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Characterization and cloning of agonistic autoantibodies specific for the PDGF receptor from the B cell repertoire of SSc patients.

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ABSTRACT

BACKGROUND

Systemic sclerosis (SSc) is an autoimmune disorder characterized by fibrosis of the skin and visceral organs. It has been recently demonstrated that serum from SSc patients contains autoantibodies specific for PDGF receptor alpha (PDGFRα) able to trigger an intracellular signaling pathway involving Ha-Ras and ERK1/2 and leading to the production of reactive oxygen species (ROS). The overproduction of ROS induces collagen gene transcription and senescence in normal fibroblasts [1]. To dissect the immunological repertoire of SSc patients, circulating B lymphocytes have been immortalized with Epstein Barr virus (EBV) and a small panel of cultures producing IgM and IgG specific for PDGFRα has been isolated on the basis of their ability to immunoprecipitate PDGFRα from the cell membrane of normal fibroblasts, to induce ROS production and to regulate the expression of genes associated with PDGFR activation.

MATERIALS AND METHODS

Three IgG- and one IgM-positive B lymphocytes oligoclonal cultures which produced antibodies showing binding capacity and biological activity on murine fibroblasts transfected with human PDGFRα (but not on mock transfected cells) have been selected. mRNA from these cells has been isolated and the sequencing of the variable regions of the immunoglobulins carried out. The identified VH and VL sequences have been cloned, variously combined and subsequently mounted in a human IgG expression vector. These constructs have been

transiently transfected in CHO cells to generate fully human monoclonal antibodies (HumAbs). The recombinant IgG have been purified from CHO cell culture supernatants and tested for selected biological properties, including PDGFRα binding capacity, ROS induction, ERK phosphorylation and collagen gene expression. Selection of stably transfected CHO cell clones is on-going.

A preliminary research for sequence homology of the variable regions of the HumAbs with already described human immunoglobulins has been performed in silico on public databases. mRNA from circulating lymphocytes obtained from a small number of sclerodermic patients and healthy subjects has been analyzed with PCR in order to investigate the presence of the CDR3 sequences of the isolated anti-PDGFRα autoantibodies.

To further characterize the selected HumAbs and to extend the analysis of the immunological repertoire of SSc patients, a recombinant C-terminal truncated isoform of the PDGFRα tagged with six histidine residues (PDGFRα-HIS, a.a. 1-834), has been stably transfected in PDGFR-negative HeLa cells and then purified from the cell lysate on a nickel-coated affinity column. The purified PDGFRα-HIS has been immobilized via its His tag on an activated sensor chip and the specific binding activity of a small panel of IgG, purified from the serum of sclerodermic and healthy individuals, was evaluated using surface plasmon resonance technology.

To determine the amount of anti-PDGFRα autoantibodies in SSc patients and controls, the PDGFRα-HIS was used as a target to set up an immunoenzymatic assay (ELISA). A panel of sera of SSc patients, healthy donors and subjects with

other selected diseases was tested in terms of binding activity to the immobilized receptor.

RESULTS

Using an improved EBV-immortalization technique [2], we isolated from the B cell repertoire of two sclerodermic patients three cultures producing IgG (namely 16F4, 13B8 and 17H8) and one producing IgM (1F5) selectively reacting with PDGFRα expressing cells and able to immunoprecipitate the receptor from human fibroblasts, therefore proving to be specific for PDGFRα. Three out of four HumAbs were able to mimic the biological activity of the natural ligand (PDGF); indeed, they stimulated ROS production through the Ras-ERK signaling cascade.

The sequence analysis of the variable regions of the IgG produced by 16F4, 13B8 and 17H8 cultures allowed to identify two VH and four VL sequences. The sequence analysis of the IgM produced by 1F5 culture showed two VL and two different VH sequences. One VH is shared by all the sequenced immunoglobulins and seems to possess unique CDR1 and CDR3 sequences. Various combinations of the identified VH and VL sequences were cloned in a human IgG expression vector and transfected in CHO cells, to generate a panel of monoclonal IgG. The recombinant IgG showed PDGFR-binding capacity and biological activities. In detail, seven IgG were able to immunoprecipitate the receptor from the membrane of human fibroblasts, six stimulated ROS production, four induced ERK1/2 phosphorylation, and two promoted collagen gene expression.

A small preliminary epidemiologic study showed that the sequence of the VLκ of the 16F4 IgG is present in seven out of nine SSc patients and in two out of six healthy individuals. A larger study is underway.

To further characterize the properties of anti-PDGFRα antibodies and to obtain a cell model useful for functional studies, HeLa cells have been transfected with a recombinant PDGFRα-HIS. Cytofluorimetric analysis confirmed that the receptor was stably expressed on the surface of transfected cells. Western blot analysis of the cell lysate and of the enriched preparations of PDGFRα-HIS obtained after affinity purification, showed that the recombinant receptor has a molecular weight of 116 kDa, accordingly with the expected molecular weight of the truncated recombinant receptor (a.a.1-834).

Surface plasmon resonance experiments demonstrated that the purified recombinant receptor was able to bind its natural ligand (PDGF) as well as a murine monoclonal antibody recognizing a conformational epitope of PDGFRα, indicating a correct folding of the recombinant molecule. The immobilized receptor was challenged with IgG from patients and healthy controls to assess the PDGFR-binding activity. The results highlighted that all of the eight SSc IgG tested were able to bind the immobilized receptor with an affinity comparable to that of the natural ligand, while all but one normal IgG showed a significantly lower binding capacity.

The PDGFRα-HIS has also been used as the target molecule to set up an ELISA test designed to investigate the presence of specific autoantibodies in human serum. The screening of human sera showed that immunoglobulins of SSc

patients have an anti-PDGFR binding capacity higher than that of healthy donors. The difference between the mean values of the two cohorts was statistically significant. Sera of patients with rheumatoid arthritis, systemic lupus erythematosus and primary Raynaud's phenomenon showed a reduced binding when compared to SSc patients, but the difference between the groups did not reach statistical significance.

A gene expression profiling study on human adult fibroblast stimulated with IgG purified from both sclerodermic and healthy individuals is in progress.

CONCLUSIONS

Stimulatory anti-PDGFRα autoantibodies isolated from the immunoglobulin repertoire of SSc patients represent a specific hallmark of systemic sclerosis and their biological activity on fibroblasts suggests an active role in the pathogenesis of the disease. Results so far obtained using affinity binding assays highlighted the presence of PDGFRα-specific antibodies in the serum of SSc patients. Anti-PDGFRα antibodies are also present in a fraction of healthy individuals; however, these antibodies did not show agonistic activity, at least in the experimental conditions adopted.

The cloned recombinant monoclonal IgG represent the formal proof that it is possible to isolate PDGFRα–specific immunoglobulin from the B cell repertoire of SSc patients. Fully human monoclonal antibodies generated from the sequences of the variable regions of the heavy and light chains of anti-PDGFRα autoantibodies have a binding capacity comparable to that of the anti-PDGFRα autoantibodies present in the serum and retain the same biological activities.

These findings strongly support the idea of the existence of PDGFRα–directed autoimmunity in SSc patients.

The identification of the specific epitopes of PDGFRα bound by the sclerodermic stimulatory autoantibodies is in progress. This analysis will allow us to further investigate the binding properties of the isolated and the recombinant anti-PDGFRα antibodies, and will open new diagnostic and therapeutic venues for the management of SSc.

The analysis of the effects of SSc autoantibodies on fibroblasts at the transcriptional level will shed light on the autoantibody-mediated activation patterns downstream of the PDGFR and on the pathogenic role of these or other autoantibodies in systemic sclerosis, possibly allowing for the discovery of novel markers of this complex disease.

1 *Introduction and background*

1.1 SYSTEMIC SCLEROSIS

Systemic sclerosis (SSc) is a rare and heterogeneous systemic autoimmune disease of unknown etiology which affects the connective tissue of the skin and internal organs. It is believed that its complex pathophysiology involves genetic and environmental factors, vascular and immune system functions, as well as fibroblasts and extracellular matrix alterations. Clinically it is characterized by the massive deposition of collagen fibers in tissues such as the gastrointestinal tract, lungs, heart and kidneys, by alterations of the microvasculature and by disturbances of the immune system at both the cellular and humoral level. Patients with SSc almost universally present numerous autoantibodies, some of which seem to be disease-specific.

Systemic sclerosis is comprised in the wider spectrum of sclerodermatous diseases, which also include morphea, pseudo-scleroderma and the overlap-syndromes with similar cutaneous and histopathologic manifestations (Figure 1)[3].

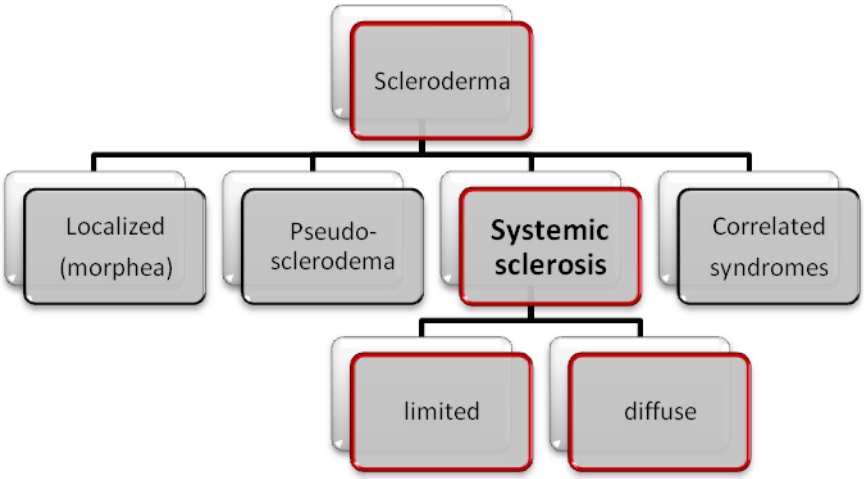

Figure 1: Clinical aspects of scleroderma.

- The American College of Rheumatology (ACR) has defined the criteria for the diagnosis of this disease, which are 97% sensitive and 98% specific for Systemic sclerosis [4].

Major criterion

- Proximal diffuse sclerosis of the truncal region (clinically manifesting as skin tightening and thickening, and non-pitting induration).

Minor criteria

- Sclerodactyly.
- Digital pitting scars or loss of substance of the digital finger pads.
- Bibasilar pulmonary fibrosis.

Patients should fulfill the major criterion or two of the three minor criteria to be diagnosed with Systemic sclerosis.

Raynaud's phenomenon, a vasospastic disorder causing discoloration of fingers and toes, is observed in 90-98% of the patients and can be considered a predictive factor for the subsequent development of the disease, in particular when it is associated with abnormal nailfold capillaries and the occurrence of anti-nuclear antibodies (ANA)[5, 6].

1.2 CLASSIFICATION AND EPIDEMIOLOGY OF SSC

Several classification systems for Systemic sclerosis have been proposed, depending on the level of cutaneous involvement at the time of clinical manifestation [7] (such is the case for CREST syndrome, clinically manifested with calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactyly, and teleangiectasia [8, 9]), on the presence of a diffuse inflammatory component at the vascular level [10], or on the association with specific autoantibodies [11].

At present, the most accurate and reliable classification of scleroderma is the one proposed by LeRoy et al., which distinguishes two main subsets of Systemic sclerosis: limited SSc and diffuse cutaneous SSc [12].

The main clinical and pathological characteristics of the two disease subsets are:

Limited cutaneous SSc

- Raynaud's phenomenon for years at presentation.
- Skin sclerosis limited to hands, feet, face, and forearms, or absent.
- Significant late incidence of pulmonary hypertension, trigeminal neuralgia, calcinosis, and teleangiectasia.
- Dilated nailfold capillary loops, usually without capillary dropouts.

Diffuse cutaneous SSc

- Onset of Raynaud's phenomenon within one year of onset of skin changes.
- Truncal and acral skin involvement.
- Presence of tendon friction rubs.
- Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, and myocardial involvement.
- Presence of anti-DNA topoisomerase I (anti-Scl-70) antibodies.
- Absence of anti-centromere antibodies.
- Nailfold capillary dilatation and destruction.

More than 50% of SSc patients fall into the subgroup of limited SSc. These patients usually present a more insidious onset of illness and a long history of Raynaud's phenomenon and swelling of digits. However, their prognosis is significantly more favorable when compared with patients with the diffuse variant. Limited SSc indeed presents a more benign disease course and a lower incidence of renal involvement and restrictive pulmonary disease [13].

SSc is relatively rare, if compared with other connective tissue diseases. However, it is believed that the true incidence is underestimated, since the early features of the disease are frequently overlooked. The prevalence of SSc reported to be between 13 to 105 and 13 to 140 per million in North America, Australia and Europe, respectively [14].

The overall female/male ratio is attested at 3:1. However, this ratio is larger in Great Britain (6:1) and in the USA (8:1) than it is in the rest of Europe. The

female preponderance is most significant in the young adult population (7:1), while it narrows to 2 - 3:1 toward the fifth decade of life. The onset of SSc occurs around the 45 years old mark in the general population, but in childbearing women the age at diagnosis can be as low as between 30 and 39 years old. Less than 10% of patients develop SSc before the age of 20 [15].

Several survival studies have indicated a distinct dependency on the level of involvement of internal organs. In general, the survival rate lies between 34 and 73% [6]. It is, however, lower and associated with a poorer prognosis in men and older patients, while women and younger patients generally present a higher survival score and better prognosis. Survival differences are also appreciable in the Caucasian population as compared to the African Americans [16].

The extent of skin sclerosis (skin score) is a useful marker of both severity and prognosis of the disease. Sclerodactyly alone is associated with a survival rate of 79-84% at 5 years post-diagnosis; the survival score lowers to 47%-75 % at 10 years. Patients diagnosed with truncal SSc have a much lower survival score: the rate is attested at 48-50% at 5 years and at 22%-26 % at 10 years [17].

The average mortality for Systemic sclerosis is reported to be between 0.9 and 3.8 per million per year [16]. Affected individuals have is a 4.6-fold risk of death compared with the general population; the risk level is even higher when only male patients with the diffuse subtype are considered. The principal causes of death for patients diagnosed with Systemic sclerosis are cardiopulmonary complications and renal failure [18]. In SSc patients, it has also been observed

an increased susceptibility to the development of cancer, mainly of the lung [14].

1.3 PATHOGENESIS OF SSC

The initial triggers and the course of the pathogenesis of SSc are still poorly understood. Associations with particular genetic backgrounds are undoubtedly important factors to consider while attempting to discern the causes of SSc pathogenesis [19]. The microvasculature compartment (endothelial cells, platelets, capillaries) is one of the first affected systems [20]; at times, events of localized vascular damage (such as Raynaud's phenomenon [21]) can precede even by years the clinical outbreak of the disease. There is also strong evidence that one of the early events that could initiate the development of the disease may be an immunological imbalance. Systemic sclerosis is an autoimmune disease and, as such, one of the distinguishing features of its pathogenic course is the presence of autoantibodies. Several classes of antibodies targeting endothelial cells and fibroblasts have been described, and few of them seem to have an agonistic role in SSc pathogenesis [22]. The humoral immune compartment is not the only one involved: T lymphocytes, in collaboration with B lymphocytes and monocytes, may act as both mediators and targets in the pathophysiological network underlying the pathogenesis of SSc [23]. The cells of the immunological compartment express adhesion molecules and release cytokines and growth factors which act upon fibroblasts, promoting their activation and proliferation. Once activated, proliferating fibroblasts have been described to behave relatively autonomously, continuously overexpressing genes encoding for extracellular matrix components. This self-maintained

imbalanced circuit ultimately causes an excessive deposition of collagen and other matrix proteins of the connective tissue, leading to the progressive fibrosis of blood vessels, skin and internal organs [24].

1.4 GENETICS OF SSC

Familial clustering of the disease [25], the high incidence of other autoimmune disorders in families of patients with scleroderma [26], and differences in phenotype among racial and ethnic groups [16, 27], suggest that genetic factors contribute to SSc. The most prominent genetic factor is gender (on average, the female population is affected twice to three times as much as the male population). There is strong evidence of linkage of certain human major histocompatibility complex (MHC) molecules to clinical phenotypes: an increased frequency of a small subset of class I and II MHC alleles were found in sclerodermic patients (i.e., HLA Bw35, DR1, DR5 or HLA1-B8-DR3) [28]. However, their nature and association with the onset of the disease is still controversial. On the other hand, two variants of DRB (DR3 and DRw52) are suggested to be the primary MHC class II alleles associated with SSc. In addition, there is an association between the development of lung fibrosis and the presence of the alleles B8-DR3 and DRw52-DQB2 [29].

Chromosomal alterations have also been described as features of Systemic sclerosis. A higher occurrence of chromosomal breakage, deletions and acentric fragments can be observed in lymphocytes and fibroblasts of SSc patients versus healthy controls (15.5% versus 1.7%). Clastogenic activity has been described in sera and cell extracts from SSc patients. The increased rate of

spontaneous and clastogen-induced chromosomal damage may indicate that lymphocytes of SSc patients may have a general susceptibility to DNA damage caused by free radicals [30].

1.5 MICROCHIMERISM

Numerous clinical features of SSc are reminiscent of those occurring in chronic Graft-versus-Host Disease (GvHD) [31], and it is likely that these features are implicated in the pathogenesis of SSc. One of the hypotheses formulated to explain these similarities is the occurrence of a phenomenon of persistent cellular microchimerism [32]. Microchimerism results from the migration of fetal cells (including stem cells) through the placenta into the maternal circulation during pregnancy [33]. These cells can survive and then proliferate in the maternal vascular system thanks to familial HLA class II compatibility. Indeed, the presence of fetal CD3-positive T lymphocytes in the maternal peripheral blood and of fetal cells in tissue samples showing signs of fibrosis typical of sclerodermic disease has been described in female SSc patients with previous pregnancies. [33-35]. The persistent microchimerism might be the initial stimulus of a fetal anti-maternal GvHD-like response that could, later on, cause the onset of SSc in certain patients [36]. However, additional stimuli are required to prevent tolerance and to convert this type of GvHD from latent to active. In male SSc patients, allogenic cells derived from a twin or from the maternal placental blood, or white blood cells surviving after blood transfusions may behave in a similar way and contribute to the pathogenic process [37].

1.6 RAYNAUD'S PHENOMENON

The most peculiar vascular dysfunction in SSc is Raynaud's phenomenon. This clinical feature is caused by the dysregulation of the vascular tone of fingers and toes. An exaggerated vasomotor response, often triggered by environmental factors such as cold or stress, causes the abrupt vasoconstriction of peripheral capillaries, leading to tissue hypoxia [38]. In Systemic sclerosis, in addition to an imbalance in endothelial signals (for example, an increased release of vasoconstrictive endothelin), other factors, including impaired vasodilatory mechanisms (such as lowering of nitric oxide levels or of endothelial-dependent relaxation factor), enhanced platelet aggregation and reduced neuropeptide levels, contribute to the vasospastic propensity. Other harmful factors, such as toxic factors, proteases (e.g., granzyme 1), lipoperoxides, and anti-endothelial autoantibodies may contribute to this process [39, 40].

1.7 THE ROLE OF TGF-β AND PDGF

There are several pathologies characterized by the progressive onset of a fibrotic phenotype. The mechanism underlying the deterioration of lungs, kidneys, gastrointestinal tract or skin in all of these diseases, is the proliferation of mesenchimal cells with characteristic fibroblastoid features and the consequent deposition of collagen and other proteins of the extracellular matrix (ECM) [41]. The gradual accumulation of ECM causes the progressive sclerosis and subsequent loss of function of the affected organs [42]. The identification of specific factors or pathways involved in the deregulation of the fibrotic process offers the potential for the development of therapeutic strategies for all fibrotic diseases.

Generally, fibrogenesis is the physiological response to tissue damage, but there are several other mechanisms able to elicit a proliferative response in mesenchimal cells, even in the absence of primitive endothelial alterations. This is the case for Systemic sclerosis: several mediators have been identified as key factors in the onset of pathological fibrosis, including transforming growth factor beta 1 (TGF-β1, or TGF-β) and platelet-derived growth factor (PDGF) [43]. PDGF is a potent mitogen for cells of mesenchimal origin; in particular, it strongly stimulates proliferation of myofibroblasts [43]. On the other hand, TGF-β mainly functions as inducing factor of collagen deposition in newly replicated myofibroblasts [41].

The dimeric isoforms of the A and B chain of PDGF (PDGF-AA, -AB, -BB) play a key role on the onset of fibrosis. These molecules are able to induce proliferation and chemotaxis of myofibroblasts, collagen production and adhesion in endothelial cells [44].

The production and secretion of PDGF is regulated by itself with an autocrine loop and, in addition, by a variety of extracellular proteins, glycoproteins and ECM proteins, whose expression is usually upregulated in patients with a progressive fibrotic disease [45]. The concentration of PDGF is increased in endothelial cells, in the serum and in the bronchoalveolar lavage of sclerodermic patients [46].

TGF-β is considered one of the most powerful pro-fibrotic cytokines, and its role in the pathogenesis of SSc and other fibroproliferative diseases is unquestionable [47]. One of the major functions of TGF-β *in vivo* is the stimulation of the ECM synthesis and the upregulation of the expression of genes encoding ECM proteins. TGF-β is also able to inhibit the synthesis of collagen-disrupting proteins, such as metalloproteinases (MMP). Exposure to even low doses of TGF-β is able to maintain fibroblasts in a state of permanent activation. TGF-β also acts as an autocrine inductor of its own production. In sclerodermic patients increased production of TGF-β, by both fibroblasts and monocytes, and up-regulation of the TGF-β-receptor on fibroblasts have been described [47, 48].

TGF-β binds the TGF-β type II receptor (TβRII) on the cell surface, forming an active complex that in turn phosphorylates the type I receptor (TβRI). The activation signal reaches the nucleus through the activation of the Smad proteins cascade; selected members of the Smad family bind directly to the DNA, promoting the transcription of collagen genes. TGF-β is able to induce collagen production not only in a direct manner, but also exploiting collateral pathways, for example stimulating the production of the connective tissue growth factor

(CTGF), and activating protein kinase C-δ (PKC-δ) or phospholipase C (PLC). All these pathways converge in the promotion of the transcription of collagen or other fibrosis-related genes [47].

The binding of TGF-β to sclerodermic fibroblasts is also able to positively regulate the production of PDGF, in a concentration-independent manner. Sclerodermic fibroblasts, in contrast with normal fibroblasts, are able to autonomously produce the pro-inflammatory cytokine IL-1α, which in turn stimulates the expression of PDGF-AA and IL-6. These cytokines induce cell proliferation and type I collagen expression, respectively, leading to hyperplastic growth and fibrosis of the tissue (Figure 2)[49].

