UNIVERSITY OF SIENA

DOCTORAL SCHOOL IN BIOMEDICINE AND IMMUNOLOGICAL SCIENCES

SECTION: CLINICAL AND EXPERIMENTAL ALLERGOLOGY AND IMMUNOLOGY

ADENOSINE A2A RECEPTOR ACTIVATION STIMULATES COLLAGEN PRODUCTION EITHER DIRECTLY AND THROUGH A CROSS-TALK WITH THE CANNABINOID SYSTEM.

AN *IN VITRO* STUDY IN SCLERODERMIC DERMAL FIBROBLASTS AND *IN VIVO* STUDY IN A MURINE MODEL OF ESTABLISHED DERMAL FIBROSIS

TUTOR:

PROF. FRANCO LAGHI-PASINI

PHD STUDENT:

ELENA GIANCHECCHI

XXIV CYCLE

ACADEMIC YEAR 2011-2012

ABSTRACT *p2*

1. INTRODUCTION *p3*

1.1 The pathogenesis of Systemic Sclerosis (SSc) and cellular determinants of fibrosis *p4*

1.2 The adenosine A2A receptor and its role in fibrogenesis in vitro and in vivo *p8*

1.3 Role of the MAP-Kinase ERK 1/2 in the regulation of fibrogenesis *p11*

1.4 Cannabinoid CB1 and CB2 receptors and their effects on regulation of extracellular matrix (ECM) production in vitro and in vivo *p13*

1.5 Physical and functional interaction between A2A and CB1 receptors *p15*

1.6 Aim of the present study *p16*

2. MATHERIALS AND METHODS *p17*

2.1 In vitro study *p18*

2.2 Treatment of mice with established dermal fibrosis induced by bleomycin *p23*

3. RESULTS *p26*

3.1 Results from in vitro study *p27*

3.2 Results from in vivo study *p32*

4. DISCUSSION *p33*

5. BIBLIOGRAPHY *p40*

6. IMAGES AND TABLES *p53*

ABSTRACT

OBJECTIVES. Systemic sclerosis (SSc) is a connective tissue disease characterised by exaggerated collagen deposition in the skin and visceral organs. Adenosine A2A receptor stimulation (A2Ar) promotes dermal fibrosis, while the cannabinoid system modulates fibrogenesis in vitro and in animal models of SSc. Moreover, evidence in central nervous system suggests that A2A and cannabinoid (CB1) receptors may physically and functionally interact. On this basis, we investigated A2Ar expression and function in modulating collagen biosynthesis from SSc dermal fibroblasts, and analysed the cross-talk with cannabinoid receptors either in vitro and in a murine model of established dermal fibrosis.

METHODS. In sclerodermic cells, A2Ar expression (RT-PCR, Western blotting) was evaluated together with the effects of A2A agonists and/or antagonists on collagen biosynthesis (EIA, Western blotting). Putative physical and functional interactions between the A2A and cannabinoid receptors were respectively assessed by co-immuno-precipitation and co-incubating the cells with the unselective cannabinoid agonist WIN55,212-2, and the selective A2A antagonist ZM-241385.

In a murine model of established dermal fibrosis induced by repeated local injections of bleomycin the anti-fibrotic effect of ZM-241385 and/or WIN55,212-2 was assessed by evaluating changes in dermal thickness (histopathologic analysis) and alpha-smooth muscle actin (α-SMA) expression (immunohistochemistry).

RESULTS. In SSc fibroblasts, (1) the A2Ar is overexpressed and its occupancy with the selective agonist CGS-21680 increases collagen production, myofibroblast trans-differentiation, and ERK-1/2 phosphorylation; (2) the A2Ar forms an heteromer with the cannabinoid CB1 receptor; and (3) following A2Ar blockade, the non-selective cannabinoid receptor stimulation with a per se ineffective dose of WIN55,212-2 results in a marked anti-fibrotic effect either in vitro and in a murine model of established dermal fibrosis.

CONCLUSIONS. A2Ar stimulation induces a pro-fibrotic phenotype in SSc dermal fibroblasts, either directly, and indirectly, by activating the CB1 cannabinoid receptor. These findings increase our knowledge of the pathophysiology of sclerodermic fibrosis also further suggesting a new therapeutic approach to the disease.

SECTION 1.

INTRODUCTION

1.1 THE PATHOGENESIS OF SYSTEMIC SCLEROSIS (SSC) AND CELLULAR DETERMINANTS OF FIBROSIS

Systemic sclerosis (SSc) is a rare connective tissue disease of unknown etiology. Based on incidence and survival rates, an estimated 75,000 - 100,000 individuals in the United States have SSc, with a higher risk in women than men (ratio ranging from 3:1 to 14:1) and in blacks than whites. SSc is characterized by the presence of vasculopathy in multiple vascular beds, immunological alterations and interstitial fibrosis; these three pathological hallmarks can be variably present in sclerodermic patients and thus explain the variable course that characterizes this pathology.

Based on the extension of fibrosis, SSc can be divided into two subgroups:

- Limited cutaneous systemic sclerosis (lcSSc): fibrosis is mainly confined to hands, arms and face. It is characterized by a relatively good prognosis of survival compared to the diffuse form of the pathology (>70% at 10 years);
- Diffuse cutaneous systemic sclerosis (dcSSc): rapidly progressing disorder that affects a larger portion of skin associated with the important and serious involvement of many internal organs like lungs, heart, gastrointestinal tract and/or kidneys. It is associated with a worse rate of survival (40-60% at 10 years).

At the moment, no effective anti-fibrogenetic therapy is available for the disease (Gabrielli et al., 2009).

Vascular damage and endothelial involvement are the primary events in the development of SSc. Activation and apoptosis of endothelial cells, perhaps caused by anti-endothelial antibody, can be revealed in the earliest stages of SSc, whereas the later phases show a progressive closure of the lumen of capillaries for proliferation and hypertrophy of endothelial and smooth muscle vascular cells. The hypoxic situation is aggravated because the capillary network rarefies (avascular fields) causing the development of ischaemic

digital ulcers. In physiologic conditions hypoxia induces the formation of new vessels by the release of vascular endothelial growth factor (VEGF) and other mediators, but in this pathologic condition angiogenesis results as alterated, and promotes the development of enlarged and giant capillaries associated with micro-haemorrhages (Varga and Abraham, 2007; Distler O, 2009; Gabrielli et al., 2009).

Autoimmunity represents the other important and unclear aspect of SSc with the involvement of both innate and acquired immune responses. Sclerodermic patients show an increased activity of T cells, more in particular an imbalance towards T helper type 2 (T_H2) cytokine immune response (as Il-4 and Il-13, known for their pro-fibrotic effects) and a decrease of type 1 activity (T_H1) (like IFN-γ and IL-10 with anti-fibrotic action). Data from *in vitro* and *in vivo* studies have demonstrated that an over-expression of Type 2 response could induce pro-fibrotic phenotypic variation in fibroblasts (Postlethwaite et al., 1992; Sime and O'Reilly, 2001; Varga and Abraham, 2007; Jinnin, 2010; Fuschiotti, 2011). On the other hand, the administration of IL-12 reduced collagen deposition in a murine model of pulmonary fibrosis induced by bleomycin both directly through the promotion of T_H1 response, and indirectly by increasing IFN-γ levels (Keane et al., 2001).

The hypothesis is that vascular damage, autoimmunity and inflammation could induce the activation of fibroblasts. In fact, in respect to healthy cells, sclerodermic fibroblasts resulted as constitutively activated to synthesize and release an exaggerated quantity of collagen-rich extracellular matrix (ECM) that substitute the normal connective architecture (LeRoy, 1972; Uitto et al., 1979; Kähäri et al., 1987; Sime and O'Reilly, 2001; Distler et al., 2007; Varga and Abraham, 2007; Varga and Trojanowska, 2008) and, moreover, their modified phenotype is maintained for many passages *in vitro* after biopsy (Jimenez et al., 1986; LeRoy, 1974; Jinnin, 2010). They even show a major expression of cell surface receptors binding fibrogenic mediators like transforming growth factor beta receptors

(TGFβ-r) (Kawakami et al., 1998; Kubo et al., 2002; Yamane et al., 2002) and constitutively secrete cytokines and chemokines (Jinnin, 2010). The elevated levels of TGFβ-r mRNA and protein were positively associated with the higher expression of α2(I) collagen mRNA and type I collagen protein (Yamane et al., 2002). Moreover the potent pro-fibrotic citokyne TGFβ (Roberts et al., 1986; Verrecchia and Mauviel, 2007) stimulates fibroblasts to trans-differentiate in myofibroblasts (Desmoulière et al., 1993), considered the principal cellular components responsible for the development of fibrosis in SSc and in other pathological conditions. Myofibroblasts show a biochemical phenotype between an α–smooth-muscle cell and a typical interstitial fibroblast. They can be specifically detected for the presence of alpha-smooth muscle actin (α-SMA), and their localization was observed in scleroderma lesions of skin and visceral organs (esophagus, liver and lung) (Sappino et al., 1990). The higher resistance of myofibroblasts to apoptosis permits their persistence in the lesional areas (Jelaska and Korn, 2000; Varga and Trojanowska, 2008). These cells, responsible for wound contraction, actively produce collagen as well as high levels of tissue inhibitor of metalloproteinase that, through the inhibition of collagen degradation, contribute to the maintenance of fibrosis (Kirk et al., 1995). Moreover myofibroblasts release fibrogenic cytokines like TGFβ, which in binding to TGFβ-r, could explain the established autocrine mechanism which maintains the persistence of the activated phenotype of fibroblasts (Kawakami et al., 1998; Varga and Trojanowska, 2008). The involvement of TGFβ on promoting myofibroblasts transdifferentiation was demonstrated in a murine model of scleroderma induced by bleomycin, which express higher levels of α–SMA that was reduced following anti-TGFβ antibody treatment (Yamamoto and Nishioka, 2002).

Moreover, various auto-antibodies have been identified in the sera of sclerodermic patients, which include anti-Scl70, anti-centromere, anti-fibroblasts, anti-endothelial cells

and anti-platelet derived growth factor (PDGF) . They may promote the development of SSc linking and constitutively activating their cellular targets (Gilliam, 2008; Bosello et al., 2011; Chizzolini et al., 2011).

1.2 THE ADENOSINE A2A RECEPTOR AND ITS ROLE IN FIBROGENESIS IN VITRO AND IN VIVO

A2A adenosine receptor (A2Ar) belongs to P1 receptors, more in detail to adenosine receptors, a family of four G protein-coupled receptors (GPCRs) (Burnstock, 2007), and depending on the tissue in which it is expressed it can links different G-coupled signal transduction proteins (Fredholm et al., 2000). One of the most potent endogenous mediators that can bind A2Ar is represented by adenosine, an ubiquitous purine nucleoside produced in physiological processes (by breakdown of ATP). In pathological conditions like stress or hypoxia, the lowering of cellular energy's levels and the increase of intracellular adenosine levels permit the extracellular release of this nucleoside (Stiles, 1992; Haskó and Cronstein, 2004). The activity of adenosine is strictly regulated by its short plasma and tissue half-life (< 10 seconds) which doesn't permit its direct quantification. More specifically, it can be transported back into the cells by a specific nucleoside transporter (Kwong et al., 1988) or can cross the cell membrane by simple diffusion and can be deaminated in receptor-inactive inosine by adenosine deaminase or phosphorylated to adenosine monophosphate (AMP) by adenosine kinases (Stiles, 1992; Chan et al., 2006b). The activation of A2Ar by adenosine can regulate many functions like neurotransmission (Gomes et al., 2011), coronary vasodilatation (Chan and Cronstein, 2010) and inhibits inflammatory response (Cronstein, 1994; Ohta and Sitkovsky, 2001; Haskó and Cronstein, 2004; Antonioli et al., 2008; Kumar and Sharma, 2009; Scheibner et al., 2009). Moreover it can accelerate wound healing through the increase of ECM production and the number of fibroblasts (Montesinos et al., 1997; Victor-Vega et al., 2002). More precisely, recent studies in cell and animal models have demonstrated that the endogenous purine adenosine permits the passage of A2Ar from an exclusively anti-inflammatory function to the promotion of the restoration of tissue integrity by modulation

of immune function, but in some cases it can also acquire a mal-adaptive role promoting fibrosis in different organs (Chan and Cronstein, 2010; Cronstein, 2011).

More in detail, selective A2A receptor agonists stimulate collagen production from rat and human hepatic stellate cell lines (Che et al., 2007) while A2A receptor antagonists selectively counteract the development of liver fibrosis in carbon tetrachloride $(CCl_4)^-$ exposed mice (Chan et al., 2006b). Moreover, A2Ar occupancy promotes collagen synthesis from peritoneal fibroblasts and, conversely, both blockage and deficiency of the A2Ar reduce the progression of peritoneal fibrosis in a murine model (Nakav et al., 2009).

