agonistic effect on many type of cells and lead to the activation of immune cells [133] as well as fibroblasts [132]. The results from the current study provide additional evidence for the hypothesis. First, I could show that anti-AT1R pAbs and mAbs generated from mice are functional autoantibodies. Second, my findings demonstrate that anti-hAT1R pAbs and mAbs are able to promote IL-8 production from monocytes. Since IL-8 has been found to be elevated in sera and in BALF during SSc process and play an important proinflammatory and profibrotic chemokine [166][158], the promoting effect of anti-hAT1R autoantibodies on IL-8 production further support a pathogenic role of those antibodies in the development of SSc.

In addition, the findings from this study also helps to explain the close relationship between autoantibodies against AT1R and ETAR in patients with SSc. Previously, Riemekasten and colleagues have demonstrated that 1) levels of autoantibodies against AT1R are highly correlated with levels of autoantibodies against ETAR in patients with SSc; 2) both anti-AT1R and anti-ETAR autoantibodies are associated with many SSc clinical sub-phenotypes such as fibrotic and vascular manifestations and overall survival; 3) the two autoantibodies are functionally related *in vitro* [131][132][133]. The high clinical and functional associations between anti-ETAR and anti-AT1R autoantibodies in SSc patients suggest that they might show high cross reactivity to antigens of each other. The findings from the current study shed some new light on this issue. Notably, the 2.2b monoclonal IgG recognize both AT1R and ETAR, while 5.2a and 7.1 monoclonal IgGs are reactive specifically to AT1R, suggesting that some monoclonal antibodies against AT1R show cross reactivity to ETAR. Moreover, monoclonal antibody 7.1 exert an unexpected agonistic effect to ETAR but not AT1R on cardiomyocytes, where mAb 7.1 decreased cell beating frequency of cardiomyocytes and the decrease was totally blocked by ETAR antagonist, BQ123. This finding suggests that at the monoclonal levels some anti-hAT1R IgGs are able to bind to hETAR or activate ETAR, which leads to the cross reactivity and functional overlap between the two

polyclonal antibodies.

It needs to be mentioned there is one possible limitation in this study. Both anti-AT1R pAbs and mAbs investigated here were generated from mice immunized with hAT1R emulsified in CFA, an adjuvant. Thus, these anti-AT1R antibodies might be different to autoantibodies against AT1R in patients with SSc which are produced spontaneously without artificial induction. Therefore, this difference needs to be taken into consideration during the clinical translation.

4.4. Outlook

Based on my initial findings presented here, a further characterization of the three monoclonal IgGs against hAT1R is required. On one hand, epitopes recognized by those monoclonal antibodies should be identified. This can be performed by using cell lines expressing hAT1R with various mutations. Moreover, the pathogenicity of the three monoclonal IgGs should be investigated *in vivo*. To do this, anti-hAT1R monoclonal IgGs can be transferred to healthy mice locally or systemically. For local transfer, antibodies would be injected intracutaneously to mouse ears, and local infiltration, vascular changes and skin fibrosis can be evaluated. For systemic transfer, antibodies can be injected intraperitoneally or intravenously, and multiple organs including skin, lung, kidney, heart, liver and muscle would be evaluated.

If transfer of anti-hAT1R monoclonal IgGs successfully induce disease symptoms in mice, this would represent a novel mouse model for SSc. Such antibody transfer-induced model could provide a new tool for the development of effective therapeutic strategies in the treatment of the disease.

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Publications

1) Zheng LC*, **Wang XQ***, Lu K*, Deng XL, Zhang CW, Luo H, Xu XD, Chen XM, Yan L, Wang YQ, Shi SL. Ephrin-B2/Fc promotes proliferation and migration, and suppresses apoptosis in human umbilical vein endothelial cells. Oncotarget. 2017; 8:41348-41363. (*Joint first authors)

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