Moreover, TGF-β has an important role in the progression of fibrosis. It is indeed responsible for the up-regulation of the expression of PDGF receptors on the cell surface of fibroblasts [45] and, therefore, for the augmented capacity of these cells to respond to the mitogenic stimuli provided by both PDGF-AA and TGF-β itself (Figure 2).

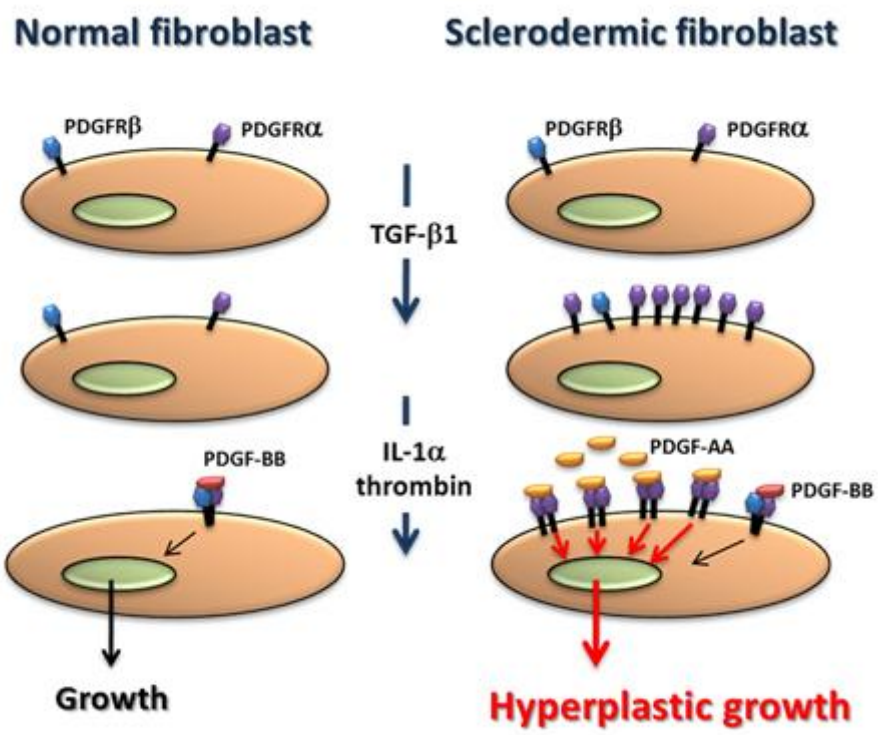

Figure 2: Differential regulation of PDGF and PDGF receptors in normal and sclerodermic fibroblasts. TGF-β and thrombin are potent inductors of PDGFRα expression on the surface of sclerodermic fibroblasts, but not of healthy ones. Following the up-regulation of the receptor, thrombin and IL-1α co-operate in the induction of an autocrine production of PDGF-AA, which in turn stimulates the typical hyperplastic growth of sclerodermic fibroblasts.

1.8 THE ROLE OF PDGF IN THE OXIDATIVE STRESS

PDGF is an important regulator of the senescence process and survival of human fibroblasts. It is indeed able to regulate the level of expression of the protein Ras through the activation of intracellular signaling pathways involving extracellular-regulated kinases 1 and 2 (ERK1/2) and the modulation of the production of reactive oxygen species (ROS) [50].

Fibroblasts are able to constitutively produce high levels of ROS through the activation of the NADPH-oxydase (NOX-1). ROS produced in this manner are able to mediate apoptotic signaling in a direct way; however, through the induction of NF-kB, they can positively modulate the expression of genes characteristic of the inflammatory response [51].

In parallel, ROS can activate the ERK1/2 pathway which, in turn, can induce the expression of the Ha-Ras gene. The expression of Ras protein is reinforced and stabilized through the activation of Raf-1, which is modulated by ROS-dependent ERK1/2 stimulation. ERK1/2 and ROS are able to stabilize the expression of Ha-Ras even at the post-translational level, inhibiting its proteasome-mediated degradation [50].

The final result of the Ras expression and stabilization is the triggering of a plethora of intracellular signaling pathways leading to a modification of the fibroblast towards a myofibroblastic phenotype, to collagen synthesis regulation and to pro-inflammatory and apoptotic responses [50].

Fibroblasts from sclerodermic patients are shown to basally express an increased number of PDGF receptors (induced by the de-regulation of TGF-β levels [45]) and an increased activation of the ERK1/2 and ROS pathways [50].

These observations, together with the occurrence of fibrotic complications in a large number of SSc patients, lead to hypothesize the existence, in the serum of sclerodermic patients, of other PDGFR-binding molecules that might be able to induce the above described signaling cascades, thus causing fibrosis of the connective tissue. In particular, the proposed theory envisages PDGFR-specific

autoantibodies as one of the primary causes of PDGFR activation, ultimately leading to fibroblast proliferation and fibrosis (Figure 3).

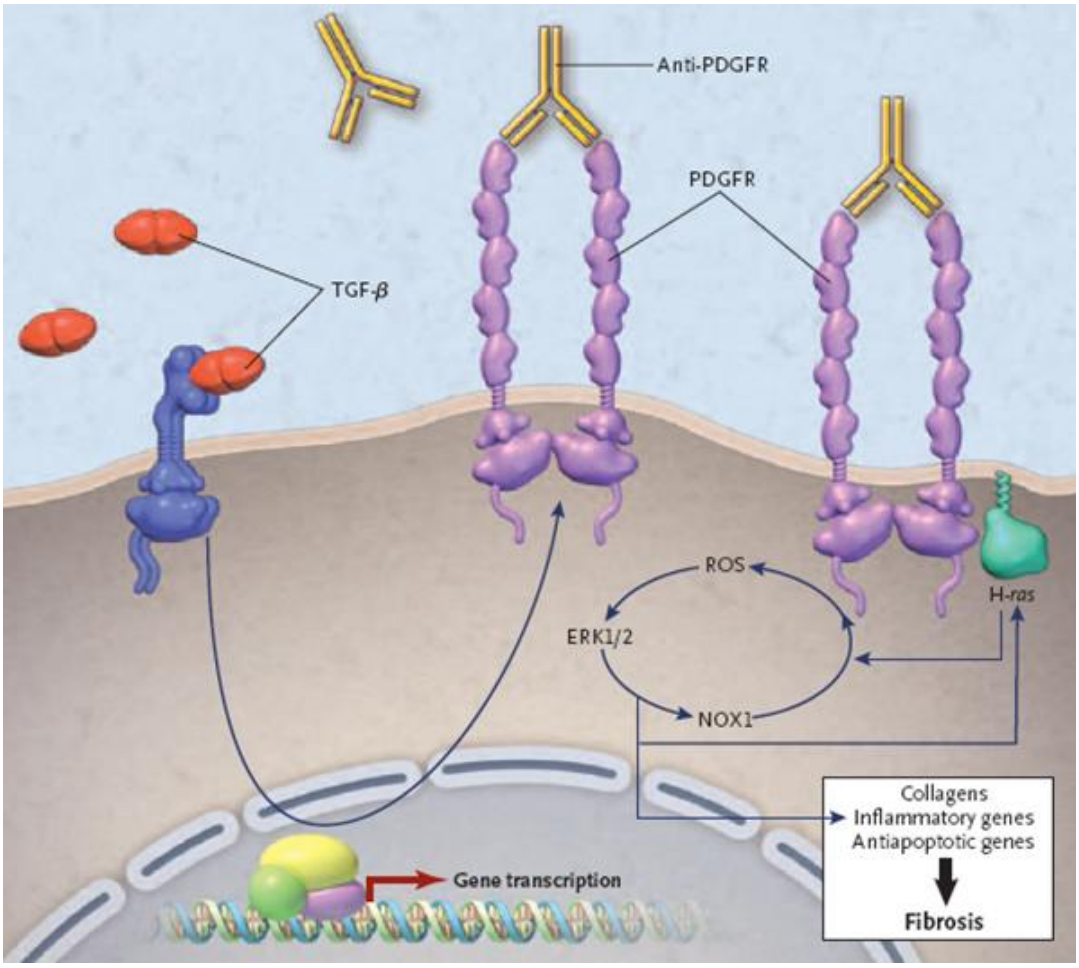

Figure 3: TGF-β induces the overexpression of PDGF receptors on the cell surface of sclerodermic fibroblasts. The cells therefore become more sensitive to the stimuli arising from this receptor in following engagement by the native ligand or by specific autoantibodies. The activation of the receptor induces NADPH-oxydase (NOX1) to produce reactive oxygen species (ROS). ROS, in turn, activate the kinases ERK1/2, which induce Ha-Ras (H-Ras) expression and stabilization, directing the cell towards collagen gene induction and expression of inflammation-related genes. This complex signaling cascade ultimately leads to extensive fibrotic transformation and subsequent endothelial damage. Autoantibodies specific for the PDGFR could constitute an additional cause for the activation of the same signaling pathway.

1.9 THE ROLE OF THE IMMUNE SYSTEM

The presence of autoantibodies is considered the principal immunological alteration in Systemic sclerosis [52]. At least 90% of sclerodermic patients presents detectable serum levels of anti-nuclear antibodies (ANA)[53, 54], anti-nucleolar antibodies (ANoA) or antibodies specific for molecules expressed on the cell surface (anti-endothelial cells antibodies, or AECA)[55, 56], even before the onset of the clinical symptoms of the disease. In the majority of cases, the specificity and concentration of these autoantibodies is strictly correlated with the severity of the symptoms and the positive or negative prognostic outcome of the pathological phenotype.

However, also the B and T cell compartments of the cellular immunity play important pathogenic roles in the onset, development and progression of the disease.

1.9.1 T lymphocytes and cytokines

The abundance of $CD4^+$ T lymphocytes in the perivasal infiltrates and in the neo-formed fibrotic tissue, commonly found in sclerodermic patients as a result of a generalized state of inflammation, suggests that these cells could hold a pathogenic role in the disease [57, 58]. $CD4^+$ cells constitute the "T helper" class of lymphocytes (Th) which, depending on which pattern of cytokines it produces, can be further distinguished into Th1 and Th2 lymphocyte subgroups. Th1 lymphocytes mainly secrete interferon gamma (INF-γ), tumor necrosis factor alpha (TNF-α) and IL-1, promoting a cellular response and inhibiting the process of fibrogenesis. Th2 are known to

produce IL-4, IL-5, IL-6, IL-10 e IL-13, favoring the activation of the humoral immunity. IL-4, IL-6 and IL-13 are also able to induce collagen production in fibroblasts [58-61]. T lymphocytes of sclerodermic patients show a remarkable increase of the Th2 subset, with consequent increased IL-4, IL-6, IL-10 e IL-13 serum levels. Moreover, the plasmatic level of monocyte chemoattractant protein 1 (MCP-1) is significantly increased during the course of scleroderma, contributing to the unbalance towards the Th2 phenotype. Indeed, MCP-1 is an important pro-fibrotic factor *in vitro* and it has been shown to regulate the migration of monocytes and Th2 lymphocytes at sites of inflammation [62].

1.9.2 B lymphocytes

There is strong evidence that B cells could play a critical and active role in the pathogenesis of Systemic sclerosis. Genome wide analysis showed up-regulation of B lymphocytes-associated genes in clinically affected skin from patients with scleroderma [63]. SSc patients also show an increased number of peripheral B lymphocytes compared to healthy controls; however, the subpopulations of B cells are quantitatively different from those of healthy individuals. The B cell repertoire in SSc patients is indeed characterized by an expanded naïve subset, a decreased (but highly activated) memory-B cell fraction and a reduced number of plasmablasts (Figure 4) [64].

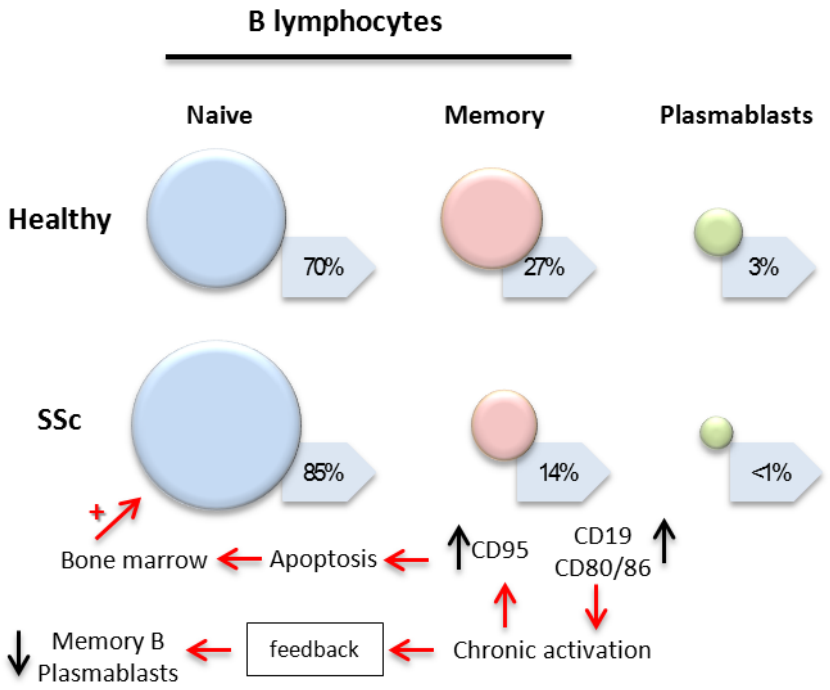

Figure 4: Impairment of B cell compartments in Systemic sclerosis and compensatory mechanisms.

CD19 results overexpressed on naïve and memory B cells from SSc patients [65]; in addition, the reduced number of memory B cells that can be found in sclerodermic patients shows high levels of expression of the co-stimulatory molecules CD80 and CD86. These finding suggest that these cells are in a state of chronic activation, as confirmed by the presence of high density of CD95 on their surface. CD95 is also considered to be the most relevant factor responsible of the enhanced susceptibility to apoptosis characterizing the sclerodermic B cells. The expansion of the naïve B cell subset is likely due to compensatory mechanisms through which bone marrow tries to balance the loss of more differentiated B cells (Figure 4) [64]. However, despite the small number, sclerodermic memory B cells show an increased propensity to

differentiate and secrete immunoglobulins [66]. These findings are confirmed by transgenic mice models in which B lymphocytes overexpress CD19: these mice exhibit hypergammaglobulinemia, loss of tolerance and spontaneous generation of autoantibodies with specificities similar to the autoantibodies repertoire of patients with Systemic sclerosis [67].

1.9.3 Autoantibodies

In Systemic sclerosis, as in the majority of the other autoimmune diseases, a spectrum of autoantibodies specific for a limited number of ubiquitously expressed antigens can be detected [55]. The pattern of specificity of these autoantibodies is strictly correlated with the pathological phenotype and can be useful in the prognosis and the prediction of disease outcome.

Sclerodermic autoantibodies can be classified in four main categories: i) anti-nuclear antibodies (ANA); ii) anti-nucleolar antibodies (ANoA); iii) anti-endothelial cells antibodies (AECA); iv) anti-fibroblast antibodies.

Anti-nuclear antibodies

ANA can be found in a consistent fraction of the sclerodermic population; they are mainly represented by anti-DNA topoisomerase I and anti-centromere antibodies (ACA, also known as anti-Scl-70) [68, 69].

The class of ACA comprises autoantibodies specific for six different centromeric proteins (CENP A-F) [70, 71]; at the clinical level, the titer of anti-Scl-70 IgG (detected with indirect immunofluorescence assays or enzyme linked immunosorbent assays) seems to positively correlate with the

patients' skin score (Figure 5). Furthermore, higher levels of ACA have been detected in patients with active disease, when compared to patients with inactive disease (Figure 6) [11, 72-74]. The frequency of ACA in sclerodermic patients ranges between 20% and 40%, and it is associated with ethnicity (ACA are more frequently found in Caucasian patients than in African-American and Hispanic subjects [75, 76]) and genotype (they are associated with HLA-DRB1*01 and HLA-DQB1*05 [76, 77]).

The presence of anti-topoisomerase 1 autoantibodies is considered mutually exclusive with ACA and it is found in approximately 20% of SSc patients. Their presence in the serum is characteristic of the diffuse cutaneous variant of scleroderma [78, 79]. It is likely that anti-topoisomerase I antibodies are the result of immunization to nuclear antigens exposed upon cell death.

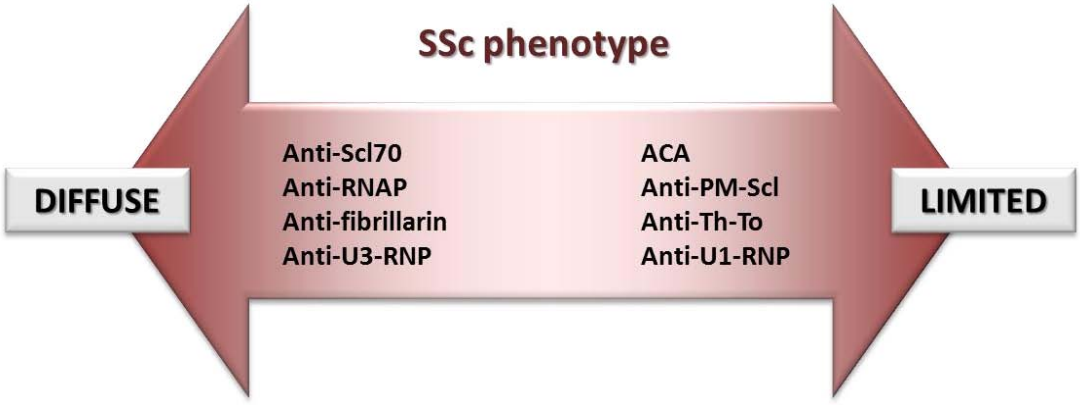

Figure 5: Association of ANA/ANoA specificity and pathological phenotype

Anti-nucleolar antibodies

ANoA comprise a series of relatively rare autoantibodies, specific for several nucleolar antigens; the more commonly found are anti-PM-Scl antibodies, anti-Th-To, anti-U3-RNP, anti-fibrillarin (AFA) and anti-RNA polymerase (anti-RNAP I-II-III) [80].

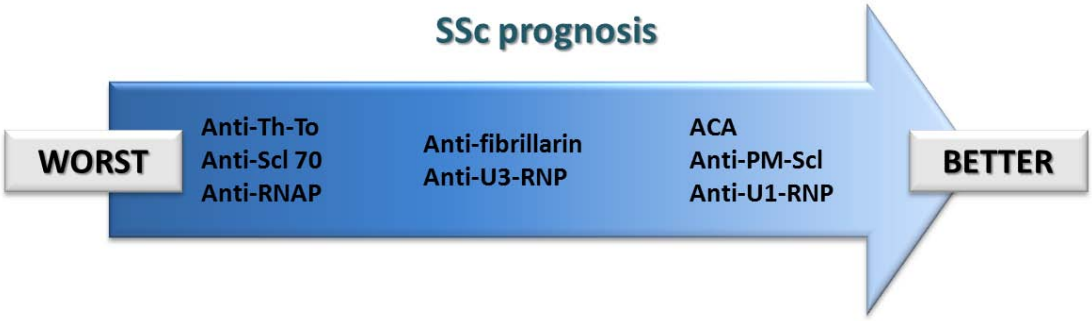

Figure 6: Association between ANA/ANoA specificity and SSc prognosis

Anti-endothelial cells antibodies

AECA have been observed in a significant fraction of sclerodermic patients. In contrast to ANA and ANoA, this class of autoantibodies seems to target antigens expressed on the cell surface and, in *in vitro* studies, it is able to induce apoptosis of human dermal microvascular endothelial cells in the presence of activated NK cells [81]. However, the specific antigens targeted by AECA and the mechanisms eliciting their production are still unknown [40, 82]. However, a likely scenario is that AECA are produced as a consequence of the exposition of cellular antigens after cell death, as it is the case for ANA and ANoA. Another possibility is that these autoantibodies are not originally directed towards self-antigens, and that their production is

initially triggered by exogenous antigens mimicking endogenous molecules. In the serum of sclerodermic patients it is indeed possible to find circulating antibodies recognizing the human cytomegalovirus (hCMV) late protein UL94. These anti-viral antibodies are also capable to bind the integrin/Nag-2 protein complex on the cell surface of human endothelial cells and activate apoptotic processes, indicating the existence of molecular mimicry between viral and endogenous antigens. The protein Nag-2 is also expressed on the surface of human dermal fibroblasts: binding of the anti-UL94 antibodies to these cells, can induce gene transcription and protein expression. The transcribed sequences include genes involved in ECM production and deposition, and genes encoding growth factors, chemokines and cytokines [83].

Anti-fibroblast antibodies

Fibroblasts have been extensively investigated in scleroderma, primarily because of their central role in the establishment and progression of the fibrotic process. Unsurprisingly, the presence of autoantibodies specific for fibroblasts has been detected in sclerodermic patients [84-86]. Antibodies targeting the C-terminal region of the protein fibrillin-1 (one of the major components of the extracellular matrix microfibrils constituting the scaffold of connective tissue) have been found in a significant fraction of sclerodermic patients [87]. These autoantibodies are able to bind human normal fibroblasts *in vitro* and reproduce the SSc-specific pattern of activation leading to fibrosis. Indeed, they are able to induce the nuclear translocation of phosphorylated Smad-3 and the subsequent expression of several ECM

components, such as collagen, metalloproteinases and metalloproteinases inhibitors (TIMPs) [88, 89].

MMPs, and in particular MMP-1 and MMP-3, have also been reported to be target of specific autoantibodies found in sclerodermic patients: they are thought to inhibit ECM degradation by blocking the action of the MMP they target, therefore allowing a worsening of the fibrosis [90, 91].

1.10 ANTI-PDGF RECEPTOR AUTOANTIBODIES

Recently, a novel class of autoantibodies targeting cell surface molecules on fibroblasts has been discovered in the serum of SSc patients [1]. These antibodies are able to bind the human PDGF receptor alpha (PDGFRα) and activate an intracellular pathway that involves ERK1/2, NADPH oxydase and Ras, stimulating the production of reactive oxygen species and inducing collagen gene transcription (Figure 7). The activation of this signaling cascade by PDGFR-specific autoantibodies ultimately results in wide-spread oxidative stress. This could lead fibroblasts to the acquisition of a myofibroblastic phenotype, that might account for a persistent profibrotic response with a subsequent formation of foci of inflammation and further spreading of the fibrotic transformation of the connective tissue [22].