The first evidence of A2Ar expression on human dermal fibroblast was reported by the group of Cronstein (Montesinos et al., 1997). Moreover, the same group (Chan et al., 2006a) showed the effects of the selective A2Ar agonist CGS-21680 on the regulation of collagen production by primary human dermal fibroblasts, in the presence or not of the selective A2Ar antagonist ZM-241385. The treatment with CGS-21680 induced an increase of collagen production, which was inhibited by incubation with ZM-241385. The same study also reported that A2Ar-deficient (A2A KO) and ZM-241385-treated mice were protected from the development of dermal fibrosis in a bleomycin-induced murine model. A2Ar modulated fibrogenesis also by regulating the recruitment of bone marrow-derived fibrocytes to the skin (Katebi et al., 2008).

In accordance with all these data, partially adenosine deaminase (ADA)-deficient mice that showed higher adenosine levels in several tissues for the reduced ability to degradate adenosine, spontaneously developed lung (Blackburn, 2003; Chunn et al., 2006), liver, renal, and dermal fibrosis associated with an increase of myofibroblasts without any administration of pro-fibrotic agents such as bleomycin (Fernàndez et al., 2008). Accordingly with these data, treating ADA-deficient mice with exogenous ADA enzyme therapy prevented lung collagen production and deposition and the increase of

myofibroblasts or reduced all these parameters in the case of established pulmonary fibrosis (Blackburn, 2003; Chunn et al., 2005).

However, no data is currently available on the expression and function of the A2Ar on human fibroblasts deriving from the skin of SSc patients.

1.3 ROLE OF THE MAP-KINASE ERK1/2 IN THE REGULATION OF FIBROGENESIS

Data from the literature demonstrated that A2Ar-dependent collagen type I production is mediated by multiple signalling pathways, including protein kinase A (PKA), src, and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) (Che et al., 2007; Chan and Cronstein, 2010).

ERK1/2 is a protein kinase constituted by two isoforms: ERK-1 ($p44^{MAPK}$) and ERK-2 ($p42^{MAPK}$) and it belongs to the mitogen-activated protein kinases (MAPKs) signalling. MAPKs represent a signalling system used by eukaryotic cells to transduce extracellular stimuli into intracellular responses through the sequential phosphorylation and activation of several cytoplasmic protein kinases. ERK1/2 is activated by the kinase immediately upstream MEK (mitogen-activated protein kinase kinase) and is involved in the regulation of many cellular processes like cell cycle progression, proliferation and differentiation (Seger and Krebs, 1995; Schulte and Fredholm, 2003). The fact that the signaling of A2Ar is mediated by the activation of ERK1/2 was demonstrated in many cellular types. Endothelial cells stimulated with adenosine showed an early (15 s -5 min) increase in phosphorylation of ERK1/2, that was reduced pretreating the cells with ZM-241385 (Wyatt et al., 2002). Moreover, siRNA - mediated knockdown of ERK1/2- reverted the effect induced by the stimulation of CGS-21680 on collagen type I from hepatic stellate cells (Che et al., 2007). Finally, studies on human dermal fibroblasts showed that stimulation with CGS-21680 increased ERK1/2 phosphorylation and induced collagen production, whereas the pharmacologic inhibition of MEK stopped the effects induced by A2Ar occupancy (Chan et al., 2006a; Chan and Cronstein, 2010).

The involvement of the MAPK pathway in the regulation of fibrogenesis was also demonstrated in a murine model of lung fibrosis induced by bleomycin, in which treatment

with inhibitors of MEK activation had the protective effects of reducing inflammation and collagen deposition (Galuppo et al., 2011).

1.4 CANNABINOID CB1 AND CB2 RECEPTORS AND THEIR EFFECTS ON REGULATION OF EXTRACELLULAR MATRIX (ECM) PRODUCTION IN VITRO AND IN VIVO

The family of cannabinoid receptors encompasses CB1 (CB1r) and CB2 receptors (CB2r) that are G-coupled protein receptors. They can be activated by three types of ligands:
(i) endocannabinoids (fatty acids produce endogenously by the human body, including anandamide), (ii) natural extracts like Δ^9-tetra-hydroxycannabinol (THC) (the main constituent of marijuana plants *Cannabis sativa*) and (iii) several synthetic derivatives. The expression of CB1r was primarily reported in the central nervous system and later detected also in endothelial cells, adipocytes, gut, liver cells (Parfieniuk and Flisiak, 2008) and dermal fibroblasts (Garcia-Gonzalez et al., 2009); whereas CB2r is expressed in immune blood cells, spleen, testis, tonsils (Parfieniuk and Flisiak, 2008), hepatic myofibroblasts (Julien et al., 2005) and dermal fibroblasts (Garcia-Gonzalez et al., 2009). A current line of research also documented that the cannabinoid system is involved not only in the regulation of inflammation, vasomotor response, proliferation, and cell death, but also has a role in the modulation of fibrogenesis (Graham et al., 2009). The available data suggest two opposite effects of receptor activation with the CB1r promoting and the CB2r inhibiting the fibrotic process, respectively. Cannabinoid system is involved in liver (Caraceni et al., 2009), pancreatic (Michalski et al., 2008) and skin fibrosis (Akhmetshina et al., 2009a). More precisely, it was demonstrated that CB1 deficient mice ($CB1^{-/-}$) were protected from bleomycin-induced dermal fibrosis compared with $CB1^{+/+}$ mice (Marquart et al., 2010), whereas mice deficient for CB2 ($CB2^{-/-}$) treated with carbon tetrachloride (CCl_4) showed significantly higher liver fibrosis than their wild-type (WT) counterparts (Julien et al., 2005) Accordingly, in two recent investigations performed in our Institution, we demonstrated that the non-selective CB1/CB2 synthetic cannabinoid agonist

WIN55,212-2 not only exerted an evident inhibiting activity on collagen biosynthesis in dermal fibroblasts from patients affected with SSc (Garcia-Gonzalez et al., 2009), but also abrogated dermal fibrosis *in vivo*, in a bleomycin murine model of scleroderma (Balistreri et al, 2011). These findings have been confirmed by Servettaz and coll. (Servettaz et al, 2010), who demonstrated that WIN55,212-2 treatment (or with the selective CB2 agonist JWH-133) prevented the development of skin and lung fibrosis in a mouse model of diffuse SSc induced by subcutaneous hypochlorite injections.

1.5 PHYSICAL AND FUNCTIONAL INTERACTION BETWEEN A2A AND CB1 RECEPTORS

GPCRs represent the more diffuse signal-transduction system in animals. Each GPCR was considered as a single monomeric receptor only able to interact with a single G protein, but a lot of recent data supports a documented new vision of G-protein coupled receptors, today considered structures able to interact actively with each other forming homo- and heteromers (Bouvier, 2001; Burnstock, 2007; Fuxe et al., 2008; Agnati et al., 2009).

In relation to adenosine and cannabinoid systems evidence in central nervous system intriguingly suggests physical and functional interactions between the A2A and the CB1 receptors, resulting in a strict dependence for CB1 signalling on A2Ar co-activation (Ferré et al, 2009). In fact, Carriba and coll. (Carriba et al., 2007) reported that CB1 and A2A receptors form heteromeric complexes in co-transfected HEK-293T cells and rat striatum, where they co-localize in fibrillar structures and co-immunoprecipitate. Moreover, in the same study the authors demonstrated that in a human neuroblastoma cell line, A2Ar blockade or incubation with adenosine deaminase counteracted the ability of a CB1 agonist to inhibit forskolin-induced cAMP accumulation. The permissive role of A2Ar for CB1 receptor-signalling is also shown in A2Ar KO mice in which the post-synaptic A2Ar deletion determined a reduction of WIN55,212-2 effects in the activation of CB1r (Tebano et al., 2009).

1.6 AIM OF THE PRESENT STUDY

We investigated A2Ar expression and function in modulating collagen biosynthesis from SSc dermal fibroblasts and analysed the cross-talk with cannabinoid receptors.

The present study was developed in two phases:

1. the first part was aimed at:
    (i) evaluating the extent of A2Ar expression (RT-PCR, Western blotting) and its role in regulating the collagen biosynthetic process in sclerodermic cells after *in vitro* treatment with A2A agonists and/or antagonists (EIA, Western blotting);
    (ii) confirming the formation of A2A-CB1 heteromers also in fibroblasts, assessed by co-immuno-precipitation;
    (iii) testing the putative synergistic anti-fibrotic effect exerted by the concomitant modulation of the purinergic and the cannabinoid systems in sclerodermic fibroblasts co-incubating the cell with the unselective cannabinoid agonist WIN55,212-2, and the selective A2A antagonist ZM-241385 (EIA).

2. the second part of the study was developed subsequently using a murine model of established dermal fibrosis induced by repeated local injections of the antitumor antibiotic bleomycin (Yamamoto et al., 1999, 2000; Yamamoto and Nishioka 2002, 2005; Yamamoto, 2006), and was aimed to confirm *in vivo* the possible effective synergistic inhibitory action evaluated in the previous *in vitro* study.

SECTION 2.

MATERIALS AND METHODS

2.1 IN VITRO STUDY

SKIN BIOPSIES

Dermal fibroblasts were obtained by 5 mm punch biopsy from the leading edge of the affected skin, on the forearm of five patients affected by anti-Scl70 positive diffuse SSc and from four healthy volunteers. All subjects gave their written informed consent to the study and the protocol was approved by the local Ethical Committee. Patients were not receiving adenosine modulating substances (drugs or xanthine-containing beverages) nor taking cannabinoids for either recreational or therapeutic use at the time of biopsy. Demography of the subjects involved in the study is depicted in Table 1.

DERMAL FIBROBLASTS ISOLATION, CULTURE AND REAGENTS

Skin specimens were digested using 1 mg/ml clostridial collagenase (Sigma-Aldrich, Milan, Italy) in phosphate-buffered saline (PBS). Cell suspensions were plated out in 10 ml of Dulbecco's modified Eagle's medium supplemented with L-glutamine (2 mM), FCS (10%), penicillin (200 U/ml), and streptomycin (200 μg/ml) in 100-mm culture dishes and incubated in a humidified atmosphere containing 5% CO_2. In order to avoid changes in the original phenotype, the experiments were conducted at the third passage in fibroblast cultures from both SSc patients and healthy controls. Except where indicated otherwise, all the reagents cited above were from Euroclone (Pero, Italy).

CULTURE STIMULATION

Primary human dermal fibroblasts of sclerodermic patients and controls in early passage were grown to near confluence prior to treatment. The adenosine A2A receptor agonist CGS-21680 (1 μM) was incubated with primary human dermal fibroblasts for 24 hours

(37°C in 5% CO_2) with or without the A2A receptor antagonist ZM-241385 (1 μM). The putative involvement of ERK1/2 pathway in CGS-21680-induced A2A receptor activation was evaluated by adding the selective ERK1/2 inhibitor FR-180204 (50 μM) to the cultures. Experiments evaluating the putative cross-talk between adenosine and cannabinoid systems were performed by a 24-h incubation of the cultures with ZM-241385 and/or the cannabinoid receptor agonist WIN55,212-2 (0.5, 1, 10 μM).

Finally, to assess the role of CB2 receptor in such a cross-talk, we added the specific CB2 receptor antagonist AM-630 at different concentrations (ranging from 80 μM to 0.1 nM) to the cultures co-incubated with WIN55,212-2 (1 μM) and ZM-241385 (1 μM).

CGS-21680, ZM-241385, WIN55,212-2, FR-180204, and AM-630 were purchased from Tocris (Bristol, UK), and solubilized in dimethyl sulfoxide (DMSO, Sigma-Aldrich, Milan, Italy). Accordingly, basal fibroblast cultures were run along with DMSO as control.

REVERSE TRANSCRIPTASE-PCR ANALYSIS

Total RNA from fibroblasts was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA). RNA (1 μg) was reverse transcribed to complementary DNA (cDNA) by incubation with Maloney murine leukaemia virus reverse transcriptase (200U/μl for sample), oligo(dT) primers (0.5 μg/μl for sample) and deoxyribonucleotide triphosphate (dNTPs; 10 mM; all Invitrogen, Carlsbad, CA, USA) in 20 μl total volume. cDNA was amplified in a 50-μl reaction mixture containing Euro Taq DNA polymerase (1.25 U for sample; Euroclone, Pero, Italy), $MgCl_2$ (5mM), dNTPs (0.25 mM), and 20 pmol of each sense and antisense primer for A2Ar: 5'-tgtcctggtcctcacgcagag-3' (forward) and 5'-cggatcctgtaggcgtagatgaagg-3' (reverse) and for β-actin: 5'-ggatcttcatgaggtagtcagtc-3' (forward) and 5'-cctgcctttgccgatcc -3' (reverse).

Polymerase chain reaction (PCR) was performed using a thermocycler ONE PERSONAL (Euroclone, Pero, Italy) for 35-40 cycles. PCR products were electrophoresed on a 2% agarose gel and stained with ethidium bromide and bands were visualised and photographed by ultraviolet transillumination.

WESTERN BLOT ANALYSIS

Dermal fibroblasts were washed twice in ice-cold PBS and lysed on ice with lysis solution: 1% Triton X-100, 150 mM NaCl, 20 mM Tris pH 8 and protease inhibitor cocktail (Sigma-Aldrich, Milan, Italy). The protein concentration of the samples was determined by the Bio-Rad protein assay (Bio-Rad Quick Start, Bio-Rad, Milan, Italy). Cell lysates were boiled at 100°C for 5 min in SDS gel loading buffer and later separated on a NuPage 3-8% Tris Acetate Gel (Invitrogen, Carlsbad, CA, USA). The proteins were then electrotransferred to nitrocellulose membranes (Pierce, Rockford, USA) for 1 h and 30 min at room temperature. The filters were blocked for 1 h at room temperature in 5% milk dissolved in Tris-buffered saline Tween (TBS-T), and then incubated (overnight, 4°C) with specific antibodies: anti-adenosine A2A receptor rabbit polyclonal IgG, anti-α1 type I collagen goat polyclonal IgG, anti-alpha-smooth muscle actin (α-SMA) mouse monoclonal IgG, anti-cannabinoid CB1 receptor goat polyclonal IgG, anti-cannabinoid CB2 receptor goat polyclonal IgG, anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, USA), anti-phospho-ERK-1/2, and anti-ERK-1/2 (both rabbit polyclonal IgG; Cell Signalling, Danvers, MA, USA). After 3 washes of 10 min each in TBS-T, the membranes were incubated with specific horseradish peroxidase-conjugated second antibodies for 45 min at room temperature and washed twice in TBS-T. Proteins were visualised using an ECL Western Blotting kit (Amersham Biosciences, Buckinghamshire, UK). GAPDH was determined in each filter in order to normalise slight variations in protein loading.

COLLAGEN SUPERNATANT ASSAY

After the different treatments, the supernatants were collected and stored at -20°C. An enzyme immunoassay (EIA) kit (Takara Bio Inc., Otsu, Japan) was used to evaluate the procollagen type I carboxy-terminal peptide (PIP) supernatant levels as an expression of the collagen release. PIP level was measured as nanograms per microgram of protein.

CO-IMMUNO-PRECIPITATION

Co-immuno-precipitation was used to study the putative A2A-CB1 and/or A2A-CB2 receptor physical interactions in dermal fibroblasts from SSc patients and controls.

The experiment was conducted in two consecutive phases: (1) the immuno-precipitation of the A2A receptor protein with anti-A2A antibody, and then (2) the immunoblotting of the precipitate with anti-CB1 or anti-CB2 antibody.

Dermal fibroblasts were lysed on ice for 30 min with buffer G (50 mM Tris HCl, 1% Triton X-100, 10% glycerol, 300 mM NaCl, 1.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM EGTA, and protease and phosphatase inhibitors) and centrifugated for 10 min at 14,000xg at 4°C. Protein concentration of the samples was assayed using the Bio-Rad protein assay.

In a preclearing phase, the resina was washed twice with Buffer G and centrifugated for 5 s at 10,600xg at 4°C. The pellet was incubated for 30 min with cell lysates under continuous shaking at 4°C and then it was centrifugated for 5 s at 10,600xg at 4°C.

The supernatants (lysates) obtained were incubated for 2 h at 4°C with anti-A2A receptor; then, they were added to the pellet (Protein A-AGAROSE, Roche Applied Science, Mannheim, Germany) previously washed repeatedly with Buffer G and incubated for 1 h at 4°C while being gently agitated. The supernatants (called "Void") were constituted by any unbound protein, and they were blotting with the total protein and the immunoprecipitates. The pellet (Protein A) was washed several times with buffer G, centrifugated for 5 s at

10,600xg at 4°C, resuspended in sample buffer 2x (SDS, 1 M pH 6.8 Tris, glycerol; B-mercaptoethanol), and then boiled for 5 min at 100°C. Western blots were performed with anti-CB1 and anti-CB2 receptor antibodies and Void was used as a control sample.

STATISTICAL ANALYSIS

The difference in A2Ar expression between patient and control fibroblasts was estimated by the two-tailed Mann-Whitney *U* test (A2Ar/GAPDH ratio, Western blotting) and paired "*t*" test (A2Ar/actin ratio, reverse transcriptase-PCR analysis (RT-PCR)) with the data not normally and normally distributed, respectively. Statistical evaluation of the effect of the different treatments (CGS-21680, ZM-241385, WIN55,212-2, FR-180204, and AM-630) was performed by the one-way analysis of variance for repeated measurements (RM-ANOVA) with the data normally distributed in all the cases. Then a "post hoc" test (Tukey-Kramer test for multiple comparisons) was employed to specifically compare the effects of each different treatment. In any case, *p* values less than 0.05 were considered significant (GraphPad-InStat, version 3.06 for Windows 2000, GraphPad, San Diego, CA, USA; Microsoft Corp., Redmond, WA).

2.2 TREATMENT OF MICE WITH ESTABLISHED DERMAL FIBROSIS INDUCED BY BLEOMYCIN

The putative effect of ZM-241385, WIN55,212-2 and ZM-241385 + WIN55,212-2 on estabilished dermal fibrosis was evaluated in a modified bleomycin murine model (Akhmetshina et al., 2009b). Skin fibrosis was induced in five groups of 4-week-old DBA/2J mice by local intracutaneous injections of 100 μl bleomycin dissolved in 0.9 % NaCl, at a concentration of 0.5 mg/ml, every other day in defined areas of 1 cm^2 on the upper back for 3 weeks. Afterwards, the mice were treated for 3 more weeks but with different injection regimens: one group with 0.9% NaCl, one with bleomycin, one with bleomycin and ZM-241385, one with bleomycin and WIN55,212-2, and the last with bleomycin and ZM-241385 + WIN55,212-2. Finally, a group of mice (the 6^{th}) were injected with 100 μl 0.9% NaCl for the whole period of 6 weeks as control. The injection regimens for the 6 different groups are schematically represented in Figure 8. Concerning the specific administration route of the different substances employed: ZM-241385 (Tocris, Missouri, USA) was administered intraperitoneally every day in a carrier consisting of 15% DMSO, 15 % Cremophor EL, and 70% water to a total injection volume of 0.1 ml at a concentration of 10 mg/kg/day; WIN55,212-2 (Tocris, Missouri, USA) was dissolved in phosphate-buffered saline/0.3% Tween80/dimethyl sulfoxide (DMSO) at a concentration of 0.25 mg/kg/day in a total volume of 100 μl, and administered every day by subcutaneous injections, in the same area as the bleomycin treatment; NaCl was administered subcutaneously (0.9%, 100 μl). After 6 weeks, all the animals were killed by cervical dislocation. The injected skin was removed and processed for analysis. A total of 36 mice were analyzed (n = 6 per group). The local ethical committee approved all animal experiments.

HISTOLOGICAL ANALYSIS

The injected skin areas were fixed in 4 % formalin and embedded in paraffin. Histologic sections (5 μm) were stained with hematoxylin-eosin and Masson's thricrome. Dermal thickness was determined at 100-fold magnification by measuring the distance between the dermal–epidermal junction and the dermal–subcutaneous fat junction (μm) in three randomly selected fields for each skin section, using a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, The Netherlands). The evaluation of dermal thickness was performed blindly by an experienced examiner. Results are expressed as mean ± SD.

IMMUNOHISTOCHEMISTRY FOR α-SMOOTH MUSCLE ACTIN (α-SMA)

Myofibroblasts were identified by staining for α-smooth muscle actin (α-SMA) in paraffin-embedded sections (5 μm) of lesional skin from the treated mice. After deparaffinization and blocking with 5% horse serum in PBS for 1 h at room temperature, skin sections were incubated with monoclonal anti–α-SMA antibody (Sigma-Aldrich, Steinheim, Germany) 1:1000 in PBS for 2 h at room temperature. Then the skin sections were blocked with 3% hydrogen peroxide in PBS for 10 min. As secondary antibody was used a polyclonal rabbit anti-mouse IgG labeled with horseradish peroxidase (HRP) (Dako, Hamburg, Germany) 1:200, diluted in 2% BSA/PBS for 1 h at room temperature. Positive cells were visualized by diaminobenzidine peroxidase substrate solution (Sigma-Aldrich, Steinheim, Germany). Counting was performed in a blinded manner by an experienced examiner at 400-fold magnification, in three different sections from each mouse. Results are expressed as mean ± SD of positive spindle-shaped fibroblastic cells per field.

STATISTICAL ANALYSIS

All the values presented are expressed as the mean ± SD. Statistical evaluation of the differences in dermal thickness and α-SMA expression among the different treatment groups was performed using the one-way analysis of variance (ANOVA) with the data not normally distributed in both the cases, followed by a "post-hoc" test (Dunn's Multiple Comparisons Test). In any case, a *p* value less than 0.05 was considered as significant (GraphPad-InStat, version 3.06 for Windows 2000, GraphPad, San Diego, CA, USA; Microsoft Corp., Redmond, WA).

SECTION 3.

RESULTS

3.1 RESULTS FROM IN VITRO STUDY

ADENOSINE A2A RECEPTOR EXPRESSION IS INCREASED IN SSC DERMAL FIBROBLASTS

Western blot and RT-PCR analyses showed that the A2Ar is expressed in dermal fibroblasts from healthy controls and SSc patients (Fig. 1a, b). Moreover, Western blot findings demonstrated that A2Ar expression was increased by about 3-fold in SSc fibroblasts compared to control cells (Fig. 1a).

ADENOSINE A2A RECEPTOR STIMULATION INCREASES COLLAGEN PRODUCTION BY SSC DERMAL FIBROBLASTS

Since adenosine A2A receptor was present and overexpressed in SSc fibroblasts compared with control cells, we then performed EIA and Western blot assays in SSc and normal fibroblasts to evaluate the effects of A2A agonists and antagonists on collagen production by these cells.

The selective adenosine A2Ar agonist, CGS-21680, increased PIP supernatant levels in SSc fibroblast cultures. Time-course experiments showed that the effect of CGS-21680 on PIP levels is early as the difference in PIP level in CGS-treated and untreated cells becomes detectable after 6 h. It reaches significance after 12 h, and then it remains almost stable up to 24 h (data not shown).

The effect of CGS-21680 was completely abrogated by the co-incubation with the A2Ar antagonist ZM-241385 (Fig. 2a). A similar behaviour was observed for collagen type I intracellular protein amount (Fig. 2b). Specifically, EIA assay revealed that CGS-21680 was able to augment PIP supernatant level near to 40%, whereas CGS-21680 + ZM-241385 co-incubation reduced the same parameter of about 50% (Fig. 2a).

On the contrary, EIA experiments performed in healthy control fibroblasts (*n*=4) did not show any significant effect of CGS-21680 and/or ZM-241385 on collagen supernatant levels (data not shown).

ADENOSINE A2A RECEPTOR ACTIVATION INDUCES MYOFIBROBLAST TRANS-DIFFERENTIATION AND ERK-1/2 PHOSPHORYLATION IN SSC DERMAL FIBROBLASTS

In order to provide more information on the possible intracellular mechanisms involved in the A2A-mediated collagen production by SSc fibroblasts, we evaluated the effect of adenosine A2A agonists and antagonists on myofibroblast trans-differentiation and ERK-1/2 phosphorylation in these cells.

Myofibroblasts are specialised fibroblasts critically involved in the abnormal collagen production characterising SSc. The expression of the cytoskeletal protein α-SMA represents the hallmark of these cells (Abraham et al., 2007). As expected, Western blot analysis revealed that the α-SMA expression, present in basal conditions only in SSc fibroblasts, increased after A2Ar stimulation with CGS-21680 (markedly in SSc cells, slightly in healthy fibroblasts) (Fig. 3).

Increasing evidence suggests that the mitogen-activated protein kinase (MAPK) ERK-1/2, specifically the activated phosphorylated form (p-ERK-1/2), plays a key role in the SSc fibroblast activation leading to collagen production (Chen et al., 2005; Baroni et al., 2006). Our Western blot experiments on SSc fibroblasts demonstrated the ability of the A2A agonist CGS-21680 in activating ERK-1/2 in a time-dependent manner with the maximal protein phosphorylation reached after 2-5 min (Fig. 4a). Moreover, pre-treating cells with the ERK-1/2 inhibitor FR-180204 at the concentration of 50 μM (proved to be effective in blocking CGS-21680-dependent ERK-1/2 activation; Fig. 4b) the stimulating effect of the

A2A agonist on collagen production, as evaluated with PIP supernatant levels, was completely prevented (Fig. 4b).