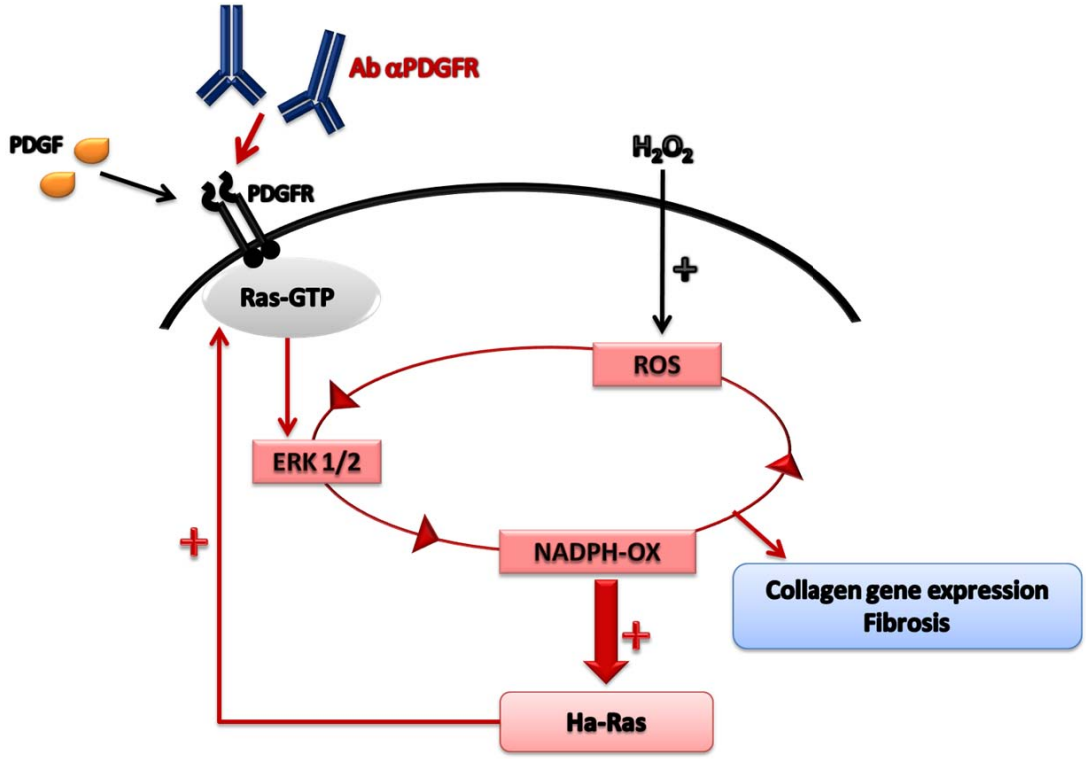

Figure 7: Diagram of the signaling cascade triggered by sclerodermic PDGFR-specific autoantibodies. The autoantibodies bind the receptor and induce its activation, with the stabilization of Ras at the membrane level. Ras stabilization induces ERK1/2 phosphorylation and consequent activation. Through the consequent involvement of NADPH oxydase, ERK1/2 induces an increase in the production of reactive oxygen species. The long term persistence of ERK1/2 in its active state and the continuous production of ROS ultimately lead to the stimulation of collagen gene expression and translation.

The observation and description of this signaling pathway constituted the starting point for the development of the work described in this thesis.

Baroni et al. [1] observed that the PDGFR-specific autoantibodies found in the immunological repertoire of sclerodermic patients can bind and immunoprecipitate the αα, αβ, and ββ PDGF receptor chains on the cell surface of PDGFR-expressing cells (Figure 8, panel A). The binding of the autoantibodies to PDGFR induces receptor dimerization and *in trans* auto-

phosphorylation of tyrosine residues in the two receptor chains composing the dimer, indicating that the specific immunoglobulins have agonistic biological properties (Figure 8, panel B).

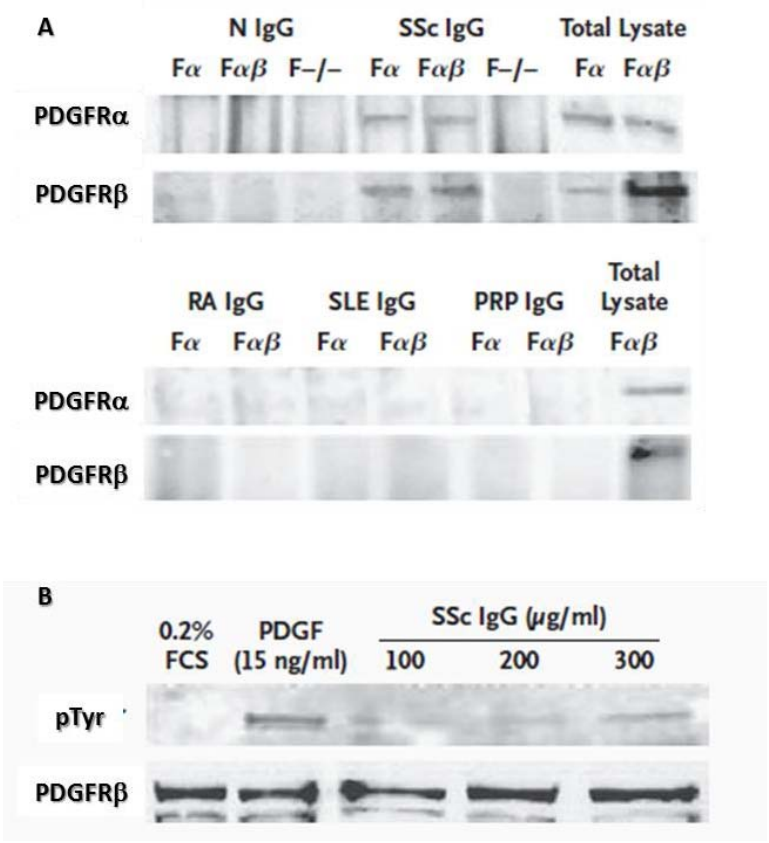

Figure 8: A) IgG purified from the serum of sclerodermic patients (SSc IgG), but not IgG of healthy controls (N IgG) are able to immunoprecipitate both the alpha and beta chains of the PDGFR from the lysate of PDGFR expressing cells (Fα and Fαβ, mouse embryo fibroblasts transfected with the human full length PDGFR chains α and αβ, respectively [92]). Immunoglobulins purified from the serum of patients with other pathologies are not able to immunoprecipitate the receptor from transfected cells (RA: Rheumatoid Arthritis, SLE: Systemic Lupus Erythematosus, PRP: Primary Raynaud's phenomenon). B) SSc IgG are able to induce the tyrosine phosphorylation of PDGFRβ.

PDGFR engagement by SSc immunoglobulins induces receptor activation through its phosphorylation. According to the previously described signaling pathway, this should stimulate the production of ROS through the activation of NADPH oxydase. Human normal fibroblasts or PDGFR-expressing cells challenged with immunoglobulins purified from the serum of sclerodermic patients (but not from healthy controls) proved to be able to induce the production of ROS. IgG purified from the serum of patients with other autoimmune diseases (i.e., Systemic Lupus Erythematosus and Rheumatoid Arthritis) or with SSc-related diseases, not of autoimmune origin (i.e., Primary Raynaud's phenomenon or Interstitial Lung Disease) were not able to induce ROS production in PDGFR-expressing cells (Figure 9).

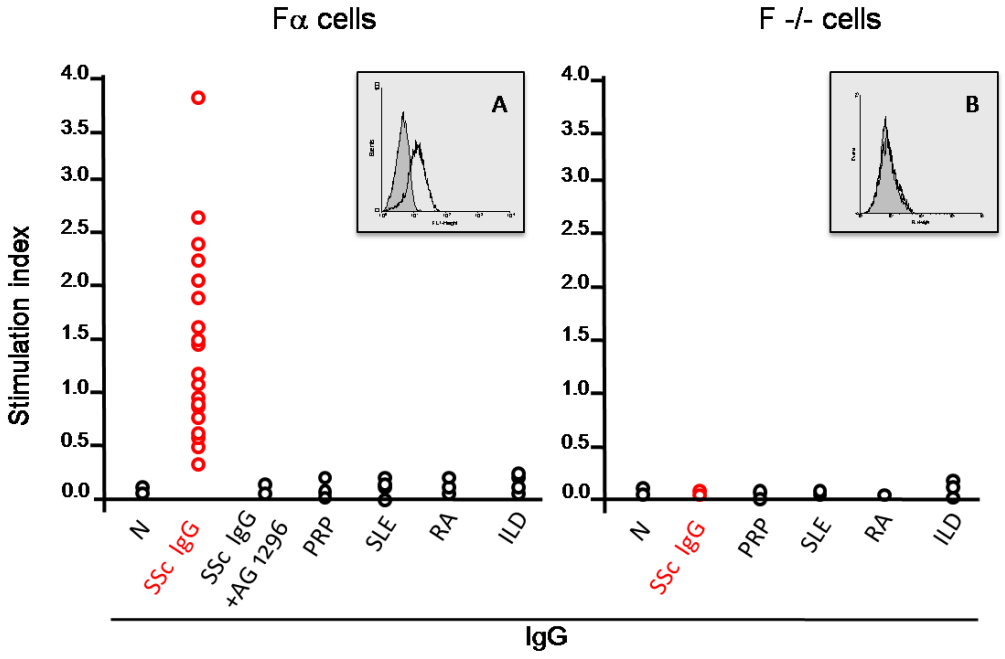

Figure 9: SSc IgG induce the production of reactive oxygen species in PDGFRα-expressing target cells (Fα) but not in mock transfected cells (F-/-). Panel A and B show the level of PDGFRα expression on Fα and F-/- cells, respectively. Immunoglobulins purified from the serum of

healthy controls and other diseases are not able to trigger oxidative stress, measured as a stimulation index of ROS in a fluorimetric assay. A specific inhibitor of the PDGFR tyrosine kinase (AG 1296) abolishes the biological activity of sclerodermic IgG. SSc IgG = sclerodermic IgG; N = IgG from healthy controls; PRP = Primary Raynaud's phenomenon; SLE = Systemic Lupus Erythematosus; RA = Rheumatoid Arthritis; ILD = Interstitial Lung Disease.

Reactive oxygen species produced following the activation of PDGFR are able to inhibit tyrosine phosphatases, therefore activating the ERK1/2 kinase that, in turn, stabilizes the GTPase Ha-Ras on the internal surface of the cell membrane (Figure 10). Physiologically, Ras is degraded via the proteasomic system shortly after its induction, in order to preserve cellular viability. In a pathological context, Ras activation following binding of PDGF or anti-PDGFR autoantibodies to the receptor induces an autocrine protective mechanism that hinders Ras degradation and keeps its cytoplasmic level at high values for a long period [50]. Ras is able to further stimulate ERK1/2, which can activate the NADPH oxydase, resulting in an even more amplified production of ROS.

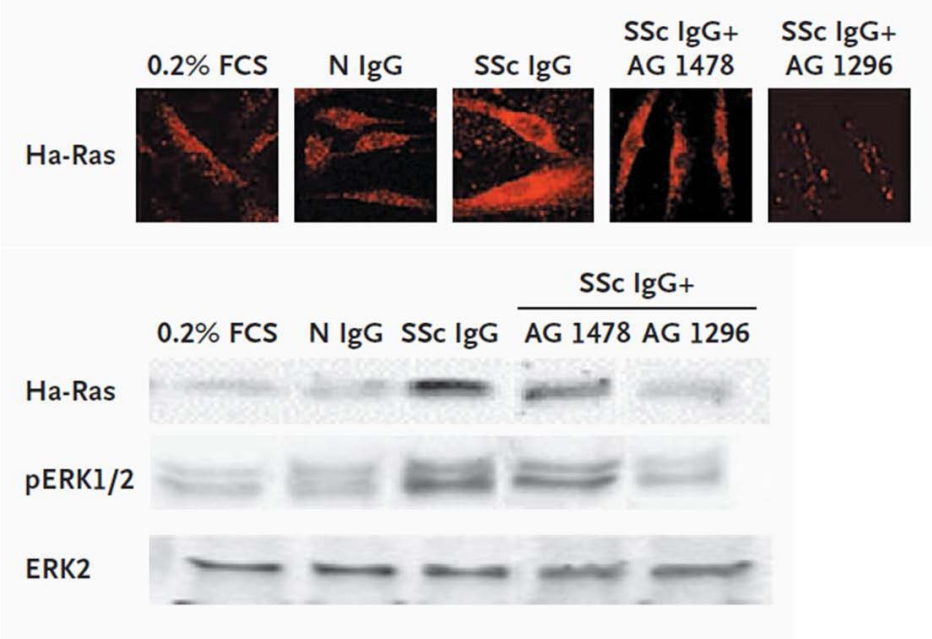

Figure 10: Sclerodermic IgG, but not IgG of healthy controls, are able to induce Ha-Ras expression and ERK1/2 phosphorylation in fibroblasts. A) Immunocytochemical analysis of Ha-Ras expression in normal fibroblasts following stimulation with SSc or normal IgG. B) Western Blot analysis of Ha-Ras expression and ERK1/2 phosphorylation. The biological effects of SSc IgG are inhibited by PDGFR tyrosine kinase-specific inhibitors (AG 1296), but not by an inhibitor of a tyrosine kinase non-correlated to the signaling pathway downstream of PDGFR (EGFR kinase-specific inhibitor, AG 1478).

Furthermore, immunoglobulins purified from the serum of SSc patients are able to induce the overexpression of two genes characterizing the sclerodermic phenotype of fibroblasts: α-smooth muscle actin (α-SMA) and type I collagen. The overexpression of α-SMA and type I collagen is appreciable both at the molecular and at the phenotypic level, as demonstrated by the increased expression of the mRNA of both genes as well as by the overexpression of the corresponding proteins into the cytoplasm of fibroblasts stimulated with SSc IgG. The expression of these genes follows the binding of sclerodermic IgG and it is not stimulated by control

immunoglobulins (Figure 11). All the biological effects that the sclerodermic immunoglobulins have on human fibroblasts can be hampered by the combined treatment with selective inhibitors of key factors in the signaling cascade downstream of PDGFR.

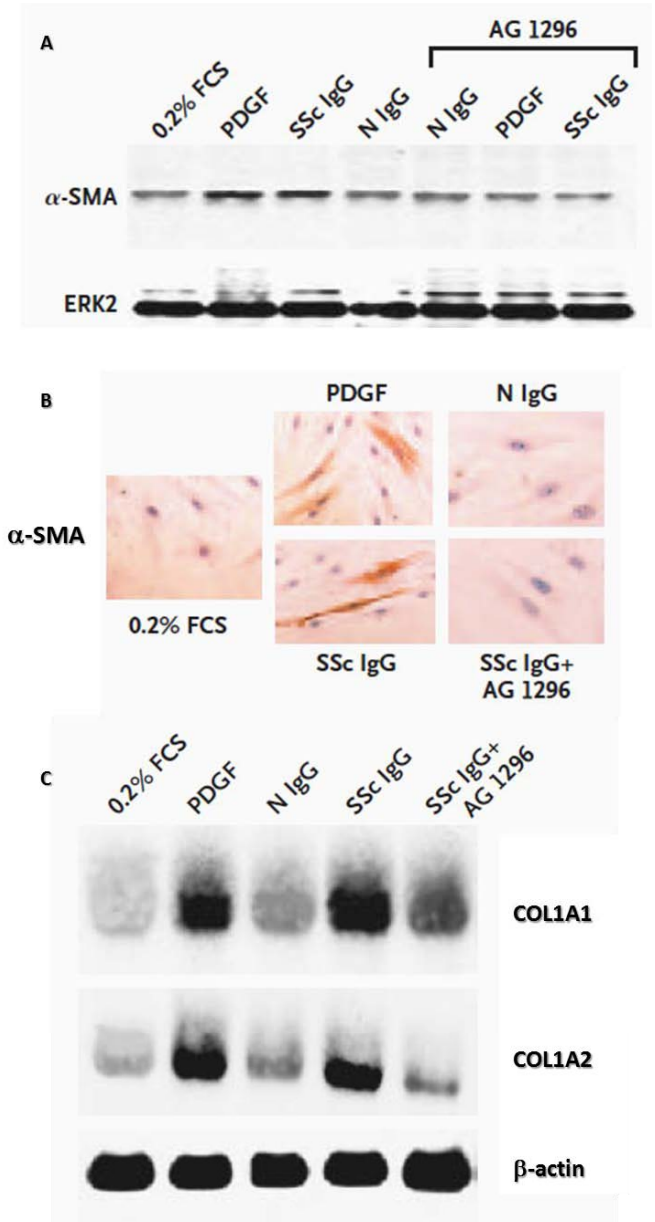

Figure 11: Sclerodermic IgG, but not IgG of healthy controls, are able to induce the expression of myofibroblastic markers in human fibroblasts, indicating a role in the fibrotic transformation. A) SSc IgG are able to induce the transcription of alpha smooth muscle actin (α-SMA), as demonstrated

by the increase of the mRNA level after stimulation of fibroblasts with IgG purified from the serum of SSc patients. The level of expression is comparable to that induced by PDGF. B) Both SSc IgG and PDGF are able to induce the expression of α-SMA protein at comparable levels. C) PCR analysis shows that SSc IgG stimulate the transcription of the α1 and α2 chains of type I collagen (COL1A1 and COL1A2) in fibroblasts. The agonistic effects of SSc IgG on both α–SMA and collagen result markedly reduced in the presence of a specific inhibitor of PDGFR activation (AG1296), indicating that the activation pathway is the one downstream of the receptor.

To further characterize the specificity and the biological properties of sclerodermic anti-PDGFR antibodies, the B cell repertoire of selected SSc patients has been immortalized with Epstein-Barr virus (EBV) and analyzed for the production of PDGFR-specific antibodies.

Two cultures secreting PDGFR-specific antibodies of IgM class (namely, ROM D2-1F5 e ROM 2C5-4G11) have been isolated on the basis of their selective binding on PDGFR-expressing cells (Figure 12, panel A). Both antibodies were able to induce the production of reactive oxygen species (Figure 12, panel B).

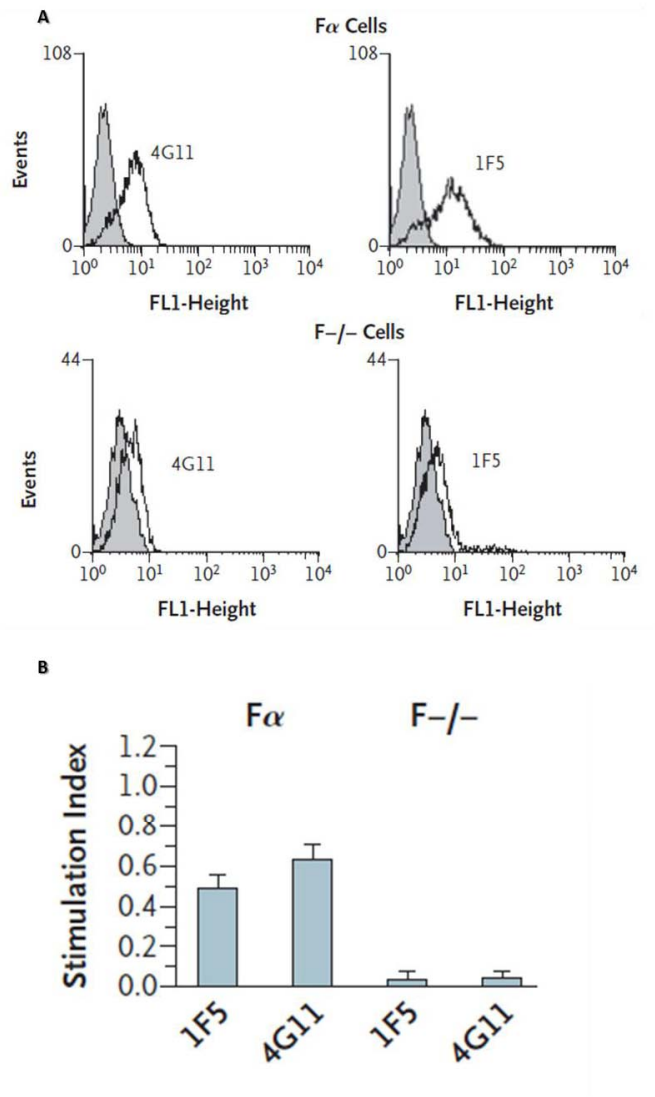

Figure 12: A) Cytofluorimetric analysis shows that isolated PDGFR-specific IgM oligoclones (4G11 and 1F5) bind selectively to PDGFR-expressing cells (Fα) but not to PDGFR-negative cells (F-/-). B) The two PDGFR-specific IgM possess the same biological activity of sclerodermic IgG purified from serum; indeed, they induce ROS production only in Fα cells.

The isolated PDGFR-specific immunoglobulins possess the same agonistic activity of the immunoglobulins purified from serum of sclerodermic patients, as underlined by their ability to induce the expression of the myofibroblastic markers α-SMA and collagen (Figure 13).

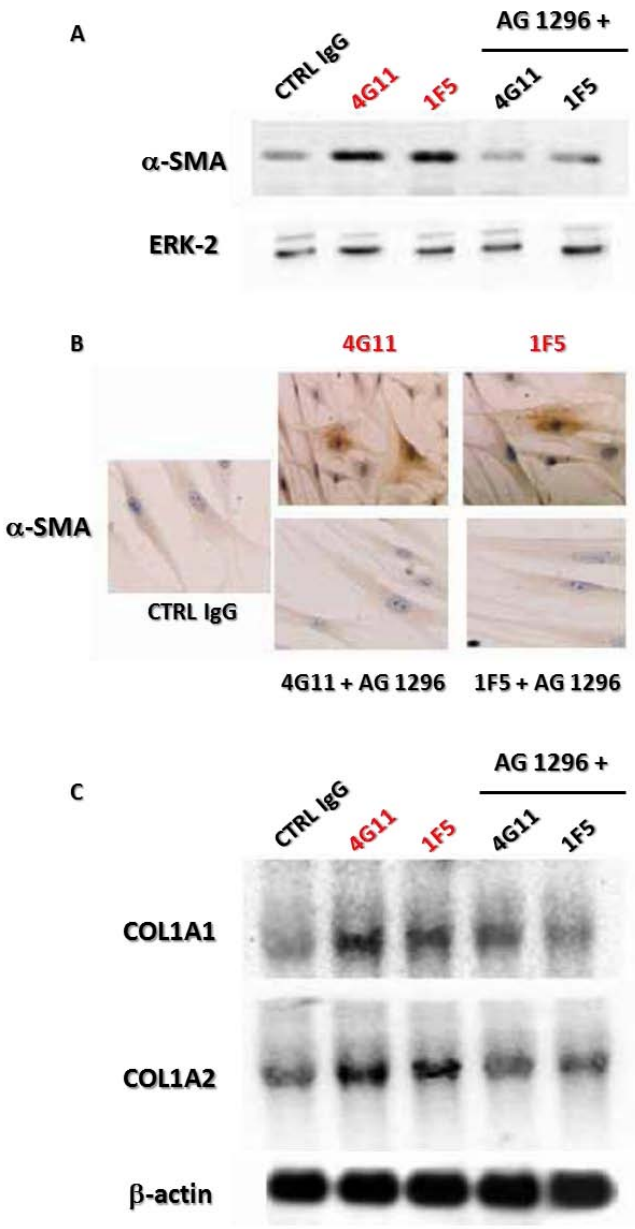

Figure 13: The PDGFR-specific IgM autoantibodies induce the expression of markers of myofibroblastic transformation in human fibroblasts. 1F5 and 4G11 IgM are able to induce the transcription of α-SMA (Panel A) and both chains of type I collagen (COL1A1 and COL1A2, panel C), as demonstrated by PCR analysis. B) Immunocytochemical analysis shows that the stimulation of the expression of α-SMA by the two IgM on fibroblasts is appreciable also at the phenotypic level. A specific inhibitor of the PDGFR activation (AG 1296) significantly inhibits the agonistic activity of the sclerodermic autoantibodies.