ADENOSINE A2A AND CANNABINOID CB1 RECEPTORS CO-IMMUNOPRECIPITATE IN SSC DERMAL FIBROBLASTS

Processing SSc fibroblasts for immuno-precipitation with an anti-A2Ar antibody and then analyzing the precipitate immunoblotted with an anti-CB1 antibody, we found a 54 kDa band corresponding to the molecular weight of the CB1 receptor (Fig. 5). The presence of the CB1 receptor in the immuno-precipitate obtained by using an anti-A2Ar antibody demonstrated that the two receptors co-immunoprecipitate, thus constituting an heteromer in SSc fibroblasts. The fact that similar results were also obtained in healthy control cells suggests that the phenomenon does not represent a peculiarity of SSc patients but, it is a general characteristic of dermal fibroblasts.

Conversely, we found that in the same cells A2Ar and CB2 receptor do not co-immuno-precipitate thus providing evidence that the A2Ar specifically interacts with the CB1 receptor only (data not shown).

CONCOMITANT ADENOSINE A2A AND CANNABINOID RECEPTORS MODULATION SYNERGISTICALLY REDUCES COLLAGEN PRODUCTION BY SSC DERMAL FIBROBLASTS

Starting from the above evidence that not only CB1/CB2 but also A2Ar are actively involved in SSc fibrogenesis, and that A2A and CB1 are physically associated in SSc fibroblasts, we explored the functional cross-talk putatively existing between these two systems in regulating collagen production by SSc cells.

Co-incubation of SSc fibroblasts with the non-selective CB1/CB2 synthetic cannabinoid agonist WIN55,212-2 and the selective A2Ar antagonist ZM-241385 exerted different effects on collagen production on the basis of the WIN55,212-2 concentration employed. In fact, on one hand WIN55,212-2 10 μM induced a marked reduction in PIP supernatant levels (-79%) which was not enhanced any further by the addition of ZM-241385 (Fig. 6a). On the other hand, despite the ineffectiveness of both WIN55,212-2 (0.5-1 μM) and ZM-241385 in modulating collagen production individually, co-incubation of 1 μM WIN55,212-2 and ZM-241385 resulted in a clear-cut inhibitory effect (-66%) similar to that observed with a high WIN55,212-2 concentration (Fig. 5b).

Co-incubation of ZM-241385 with a lower WIN55,212-2 concentration (0.5 μM) led to an apparent decrease in the collagen production from the cells, but such a change did not reach statistical significance (Fig. 6b).

CANNABINOID CB2 RECEPTOR SELECTIVE ACTIVATION IS THE MAIN MECHANISM MEDIATING THE SYNERGISTIC INHIBITORY EFFECT OF WIN55,212-2 AND ZM-241385 CO-INCUBATION ON COLLAGEN PRODUCTION BY SSC DERMAL FIBROBLASTS

The synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production is likely the final result of a net cannabinoid CB2-stimulating effect. In fact, in these conditions WIN55,212-2 can act on the cannabinoid CB2 receptor only since the CB1 receptor is indirectly blocked by ZM-241385. To address this issue, we evaluated whether the specific CB2 antagonist AM-630 was able to remove such an inhibitory effect. AM-630 was originally referred to as CB2 cannabinoid receptor antagonist but, it has now become clear that this compound causes activation of the CB2 receptor when employed at

high concentrations (particularly in the absence of agonist binding) with opposite effects on downstream signaling cascades as those of the agonists. Accordingly, AM-630 is now referred to as cannabinoid receptor antagonists/inverse agonists (Pertwee, 1999). On this basis, we performed a preliminary dose-finding study by adding AM-630 at different concentrations (ranging from 80 μM to 0.1 nM) to the cultures co-incubated with WIN55,212-2 and ZM-241385. As expected, we obtained a sigmoid curve in which the inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production was: (1) enhanced at higher AM-630 concentrations (80 – 20 μM), likely as a result of the dominant CB2 agonist-like effect of the molecule; (2) unaffected at the intermediate concentrations (10 μM – 5 nM), as the CB2 agonist-like and antagonist activities were substantially equivalent; (3) reduced at lower concentrations (1 - 0.1 nM) as a result of a dominant CB2-antagonist effect of the molecule (Fig. 7a). In particular, in cultures treated with the AM-630 concentration of 1 nM as from dose-finding experiments, collagen production was significantly increased with respect to AM-630-untreated cultures with an almost complete restoration of baseline values (Fig 7b).

3.2 RESULTS FROM IN VIVO STUDY

COMBINED ZM-241385 + WIN55,212-2 TREATMENT PREVENTS DERMAL FIBROSIS PROGRESSION AND MYOFIBROBLAST TRANS-DIFFERENTIATION IN A MURINE MODEL OF ESTABLISHED FIBROSIS INDUCED BY BLEOMYCIN

The efficacy of combined ZM-241385 + WIN55,212-2 treatment was analyzed in a murine model of established dermal fibrosis induced by bleomycin by evaluating modifications in dermal thickness and α-SMA expression.

Dermal fibrosis was established treating mice with bleomycin for 3 weeks. Starting from this condition, a following 3 weeks-period of bleomycin resulted in a significant progression of dermal fibrosis as demonstrated by the further increase in dermal thickness (Fig. 9) and α-SMA expression (Fig.10) (29% and 92%, respectively) observed. Such a fibrosis progression was not significantly affected by separately adding ZM-241385 or WIN55,212-2 in the last 3 weeks of treatment, although a slight decrease in dermal thickness (Fig. 9) as well as in myofibroblast number (Fig.10) were observed. On the contrary, fibrosis progression as determined by dermal thickness (Fig.9) and α-SMA (Fig.10) expression was completely blocked treating mice for 3 weeks with ZM-241385 + WIN55,212-2.

Notably, combined treatment produced a significant reduction in the number of α-SMA positive fibroblasts also in comparison with 3 weeks-bleomycin treated mice, thereby almost completely restoring basal conditions (Fig 10).

SECTION 4.

DISCUSSION

4. DISCUSSION

The main findings of the present study are the following: (1) the adenosine A2Ar is overexpressed in SSc fibroblasts compared with control cells and its occupancy increases collagen production, α-SMA expression and ERK-1/2 phosphorylation in SSc fibroblasts. Moreover, in SSc and control cells (2) the A2Ar forms an heteromer with the cannabinoid CB1 receptor, and (3) in SSc fibroblasts, following A2Ar blockade, the non-selective cannabinoid receptor stimulation with a per se ineffective dose of WIN55,212-2 results in a marked anti-fibrotic effect either *in vitro* and in a murine model of established fibrosis induced by bleomycin.

Such results, considered as a whole, suggest that in SSc fibroblasts A2Ar activation may exert a pro-fibrotic activity either directly, and indirectly, via a cross-talk with the cannabinoid receptor system.

Recent evidence suggests that adenosine A2Ar plays a key role in the development of dermal fibrosis. Chan and coll. (Chan et al., 2006a) demonstrated that in primary human dermal fibroblasts A2Ar stimulation promoted collagen production and, this process, in part working via the MEK-1/2/ERK pathway activation, was selectively blocked by A2A receptor antagonism. Moreover, in ADA-deficient mice, the high tissue adenosine levels deriving from the genetic defect are associated with elevated concentrations of the pro-fibrotic mediators TGF-β, CTGF, IL-6, IL-13, and PDGF in the skin as well as the development of dermal fibrosis. Both these phenomena were prevented by the pharmacological treatment with the A2A antagonist ZM-241385 (Fernández et al., 2008). Accordingly, in a murine model of scleroderma, ZM-241385 administration as well as A2Ar deletion were able to markedly attenuate bleomycin-induced dermal fibrosis as demonstrated by the reduced skin thickness, collagen content and fibrocyte accumulation (Katebi et al., 2008).

These findings are strengthened and expanded by the results of the present study which confirms that the A2A-mediated activation of the fibrotic process is also relevant in human fibroblasts deriving from patients affected with SSc. In fact, in these cells, A2Ar agonism resulted in increased collagen production, α-SMA expression and ERK-1/2 phosphorylation, thereby suggesting that A2Ar stimulation may favour fibroblast trans-differentiation towards a fibrogenic phenotype (myofibroblasts) by activating the MAPKs pathway. Moreover, the fact that A2Ar agonist/antagonists employment did not result in an evident modulation of collagen production in fibroblast cultures from healthy controls suggests that in SSc cells the adenosinergic system has a particular relevance. In accordance with such considerations, we have provided evidence for the first time that the A2Ar is overexpressed in SSc with respect to control fibroblasts, thereby possibly explaining the different agonist/antagonists response that we have found. This latter finding is intriguing and, although the possibility that it represents a primitive abnormality of SSc fibroblasts cannot be ruled out, it may be likely interpreted as an adaptative response to the chronic immuno-inflammatory activation characterising the disease. In fact, a large body of evidence indicates that adenosine, by stimulating A2Ar exerts relevant anti-inflammatory and immuno-modulating activities including inhibition of pro-inflammatory cytokine release from macrophages, decrease of adhesion molecules on neutrophils and induction of an anti-inflammatory dendritic cell phenotype driving T cell responses towards a T_H2 profile (Haskó et al., 2008). It has been demonstrated that pro-inflammatory cytokines, particularly TNF-α, are able to up-regulate the A2Ar in different cell types (endothelial cells, peripheral blood mononuclear cells, neutrophils, and lung epithelial cells) (Nguyen et al., 2003; Capecchi et al., 2005; Fortin et al., 2006; Morello et al., 2006) as an expression of a possible self-regulatory mechanism. Accordingly, patients with rheumatoid arthritis show an A2Ar up-regulation in lymphocytes and neutrophils (about 2-

3-fold) which is normalised by the treatment with anti-TNFα drugs (Varani et al., 2009). On this basis, it is conceivable that also the A2Ar overexpression observed in SSc fibroblasts may be the consequence of a compensatory mechanism primarily directed to dumping immuno-inflammatory activation. However, being the receptor also able to enhance collagen production, A2Ar up-regulation may be actually maladaptative for SSc dermal fibroblasts, thereby further promoting the imbalance of these cells towards a pro-fibrotic phenotype.

The other relevant finding arising from our research is that in SSc fibroblasts a physical and functional relationship exists between the adenosine A2A and the cannabinoid CB1 receptors, resulting in a cross-talk possibly involved in a critical manner in the regulation of collagen production by these cells. The evidence here provided that the A2A and the CB1 receptors co-immuno-precipitate in SSc fibroblasts (but also in fibroblasts from healthy controls) is in agreement with previous studies demonstrating that these receptors form an heteromer in the central nervous system (Carriba et al., 2007; Ferré et al., 2009). In the same studies, the authors found that CB1 receptor function is completely dependent on A2Ar co-activation: more particularly, A2Ar activation is a necessary condition for CB1 receptor signalling may occur. Our data seem to indicate that such a functional receptor relationship also operates in sclerodermic cells. In fact, we demonstrated how a relatively low concentration (1 μM) of the unselective cannabinoid CB1/CB2 agonist WIN55,212-2, per se ineffective in influencing collagen production from SSc fibroblasts, acquired a strong inhibitory effect after cell exposure to the A2A receptor antagonist ZM-241385 (also ineffective when used alone). It is conceivable that, in this condition, A2Ar blockage renders the CB1 receptor unresponsive to the agonistic effect of WIN55,212-2, thus leaving the molecule free to act on the CB2 receptor only, with the net result of a marked anti-fibrotic effect. Such a hypothesis was indeed confirmed by the fact that the

CB2 receptor selective antagonist AM-630 was effective in removing the synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production by SSc dermal fibroblasts.

It should be noted that in the present study we also show how the collagen production inhibiting activity of WIN55,212-2 at a concentration as high as 10 μM is not enhanced by ZM-241385, as a possible expression of a maximal effect achieved at that concentration of WIN55,212-2, with no chance for any further effect. In this view, it may be also conceivable that a WIN55,212-2 concentration as low as 1 μM could be unable to produce significant receptor stimulation when the agonist is employed alone. However, our results suggest an alternative interpretation of the phenomenon. In fact, the lack of a detectable anti-fibrotic effect of 1 μM WIN55,212-2 would not be related to an insufficient CB1/CB2 receptor occupancy, but to a balanced stimulation of two receptors (CB1 and CB2) exerting opposite, mutually neutralising effects on collagen production. In this context, the indirect CB1 blockade by the A2Ar antagonist ZM-241385 could allow the appearance of a relevant, previously masked anti-fibrotic effect of the molecule. Although WIN55,212-2 is a non-selective CB1/CB2 agonist, it has a slightly greater affinity for the CB2 rather than the CB1 receptor (Howlett et al., 2002). Thus, it is possible that a CB2 stimulation-mediated effect may arise in a significant manner for higher concentrations of WIN55,212-2 whereas it is negligible at a concentration of the agonist as low as 1 μM. If this is the case, the clear-cut anti-fibrotic effect of WIN55,212-2 at a concentration as high as 10 μM would have a clear explanation.

This view is further suggested by the results of the *in vivo* study, specifically designed to confirm in animals the existence of a cross-talk between A2A and cannabinoid receptors able to significantly modulate fibrogenesis.

In fact, on the basis of a previous study on mice demonstrating as a WIN55,212-2 dose of 1 mg/kg/day (Balistreri et al., 2011) was able to completely prevent bleomycin-induced dermal fibrosis, we chose to treat mice with a 4-fold lower dose of WIN55,212-2 to render detectable a possible synergistic effect with ZM-241385 in the combined treatment.

In accordance with our *in vitro* findings, while WIN55,212-2 and ZM-241385 alone did not produce any significant effect in our murine model, the combination of the two molecules significantly blocked the progression of the established dermal fibrosis.

On the contrary, the combined treatment did not show any activity in promoting regression of established fibrosis in terms of dermal thickness, even though in these mice the extent of myofibroblast transdifferentiation was markedly reduced, until reaching near-control levels. These data indicated that also *in vivo* the WIN55,212-2 + ZM-241385 association exerted a potent inhibitory effect on fibroblast activation.

However, our data also suggest that such an activity may lead to a clinically significant anti-fibrotic effect only when the treatment is employed before that collagen deposition takes place, i.e. translating these results to the human disease, in the early phases of systemic sclerosis.

In conclusion, our data suggest that adenosine A2Ar occupancy is able to induce a pro-fibrotic phenotype in sclerodermic dermal fibroblasts, either directly, and indirectly, by permitting CB1 cannabinoid receptor activation and conversely, that pharmacologic inhibition of A2Ar reduces fibrogenesis both directly and indirectly, blocking the pro-fibrotic effect of CB1r. These findings increase our knowledge on the pathophysiology of sclerodermic fibrosis also further substantiating the hypothesis that drugs specifically blocking the A2Ar may be useful in the treatment of SSc. Moreover, the evidence of a synergistic anti-fibrotic effect of the concomitant modulation of the adenosinergic and the cannabinoid systems in sclerodermic dermal fibroblasts and in the murine model of

fibrosis, suggests that a multi-target therapeutic approach to SSc may be of particular efficacy and possibly loaded with less side effects in the view of a dose-sparing effect.

SECTION 5.

BIBLIOGRAPHY

Abraham DJ, Eckes B, Rajkumar V, Krieg T (2007) New developments in fibroblast and myofibroblast biology: implications for fibrosis and scleroderma. *Curr Rheumatol Rep* 9:136–143.

Agnati LF, Fuxe K, Woods AS, Genedani S, Guidolin D (2009) Theoretical considerations on the topological organization of receptor mosaics. *Curr Protein Pept Sci* 10:559-569.

Akhmetshina A, Dees C, Busch N, Beer J, Sarter K, Zwerina J, Zimmer A, Distler O, Schett G, Distler JH (2009a) The cannabinoid receptor CB2 exerts antifibrotic effects in experimental dermal fibrosis. *Arthritis Rheum* 60:1129-1136.

Akhmetshina A, Venalis P, Dees C, Busch N, Zwerina J, Schett G, Distler O, Distler JH (2009b) Treatment with imatinib prevents fibrosis in different preclinical models of systemic sclerosis and induces regression of established fibrosis. *Arthritis Rheum* 60:219-224.

Antonioli L, Fornai M, Colucci R, Ghisu N, Tuccori M, Del Tacca M, Blandizzi C (2008) Pharmacological modulation of adenosine system: novel options for treatment of inflammatory bowel diseases. *Inflamm Bowel Dis* 14:566-574.

Balistreri E, Garcia-Gonzalez E, Selvi E, Akhmetshina A, Palumbo K, Lorenzini S, Maggio R, Lucattelli M, Galeazzi M, Distler JH (2011) The cannabinoid WIN55,212-2 abrogates dermal fibrosis in scleroderma bleomycin model. *Ann Rheum Dis* 70:695-699.

Baroni SS, Santillo M, Bevilacqua F, Luchetti M, Spadoni T, Mancini M, Fraticelli P, Sambo P, Funaro A, Kazlauskas A, Avvedimento EV, Gabrielli A (2006) Stimulatory autoantibodies to the PDGF receptor in systemic sclerosis. *N Engl J Med* 354:2667–2676.

Blackburn MR (2003) Too much of a good thing: adenosine overload in adenosine-deaminase–deficient mice. *Trends Pharmacol Sci* 24:66-70

Bosello S, De Luca G, Tolusso B, Lama G, Angelucci C, Sica G, Ferraccioli G (2011) B cells in systemic sclerosis: a possible target for therapy. *Autoimmun Rev* 10:624-630.

Bouvier M (2001) Oligomerization of G-protein-coupled transmitter receptors. *Nat Rev Neurosci* 2: 274-286.

Burnstock G (2007) Purine and pyrimidine receptors. *Cell Mol Life Sci* 64:1471-1483.

Capecchi PL, Camurri A, Pompella G, Mazzola A, Maccherini M, Diciolla F, Lazzerini PE, Abbracchio MP, Laghi-Pasini F (2005) Upregulation of A2A adenosine receptor expression by TNF-alpha in PBMC of patients with CHF: a regulatory mechanism of inflammation. *J Card Fail* 11:67-73.

Caraceni P, Domenicali M, Giannone F, Bernardi M (2009) The role of the endocannabinoid system in liver diseases. *Best Pract Res Clin Endocrinol Metab* 23:65-77.

Carriba P, Ortiz O, Patkar K, Justinova Z, Stroik J, Themann A, Müller C, Woods AS, Hope BT, Ciruela F, Casadó V, Canela EI, Lluis C, Goldberg SR, Moratalla R, Franco R, Ferré S (2007) Striatal adenosine A2A and cannabinoid CB1 receptors form functional heteromeric complexes that mediate the motor effects of cannabinoids. *Neuropsychopharmacology* 32:2249-2259.

Chan ES, Fernandez P, Merchant AA, Montesinos MC, Trzaska S, Desai A, Tung CF, Khoa DN, Pillinger MH, Reiss AB, Tomic-Canic M, Chen JF, Schwarzschild MA, Cronstein BN (2006a) Adenosine A2A receptors in diffuse dermal fibrosis: pathogenic role in human dermal fibroblasts and in a murine model of scleroderma. *Arthritis Rheum* 54:2632-2642.

Chan ES, Montesinos MC, Fernandez P, Desai A, Delano DL, Yee H, Reiss AB, Pillinger MH, Chen JF, Schwarzschild MA, Friedman SL, Cronstein BN (2006b) Adenosine A(2A) receptors play a role in the pathogenesis of hepatic cirrhosis. *Br J Pharmacol* 148:1144-1155.

Chan ES and Cronstein BN (2010) Adenosine in fibrosis. *Mod Rheumatol* 20: 114-122.

Che J, Chan ES, Cronstein BN (2007) Adenosine A2A receptor occupancy stimulates collagen expression by hepatic stellate cells via pathways involving protein kinase A, Src, and extracellular signal-regulated kinases 1/2 signaling cascade or p38 mitogen-activated protein kinase signaling pathway. *Mol Pharmacol* 72:1626-1636.

Chen Y, Shi-Wen X, van Beek J, Kennedy L, McLeod M, Renzoni EA, Bou-Gharios G, Wilcox-Adelman S, Goetinck PF, Eastwood M, Black CM, Abraham DJ, Leask A. (2005) Matrix contraction by dermal fibroblasts requires transforming growth factor-beta/activin-linked kinase 5, heparan sulfate-containing proteoglycans, and MEK/ERK: insights into pathological scarring in chronic fibrotic disease. *Am J Pathol* 167:1699–1711.

Chizzolini C, Brembilla NC, Montanari E, Truchetet ME (2011) Fibrosis and immune dysregulation in systemic sclerosis. *Autoimmun Rev* 10:276-281.

Chunn JL, Molina JG, Mi T, Xia Y, Kellems RE, Blackburn MR (2005) Adenosine-dependent pulmonary fibrosis in adenosine deaminase-deficient mice. *J Immunol* 175:1937-1946.

Chunn JL, Mohsenin A, Young HW, Lee CG, Elias JA, Kellems RE, Blackburn MR (2006) Partially adenosine deaminase-deficient mice develop pulmonary fibrosis in association with adenosine elevations. *Am J Physiol Lung Cell Mol Physiol* 290:L579-587.

Cronstein BN (1994) Adenosine, an endogenous anti-inflammatory agent. *J Appl Physiol* 76:5-13.

Cronstein BN (2011) Adenosine receptors and fibrosis: a translational review *F1000 Biol Rep* 3:21.

Desmoulière A, Geinoz A, Gabbiani F, Gabbiani G (1993) Transforming growth factor-beta 1 induces alpha-smooth muscle actin expression in granulation tissue myofibroblasts and in quiescent and growing cultured fibroblasts. *J Cell Biol* 122: 103-111.

Distler JH, Jüngel A, Pileckyte M, Zwerina J, Michel BA, Gay RE, Kowal-Bielecka O, Matucci-Cerinic M, Schett G, Marti HH, Gay S, Distler O (2007) Hypoxia-induced increase in the production of extracellular matrix proteins in systemic sclerosis. *Arthritis Rheum* 56:4203-4215.

Distler O (2009) Scleroderma – modern aspects of pathogenesis, diagnosis and therapy. UNI-MED SCIENCE

Fernández P, Trzaska S, Wilder T, Chiriboga L, Blackburn MR, Cronstein BN, Chan ES (2008) Pharmacological blockade of A2A receptors prevents dermal fibrosis in a model of elevated tissue adenosine. *Am J Pathol* 172:1675-1682.

Ferré S, Goldberg SR, Lluis C, Franco R (2009) Looking for the role of cannabinoid receptor heteromers in striatal function. *Neuropharmacology* 56:226-234.

Fortin A, Harbour D, Fernandes M, Borgeat P, Bourgoin S (2006) Differential expression of adenosine receptors in human neutrophils: up-regulation by specific Th1 cytokines and lipopolysaccharide. *J Leukoc Biol* 79:574-585.

Fredholm BB, Arslan G, Halldner L, Kull B, Schulte G, Wasserman W (2000) Structure and function of adenosine receptors and their genes. *Naunyn Schmiedebergs Arch Pharmacol* 362:364-374.

Fuschiotti P (2011) Role of IL-13 in systemic sclerosis. *Cytokine* 56:544-549.

Fuxe K, Marcellino D, Guidolin D, Woods AS, Agnati LF (2008) Heterodimers and receptor mosaics of different types of G-protein-coupled receptors. *Physiology* 23:322-332.

Gabrielli A, Avvedimento EV, Krieg T (2009) Scleroderma. *N Engl J Med* 360:1989-2003.

Galuppo M, Esposito E, Mazzon E, Di Paola R, Paterniti I, Impellizzeri D, Cuzzocrea S (2011) MEK inhibition suppresses the development of lung fibrosis in the bleomycin model. *Naunyn Schmiedebergs Arch Pharmacol* 384:21-37.

Garcia-Gonzalez E, Selvi E, Balistreri E, Lorenzini S, Maggio R, Natale MR, Capecchi PL, Lazzerini PE, Bardelli M, Laghi-Pasini F, Galeazzi M (2009) Cannabinoids inhibit fibrogenesis in diffuse systemic sclerosis fibroblasts. *Rheumatology* 48:1050-1056.

Gilliam AC (2008) Scleroderma. *Derm Imm* 10: 258-279.

Gomes CV, Kaster MP, Tomé AR, Agostinho PM, Cunha RA (2011) Adenosine receptors and brain diseases: neuroprotection and neurodegeneration. *Biochim Biophys Acta* 1808:1380-1399.

Graham ES, Ashton JC, Glass M (2009) Cannabinoid receptors: a brief history and "what's hot" *Front Biosci* 14:944-957.

Haskó G and Cronstein BN (2004) Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* 25: 33-39.

Haskó G, Linden J, Cronstein BN, Pacher P (2008) Adenosine receptors: therapeutic aspects for inflammatory and immune diseases. *Nat Rev Drug Discov* 7:759-770.

Howlett AC, Barth F, Bonner TI, Cabral G, Casellas P, Devane WA, Felder CC, Herkenham M, Mackie K, Martin BR, Mechoulam R, Pertwee RG (2002) International Union of Pharmacology. XXVII. Classification of cannabinoid receptors. *Pharmacol Rev* 54:161-202.

Jelaska A and Korn JH (2000) Role of apoptosis and transforming growth factor beta 1 in fibroblast selection and activation in systemic sclerosis. *Arthritis Rheum* 43:2230-2239.

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

Jinnin M (2010) Mechanisms of skin fibrosis in systemic sclerosis. *J Dermatol* 37:11-25.

Julien B, Grenard P, Teixeira-Clerc F, Van Nhieu JT, Li L, Karsak M, Zimmer A, Mallat A, Lotersztajn S (2005) Antifibrogenic role of the cannabinoid receptor CB2 in the liver. *Gastroenterology* 128:742-755.