2 *Aim of the study*

The scientific background of the work discussed in this thesis was the discovery that agonistic autoantibodies specific for PDGFRα are present in the serum of patients with Systemic sclerosis [1].

Sclerodermic anti-PDGFRα autoantibodies are able to activate a distinct signaling pathway downstream of PDGFR, which involves the activation of NADPH oxydase, ERK1/2 phosphorylation and Ha-Ras expression. The consequences of the activation of this pathway in human fibroblasts are the overproduction of reactive oxygen species (ROS) and the up-regulation of collagen and smooth muscle actin genes. The induction of oxidative stress and of the transcription of genes characteristic of a pro-fibrotic phenotype in fibroblasts, argues for a potential pathogenic role of SSc autoantibodies in the onset and progression of fibrosis in Systemic sclerosis [50].

The aims of this work were:

- isolation and immortalization, from the immunological repertoire of Sclerodermic patients, of the B cells responsible for the production of anti-PDGFRα antibodies;
- characterization of the binding capacity, affinity and biological activities of the isolated anti-PDGFRα autoantibodies;
- analysis and cloning of the heavy and light chain variable regions of the isolated anti-PDGFRα autoantibodies;

- production and characterization of human recombinant anti-PDGFRα IgG;
- set-up of a binding assay for large-scale screening of the presence of anti-PDGFRα autoantibodies in human serum, with potential diagnostic purposes.

Future objectives comprise: i) in-depth characterization of the recombinant anti-PDGFRα antibodies, to confirm the pathogenic role of agonistic autoantibodies in SSc; ii) identification of the epitopes of PDGFRα bound by the agonistic anti-PDGFR autoantibodies; iii) analysis of the variations in the transcriptional profile of human fibroblasts following stimulation with sclerodermic PDGFR-specific autoantibodies.

3 *Materials and methods*

3.1 CELL CULTURES

Immortalized B cells and allogenic feeder layer

A peripheral blood sample from a sclerodermic patient, obtained after written consent, was subjected to separation on a density gradient (Ficoll-Hypaque, GE Healthcare) in order to obtain a purified population of peripheral blood mononuclear cells (PBMC). The obtained PBMC were incubated for 15 minutes at 4°C with magnetic beads conjugated with anti-human CD22 antibodies, and the $CD22^+$ population was isolated with a magnetic column. The $CD22^+/IgG^+$ population was obtained through a second magnetic selection with beads coated with anti-human IgG antibodies (MACS protocol for magnetic cell sorting, Miltenyi Biotech).

Purified $CD22^+/IgG^+$ cells were immortalized with Epstein Barr Virus [2]. Briefly, the purified cells were infected by incubation with culture supernatant obtained from the EBV-producing marmoset B-lymphoblastoid cell line B95-8 (30% v/v for 16h at 37°C, 95% CO_2). The infected cell culture was propagated *in vitro* in complete RPMI-1640 medium (RPMI, Sigma Aldrich) supplemented with 10% fetal calf serum (FCS, Biochrome), in presence of CpG (ODN2006, 1 μg/ml, Coley Pharmaceutical Group) and IL-2 (200 U/ml, Roche) for 15 days.

After *in vitro* expansion, cells were seeded in 96 well plates (Corning Inc.) at decreasing concentrations (10-5 cells/well) in complete RPMI medium supplemented with 10% FCS.

Cell growth of EBV oligoclonal cultures was supported with irradiated (30 Gy) allogenic PBMC used as feeder layer. PBMC were purified with density gradient centrifugation from the buffy coat fractions of peripheral blood donations obtained from a public blood donors bank (AVIS). Irradiated PBMC were plated in complete RPMI medium at a concentration of 35-50,000 cells per well in 96 well plates, prior to the seeding of EBV-immortalized cells.

The RPMI medium used in all above procedures was additioned with penicillin (100 U\ml, Sigma-Aldrich), streptomycin (100 μg\ml, Sigma-Aldrich), L-glutamine (5mM, Sigma-Aldrich), B amphotericin (0.5 μg\ml, Sigma-Aldrich), sodium bicarbonate (1mM, Sigma-Aldrich) and sodium pyruvate (1mM, Sigma-Aldrich).

Fα and F-/- cells

Mouse embryo fibroblasts knock-out for murine PDGFR and transfected with the human full length PDGFRα (Fα), and the corresponding mock transfected cells (F -/-)[92] were maintained in adherent culture in complete DMEM medium (Dulbecco's modified Eagle's medium, EuroClone) supplemented with 10% FCS. Histidinol (5mM, Sigma-Aldrich) was added to the Fα cell culture medium in order to maintain the selection of stably transfected cells.

HeLa cells

HeLa cells were propagated *in vitro* in complete RPMI medium additioned with 10% FCS.

CHO cells

CHO cells were propagated *in vitro* in complete M199 medium (Sigma-Aldrich) supplemented with 10% FCS.

Human normal fibroblasts

Human normal fibroblasts were expanded *in vitro* from dermal biopsies samples obtained from the forearm skin of healthy volunteers. Fibroblasts were cultivated in complete RPMI medium additioned with 10% FCS and utilized for experiments within the fourth-seventh passage.

When necessary, the adherent cell cultures were detached from culture vessels with trypsin-EDTA (Sigma-Aldrich) or with a solution of ethylenediaminetetraacetic acid (EDTA, 2mM) in phosphate buffer saline (PBS).

3.2 IgG REPERTOIRE ANALYSIS

To evaluate the presence of PDGFRα-specific IgG in the cell culture supernatants obtained following immortalization of the B cell repertoire of sclerodermic patients, Fα and F-/- cells were used as target cells in immunofluorescence analysis. Fα cells (10^5 cells/50 μl) were incubated with 100 μl of culture supernatant of single wells for 1 hour at 4°C, washed twice in PBS and then probed with an anti-human IgG conjugated with a fluorescent probe (rabbit anti-human IgG-FITC, Jackson Immunoresearch Laboratories) for 40 minutes at

4°C. The reactivity of each supernatant on target cells was evaluated with a fluorescence microscope and confirmed with cytofluorimetric analysis (FACSCalibur, Becton Dickinson, CellQuest and WinMDI v.2.9 software). Cell culture supernatants showing reactivity on Fα cells were counter-selected on F–/– cells using the same protocol.

3.3 IgG PURIFICATION

Total IgG were purified from the serum of sclerodermic patients and controls on affinity chromatography columns packed with Protein A/G-coupled agarose (Pierce).

Immortalized B cell cultures showing reactivity on Fα cells but not on mock cells were expanded *in vitro* in complete RPMI medium supplemented with 10% FCS, then adapted for growth in synthetic serum-free medium (Hybri-SFM, Invitrogen). Cell culture supernatant was collected and the anti-PDGFR IgG were purified on affinity chromatography columns packed with Protein A -coupled agarose.

3.4 ANALYSIS OF ROS INDUCTION

Fα cells and early-passage human fibroblasts ($2x10^4$/well) were seeded in 24 well plates (Nunc) and starved in DMEM medium additioned with 0.2% FCS for 24 hours. Cells were treated with recombinant PDGF-BB (15 ng/ml, R&D Systems) or purified IgG (at the indicated concentrations) for 15 minutes at 37°C. The biological activity of the purified IgG on adherent Fα cells was tested by fluorimetric determination of ROS production following addition of 2'-7'-dichlorofluorescein (DCF, 10 μM, Molecular Probes). The results were expressed

as a stimulation index, corresponding to (S - C)/(P - C), where S is the DCF fluorescence intensity of the test IgG, C is the DCF fluorescence intensity of a negative control obtained by culturing cells without IgG, and P is the DCF fluorescence intensity of PDGF.

3.5 IMMUNOPRECIPITATION

Human normal fibroblasts were lysed with RIPA buffer (PBS + Nonidet P-40 1% + Sodium deoxycholate 0.5% + SDS 0.1% + Sodium orthovanadate 2mM + PMSF 1mM) and cellular debris was eliminated by centrifugation at 14,000 rpm for 15 minutes. The cell lysates were incubated with purified IgG (at the indicated concentrations) and the immune complexes were precipitated with agarose beads coupled with Protein G. The immune complexes were subjected to SDS-page gel electrophoresis and transferred onto PVDF membranes. The membranes were blocked with a solution of 5% nonfat milk and blotted with a rabbit polyclonal antibody specific for the N-terminal domain of PDGFRα (sc-338, 1 μg/ml, Santa Cruz Biotechnology) and with a horseradish peroxidase-conjugated secondary antibody (anti-rabbit IgG-HRP, 1:5,000, Santa Cruz Biotechnology). The reaction was developed with a chemiluminescent substrate (Pierce).

3.6 IMMUNOGLOBULIN SEQUENCING AND CLONING

The variable regions of the heavy (VH) and light (VL) chains of the isolated anti-PDGFRα IgG (i.e., PAM 16F4, PAM 13B8 and PAM 17H8) and IgM (i.e., ROM D2/1F5) were sequenced with a commercial vector (TOPO TA cloning® kit for sequencing, Invitrogen). Briefly, total mRNA was extracted from frozen cell

pellets of the oligoclonal EBV-immortalized cells and cDNA was prepared by reverse-transcription with the RNeasy kit (Qiagen), following the manufacturers' protocol. The sequences of the VH, VLκ and VLλ chains of the isolated immunoglobulins were amplified with a PCR carried out with a mix of primers specifically designed to amplify the whole human immunoglobulin repertoire (Table 1). The obtained amplified constructs were cloned into the pCR 2.1 TOPO® vector (Invitrogen) and sequenced.

IgG			IgM		
IgG1	×	ConG1-3	IgM	×	ConM
IgG2	×	ConG1-3	CHM	×	VH1
IgG3	×	ConG1-3	CHM	×	VH2
IgG4	×	ConG4	CHM	×	VH3
CHG	×	VH1	ConKfw	×	ConKrev
CHG	×	VH2	ConLfw	×	ConLrev
CHG	×	VH3	CK	×	VK1/4
ConKfw	×	ConKrev	CK	×	VK2
ConLfw	×	ConLrev	CK	×	VK3
CK	×	VK1/4	CL	×	VL1
CK	×	VK2	CL	×	VL2/5
CK	×	VK3	CL	×	VL3
CL	×	VL1	CL	×	VL4a
CL	×	VL2/5	CL	×	VL4b
CL	×	VL3	CL	×	VL6
CL	×	VL4a			
CL	×	VL4b			
CL	×	VL6			

Table 1: PCR primer pairings designed to screen the immunological repertoire of the isolated B cell oligoclones. C indicates the constant region of the immunoglobulin, V indicates the variable region. The designed panel of primers for IgG genes covers all IgG subclasses (IgG1, IgG2, IgG3 and IgG4), every subclass of the variable region heavy (VH) and light chains (VL, kappa and lambda). The panel of primers for IgM genes was designed with the same method.

The nucleotide sequences obtained after PCR amplification of the VH and VL regions of the selected immunoglobulins were independently grafted to replace the heavy chain complementarity-determining regions of antibody b12 (kindly provided by Dr. Dennis Burton, The Scripps Research Institute, La Jolla, CA), by using a three-step overlap extension PCR [93].

The VH and VL (κ or λ) sequences were paired in all their possible combinations (Table 3) and sub-cloned into the pDR12 expression vector (Figure 14) [94], containing the parental b12 light chain gene, for expression as recombinant human IgG1 .

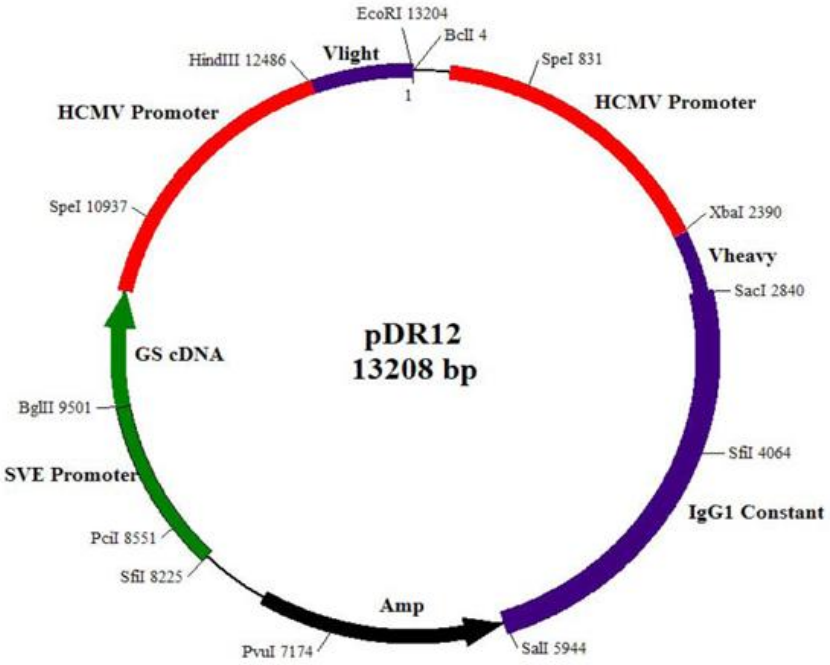

Figure 14: Map of the pDR12 expression vector. All the VH/VL pairs obtained from the sequences of the variable heavy and light regions of the anti-PDGFRα immunoglobulins isolated from the sclerodermic patients were subcloned into this vector for the production of human recombinant anti-PDGFRα IgG1.

Briefly, the sequences of all the paired VH and VL chains were inserted into the restriction sites of *Xba I-Sac I* and *Hind 3-Eco RI,* respectively, under the control of the HCMV promoter. The coding sequence for the leader peptide (necessary for the post-translational secretion of the encoded protein) was added to each pair with a three-step PCR carried out with partially overlapping primer pairs. The VH fragment was cloned adjacently to the *Sac I* restriction site and was fused with the IgG1 constant fragment encoded by the pDR12 vector. The VL fragments were amplified separately and added to the VH chains with an "overlap" PCR passage (Figure 15).

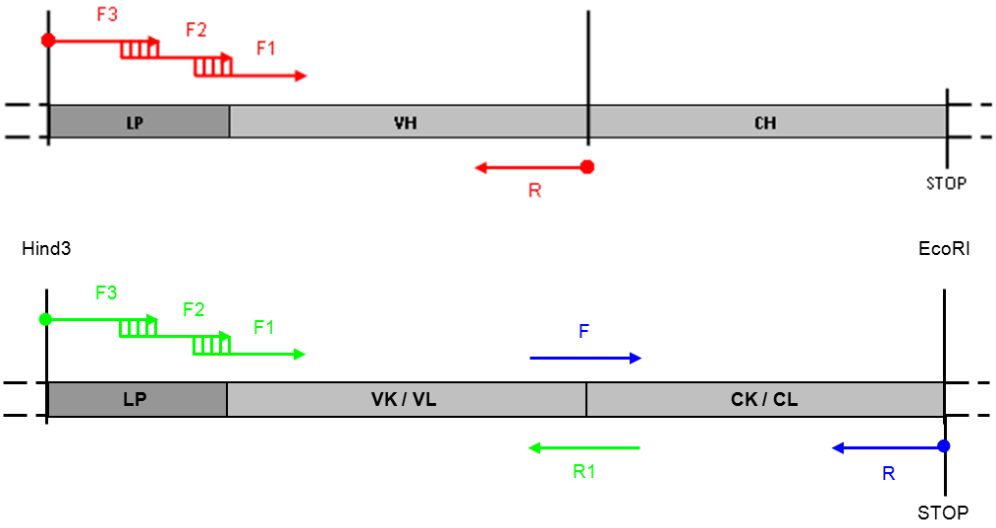

Figure 15: Schematic representation of the "overlap" PCR performed in order to adapt the VH and VL regions of the anti-PDGFRα autoantibodies to the cloning into pDR12 expression vector. VH: variable heavy chain; CH: constant heavy chain; VK/VL: variable light chain kappa or lambda; CK/CL: constant light chain kappa or lambda; LP: leading peptide; F1-F3: overlapping forward primers; F: forward primer; R: reverse primers.

The PCR for the amplification of the VH sequence was performed as follows:

1. F1 × R Template: DNA of VH gene cloned in TOPO vector;
2. F2 × R Template: PCR product of point 1;

3. F3 × R Template: PCR product of point 2.

The PCR for the amplification of the VL sequences was performed as follows:

4. F1 × R1 Template: DNA of VLκ or VLλ genes cloned in TOPO vector;
5. F2 × R1 Template: PCR product of point 4;
6. F3 × R1 Template: PCR product of point 5.

The constant region of the light chains was amplified as follows:

7. F × R Template: pDR12.

The "overlap" PCR between the sequences of the VH and VL sequences was performed as follows:

8. NO primer Template: PCR product of point 6 + PCR product of point 7;
9. F3 × R Template: PCR product of point 8.

All PCR were performed with 100 ng of DNA as template and Platinum Taq DNA Polymerase High Fidelity (Invitrogen).

The recombinant IgG constructs cloned in the pDR12 expression vectors were sequenced in order to verify whether the grafted VH and VL sequences were correct.

This part of the work was performed at the Molecular Medicine Lab, Dipartimento di Scienze Mediche e Chirurgiche, Università Politecnica delle Marche, AN.

Each recombinant IgG construct was transfected in CHO cells [95] in order to obtain recombinant IgG-producing cell lines. Briefly, the vector carrying each recombinant IgG was linearized by digestion with *Sal I* enzyme, purified with ethanol and transfected in CHO cells with the Lipofectamine-2000 reagent (Invitrogen), following the manufacturers' protocol.

Stably transfected monoclonal cell lines were selected with methionine sulphoximine (MSX, 120 μM) and the production of the transfected recombinant IgG in the cell culture medium was assessed with a quantitative ELISA assay for human IgG.

High-yielding transfected CHO clones were expanded *in vitro* and adapted for suspension growth in disposable bioreactors (CELLine CL1000, Integra Biosciences). Briefly, CHO cells (3 x 10^6 cells/ml) were suspended in complete M199 medium additioned with 10% Ultra-Low IgG FCS (Invitrogen). Dextran-coated microcarriers (Cytodex-1, 5 mg/ml, Sigma Aldrich) were added to the cell suspension and the mixture was inoculated in the cultivation chamber of the CELLine system. Cells were allowed to adhere to the surface of the carriers for 90 minutes at 37°C and then the medium reservoir chamber of the CELLine system was filled with complete M199 medium. Cell culture supernatant was harvested from the cultivation chamber every 7 days after inoculation and IgG production was monitored with a quantitative ELISA assay for human IgG.

3.7 SCREENING OF CDR EXPRESSION

PBMC were purified from blood samples of SSc patients and controls afferent to the clinical section of the S.O.D. Clinica Medica, Ospedali Riuniti Ancona, by density gradient centrifugation. Total mRNA was extracted from frozen cell pellets of the isolated PBMC with RNeasy kit (Qiagen), following the manufacturers' protocol.

Primers and oligonucleotide probes were designed to specifically anneal to the CDR sequences of the variable regions of the kappa light chain of one isolated SSc IgG (i.e., VLκ PAM 16F4). These primers were utilized to set up a real-time

PCR with TaqMan methodology on the mRNA extracted from PBMC of SSc patients and controls. Genes encoding constant antibody regions (IgM and IgG Fc regions) were used as housekeeping genes in order to normalize the expression levels of the fragments amplified by PCR.

3.8 PHOSPHORYLATION ASSAYS

Human normal fibroblasts were cultured *in vitro* in 60 mm Petri dishes as described. Sub-confluent cultures were starved in RPMI medium containing 0.2% FCS for 4 hours, then washed and recombinant PDGF-BB (15 ng/ml, R&D Systems) and purified recombinant human monoclonal antibodies (from 3 to 10 μg/ml) were added to the wells for 15 minutes at 37°C. After stimulation, cells were washed and lysed with RIPA buffer. Cell debris was eliminated by centrifugation at 10,000 rpm at 4°C. Samples from each experimental condition were subjected to SDS page and immunoblot with anti-pERK antibody (1:1,000, Santa Cruz Biotechnology).

3.9 COLLAGEN GENE EXPRESSION ANALYSIS

Early-passage human fibroblasts, cultured *in vitro* as described, were washed and incubated for one hour with purified human recombinant monoclonal antibodies (at concentration ranging between 3 and 10 μg/ml). Total RNA was extracted from the fibroblasts before and after stimulation using Aurum total RNA mini Kit (Bio-Rad) following the manufacturers' protocol. Total RNA from each experimental sample (500 ng) was reverse transcribed at 42°C for 45 minutes using the iScript cDNA synthesis kit (Bio-Rad), according to the manufacturers' protocol. Expression levels of type I collagen A1 and A2 chains

(COL1A1 and COL1A2) were determined by real-time PCR using the iQ SYBR Green Supermix (Bio-Rad) and the following primers:

COL1 (I) FW	5'-AGGGCCAAGACGAAGACATC-3'
COL1 (I) REV	5'-AGATCACGTCATCGCACAACA-3'
COL2 (I) FW	5'-AGGTCAAACAGGAGCCCGTGGG-3'
COL2 (I) REV	5'-GCACCTGGGAAGCCTGGAGGG-3'

Glyceraldehyde 3-phosphate dehydrogenase (GADPH) gene was used as reference gene for normalization. Collagen gene expression was expressed as percentage of the baseline level.

3.10 RECOMBINANT PDGF RECEPTOR ALPHA

A C-terminal truncated isoform of the human PDGFRα (a.a. 1-834) tagged with six histidine residues at the C terminus has been stably transfected in HeLa cells.

The cDNA of the recombinant receptor, cloned in an expression vector carrying the geneticin resistance gene, was kindly provided by Prof. Avvedimento (University of Napoli "Federico II", NA).

The pcDNA 3.1/V5-HIS/PDGFRα construct was obtained cloning the cDNA encoding aminoacids 1-834 of human PDGFRα (comprising the extracellular C-terminal domain, the transmembrane domain and a portion of the intracellular N-terminal domain) into the plasmid vector pcDNA™ 3.1/V5-HIS A (Invitrogen, Figure 16), following the manufacturer's protocol.

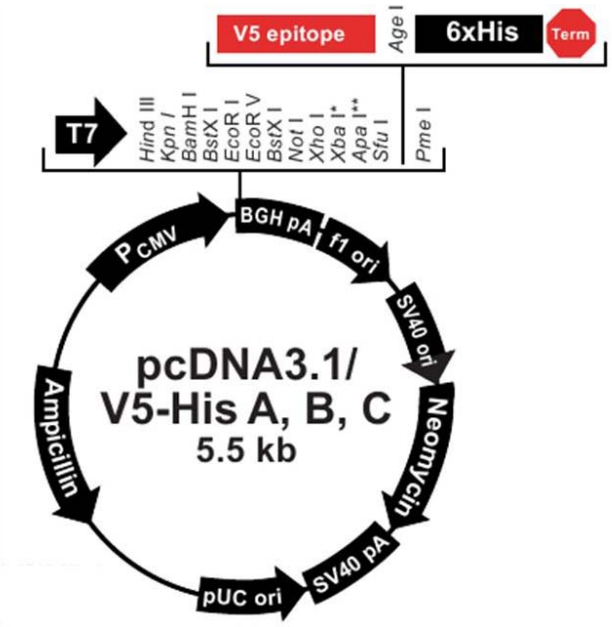

Figure 16: Map of the pcDNA 3.1/V5-HIS expression vector for the expression of His-tagged recombinant proteins. The PDGFRα insert (a.a. 1-834) was obtained from the pBluescriptSK vector containing the whole alpha chain of human PDGFRα, after digestion with *Not I* and *Bgl II* enzymes. The pcDNA 3.1/V5-His A expression vector was linearized with *Hind III* and *BamH I* enzymes. The two digestion products were rendered compatible with the addition of the Klenow fragment (Fermentas) and the complete expression vector was transformed into JM 109 High Efficiency competent cells (Promega). The amplified plasmid was extracted with a miniprep kit (Macherey-Nagel) and transfected in HeLa cells.

PDGFR-negative HeLa cells were seeded in 6mm Petri dishes (6 x 10^5 cells in 7 ml of antibiotic-free RPMI medium supplemented with 5% FCS). Sub-confluent cells were washed twice with serum-free RPMI and transfected with 9 μg of 3.1/V5-HIS/PDGFRα plasmid DNA with the Lipofectamine-2000 reagent (Invitrogen), following the manufacturers' protocol.

Following transfection, HeLa cells were expanded *in vitro* in antibiotic-free RPMI medium additioned with 10% FCS and 1 mg/ml of geneticin (G418, Sigma-Aldrich) for selection of stably transfected cells, for at least two weeks.

Then, PDGFR-transfected HeLa cells were seeded in 96 well plates at a concentration of 1 cell per well. Single colonies were expanded and tested for the expression of PDGFRα on the cell surface by means of immunofluorescence.

Transfected HeLa cells clones were suspended in PBS and probed with mouse monoclonal antibodies specific for the extracellular portion of human PDGFRα (mAb 1264 and mAb 322, 1μg/ml, R&D Systems) for 1h at 4°C, washed twice in PBS and then incubated with an anti-mouse IgG antibody conjugated with a fluorescent probe (anti-mouse IgG-FITC, Jackson Immunoresearch Laboratories) for 40 minutes at 4°C. The fluorescence was analyzed with cytofluorimetric analysis.

Cell cultures expressing the receptor at high density on the cell surface of 100% of the population were selected. Then, the recombinant receptor has been analyzed by means of Western blot. Cells were lysed with RIPA buffer and cellular debris was removed by centrifugation at 14,000 rpm at 4°C. Whole cell lysate samples were subjected to SDS-page and transferred onto PVDF membranes. The membranes were blotted with a rabbit polyclonal antibody specific for the extracellular domain of PDGFRα (1 μg/ml, Abnova) and with a horseradish-peroxidase conjugate secondary antibody (anti-rabbit IgG-HRP, Santa Cruz Biotechnology, 1:5,000). The reaction has been developed with a chemiluminescent substrate (Perkin-Elmer).

3.11 PURIFICATION OF RECOMBINANT PDGFRα-HIS

For the purification of the HIS-tagged recombinant PDGFR from the cell lysate of transfected HeLa cells, cells were harvested by trypsinization and lysed with a buffer suitable for affinity purification by metal ion chromatography (Tris HCl

50mM + NaCl 50mM + Glycerol 5% + Triton-X 100 1% + β-mercaptoethanol [1:160] + imidazole 5mM). Cell debris was eliminated by centrifugation at 14,000 rpm.

The recombinant PDGFRα-HIS was purified from the cell lysate with a nickel-activated HiTrap Chelating HP column (GE Healthcare). The iminodiacetic acid-cross-linked agarose beads packed into the HiTrap column were washed with 10 column volumes (CV) of deionized water; the chelating resin was charged with Ni^{2+} cations by loading 5 CV of a 0.1M $NiCl_2$ solution.

After a washing step, total lysate of HeLa PDGFRα-HIS cells was loaded onto the column until saturation. Unbound proteins were washed with 10 CV of washing buffer ($Na_2H_2PO_4$ 0.02M + NaCl 0.5M + imidazole 10mM). PDGFRα-HIS was recovered from the column in sequential elution steps, performed with a solution of $Na_2H_2PO_4$ 0.02M + NaCl 0.5M supplemented with increasing concentrations of imidazole (100 mM and 150 mM).

The protein concentration of the eluted fraction was evaluated with Lowry method [96]; the eluted fractions were dialyzed overnight against PBS in order to eliminate imidazole and residual salts, and then lyophilized with a centrifugal concentrator.

The purified receptor was analyzed by means of Western Blot following the same protocol used for the detection of the recombinant PDGFR in the whole-cell lysate.

3.12 SURFACE PLASMON RESONANCE BINDING ASSAYS

The purified PDGFRα-HIS was used as the target molecule in the set-up of an affinity binding assay with surface plasmon resonance technology (SPR) in an IAsys system (Interaction Analysis system). This part of the work was performed in collaboration with the group of Dr. Angeletti at the University of Camerino, MC.

The purified PDGFRα-HIS was immobilized on the activated surface of a sensor chip via its histidine tag. Briefly, the metal surface of the sensor chip was washed with a solution of PBS + 0.05% Tween 20 and equilibrated with PBS at 25°C. PDGFRα-HIS, resuspended in a buffer containing 10mM CH_3COONa, was covalently bound to the free carboxyl groups on the surface of the sensor chip in the presence of the EDC/NHS activating solution (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride + *N*-hydroxysuccinimide). The chip was washed with PBS to eliminate any trace of unbound receptor and the residuals free carboxyl groups on the chip were inactivated with 1M ethanolamine [97].

Binding analysis on the immobilized receptor was performed with eight IgG samples purified from the serum of SSc patients and eight IgG samples from healthy controls (120 μg/ml in PBS). The change in refractive index, proportional to the concentration of the interacting molecules was measured by an optical sensor. The binding response of each IgG sample was expressed with a sensorgram displaying the change of the angle of the binding curve over time (arcsin x sec^{-1}, or arcsec). The cut-off which discriminates between positive and

negative binders was calculated as the mean binding value of the control IgG (in arcsec) plus 3 standard deviations.

The binding affinity of a recombinant monoclonal IgG purified from the cell culture supernatant of transfected CHO cultures was evaluated with the same method. The dissociation constant (Kd) of the tested immunoglobulin was determined using the IAsys software.

3.13 IMMUNOENZYMATIC ASSAY

The purified PDGFRα-HIS was immobilized on solid phase in order to develop an enzyme-linked immunosorbent assay (ELISA) able to detect the presence of anti-PDGFR autoantibodies in the serum of sclerodermic patients and controls.

Two methods were set up.

- 300 ng of purified receptor were immobilized on high-capacity 96 well plates (Nunc) in PBS, overnight at 4°C. Wells were washed with washing buffer (PBS + 0.05% Tween 20) and blocked with blocking buffer (PBS + 0.05% Tween 20 + 1% nonfat milk). Sera of patients and controls were tested for binding to the immobilized receptor at a dilution of 1:100 in blocking buffer. Quality control assessment was carried out testing the reactivity of the following control antibodies: monoclonal mouse anti-PDGFRα antibodies (mAb 1264 and mAb 322, 1 μg/ml, R&D Systems), monoclonal mouse anti-HLA class I antibody (01.65, IgG2a), X63 IgG [98], polyclonal rabbit anti-PDGFRα (Abnova, 1:1,000) polyclonal rabbit anti-JNK (Santa Cruz Biotechnology, 1:1,000). The primary antibodies were incubated 2 hours at 37°C and plates were washed five times with

washing buffer. Horseradish peroxidase-conjugated secondary antibodies (anti-human Ig-HRP, Cappel, 1:10,000, anti-mouse IgG-HRP, Perkin-Elmer, 1:5,000 and anti-rabbit IgG-HRP, Santa Cruz Biotechnology, 1:5,000) were matched appropriately and incubated 1 hour at 37°C. Plates were washed five times with washing buffer and the reaction was developed with tetramethylbenzidine substrate (TMB, BioFX) for 10 minutes. The reaction was arrested with a stop solution (H_2SO_4 0.5M) and the plates were read in a spectrophotometer at a wavelength of 450 nanometers.

- 300 ng of purified receptor were immobilized on 96 well HIS-Grab™ nickel coated plates (Pierce) in a Tris-based buffer saline (TBS), for 2 hours at 37°C. Plates were washed once in TBS additoned with 0.05% Tween 20 (TBS-T) and the wells were blocked with TBS-T supplemented with 1% nonfat milk for 2 hours at 37°C. Plates were washed three times in TBS-T. Sera of patients and controls were diluted 1:100 in TBS-T + 1% nonfat milk; diluted sera and control antibodies were incubated 2 hours at 37°C. Plates were washed five times with TBS-T. HRP-conjugated secondary antibodies were added and incubated 1 hour at 37°C, plates were washed five times with TBS-T and the reaction was developed with TMB for 10 minutes. The reaction was stopped and the plate was read in a spectrophotometer at a wavelength of 450 nm.

Sera included in the study comprised:

- 22 sclerodermic patients
- 94 healthy controls

- 6 individuals with Raynaud's phenomenon
- 7 Rheumatoid Arthritis patients
- 13 Systemic Lupus Erythematosus patients

One-way ANOVA statistical analysis (Kruskall-Wallis non-parametric test with Dunn's comparison test, 95% C.I.) was performed on data obtained from the ELISA tests using Graph Pad Prism software (GraphPad Inc.).

4 Results

4.1 Analysis of the SSc immunological repertoire

The identification of the presence of agonistic autoantibodies specific for the alpha chain of PDGF receptor (PDGFRα) in the serum of patients with Systemic sclerosis constituted the rationale for the design of this research project.

Recently it has been demonstrated that anti-PDGFRα autoantibodies may have a causal role in the development of the pro-fibrotic phenotype characterizing sclerodermic fibroblasts. Indeed, they can induce oxidative stress in human normal fibroblasts, with production of reactive oxygen species and over-expression of collagen genes, ultimately leading to tissue fibrosis [1].

The first step has been the isolation of the B cells responsible for the production of anti-PDGFRα antibodies from the B cell repertoire of patients with Systemic sclerosis. The IgG-producing B cell subpopulation ($CD22^+/IgG^+$) of a sclerodermic patient whose serum immunoglobulins showed biological activity (namely, the ability to induce ROS production) has been isolated with immunomagnetic selection, expanded *in vitro* in the presence of CpG and IL-2 and immortalized with Epstein Barr Virus [2]. The immortalized cells have been cultured in 96 well plates at low concentrations (5-10 cells/well). The resulting cell culture supernatants have been tested for the presence of anti-PDGFR antibodies by means of immunofluorescence, using mouse embryo fibroblasts transfected with the full-length human PDGFRα (Fα) as a target. Supernatants showing reactivity on Fα cells were counter-selected on mock-transfected cells

(F-/-). Three cultures producing class G immunoglobulins selectively reacting with Fα cells were isolated (namely, PAM 13B8, PAM 16F4 and PAM 17H8) (Figure 17). The reactivity of these IgG autoantibodies was comparable to that of the ROM D2/1F5 (IgM) human autoantibody which had been isolated previously with a similar method (see Introduction).

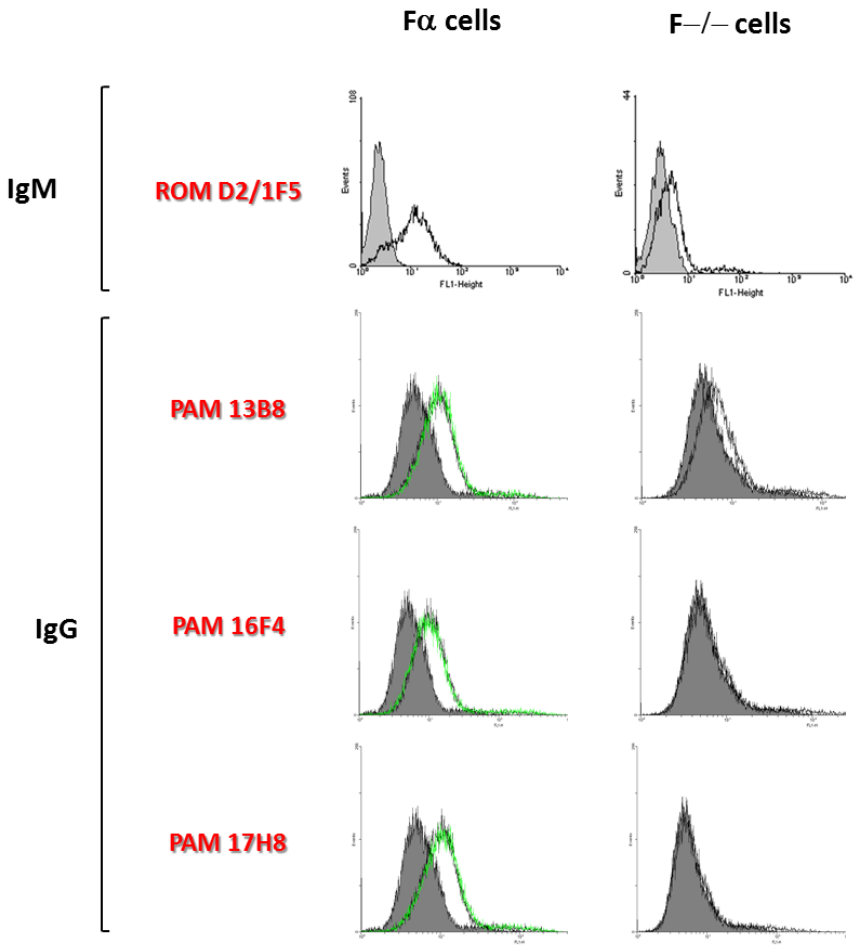

Figure 17: PDGFRα-expressing (Fα) and mock transfected cells (F-/-) were incubated with the cell culture supernatant of oligoclonal cultures of immortalized B cells of sclerodermic patients, and subjected to FACS analysis. The black lines represent the binding of the selected human immunoglobulins; the green lines correspond to the binding of a control monoclonal murine antibody specific for human PDGFRα. The shaded profiles correspond to the isotype-matched negative control.

Fibroblasts are considered the main physiological target of circulating anti-PDGFR autoantibodies. In order to confirm the specificity of the selected anti-PDGFR immunoglobulins, immunoprecipitation experiments were set up using human fibroblasts as target cells.

The anti-PDGFR IgG were purified from the supernatant of the oligoclonal cultures with affinity chromatography on Protein A/G, and utilized as the immunoprecipitating agent from total lysates of human normal fibroblasts.

All of the three selected IgG were able to immunoprecipitate the full length receptor from human fibroblasts, thus confirming their specificity for PDGFRα (Figure 18).

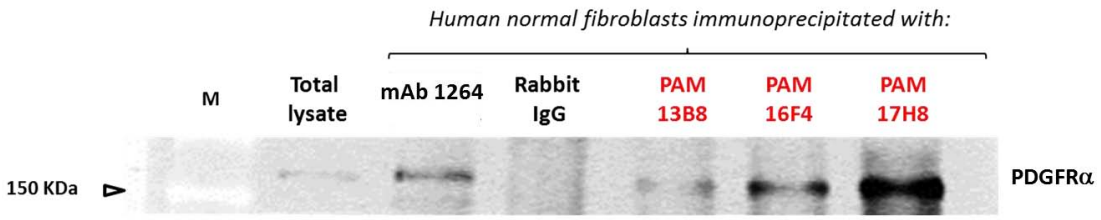

Figure 18: Human fibroblast lysates were immunoprecipitated with IgG purified from the cell culture supernatant of PDGFR-specific oligoclones (lanes 5-7). The immune complexes were subjected to SDS-page and blotted with a polyclonal antibody specific for PDGFRα. Control immunoprecipitation experiments were performed with a commercial anti-PDGFRα (mAb 1264) monoclonal antibody and with an isotype-matched rabbit immunoglobulin (Rabbit IgG).

It has been demonstrated that serum immunoglobulins from sclerodermic patients exert a specific agonistic activity on PDGFR [1], as indicated by their ability to induce the production of reactive oxygen species after binding to the

receptor. The resulting oxidative stress is thought to be the principal cause of fibroblast activation.

In order to verify whether the isolated anti-PDGFR autoantibodies maintained their agonistic activity on PDGFR, the selected immunoglobulins were tested for their capacity to induce the production of ROS in target cells. The IgM (ROM D2/1F5) and two out of three IgG (PAM 13B8 and PAM 16F4) retained the capacity to stimulate ROS production in Fα cells but not in F−/− cells. One IgG (i.e., PAM 17H8) was not able to induce oxidative stress (Figure 19, panel A).

The levels of ROS induced by the two biologically active IgG were within the range of those induced by total IgG (200 μg/ml) purified from the serum of sclerodermic patients (Figure 19, panel B).

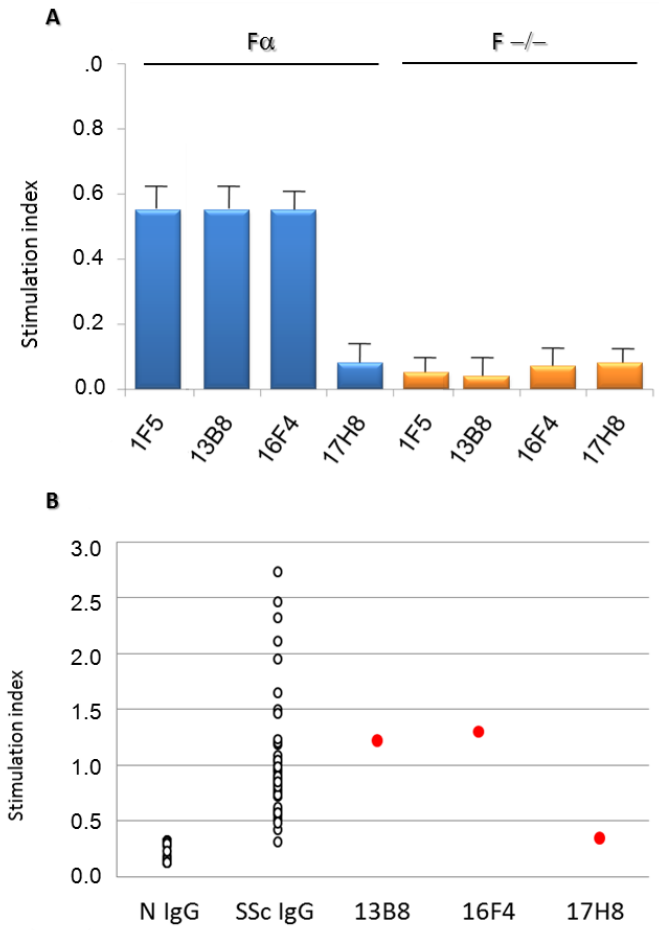

Figure 19: Panel A shows the levels of ROS induced by isolated sclerodermic anti-PDGFRα immunoglobulins on Fα and F -/- cells. Panel B shows ROS induction levels of the three isolated IgG relatively to the levels of ROS induced by total IgG purified from the serum of sclerodermic patients (SSC IgG) and healthy controls (N IgG).

4.2 ANTI-PDGFRα ANTIBODIES SEQUENCING AND CLONING

In order to better analyze the specificity and the biological properties of the sclerodermic anti-PDGFRα autoantibodies, and to evaluate their frequency of occurrence in the population, sequence analysis of the immunoglobulins isolated from the B cell repertoire of sclerodermic patients was carried out.

Total mRNA was extracted from the isolated B cell oligoclones producing the IgM (ROM D2/1F5) and the three IgG (PAM 16F4, PAM 13B8, PAM 17H8). cDNA was obtained from each mRNA sample by reverse-transcription, and the variable regions of the heavy (VH) and light (VLκ and VLλ) chains of each isolated immunoglobulin were sequenced.

The panel of VH and VL sequences obtained from the analysis of the isolated anti-PDGFRα immunoglobulins is detailed in Table 2.

IgG									IgM		
PAM 13B8			PAM 16F4			PAM 17H8			ROM D2/1F5		
VH	VL		VH	VL		VH	VL		VH	VL	
	Kappa	Lambda		Kappa	Lambda		Kappa	Lambda		Kappa	Lambda
VH2	VLκ1/4	VLλ1	VH2	VLκ1/4	VLλ1	VH2	VLκ1/4	VLλ1	VH1	VLκ1/4	VLλ1
VH3	VLκ3	VLλ3			VLλ3	VH3		VLλ3	VH2		
		VLλ4a			VLλ4a			VLλ4a	VH3		
		VLλ6			VLλ2/5			VLλ2/5			

Table 2: Panel of variable heavy (VH), variable light kappa (VL kappa, VLκ) and variable lambda (VL lambda, VLλ) chain sequences obtained from the sequence analysis of the anti-PDGFRα IgG (PAM 13B8, PAM 16F4 and PAM 17H8) and IgM (ROM D2/1F5) isolated from the immunological repertoire of two SSc patients.

The analysis of all the selected immunoglobulins reported more than one VL and one VH sequence each, indicating that each B cell culture was oligoclonal and produced more than one immunoglobulin.

The sequence analysis of the IgM produced by ROM D2/1F5 reported the presence of three VH sequences (namely, VH1, VH2 and VH3) and two VL sequences (one kappa and one lambda; namely VLκ1/4 and VLλ1). VH1 and VH3 sequences resulted identical. Therefore, the IgM produced by ROM D2/1F5 accounts for two VH chains and two VL chains (one lambda and one kappa).

PAM 13B8 IgG reported two identical VH sequences (namely, VH2 and VH3) and six VL sequences (two kappa and four lambda; namely, VLκ1/4, VLκ3, VLλ1, VLλ3, VLλ4a and VLλ6). The sequences of the two kappa and of the four lambda VL chains were identical. Therefore, PAM 13B8 accounts for one VH chain and two VL chains (one lambda and one kappa).