Kähäri VM, Multimäki P, Vuorio E (1987) Elevated pro alpha 2(I) collagen mRNA levels in cultured scleroderma fibroblasts result from an increased transcription rate of the corresponding gene. FEBS Lett 215:331-334.

Katebi M, Fernandez P, Chan ES, Cronstein BN (2008) Adenosine A2A receptor blockade or deletion diminishes fibrocyte accumulation in the skin in a murine model of scleroderma, bleomycin-induced fibrosis. *Inflammation* 31:299-303.

Kawakami T, Ihn H, Xu W, Smith E, LeRoy C, Trojanowska M (1998) Increased expression of TGF-beta receptors by scleroderma fibroblasts: evidence for contribution of autocrine TGF-beta signaling to scleroderma phenotype. *J Invest Dermatol* 110: 47-51.

Keane MP, Belperio JA, Burdick MD, Strieter RM (2001) IL-12 attenuates bleomycin-induced pulmonary fibrosis. *Am J Physiol Lung Cell Mol Physiol* 281:L92-97.

Kirk TZ, Mark ME, Chua CC, Chua BH, Mayes MD (1995) Myofibroblasts from scleroderma skin synthesize elevated levels of collagen and tissue inhibitor of metalloproteinase (TIMP-1) with two forms of TIMP-1. *J Biol Chem* 270:3423-3428.

Kubo M, Ihn H, Yamane K, Tamaki K (2002) Upregulated expression of transforming growth factor-beta receptors in dermal fibroblasts of skin sections from patients with systemic sclerosis. *J Rheumatol* 29:2558-2564.

Kumar V and Sharma A (2009) Adenosine: an endogenous modulator of innate immune system with therapeutic potential. *Eur J Pharmacol* 616:7-15.

Kwong FY, Davies A, Tse CM, Young JD, Henderson PJ, Baldwin SA (1988) Purification of the human erythrocyte nucleoside transporter by immunoaffinity chromatography. *Biochem J* 255:243-249.

LeRoy EC (1972) Connective tissue synthesis by scleroderma skin fibroblasts in cell culture. *J Exp Med* 135:1351-1362.

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

Marquart S, Zerr P, Akhmetshina A, Palumbo K, Reich N, Tomcik M, Horn A, Dees C, Engel M, Zwerina J, Distler O, Schett G, Distler JH (2010) Inactivation of the cannabinoid receptor CB1 prevents leukocyte infiltration and experimental fibrosis. *Arthritis Rheum* 62:3467–3476.

Michalski CW, Maier M, Erkan M, Sauliunaite D, Bergmann F, Pacher P, Batkai S, Giese NA, Giese T, Friess H, Kleeff J (2008) Cannabinoids reduce markers of inflammation and fibrosis in pancreatic stellate cells. *PLoS One* 3:e1701.

Montesinos MC, Gadangi P, Longaker M, Sung J, Levine J, Nilsen D, Reibman J, Li M, Jiang CK, Hirschhorn R, Recht PA, Ostad E, Levin RI, Cronstein BN (1997) Wound healing is accelerated by agonists of adenosine A2 (G alpha s –linked) receptors. *J Exp Med* 186:1615-1620.

Morello S, Ito K, Yamamura S, Lee KY, Jazrawi E, Desouza P, Barnes P, Cicala C, Adcock IM (2006) IL-1 beta and TNF-alpha regulation of the adenosine receptor (A2A) expression: differential requirement for NF-kappa B binding to the proximal promoter. *J Immunol* 177:7173-7183.

Nakav S, Kachko L, Vorobiov M, Rogachev B, Chaimovitz C, Zlotnik M, Douvdevani A (2009) Blocking adenosine A2A receptor reduces peritoneal fibrosis in two independent experimental models. *Nephrol Dial Transplant* 24:2392-2399.

Nguyen DK, Montesinos MC, Williams AJ, Kelly M, Cronstein BN (2003) Th1 cytokines regulate adenosine receptors and their downstream signaling elements in human microvascular endothelial cells. *J Immunol* 171:3991-3998.

Núñez E, Benito C, Pazos MR, Barbachano A, Fajardo O, González S, Tolón RM, Romero J (2004) Cannabinoid CB2 receptors are expressed by perivascular microglial cells in the human brain: an immunohistochemical study. *Synapse* 53:208-213.

Ohta A and Sitkovsky M (2001) Role of G-protein-coupled adenosine receptors in downregulation of inflammation and protection from tissue damage. *Nature* 414:916-920.

Parfieniuk A and Flisiak R (2008) Role of cannabinoids in chronic liver diseases. *World J Gastroenterol* 14:6109-6114.

Pertwee RG *(*1999) Pharmacology of cannabinoid receptor ligands. *Curr Med Chem* 6:635–664.

Postlethwaite AE, Holness MA, Katai H, Raghow R (1992) Human fibroblasts synthesize elevated levels of extracellular matrix proteins in response to interleukin 4. *J Clin Invest* 90:1479-1485.

Roberts AB, Sporn MB, Assoian RK, Smith JM, Roche NS, Wakefield LM, Heine UI, Liotta LA, Falanga V, Kehrl JH, Fauci AS (1986) Transforming growth factor type beta: rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. *Proc Natl Acad Sci U S A* 83:4167-4171.

Sappino AP, Masouyé I, Saurat JH, Gabbiani G (1990) Smooth muscle differentiation in scleroderma fibroblastic cells. *Am J Pathol* 137:585-591.

Scheibner KA, Boodoo S, Collins S, Black KE, Chan-Li Y, Zarek P, Powell JD, Horton MR (2009) The adenosine A2A receptor inhibits matrix-induced inflammation in a novel fashion. *Am J Respir Cell Mol Biol* 40:251-259.

Schulte G and Fredholm BB (2003) Signalling from adenosine receptors to mitogen-activated protein kinases. *Cell Signal* 15:813-827.

Servettaz A, Kavian N, Nicco C, Deveaux V, Chéreau C, Wang A, Zimmer A, Lotersztajn S, Weill B, Batteux F (2010) Targeting the cannabinoid pathway limits the development of fibrosis and autoimmunity in a mouse model of systemic sclerosis. *Am J Pathol* 177:187-196.

Sime PJ and O'Reilly KM (2001) Fibrosis of the lung and other tissues: new concepts in pathogenesis and treatment. *Clin Immunol* 99:308-319.

Stiles GL (1992) Adenosine receptors. *J Biol Chem* 267:6451-6454.

Subcommittee for sclerodermia criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980) Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 23:581–590.

Tebano MT, Martire A, Chiodi V, Pepponi R, Ferrante A, Domenici MR, Frank C, Chen JF, Ledent C, Popoli P (2009) Adenosine A2A receptors enable the synaptic effects of cannabinoid CB1 receptors in the rodent striatum. *J Neurochem* 110:1921-1930.

Uitto J, Bauer EA, Eisen AZ (1979) Scleroderma: increased biosynthesis of triple-helical type I and type III procollagens associated with unaltered expression of collagenase by skin fibroblasts in culture. *J Clin Invest* 64:921-930.

Varani K, Massara A, Vincenzi F, Tosi A, Padovan M, Trotta F, Borea PA (2009) Normalization of A2A and A3 adenosine receptor up-regulation in rheumatoid arthritis patients by treatment with anti-tumor necrosis factor alpha but not methotrexate. *Arthritis Rheum* 60: 2880-2891.

Varga J and Abraham D (2007) Systemic sclerosis: a prototypic multisystem fibrotic disorder. *J Clin Invest* 117:557-567.

Varga JA and Trojanowska M (2008) Fibrosis in systemic sclerosis. *Rheum Dis Clin North Am* 34:115-143.

Verrecchia F and Mauviel A (2007) Transforming growth factor-beta and fibrosis. *World J Gastroenterol* 13:3056-3062.

Victor-Vega C, Desai A, Montesinos MC, Cronstein B (2002) Adenosine A2A receptor agonists promote more rapid wound healing than recombinant human platelet-derived growth factor (Becaplermin gel). *Inflammation* 26:19-24.

Wyatt AW, Steinert JR, Wheeler-Jones CP, Morgan AJ, Sugden D, Pearson JD, Sobrevia L, Mann GE (2002) Early activation of the p42/p44MAPK pathway mediates adenosine-induced nitric oxide production in human endothelial cells: a novel calcium-insensitive mechanism. *FASEB J* 16:1584-1594.

Yamamoto T, Takagawa S, Katayama I, Yamazaki K, Hamazaki Y, Shinkai H, Nishioka K (1999) Animal model of sclerotic skin. I: Local injections of bleomycin induce sclerotic skin mimicking scleroderma. *J Invest Dermatol* 112:456-462.

Yamamoto T, Eckes B, Krieg T (2000) Bleomycin increases steady-state levels of type I collagen, fibronectin and decorin mRNAs in human skin fibroblasts. *Arch Dermatol Res* 292:556-561.

Yamamoto T and Nishioka K (2002) Animal model of sclerotic skin. V: Increased expression of alpha-smooth muscle actin in fibroblastic cells in bleomycin-induced scleroderma. *Clin Immunol* 102:77-83.

Yamamoto T and Nishioka K (2005) Cellular and molecular mechanisms of bleomycin-induced murine scleroderma: current update and future perspective. *Exp Dermatol* 14:81-95.

Yamamoto T (2006) Bleomycin and the skin *Br J Dermatol* 155:869-875.

Yamane K, Ihn H, Kubo M, Tamaki K (2002) Increased transcriptional activities of transforming growth factor beta receptors in scleroderma fibroblasts. *Arthritis Rheum* 46:2421-2428.

SECTION 6.

IMAGES AND TABLES

	SSc patients (*n*=5)	Healthy controls (*n*=4)
Sex (F/M, *n*)	**4/1**	**2/2**
Age (median (range), years)	**49.0 (32-69)**	**47.5 (31-65)**
Disease duration[a] (median (range), years)	**5 (4-15)**	**-**
Disease subset (limited/diffuse, *n*)	**0/5**	**-**
Anti-Scl-70 (positive/negative, *n*)	**5/0**	**-**
Treatment (*n*)		
Prostacyclin analogues	**5**	**-**
Endothelin-1 receptor antagonists	**3**	**-**
Calcium-channel blockers	**3**	**-**
Steroids	**2**	**-**

Table 1 Demography and clinical characteristics of SSc patients and healthy controls

SSc was determined according to the Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee (1980).

[a]From the first non-Raynaud's manifestation

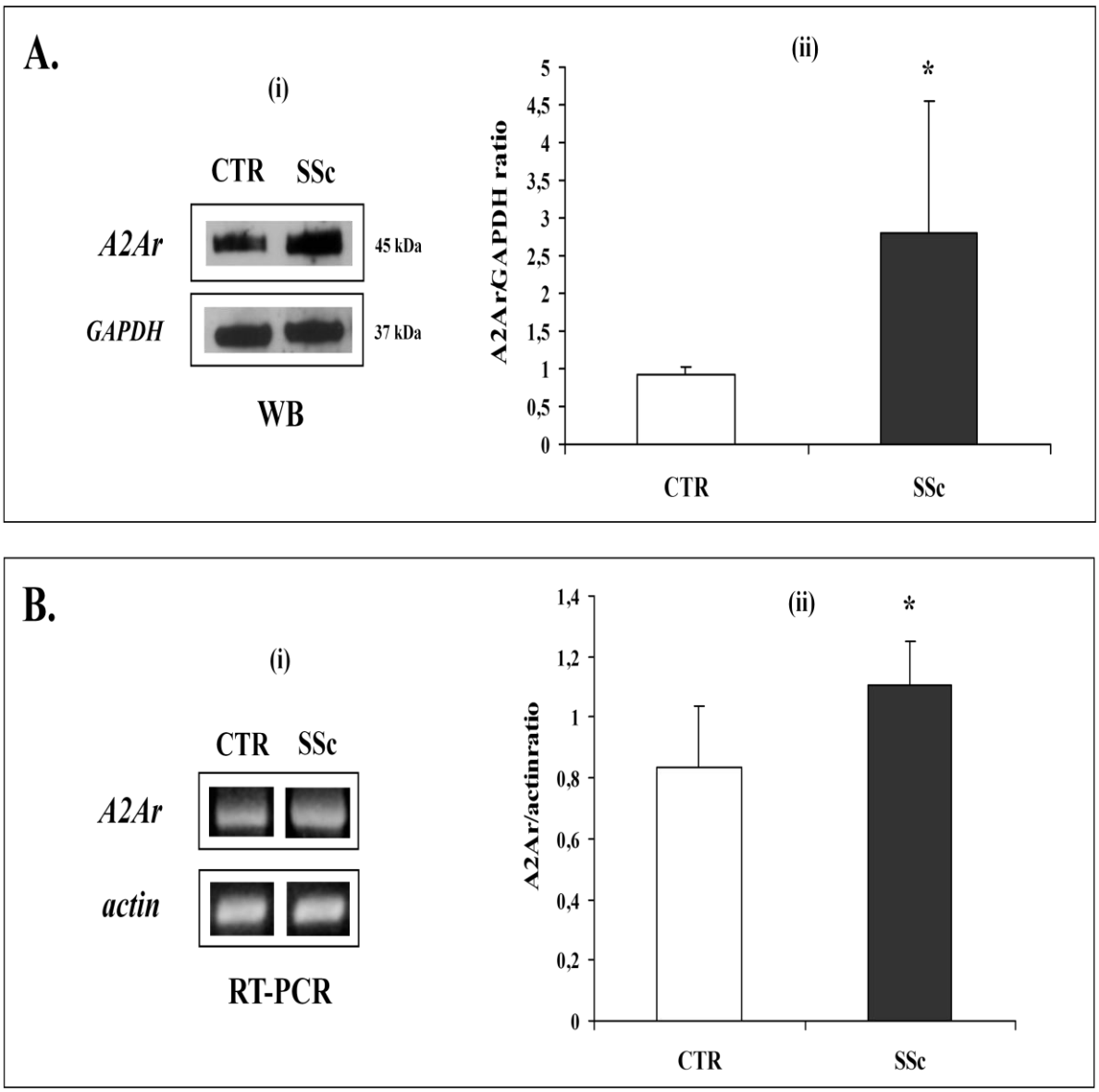

Fig. 1 Adenosine A2A receptor expression is increased in SSc dermal fibroblasts.