PAM 16F4 IgG reported one VH sequence (VH2) and five VL sequences (one kappa and four lambda; namely, VLκ1/4, VLλ1, VLλ2/5, VLλ3 and VLλ4a). The sequences of VLλ1, VLλ3 and VLλ4a resulted identical. The IgG produced by PAM 16F4 B cell oligoculture are composed by one VH and three VL chains (one kappa and two lambda).

Sequence analysis of PAM 17H8 IgG reported two identical VH sequences (VH2 and VH3) and five VL sequences (one kappa and four lambda; namely, VLκ1/4, VLλ1, VLλ2/5, VLλ3, VLλ4a). The sequences of the four VL lambda chains were identical. The oligoclonal PAM 17H8 IgG therefore comprise one VH chain and two VL chains (one kappa and one lambda).

The nucleotide sequences of all the identified VH and VL chains are the subject of a pending patent, and for this reason they cannot be disclosed.

One VH sequence (VH2) is shared among all the isolated IgG, suggesting that at least one of the antibodies present in the IgG-producing oligoclonal cultures could possess the same specificity.

The sequences of the complementarity-determining regions 1 and 3 (CDR1 and CDR3) of the common VH2 chain were aligned and compared with known sequences available in public databases, in order to determine whether they had already been annotated. No match for the VH CDR1 and CDR3 sequences was found, suggesting that the identified VH chain could indeed belong to a novel autoantibody.

In order to evaluate the presence and the frequency of occurrence of the CDR sequences of the selected anti-PDGFRα immunoglobulins, real-time PCR analysis was carried out with RNA of SSc patients and controls.

Total RNA was extracted and reverse-transcribed from PBMC purified from blood samples of nine SSc patients and six healthy controls, following a protocol that eliminates interfering genomic material and RNA of erythrocyte origin. Genes encoding constant antibody regions, such as the Fc regions of IgG or IgM were used as housekeeping genes in order to rule out that any increase in selected CDR gene expression may be due to increased total Ig expression levels. Primers for PCR analysis and oligonucleotide probes were designed to anneal specifically to the CDR sequences of the VL kappa chain of the PAM 16F4 IgG (VLκ1/4) and PCR was performed with a multiplex TaqMan assay.

PCR analysis of the expression of the CDR regions of the VLκ chain of PAM 16F4 IgG reported that the sequence is expressed in 7 out of 9 SSc patients (77.7%) and in 2 out of 6 healthy subjects (33.3%) (Figure 20).

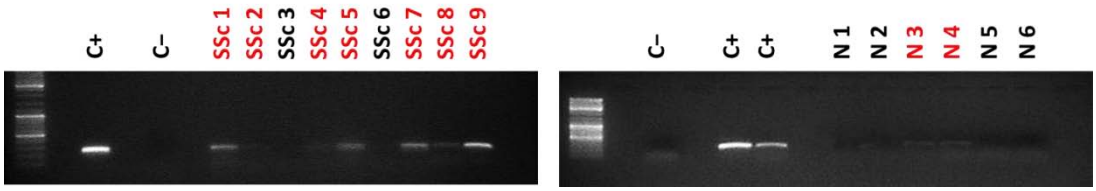

Figure 20: PCR analysis of CDR expression of VLκ from PAM 16F4 IgG in PBMC purified from sclerodermic patients (SSc 1-9) and healthy subjects (N 1-6). As a positive PCR control (C+) cDNA from PAM 16F4 cells was used.

The sequences of the VH and VL chains of the isolated autoantibodies were used to engineer fully human monoclonal anti-PDGFRα antibodies.

The identified DNA sequences of VH and VL chains were paired in order to produce all the possible combinations between the variable regions of the heavy and light chains of the anti-PDGFRα immunoglobulins produced by the isolated B cell oligoclones ROM D2/1F5, PAM 13B8, PAM 16F4 and PAM 17H8.

The VH2 sequence shared by all the isolated anti-PDGFRα IgG was paired with two VL kappa sequences (VLκ1/4, shared by PAM 13B8 and PAM 17H8; VLκ1/4, specific of PAM 16F4) and two VL lambda sequences (VLλ1, shared by PAM 13B8 and PAM 17H8; VLλ2/5, specific of PAM 16F4).

The VH2 sequence originated from ROM D2/1F5 was coupled with the VLκ1/4 shared by PAM 13B8 and PAM 17H8 and with the VLλ1, specific of ROM D2/1F5.

The VH1 sequence, specific of ROM D2/1F5 was paired with the VLκ1/4 shared by PAM 13B8 and PAM 17H8.

The obtained combinations encode for seven different VH/VL pairs; the combinations and the monoclonal antibodies that they originate are listed in Table 3.

Recombinant Antibody	VH chain	VL chain
Vκ 13B8	VH2 *(shared)*	VLκ1/4 *(PAM 13B8/ PAM 17H8)*
Vλ 13B8	VH2 *(shared)*	VLλ1 *(PAM 13B8/PAM 17H8)*
Vκ 16F4	VH2 *(shared)*	VLκ1/4 *(PAM 16F4)*
Vλ 16F4	VH2 *(shared)*	VLλ2/5 *(PAM 16F4)*
VH1κ 13B8	VH1 *(ROM D2/1F5)*	VLκ 1/4 *(PAM 13B8/PAM 17H8)*
VH2κ 13B8	VH2 *(ROM D2/1F5)*	VLκ 1/4 *(PAM 13B8/PAM 17H8)*
VH2 λ1	VH2 *(ROM D2/1F5)*	VLλ1 *(ROM D2/1F5)*

Table 3: Panel of recombinant antibodies generated from the VH and VL sequences of the isolated anti-PDGFRα autoantibodies.

The DNA sequences of the obtained combinations of heavy and light variable chains were cloned in an expression vector suitable for the production of recombinant human IgG in eukaryotic cells. Briefly, nucleotide sequences encoding the selected pairs of VH and VL chains were adapted to be cloned into the IgG expression vector by PCR addition of restriction sites homologous to those present in the vector, and of leader peptide sequences necessary to direct

the IgG to the cell membrane for secretion in the culture supernatant. The recombinant constructs were transfected in CHO cells and the monoclonal IgG were purified from the cell culture supernatant with affinity chromatography on Protein A.

4.3 CHARACTERIZATION OF THE RECOMBINANT ANTI-PDGFRα IGG

CHO cells were transiently transfected with the seven recombinant IgG constructs obtained as described above. The recombinant monoclonal IgG secreted into the cell culture medium were purified with affinity chromatography on Protein A/G. In order to assess the specificity of the purified IgG for PDGFRα, the immunoglobulins were used to immunoprecipitate the receptor from the total lysate of human fibroblasts.

Seven IgG (Vκ 13B8, Vλ 13B8, Vκ 16F4, Vλ 16F4, VH1κ 13B8, VH2κ 13B8 and VH2 λ1) immunoprecipitated the PDGFRα from human fibroblasts (Figure 21), although one seemed to possess a lower binding capacity (i.e., Vλ 13B8). This data confirms that the recombinant human monoclonal IgG generated through grafting of the CDR regions of sclerodermic anti-PDGFR autoantibodies retained the specificity for the receptor.

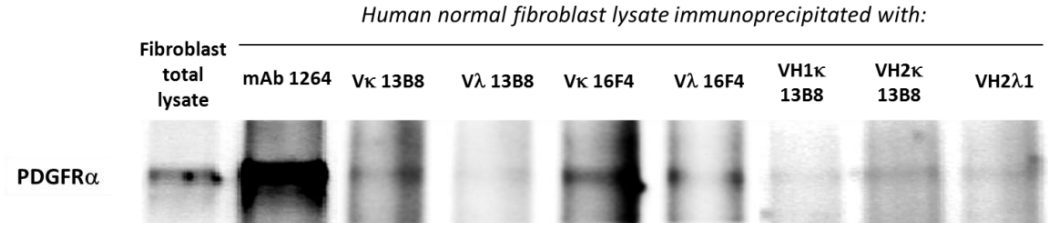

Figure 21: Total lysate of human normal fibroblasts was immunoprecipitated with 3-10 μg/ml of purified recombinant human IgG (lanes 3-9) A monoclonal murine antibody specific for

PDGFRα (mAb 1264) was used as a control. The immune complexes were run on SDS-page gel and the immunoprecipitated receptor was blotted and detected on the membranes with a polyclonal antibody specific for PDGFRα. "Fibroblast total lysate" indicates immunoblot of total proteins.

In autoimmunity, the B cell response directed against auto-antigens involved in the pathogenesis of the disease comprises heterogeneous subsets of autoantibodies with different functional properties. To further characterize the recombinant IgG, we evaluated whether they had biological properties reflecting those of the immunoglobulins purified from the serum of the same patient. Further insights into functional properties were gathered by analyzing the ability of the recombinant IgG to induce ERK1/2 phosphorylation, ROS production and collagen gene transcription in primary human fibroblasts.

First, we examined ERK1/2 phosphorylation induced on human fibroblasts by the recombinant IgG. Experiments were performed in parallel using PDGF as control. Western blot analysis of phosphorylated ERK1/2 after 15 minute stimulation at 37°C, demonstrated that four recombinant IgG (Vκ 16F4, Vλ 16F4, VH1κ 13B8 and VH2κ 13B8) were able to induce an increase of the phosphorylation level of ERK1/2 (Figure 22, panel A).

Next, the ability of the recombinant IgG to stimulate the production of ROS was evaluated in a standard biological fluorimetric assay. Six out of seven IgG (Vκ 13B8, Vκ 16F4, Vλ 16F4, VH1κ 13B8, VH2κ 13B8 and VH2 λ1) proved to be able to induce oxidative stress in human fibroblasts producing significant amounts of ROS compared with that produced by PDGF binding to its receptor (Figure 22, panel B),

Finally, the influence of the recombinant IgG in promoting fibroblast activation was monitored by PCR analysis of collagen gene transcription. Vκ 16F4 IgG was able to induce the transcription of both the alpha 1 and alpha 2 chains of type I collagen (COL1A1 and COL1A2), a typical myofibroblastic marker. VH2 λ1 was able to upregulate the transcription of the alpha 1 chain of type I collagen (Figure 22, panel C and Figure 23).

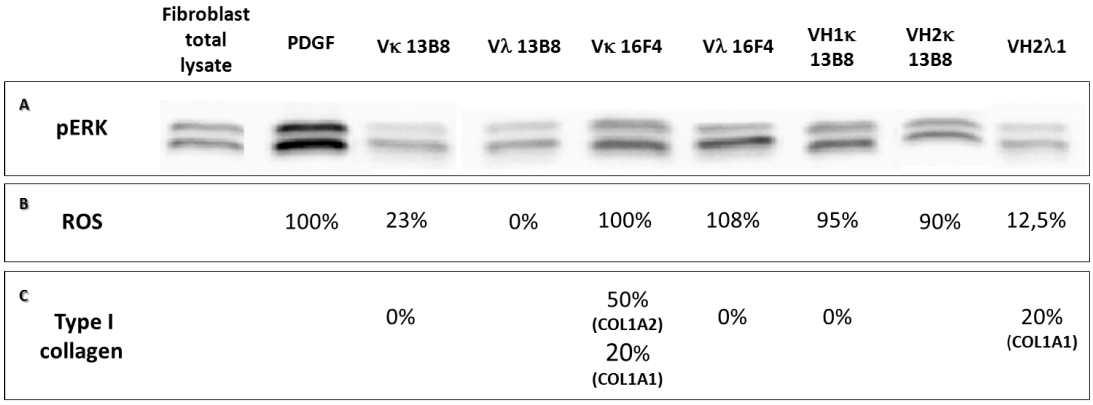

Figure 22: Analysis of the biological activity of the recombinant monoclonal IgG. Human normal fibroblasts were incubated with IgG purified from the cell culture supernatant of transiently transfected CHO cells (at concentrations ranging between 3-10 μg/ml) or 15 ng/ml of PDGF as a positive control. Panel A shows Western Blot analysis of phosphorylation levels of ERK1/2 following stimulation with IgG (lanes 3-9) or PDGF (lane 2). Lane 1 represents the immunoblots of the basal ERK1/2 phosphorylation level in non-stimulated fibroblasts. Panel B shows the levels of reactive oxygen species induced by stimulation with the recombinant IgG detected with fluorimetric analysis. ROS induction levels are expressed as a percentage of the control (stimulation with PDGF = 100%). Panel C shows the percentage of upregulation of type I collagen gene transcription in relation to baseline measured with real-time PCR.

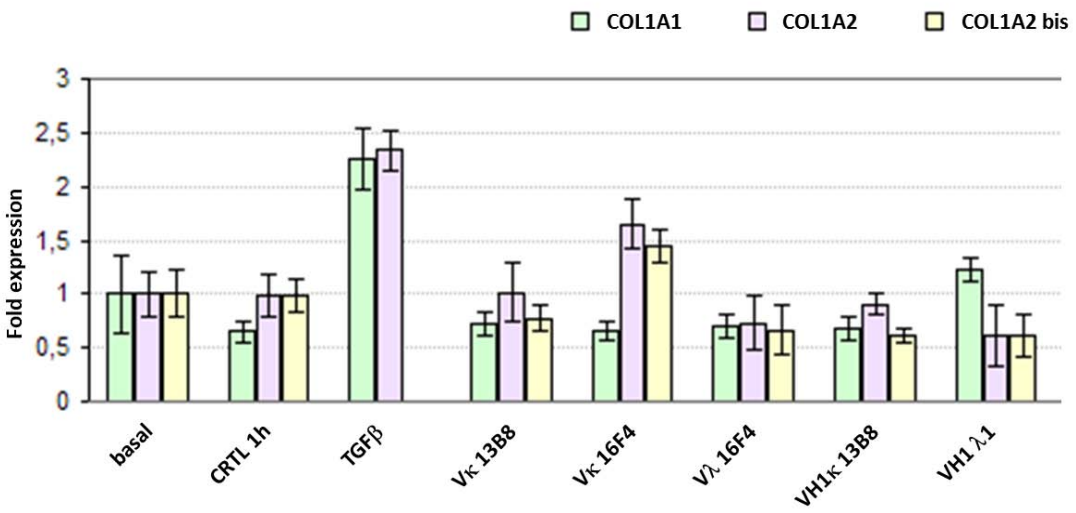

Figure 23: Analysis of collagen gene expression induced by recombinant monoclonal IgG. Levels of expression of type I collagen alpha chain 1 (COL1A1, in green) and alpha chain 2 (COL1A2, in pink and yellow, tested in duplicate with two different primer pairs for COL1A2) in normal fibroblasts were determined by real-time PCR after stimulation with purified recombinant IgG (3-10 µg/ml for 15 minutes) or TGF-β as a positive control.

Data obtained with functional assays indicate that i) the human recombinant IgG derived from the anti-PDGFRα autoantibodies of sclerodermic patients maintained the original binding specificity, ii) the selected anti-PDGFRα recombinant antibodies are functionally active.

The preliminary characterization of the recombinant IgG suggested that they could be a good model to understand the involvement of anti-PDGFRα autoantibodies in the pathogenesis of SSc.

Therefore, we have generated CHO cell lines stably transfected with the recombinant IgG constructs. The cells have been cloned by limiting dilution in order to select clones with an efficient IgG secretion. High-yield clones have been selected with a conventional quantitative ELISA test and expanded *in vitro*.

All the analyzed clones were found to produce 6-60 nanograms of IgG per ml in monolayer culture vessels. Such low levels of IgG production would not allow the purification of amounts of IgG sufficient to perform further characterization studies. To increase the yield of recombinant monoclonal IgG, CHO clones secreting Vκ 13B8 and Vκ 16F4 immunoglobulins have been adapted for growth in disposable bioreactor modules.

We set up high-density cultures of the two selected CHO transfectants allowing the cells to adhere to microcarriers, which provide extensive adhesion surface for the growth of anchorage-dependent cells in suspension culture systems. Using this method, we were able to increase the concentration of the recombinant IgG secreted in the culture medium from nanograms to micrograms per milliliter. In detail, the IgG concentration of the Vκ 13B8 IgG increased from 6 ng/ml to 3.5 μg/ml, while the yield of Vκ 16F4 increased from 55 ng/ml to 15 μg/ml. To avoid any interference in the IgG purification process, due to bovine IgG present in FCS, cells were grown in M199 medium additioned with 10% Ultra-Low IgG FCS. The recombinant monoclonal immunoglobulins will be purified from the culture medium with affinity chromatography on protein A and utilized for further studies.

4.4 RECOMBINANT PDGFRα AND CELL MODEL

To further characterize the properties of the anti-PDGFRα autoantibodies isolated from the immunoglobulin repertoire of SSc patients, and to obtain a tool suitable for functional studies, we constructed a PDGFR-expressing cell model.

PDGFR-negative HeLa cells have been transfected with a plasmid encoding a recombinant truncated PDGFR tagged with six histidine residues (PDGFRα-HIS, see Materials and Methods).

HeLa transfected cells uniformly expressed high levels of PDGFRα on the cell surface, as demonstrated by cytofluorimetric analysis. The recombinant receptor *bona fide* maintained the correct folding of the native molecule, since it was recognized by a neutralizing monoclonal antibody specific for a portion of the Ig-like domains 1-3 of PDGFRα, comprising a conformational epitope which is involved in the binding with PDGF (Figure 24, panel A).

Western Blot analysis highlighted a single band at approximately 116 kDa in the lysate of the transfected cells, a molecular weight which is consistent with the expected size of the transfected construct, as predicted by its length of 834 amino acids (Figure 24, panel B).

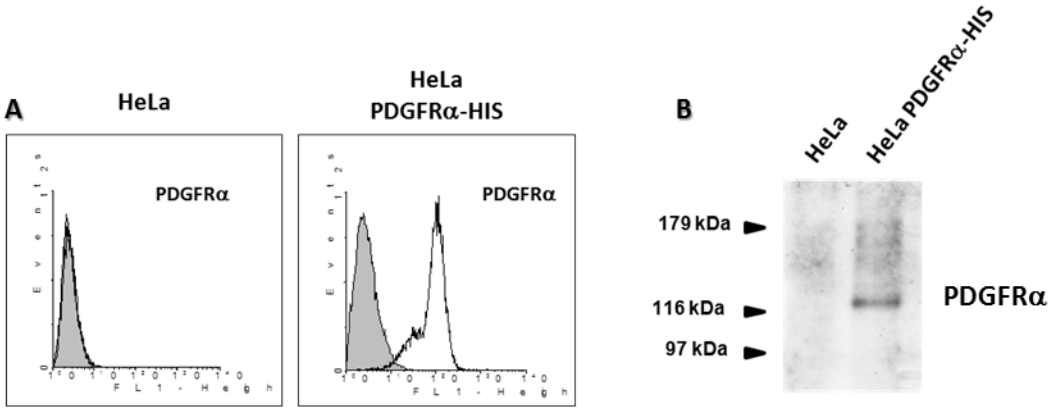

Figure 24: A) Expression of the recombinant PDGFRα-HIS on the cell surface of transfected HeLa cells (HeLa PDGFRα-HIS, white histogram) compared with the non-transfected control (HeLa, shaded histogram). The expression was assessed by immunofluorescence staining of cells with a murine monoclonal antibody specific for a conformational epitope of human PDGFRα (mAb 322). B) Western Blot analysis of 20 μg of total lysate of the transfected and control cells. The band correspondent to the recombinant PDGFRα-HIS was highlighted by staining with a rabbit polyclonal antibody specific for the extracellular portion of human PDGFRα.

The six histidine residues encoded at the C-terminus of the recombinant PDGFRα have been exploited for the purification of the receptor from the lysate of transfected HeLa by means of metal ion affinity chromatography on nickel-chelated resin. The oligo-histidine tag allowed for the binding of the PDGFRα-HIS to the nickel cations chelated on solid-phase agarose packed in a chromatography column. Sequential washing steps insured the elimination of all the proteins non-specifically bound to the chelated resin. The His-tagged recombinant receptor was recovered from the column by adding increasing concentrations of imidazole in the elution buffers. Imidazole competes with the histidine residues for binding to the nickel chelates, allowing for the selective detachment of the molecule from the column.

Fractions enriched in the recombinant PDGFRα-HIS, were dialyzed against PBS in order to eliminate the imidazole and any residual salt, which could damage the purified receptor and compromise its stability.

A quality control of the purity of the receptor was performed by subjecting the elution fractions to SDS-page and Western Blot analysis (not shown). The fraction showing the lowest number of bands due to contamination by proteins other than PDGFRα-HIS was selected for further uses.

Western Blot analysis of the selected fraction, performed with a polyclonal antibody specific for PDGFRα, showed two bands: one of 116 kDa, as expected, and the other of approximately 100 kDa (Figure 25). The nature of the band of lower molecular weight is so far unknown; a likely interpretation is that it represents a deglycosylated form of the recombinant receptor or a slightly degraded form due to the stringent conditions of the purification process.

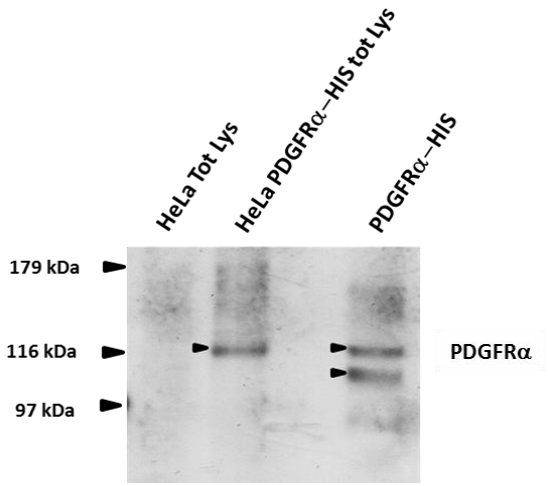

Figure 25: Western Blot analysis of the enriched fraction of recombinant PDGFRα-HIS. 20 μg of total lysate of non-transfected (lane 1) and transfected cells (lane 2) and 1 μg of purified recombinant receptor (lane 3) were blotted with a polyclonal PDGFRα-specific rabbit antibody.

4.5 PDGFRα SOLID-PHASE BINDING ASSAYS

The purified PDGFRα-HIS was used as target molecule to setup solid-phase binding assays for the evaluation of the binding capacity of PDGFR-specific immunoglobulins of sclerodermic patients. In detail, we have developed two different methods: surface plasmon resonance (SPR) and enzyme-linked immunosorbent assays (ELISA).

Surface plasmon resonance assay

For the SPR assay, the recombinant PDGFRα-HIS has been immobilized via its oligo-histidine tag onto a metal-charged sensor chip which was in contact with the flow cell carrying the IgG samples. The interaction of specific antibodies directed against PDGFα receptor bound to the sensor chip caused a change in the refractive index at the surface of the chip, which was detected by an optical sensor.

The density of the PDGFRα-HIS immobilized on the surface of the sensor chip was evaluated measuring directly the variation in the resonance angle (ΔR) detected by the biosensor during the sensor-activation procedure (Figure 26). The sensor detected a variation of 600 arcsec after receptor immobilization (ΔR=600 arcsec), indicating a superficial PDGFRα-HIS density of 1 ng/mm^2, corresponding to a concentration of approximately 5 mg/ml of bound receptor.

To test the binding capacity of the system, the natural ligand (PDGF) or a mouse monoclonal antibody recognizing a conformational epitope located at the N-terminal of the PDGFRα (mAb 322) were used (Figure 27). The binding curve

obtained with both ligands indicated the proper folding, in a native-like conformation, of the PDGFRα-HIS molecule.

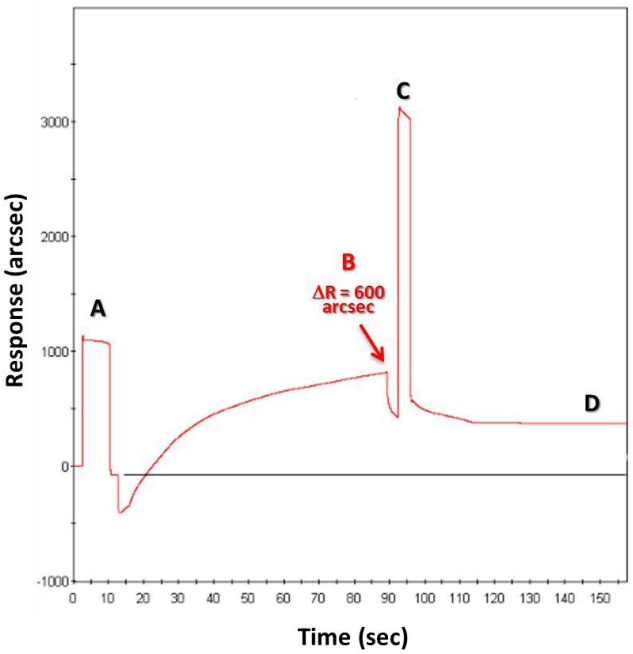

Figure 26: Sensorgram describing the variations in the resonance angle during the activation and receptor immobilization procedures. A: ECD/NHS activation of the sensor chip surface; B: PDGFRα-HIS immobilization; C: inactivation of free carboxyl groups; D: baseline.

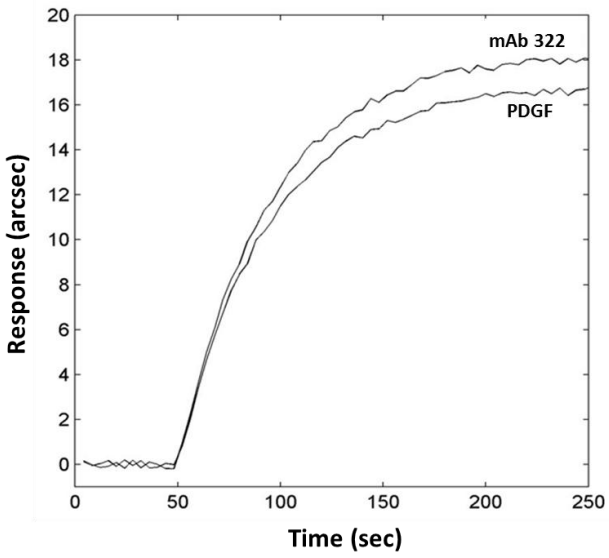

Figure 27: Sensorgram depicting the binding of PDGF and of a conformational antibody specific for PDGFRα (mAb 322) to the recombinant PDGFRα-HIS immobilized on the sensor chip.

SPR binding assays were performed to assess the PDGFRα-binding capacity of IgG purified from the serum of sclerodermic patients and healthy controls. The PDGFRα-HIS was immobilized on the surface of the sensor chip and the sensor traces obtained with IgG samples purified from eight SSc patients and eight healthy controls were recorded. The resulting sensorgrams showed that all eight sclerodermic IgG bound to the immobilized PDGFR, while only 1/8 control IgG determined a sensor trace overlapping with that of SSc IgG. The mean binding of control IgG was 8.01 ± 1.89 arcsec. The average value of controls + 3SD (9.9 arcsec) was selected as the cut-off value which discriminates between positive and negative binders (Figure 28).

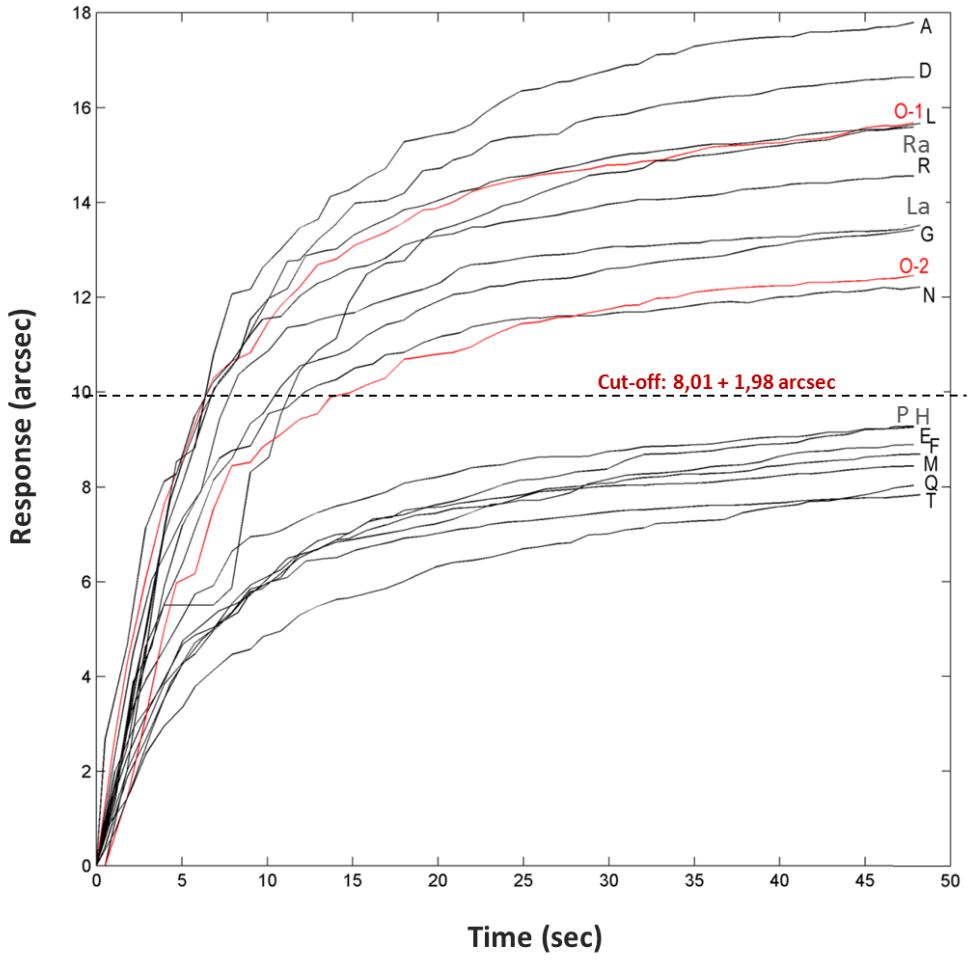

Figure 28: Sensorgram depicting the binding response of total IgG purified from the serum of eight sclerodermic patients (A, D, L, La, R, Ra, G, N) and eight healthy controls (O-1/O-2, P, H, E, F, M, Q, T) to PDGFRα-HIS immobilized on the sensor chip surface. The cut-off threshold discriminating between positive and negative samples was calculated as the average of the response values of IgG purified from the serum of healthy subjects plus three standard deviations (8.01 ± 1.89 arcsec). The sample O (tested in duplicate, in red) was the only control IgG showing a binding response higher than the cut-off value.

In order to verify whether the binding responses of the IgG samples to the immobilized PDGFRα were only attributable to the presence of PDGFRα-specific autoantibodies and not to residual contaminants derived from the IgG purification process (for example, residual PDGF), a qualitative control was

carried out for each IgG preparation. IgG samples were run on SDS-page and tested for the presence of any traces of PDGF which could bind to the immobilized receptor during the SPR assay leading to a binding trace. Western Blot analyis (with a detection limit of 1 ng of recombinant PDGF) showed that no PDGF was detectable in the purified IgG preparations (Figure 29).

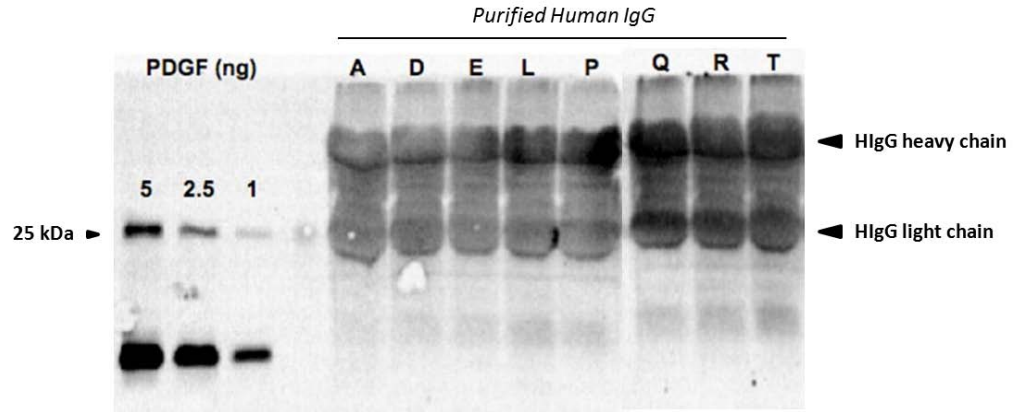

Figure 29: Decreasing concentrations of PDGF (5, 2.5, 1 ng) and purified IgG (150 μg) from each preparation used to perform SPR binding assays (lanes A –T, representative of all the purified IgG preparations) was run on polyacrylamide gel under reducing conditions. The membranes were blotted with a biotinylated anti-PDGF antibody (detecting reduced PDGF at 18 and 27 kDa) and the specific bands were highlighted with streptavidin-HRP.

Surface plasmon resonance affinity assays were also conducted to assess the PDGFRα-binding capacity of the recombinant monoclonal IgG obtained through cloning the VH and VL regions of the sclerodermic anti-PDGFRα autoantibodies.

PDGFRα-HIS, immobilized on the surface of a sensor chip, was challenged with IgG purified from the cell culture supernatant of CHO cells transiently transfected with the Vκ 16F4, Vκ 13B8 and Vλ 13B8 constructs.

The sensorgrams resulting from the binding of the IgG were recorded and the kinetic rate constants were determined. The dissociation constant (Kd) of the Vκ 16F4 IgG was measured at three different antibody concentrations, and was determined at 71.4 nM (Figure 30).

The Vλ 13B8 IgG showed a very low binding curve on PDGFRα (Figure 30). This result is consistent with the poor binding capacity demonstrated by the same IgG in the immunoprecipitation experiment described before (Figure 21).

In order to evaluate the specificity of the binding of the recombinant immunoglobulins to PDGFRα, a competition experiment was performed. The immobilized PDGFRα-HIS was first saturated with the Vκ 13B8 IgG and then challenged with the Vκ 16F4 IgG. The resulting sensorgram showed that saturation of the receptor with Vκ 13B8 induces only a limited shift in the binding curve of Vκ 16F4, indicating that the two immunoglobulins bind to two different epitopes of PDGFRα (Figure 30).

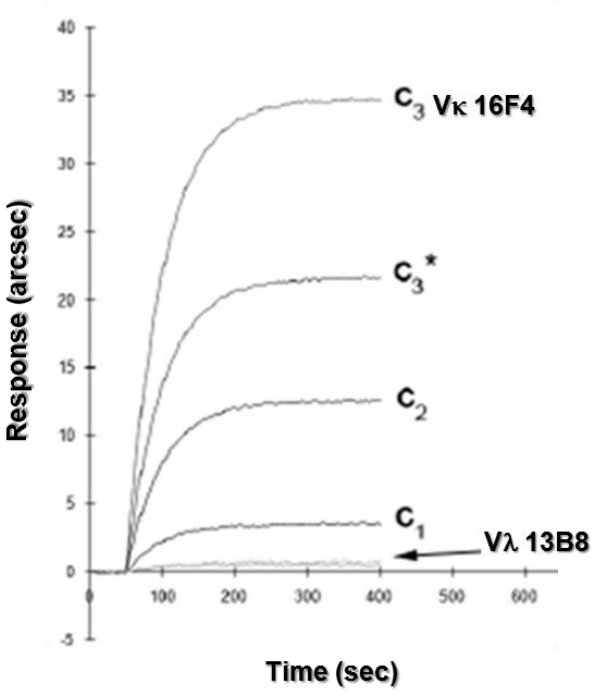

Figure 30: Sensorgram showing the binding response of purified recombinant monoclonal IgG to PDGFRα-HIS immobilized on the sensor chip surface. The binding curve of Vλ 13B8 IgG is indicated by the arrow. Vκ 16F4 IgG was tested at three different concentrations (C_1, C_2 and C_3) in order to calculate the Kd of the antibody. C_3* curve indicates the binding capacity to PDGFRα of Vκ 16F4 IgG after the receptor had been saturated with the Vκ 13B8 IgG.

ELISA

Surface plasmon resonance assays provided a detailed analysis of the binding capacity of the anti-PDGFRα autoantibodies present among the total IgG repertoire from sclerodermic patients. However, SPR technique is not easily transferable to the clinic, for the detection of autoantibodies in the serum of patients. For this reason, we developed a more versatile ELISA assay for the rapid detection of anti-PDGFRα antibodies in the serum.

The purified recombinant PDGFRα-HIS was immobilized onto the surface of high capacity polystyrene microtitre 96-well plates. The specificity of the assay was evaluated testing the binding capacity on the coated PDGFRα-HIS of three anti-PDGFRα commercial antibodies specific for different epitopes ad three irrelevant isotype-matched controls. All the PDGFRα-specific antibodies bound to the immobilized receptor while none of the control antibodies showed reactivity on the antigen (Figure 31). The proper folding of the recombinant receptor was confirmed by its ability to bind two monoclonal anti-human PDGFRα-specific antibodies.

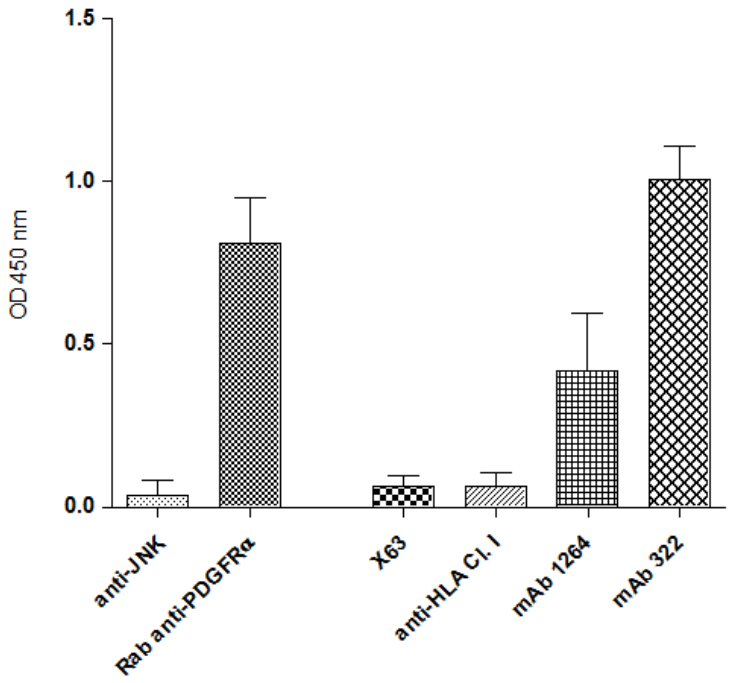

Figure 31: Quality assessment of anti-PDGFRα ELISA test. PDGFRα-HIS-coated microtiter wells were incubated with anti-PDGFRα and control antibodies, at a concentration of 1 μg/ml. The antibodies tested were: rabbit polyclonal anti-JNK (anti–JNK), rabbit polyclonal anti-PDGFRα, irrelevant mouse monoclonal IgG (X63), mouse monoclonal anti-HLA class I, mouse monoclonal anti-PDGFRα (mAb 1264) and mouse monoclonal conformational anti-PDGFRα (mAb 322).

The immobilized PDGFRα-HIS was challenged with diluted samples of serum obtained from 22 sclerodermic patients and 120 controls. The control groups comprised 94 healthy individuals and 26 patients with other selected diseases (in detail, 6 individuals with primary Raynaud's phenomenon, 7 patients with Rheumatoid Arthritis and 13 patients with Systemic Lupus Erythematosus).

A titration curve indicated that the optimal concentration of recombinant PDGFRα-HIS for the coating was 300 ng. A parallel titration assay was performed in order to determine the optimal dilution at which the sera should

be tested. The dilution of 1:100 was chosen because it reported the highest ratio between specific binding and background signal (not shown).

To exclude any interference due to "non-specific" binding of serum immunoglobulin to proteins of the milk used as a saturating agent, all samples were tested for reactivity against milk. The actual optical density of each serum was calculated following the formula: (OD on PDGFRα-coated wells) – (OD on milk-coated wells).

As a further control, all sera were tested for reactivity on 96-well plates coated with an HIS-tagged scrambled polypeptide. None of the sera showed significant binding to the irrelevant molecule (not shown).

The average optical density of sera from patients with Systemic sclerosis was 0.247 ± 0.202 (mean ± SD), whereas the mean optical density of sera from healthy controls was 0.107 ± 0.105. The difference between the mean OD of sera from SSc patients and the healthy subjects cohorts was statistically significant ($p < 0.0001$).

Sera of individuals of the other control groups showed OD values lower than those of sclerodermic patients: Primary Raynaud's phenomenon: mean 0.237 ± 0.111; Rheumatoid Arthritis: mean 0.147 ± 0.063; Systemic Lupus Erythematosus: mean 0.152 ± 0.155). However, the difference between the SSc cohort and these control groups did not reach statistical significance (Figure 32).

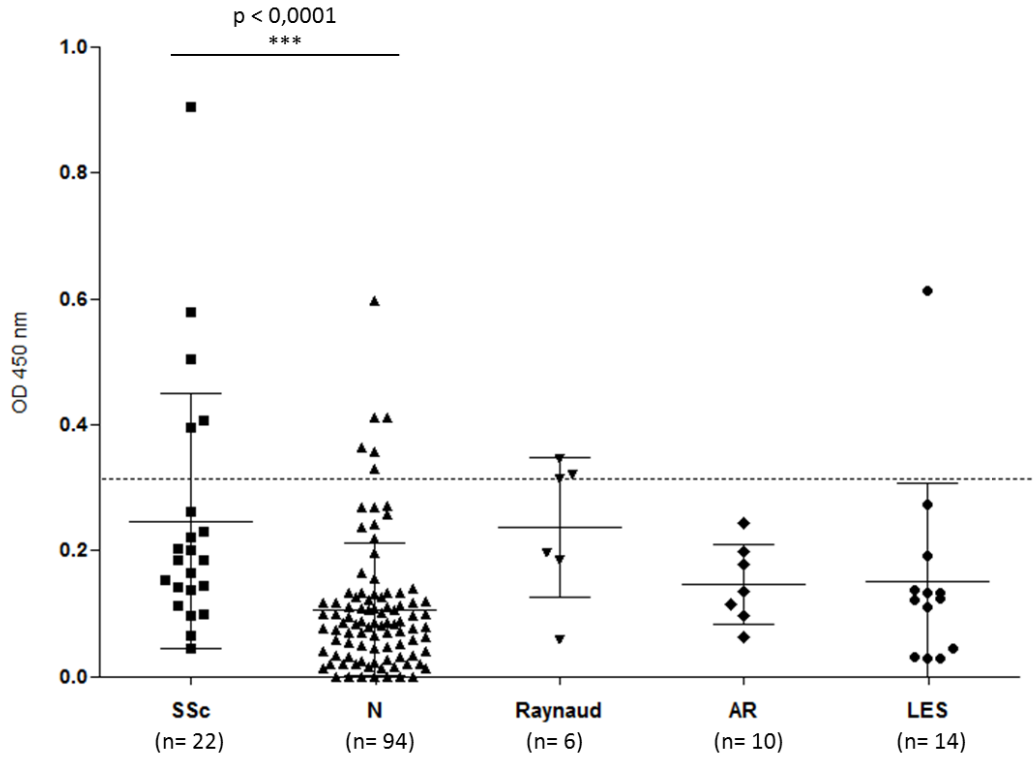

Figure 32: Serum levels of anti-PDGFRα autoantibodies in patients with Systemic sclerosis and control groups, determined by ELISA. Each plot indicates data from single patients. Bars represent the mean value ± standard deviation (mean ± SD). The dotted line indicates the cut-off value between positive and negative samples in terms of binding to PDGFRα, calculated as the mean value of healthy controls + 2 SD. SSc= Systemic sclerosis; N= healthy subjects; Raynaud= Primary Raynaud's phenomenon; AR= Rheumatoid Arthritis; LES= Systemic Lupus Erythematosus.

Sera were considered positive for the presence of anti-PDGFRα autoantibodies when their optical density was higher than the cut-off value of 0.317, calculated as the mean optical density of the healthy control group plus two standard deviations (0.107 + 0.210).

23% (5/22 samples) of patients with Systemic sclerosis resulted positive for anti-PDGFRα antibodies, whereas only 7% (6/94 samples) of healthy controls were above the cut-off threshold. Positivity scores for the other control groups

were: 50% for patients with Raynaud's phenomenon (3/6), 0% for patients with Rheumatoid Arthritis (0/7) and 8% for patients with Systemic Lupus Erythematosus (1/12) (Table 4).

	Anti-PDGFRα antibodies	
	% positive (positive/total)	**% negative (negative/total)**
Systemic Sclerosis	23% (5/22)	77% (17/22)
Healthy	7% (6/94)	93% (88/94)
Raynaud's phenomenon	50% (3/6)	50% (3/6)
Rheumatoid Arthritis	0% (O/7)	100% (7/7)
Systemic Lupus Erhytematosus	8% (1/12)	92% (12/12)

Table 4: Positivity scores of tested sera for anti-PDGFRα antibodies.