a. A2Ar protein expression in SSc vs. healthy dermal fibroblasts, as determined by WB analysis: (*i*) illustrative experiment and (*ii*) comparison between mean densitometric values. SSc patients, *n*=5; healthy controls, *n*=4. **p*=0.01, two-tailed Mann-Whitney *U* test.

b. Expression of messenger RNA for A2Ar in SSc vs. healthy dermal fibroblasts, as determined by RT-PCR analysis: (*i*) illustrative experiment and (*ii*) comparison between mean densitometric values. SSc patients, *n*=3; healthy controls, *n*=3. **p*=0.01, two-tailed paired "*t*" test.

SSc systemic sclerosis, *CTR* control, *A2Ar* adenosine A2A receptor, *RT-PCR* reverse transcription-polymerase chain reaction, *WB* Western blotting

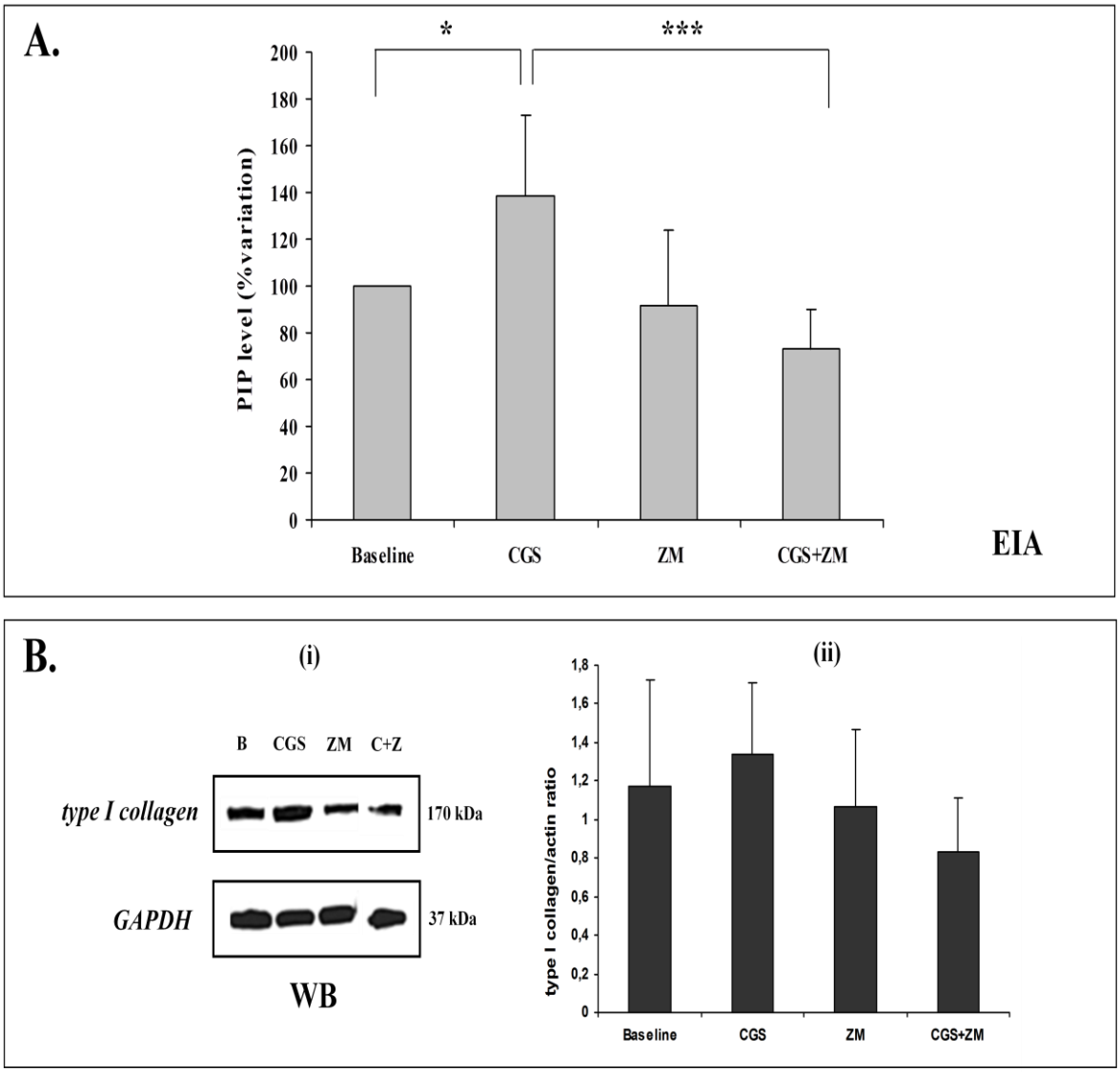

Fig. 2 Adenosine A2A receptor stimulation increases collagen production by SSc dermal fibroblasts.

a. Effect of A2Ar agonist/antagonists (CGS-21680 1 μM/ZM-241385 1 μM) on PIP supernatant levels in SSc dermal fibroblasts, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline=100%). SSc patients, *n*=5; RM-ANOVA: p=0.001; **p*<0.05, ****p*<0.001, post-hoc Tukey-Kramer multiple comparison test.

b. Effect of A2Ar agonist/antagonists on collagen type I intracellular protein amount in SSc dermal fibroblasts, as determined by WB analysis: (*i*) illustrative experiment and (*ii*) comparison between mean densitometric values. SSc patients, *n*=2.

SSc systemic sclerosis, *A2Ar* adenosine A2A receptor, *PIP* procollagen type I carboxy-terminal peptide, *WB* Western blotting

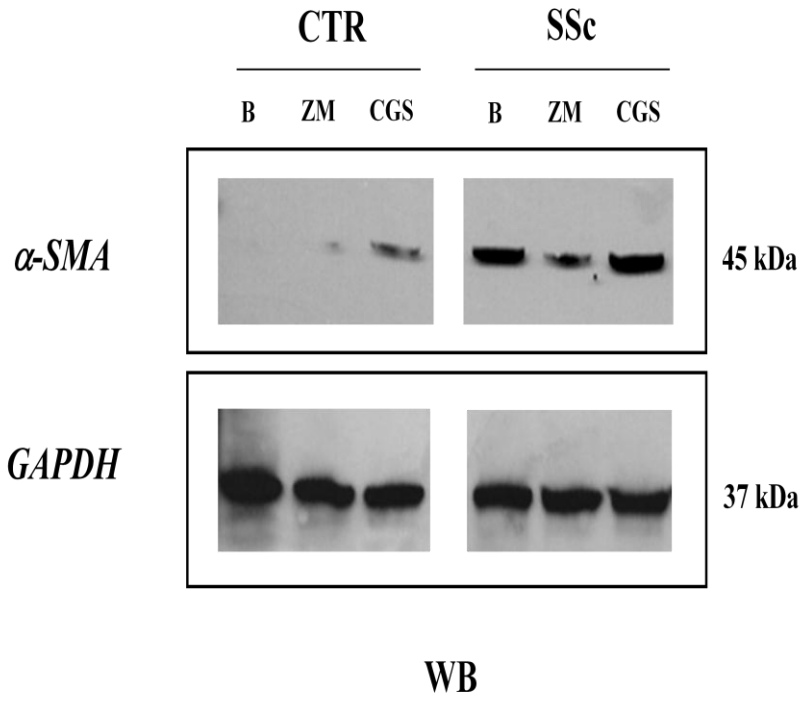

Fig. 3 Adenosine A2A receptor activation induces myofibroblast trans-differentiation in SSc dermal fibroblasts.

Effect of A2Ar agonist/antagonists (CGS-21680 1 μM/ZM-241385 1 μM) on α-SMA expression in SSc and healthy dermal fibroblasts, as determined by WB analysis.

SSc systemic sclerosis, *CTR* control, *A2Ar* adenosine A2A receptor, *α-SMA* α-smooth muscle actin, *WB* Western blotting

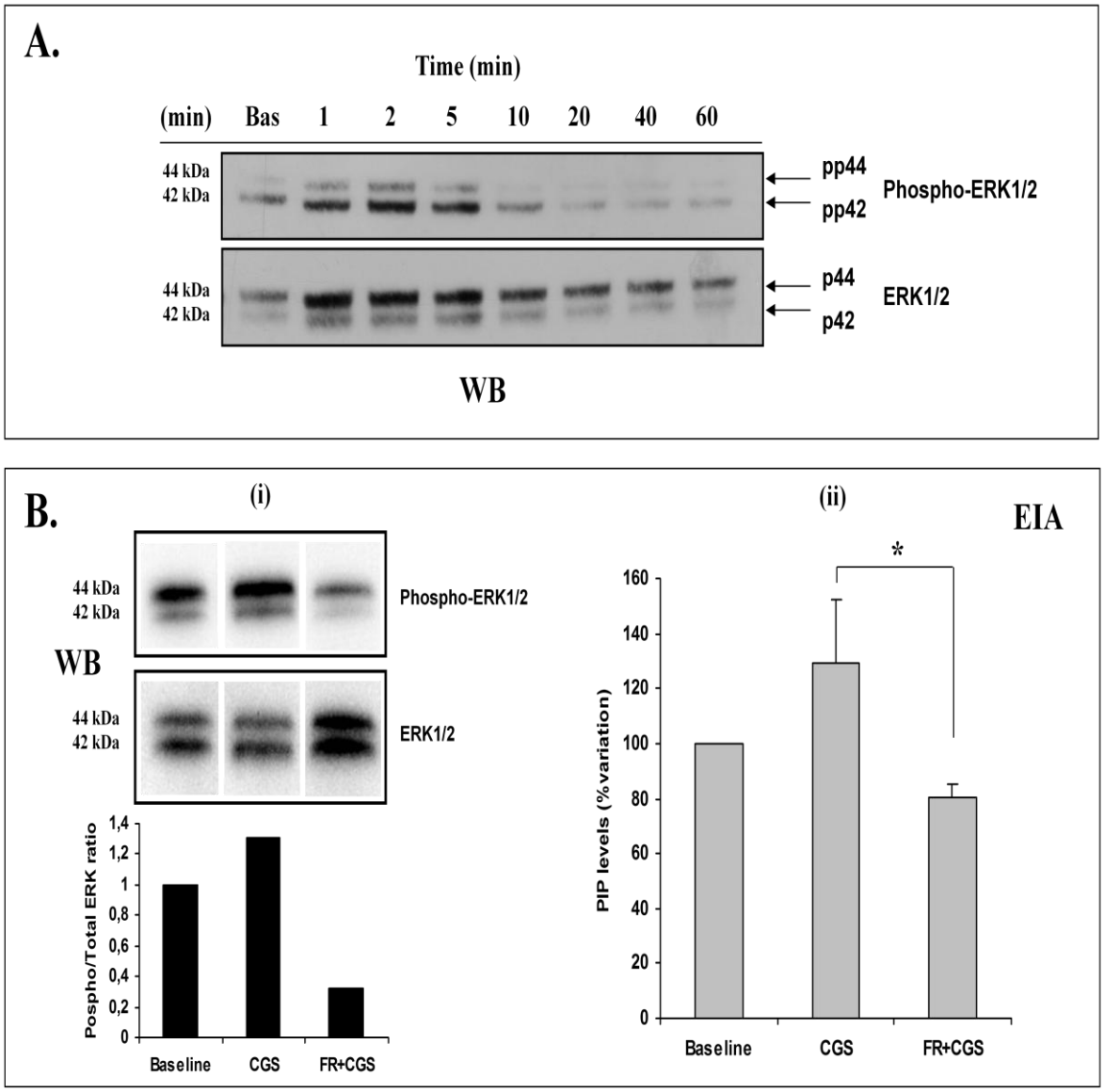

Fig. 4 Adenosine A2A receptor activation induces ERK-1/2 phosphorylation in SSc dermal fibroblasts.

a. Time course of A2Ar agonist-induced ERK-1/2 phosphorylation in SSc dermal fibroblasts, as determined by WB analysis. Serum-starved fibroblasts with 1 μM CGS-21680 for the indicated periods of time.

b. Effect of ERK-1/2 inhibitor FR-180204 (50 μM) on: (*i*) CGS-21680-induced ERK-1/2 phosphorylation, as determined by WB analysis and (*ii*) CGS-21680-induced PIP supernatant levels, as determined by EIA assay, in SSc dermal fibroblasts. Serum-starved fibroblasts with 1 μM CGS-21680 for 5 min. SSc patients, n=3; RM-ANOVA: p=0.03; *p<0.05, post-hoc Tukey-Kramer multiple comparison test.