The PDGFRα-binding capacity of IgG from sera of SSc patients and controls was also assessed in a second ELISA test, in which we used a different immobilization technique. We exploited the affinity of the oligo-histidine tag for metal ions to immobilize the receptor on 96-well plates coated with nickel. This coating approach allows for all the PDGFRα-HIS molecules to be immobilized by the tag tail, leaving all the functional epitopes of the extracellular domain available to interact with the specific antibodies. However, this assay proved to be more sensitive but less reproducible than the conventional ELISA assay. The binding characteristics of each group mirrored those observed with the conventional ELISA described before: sera of SSc patients showed an increased binding capacity to the receptor, when compared with sera of healthy controls and patients with other pathologies. However, the difference in the binding values between the cohorts did not reach statistical significance (not shown).

5 Discussion

Systemic sclerosis is a rare autoimmune disease characterized by extensive fibrosis of skin and visceral organs. SSc is denoted by high morbidity and mortality rates, mostly due to late diagnosis and insufficiency of currently available pharmacological treatments [6, 14]. One of the main hallmarks of SSc is the overproduction and accumulation of collagen and other ECM proteins, resulting in thickening of the skin and fibrosis of the affected organs (e.g., the gastrointestinal tract, lung, heart and kidney) [99, 100]. The dominant pathophysiological processes of SSc onset are the involvement of the vascular system and the activation of the immune system, culminating in the unbalancing of the connective tissue homeostasis towards a pro-inflammatory phenotype [23].

Fibroblasts constitute one of the main targets of tissue alterations detectable during the course of SSc. Indeed, sclerodermic fibroblasts show an increased susceptibility to apoptosis [101], a marked tendency to develop genetic abnormalities [102] and a significant increase of the transcription of collagen and ECM protein genes [99, 100].

Similarly to other autoimmune diseases, SSc is characterized by the presence of several classes of autoantibodies in the serum. Sclerodermic autoantibodies can be classified in two main categories: autoantibodies specific for nuclear and endocellular antigens (ANA and ANoA) and autoantibodies specific for cell surface antigens mainly expressed by endothelial cells and fibroblasts (AECA and anti-fibroblast antibodies) [55].

The pathological implication of the autoimmune response that characterizes SSc pathogenesis, however, is still largely unknown.

Recent studies have demonstrated that fibroblasts of sclerodermic patients produce, *in vitro*, high levels of reactive oxygen species (ROS). The resulting oxidative stress induces the activation of fibroblasts, resulting in an increased proliferation rate and augmented transcription of collagen genes. ROS are also able to induce the expression and stabilization of Ras protein, through the activation of extracellular-signal-regulated kinases (ERK1/2) [51].

It is known that the production of ROS can also be triggered by the binding of PDGF to its receptor expressed on the cell surface of fibroblasts, and by the subsequent activation of NADPH oxydase [50]. The signaling pathway downstream of PDGFR activation is self-maintaining and it is able to sustain the production of ROS for a long time. ERK1/2 and NADPH oxydase activation, together with Ras stabilization, are responsible for the stimulation of collagen genes transcription. The persistent condition of oxidative stress of sclerodermic fibroblasts therefore results in a massive accumulation of collagen in connective tissues, which is the main phenotypic characteristic of Systemic sclerosis.

A novel class of agonistic autoantibodies targeting the alpha subunit of PDGFR (PDGFRα) has been recently discovered in the serum of patients with Systemic sclerosis [1]. The binding of sclerodermic anti-PDGFRα autoantibodies to the receptor on fibroblasts is able to induce, *in vitro*, the activation of the signaling pathway involving ERK1/2, NADPH oxydase and Ras, resulting in an increased production of ROS and collagen gene transcription (Figure 3).

Sclerodermic anti-PDGFRα autoantibodies are able to bind the receptor and induce its phosphorylation. The activated PDGFRα is able to stimulate ROS production through the activation of NADPH oxydase. The increased level of ROS induces the phosphorylation and activation of ERK1/2 which, in turn, stabilizes the expression of Ras. This signaling cascade culminates with the long term induction of the expression of collagen genes and pro-inflammatory molecules such as smooth muscle actin (α-SMA), both characteristic of fibrotic transformation processes typical of Systemic sclerosis [1].

The agonistic activity of sclerodermic anti-PDGFRα autoantibodies argues for a causal role in the pathogenesis and progression of the disease. Therefore, a more in-depth characterization of their specificity, affinity and biological activities is urgently needed.

The first part of this project was aimed to confirm the presence of PDGFRα-targeted autoimmune activation in patients with Systemic sclerosis. This goal was achieved with the isolation of the single B cells responsible for the production of the agonistic anti-PDGFRα immunoglobulins from the immunological repertoire of sclerodermic patients.

For this purpose, the memory-B cell subpopulation of selected SSc patients was immortalized with Epstein Barr virus [2] and cells were cultured at low concentrations. Four PDGFRα specific immunoglobulins, produced by immortalized sclerodermic B cell oligoclones, were selected on an cell model constituted by a murine fibroblast transfected with the human PDGFRα gene (Fα) and the correspondent mock-transfected control (F-/-) [92].

The specificity for PDGFRα of the isolated IgM (ROM D2/1F5) and the three IgG (i.e., PAM 16F4, PAM 13B8 and PAM 17H8) was confirmed by immunoprecipitation experiments. All the isolated immunoglobulins were able to bind and immunoprecipitate PDGFRα constitutively expressed by human fibroblasts.

The isolation of anti-PDGFRα immunoglobulins (one IgM and three IgG) supports the hypothesis that there indeed is an autoimmune response directed to PDGFRα in patients with Systemic sclerosis.

The observation that sclerodermic serum IgG exert an agonistic activity on PDGFRα, stimulating the production of reactive oxygen species [1], prompted us to evaluate whether the isolated anti-PDGFRα autoantibodies retained the same biological activity. To this aim, we assessed their capacity to induce ROS production in the Fα/F-/- cell model.

The IgM (ROM D2/1F5) and two of the three IgG (PAM 16F4 and PAM 13B8) were able to induce the production of ROS in Fα cells but not in F-/- cells used as a control.

The observation that the isolated sclerodermic immunoglobulins possess an agonistic activity leading to an increased oxidative stress level in target cells, supports the hypothesis of an active role of anti-PDGFRα autoantibodies in the onset of Systemic sclerosis.

One of the three isolated IgG (PAM 17H8) did not possess biological activity on PDGFRα; indeed, despite being able to immunoprecipitate the PDGFRα, PAM 17H8 did not induce a significant ROS production in Fα cells.

It is conceivable that the B cell response against PDGFRα comprises a heterogeneous subset of autoantibodies with different functional properties. Another possibility is that the lack of biological activity was due to the low binding affinity of the antibody.

A realistic hypothesis is that the agonistic immunoglobulins (e.g., PAM 13B8 and PAM 16F4) could recognize the same epitope involved in the binding with PDGF, since the physiological interaction between PDGF and its receptor is followed by receptor activation. The hypothesis that the isolated autoantibodies share the same binding site of the natural ligand is supported by the results of competition assays showing that pre-incubation of PDGFRα-expressing cells with PDGF significantly interferes with the binding of sclerodermic anti-PDGFRα autoantibodies to the same cells [1]. Moreover, the isolated sclerodermic anti-PDGFRα antibodies activate the same signaling pathway triggered by PDGF, culminating with ROS production and collagen gene transcription.

It is likely that the inactive immunoglobulins (e.g., PAM 17H8) recognize a different epitope on PDGFR, perhaps not involved in the initiation of PDGF-dependent intracellular signaling (Figure 33). The existence in the immunological repertoire of autoantibodies recognizing different epitopes of PDGFRα is consistent with the results of SPR binding assay performed with the recombinant anti-PDGFRα IgG (Figure 28 and 30).

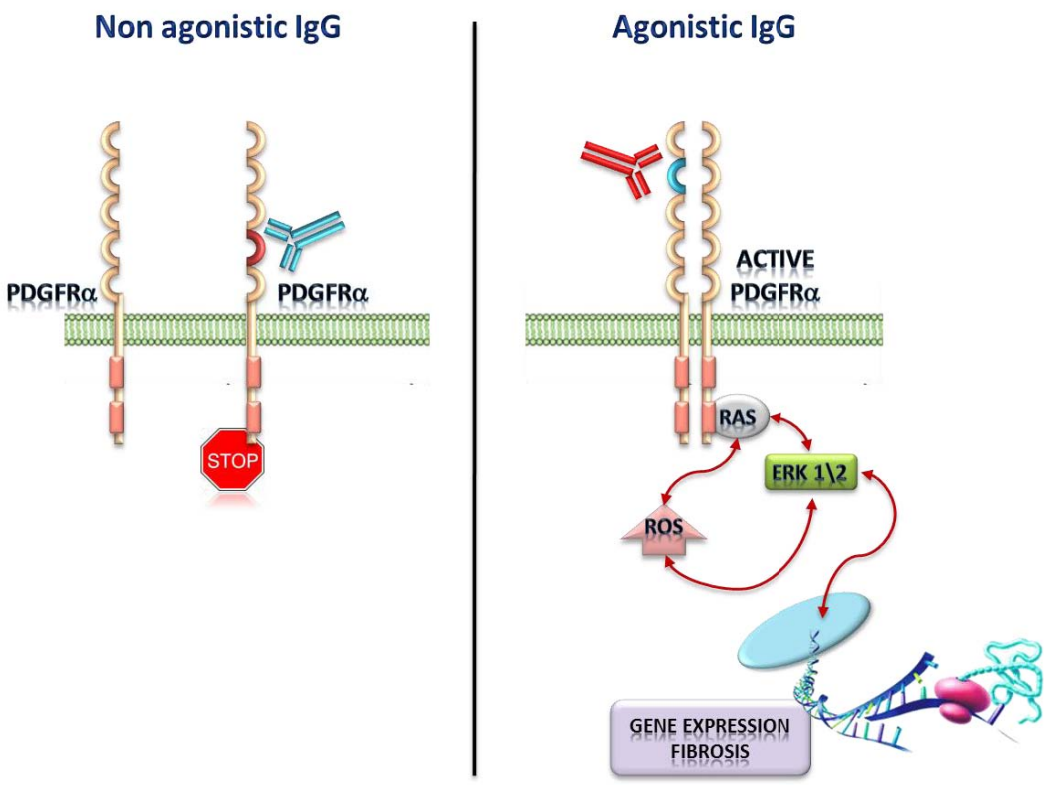

Figure 33: Agonistic and non-agonistic anti-PDGFRα autoantibodies could bind two different epitopes on the receptor. The agonistic autoantibodies (e.g, sclerodermic anti-PDGFRα autoantibodies such as PAM 16F4 and PAM 13B8) could bind an epitope able to trigger an intracellular signaling cascade (right) while non-agonistic immunoglobulins (e.g., PAM 17H8) could be specific for an epitope not involved in PDGFRα activation (left). This interpretation of differential specificity could also explain the different biological activity of sclerodermic and non-sclerodermic anti-PDGFRα autoantibodies.

Another possible interpretation of the differences between the isolated anti-PDGFRα immunoglobulins in terms of biological activity is that the primary molecular target of the non-agonistic autoantibodies is not PDGFRα, but an unknown exogenous antigen sharing linear or conformational epitopes with PDGFRα. The presence of cross-reactive autoantibodies has already been described in Systemic sclerosis and other autoimmune diseases; for example, a subset of antibodies directed against the hCMV-derived protein UL94 is able to

bind dermal fibroblasts through the surface receptor NAG-2, as a result of an event of molecular mimicry [83].

The analysis of the binding specificity of agonistic and non-agonistic sclerodermic autoantibodies on a peptide library encompassing the extracellular domain of PDGFRα (currently underway) will shed light on this aspect. Once the PDGFRα epitopes targeted by sclerodermic autoantibodies will be identified, it will be possible to determine the differences, in terms of specificity, between agonistic and non-agonistic autoantibodies. Moreover, PDGFRα epitope-mapping will allow us to analyse any similarities between the identified epitopes and potential exogenous antigens, which could explain the presence of non-agonistic, cross-reactive antibodies in healthy subjects.

To further characterize the sclerodermic PDGFRα-specific autoantibodies, sequence analysis was performed for the heavy and light chains of the variable regions of the isolated IgM (ROM D2/1F5) and IgG (PAM 13B8, PAM 16F4 and PAM 17H8).

The analysis of the sequence of the isolated immunoglobulins allowed us to identify several VH and VL sequences because the originating cultures were not clonal. One sequence for the variable heavy region (VH2) resulted identical among all the sequenced IgG.

A small epidemiologic study was performed in order to assess the frequency of expression of the complementarity-determining regions (CDR) of the kappa chain of the light variable region of PAM 16F4 IgG (selected because of its functional properties). The CDR was detected in the majority of the analyzed sclerodermic patients and in a small proportion of the healthy controls. This

preliminary evidence further supports the notion that anti-PDGFRα autoantibodies are present in the immunological repertoire of a significant number of sclerodermic patients. However, the small number of subjects (n = 8) tested in this preliminary study cannot be considered representative of the entire cohort of sclerodermic patients. A larger study aimed at a more precise assessment of the frequency of expression of the CDR of anti-PDGFRα autoantibodies in a larger cohort of patients and controls is on-going.

To obtain human monoclonal IgG specific for PDGFRα, we have cloned the cDNA sequences obtained from the oligoclonal immunoglobulins in a human IgG expression vector [93]. The VH and VL sequences obtained from the four isolated immunoglobulins, variously combined among themselves, allowed us to build a small panel of recombinant monoclonal IgG. The obtained cDNA constructs have been engineered to contain all the possible pairings between heavy and light chain variable regions encountered in each isolated oligoclonal B cell line. The monoclonal IgG constructs have then been transfected in CHO cells, and the resulting IgG were purified from the cell culture supernatant and further characterized.

First, the recombinant monoclonal antibodies were tested for specificity and biological activity on PDGFRα, using human normal fibroblasts as target cells. The recombinant IgG were able to immunoprecipitate PDGFRα from human fibroblasts, demonstrating that the variable region sequences of the isolated sclerodermic anti-PDGFRα autoantibodies, once grafted in the recombinant immunoglobulins, maintain their specificity for the receptor.

Next, the biological properties of the recombinant monoclonal IgG were evaluated testing their capacity to induce ROS production, ERK1/2 activation and collagen gene transcription, using human fibroblasts as target cells. The recombinant IgG proved able to recapitulate the agonistic characteristics of the anti-PDGFRα antibodies present in the serum of sclerodermic patients. Indeed, four recombinant IgG were able to induce phosphorylation of ERK1/2, six IgG induced ROS production and two IgG were able to induce the transcription of type I collagen.

A surface plasmon resonance assay performed on immobilized PDGFRα with one of the recombinant monoclonal IgG (Vκ 16F4), selected for its ability to recapitulate the functional effects attributed to the agonistic sclerodermic IgG, allowed us to calculate its dissociation constant, and placed it in the low nanomolar range. This indicates that this recombinant IgG is able to bind the receptor with high affinity and motivates the good performance of this IgG in all the functional assays.

A competition experiment, performed with two recombinant IgG (Vκ 16F4 and Vκ 13B8), showed that they bind two different epitopes on the receptor, confirming the observation that there is more than one site of PDGFRα targeted by sclerodermic autoimmunity.

In summary, the cloned recombinant monoclonal IgG show that it is possible to generate PDGFR-specific, fully human antibodies from the sequences of the variable regions of the heavy and light chains of anti-PDGFRα autoantibodies isolated from SSc patients. The recombinant antibodies, generated through grafting of the variable regions of anti-PDGFRα autoantibodies, have a binding

capacity comparable to that of the immunoglobulins from which they are derived and recapitulate, at least in part, their biological activities. This evidence further corroborates the hypothesis of the existence of PDGFRα-directed autoimmunity in Systemic sclerosis.

One of the main outcomes of this research project is the generation of reproducible, high-throughput, low cost novel diagnostic assay enabling the early identification of patients affected by SSc. Expectations for early diagnosis assays and novel therapies are high among sclerodermic patients and their families. Unfortunately, clinical trials to find new treatments are hampered by several hassles such as the inability i) to correctly identify patients with early stage scleroderma; ii) to monitor disease activity and progression; iii) to group homogenous subsets of patients; iv) to identify critical pathogenic steps.

To this purpose, a truncated receptor comprising the whole extracellular region of PDGFRα, tagged with six histidine residues, was stably expressed in HeLa cells and purified from cell lysate by metal ion affinity chromatography on nickel-chelated resin. The recombinant receptor was used as the target molecule in affinity binding assays.

The first method we implemented was surface plasmon resonance. SPR was utilized to assess the presence and the binding affinity of anti-PDGFRα antibodies present in IgG samples purified from the serum of sclerodermic patients and healthy controls.

The SPR affinity binding assay highlighted the presence of anti-PDGFRα autoantibodies in all the sclerodermic IgG samples so far analyzed, further

corroborating the initial hypothesis that autoantibodies against PDGFRα may represent a hallmark of Systemic sclerosis.

All but one of the IgG purified from the serum of healthy controls did not show significant binding to the receptor, whereas only one control IgG showed binding capacity to PDGFRα comparable to that of the sclerodermic IgG.

An antigen-based enzyme-linked immunosorbent assay was then designed to develop a large-scale binding assay for the early detection of anti-PDGFRα autoantibodies in the serum.

The ELISA test highlighted the presence of anti-PDGFRα autoantibodies in the serum of 23% of the tested sclerodermic patients and 7% sera from healthy subjects. The difference in the mean binding values between the SSc cohort and the healthy control group is statistically significant. This indicates a higher prevalence of anti-PDGFRα antibodies in the sclerodermic population, consistently with the data obtained from the epidemiological study performed to determine the frequency of expression of the CDR of the anti-PDGFRα immunoglobulins isolated from the repertoire of SSc patients.

The detection of anti-PDGFRα activity in a small number of normal sera is in contrast with previous results [1], where anti-PDGFRα immunoglobulins were not found in the sera from healthy subjects. This discrepancy could be attributed to the assay methods we used. Indeed, the ELISA method has a higher sensitivity than the immunoprecipitation assay used by Svegliati-Baroni et al. [1]. It is likely that the immunoprecipitation assay was unable to detect anti-PDGFRα antibodies with low affinity.

Sera from patients with other selected diseases, tested as control groups, scored lower positivity levels to PDGFRα when compared to the SSc cohort, although the differences did not reach statistical significance. None of the patients with Rheumatoid Arthritis was positive for anti-PDGFRα antibodies.

Three out of six (50%) sera from patients with Primary Raynaud's phenomenon scored above the cut-off value selected to determine positivity for the presence of anti-PDGFRα autoantibodies. Primary Raynaud's phenomenon is considered to be one of the earliest clinical manifestations of Systemic sclerosis [38]. All the sera from the group of patients with Primary Raynaud's phenomenon that we included in the ELISA were collected from patients showing no clinical signs of scleroderma at diagnosis, as dictated by ACR criteria for SSc diagnosis. However, several years after her serum was collected, one patient within this group has developed Systemic sclerosis. Interestingly, the serum from this patient is the one showing the highest optical density value in the group of patients with Raynaud's phenomenon. This observation, though it is anecdotal, suggests that anti-PDGFRα autoantibodies could indeed represent an early molecular marker for the diagnosis of Systemic sclerosis. It is tempting to speculate that anti-PDGFRα autoantibodies could represent a marker that will allow the monitoring of the clinical history of subjects whose sera show PDGFRα-specific binding.

In the group of patients with Systemic Lupus Erythematosus, 8% of the samples were found to have anti-PDGFRα antibodies. Although the interpretation of these results requires a note of caution due to the small number of SLE patients so far tested, this result is consistent with those recently reported by Kurasawa et al. [103] demonstrating the presence of PDGFRα–specific antibodies in a

subset of SLE patients showing active disease. In agreement with previous results [104, 105], these anti-PDGFRα IgG failed to demonstrate agonistic or antagonistic activity on PDGFR signaling. Taken together, these results further indicate that anti-PDGFRα antibodies could exist in a reduced number of sera from normal individuals and autoimmune patients, however, they are nonfunctional.

Sclerodermic patients included in the study have been diagnosed according to the ACR criteria for SSc [4]. However, no distinction was made between patients with active or latent disease at the time of serum collection. One could envision that SSc sera showing low levels of anti-PDGFRα autoantibodies reflect a phase of latent disease, in which the reduced concentration of the agonistic IgG is associated with a remissive phase of the disease. The evaluation of changes in the serum level of anti-PDGFRα autoantibodies over the course of the sclerodermic disease deserves further investigation.

In summary, we have determined that anti-PDGFRα antibodies may also be detected in the serum of subjects not affected by Systemic sclerosis, perhaps as a result of an event of molecular mimicry between PDGFRα and an unknown exogenous antigen. However, the biological properties of the autoantibodies (both the ones isolated from the serum of SSc patients and the recombinant ones we constructed) proved to be a unique characteristic of anti-PDGFRα autoantibodies present in the serum of patients with Systemic sclerosis. Indeed, only the isolated sclerodermic IgG proved to be stimulatory, since they induced myofibroblastic conversion, type I collagen expression and ROS production in normal fibroblasts. Stimulatory autoantibodies were not

detected in patients with primary Raynaud's phenomenon, Systemic Lupus Erythematosus and Rheumatoid Arthritis.

The agonistic activities selectively exerted by sclerodermic anti-PDGFRα autoantibodies emphasize the potential contribution of these autoantibodies to the pathogenic process that leads to the sclerodermic disease.

6 *Future perspectives*

The production and purification of adequate amounts of human recombinant anti-PDGFRα IgG is the first task scheduled for the immediate development of the work discussed in this thesis. The recombinant IgG represent a useful tool for the completion of a comprehensive characterization of the PDGFRα-directed autoimmune response in Systemic sclerosis. The binding properties and biological activities of the purified IgG will be confirmed with thorough affinity binding assays and a detailed analysis of their agonistic properties.

Moreover, the recombinant IgG will be used to determine the specific epitopes of PDGFRα targeted by SSc autoimmunity through the screening of a peptide library which encompasses all the potential binding targets of the receptor.

In parallel, the research of the CDR sequences of the isolated anti-PDGFRα autoantibodies will be extended to a broader cohort of Sclerodermic patients and controls, in order to determine whether the observed difference in the expression of the sequences is statistically significant.

An expansion in the number of test samples is also needed in order to confirm and refine the results of the ELISA test on PDGFRα. Particular attention will be given to the clinical history of the subjects whose sera show noteworthy PDGFRα-binding properties (e.g., negative subjects in the SSc cohort and positive subjects in the control groups) in order to determine whether there could be an association between the presence of anti-PDGFRα autoantibodies and a definite phase of disease course.

Furthermore, gene array technology will be employed to analyze variations in the transcriptional profile of human normal adult fibroblasts from skin biopsies following stimulation with immunoglobulins from SSc patients or healthy controls, in order to shed light on the autoantibody-mediated activation patterns downstream of the PDGFRα and on the pathogenic role of the anti-PDGFRα or other autoantibodies in Systemic sclerosis, possibly allowing for the discovery of novel markers of this complex disease.

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