SSc systemic sclerosis, *CTR* control, *A2Ar* adenosine *A2A* receptor, *PIP* procollagen type I carboxy-terminal peptide, *WB* Western blotting

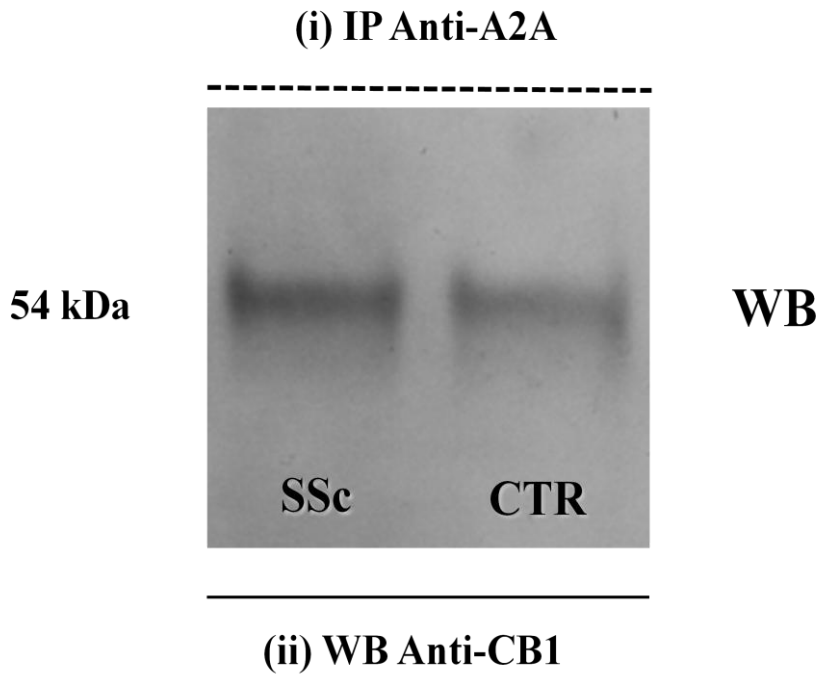

Fig. 5 Adenosine A2A and cannabinoid CB1 receptors co-immuno-precipitate in SSc and healthy dermal fibroblasts .

(*i*) Dermal fibroblasts were processed for immuno-precipitation with the anti-A2A receptor antibody; (*ii*) immunoprecipitates were analysed by SDS-PAGE and immunoblotted with the anti-CB1 antibody. The band of 54 kDa indicates the position corresponding to the CB1 receptor. The experiment has been repeated three times.

SSc systemic sclerosis, *CTR* control, *A2Ar* adenosine A2A receptor, *IP* immuno-precipitation, *CB1* cannabinoid CB1 receptor, *WB* Western blotting

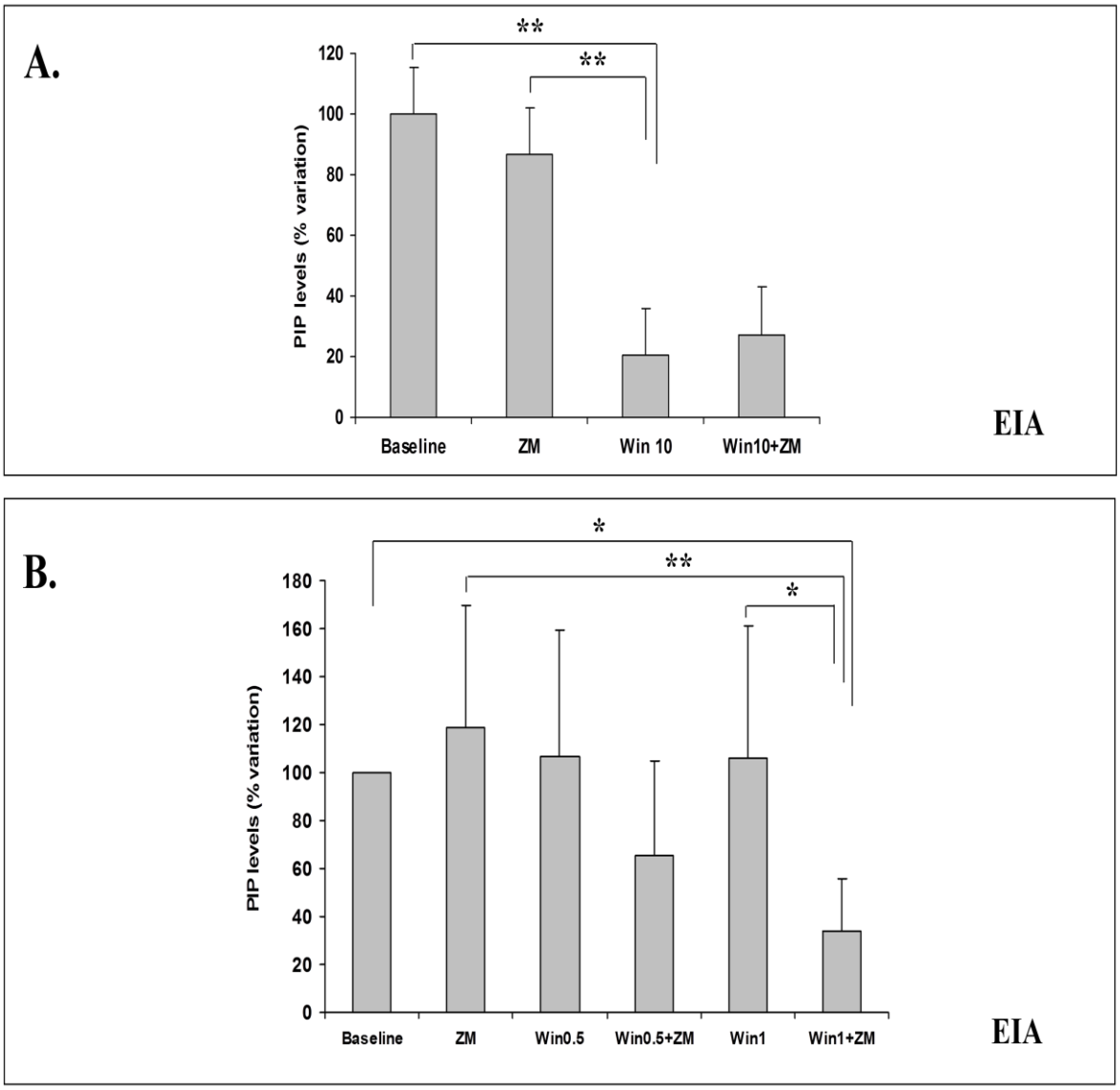

Fig. 6 Concomitant adenosine A2A and cannabinoid receptor modulation synergistically reduces collagen production by SSc dermal fibroblasts.

a. Effect of non-selective CB1/CB2 cannabinoid agonist WIN55,212-2 (10 μM) and/or A2Ar antagonist ZM-241385 (1 μM) on PIP supernatant levels in SSc dermal fibroblasts, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline=100%). SSc patients, n=5; RM-ANOVA: p<0.001; **p<0.01, post-hoc Tukey-Kramer multiple comparison test.

b. Effect of non-selective CB1/CB2 cannabinoid agonist WIN55,212-2 (0.5-1 μM) and/or A2Ar antagonist ZM-241385 (1 μM) on PIP supernatant levels in SSc dermal fibroblasts, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline=100%). SSc patients, n=5; RM-ANOVA: p=0.003; *p<0.05, **p<0.01, post-hoc Tukey-Kramer multiple comparison test.

SSc systemic sclerosis, *A2Ar* adenosine A2A receptor, *EIA* enzyme immunoassay, *PIP* procollagen type I carboxy-terminal peptide

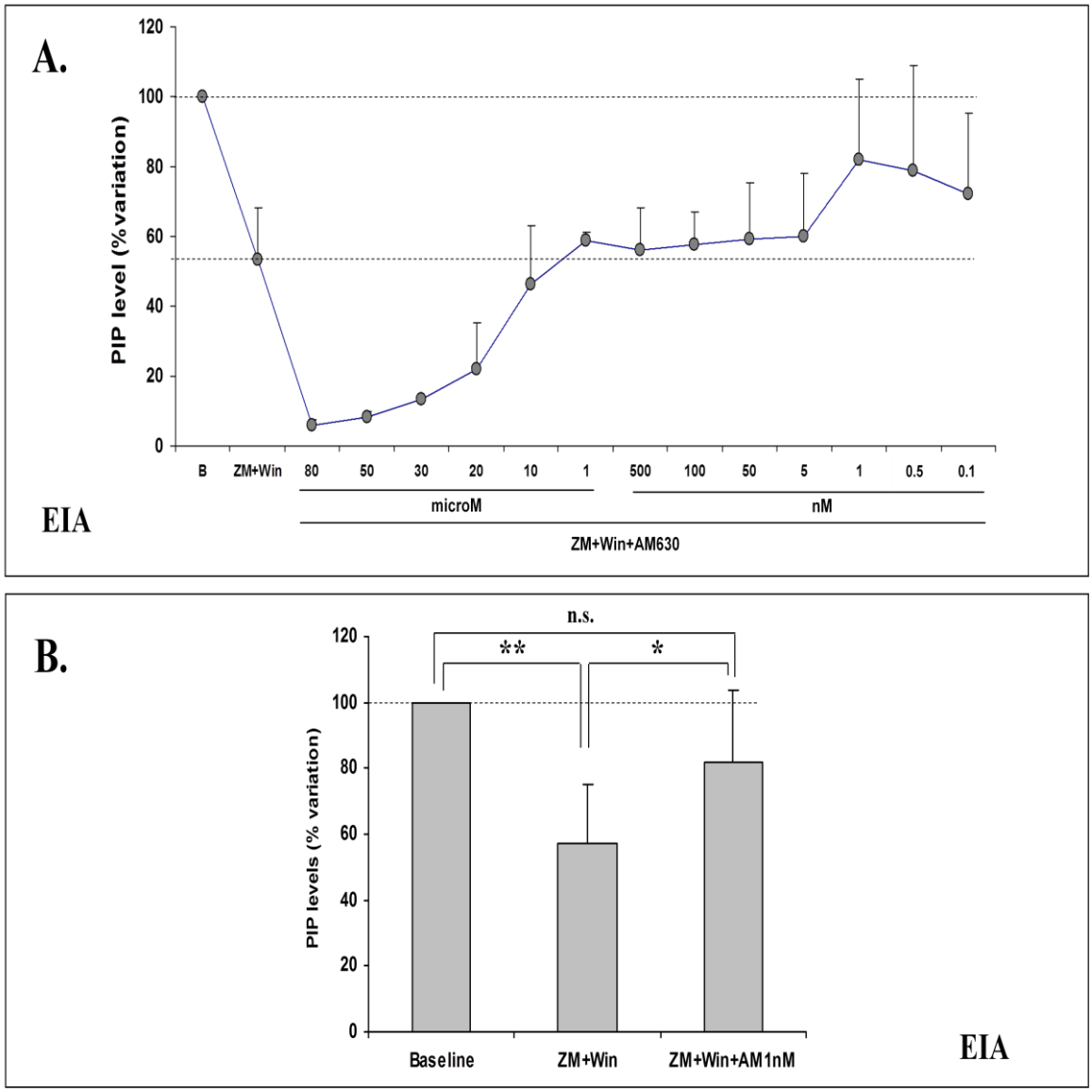

Fig. 7 Cannabinoid CB2 receptor selective activation is the main mechanism mediating the synergistic inhibitory effect of WIN55,212-2 and ZM-241385 co-incubation on collagen production by SSc dermal fibroblasts.

a. Dose-finding for maximal CB2-antagonist activity of AM-630: effect on PIP supernatant levels of different concentrations of AM-630 (from 80 μM to 0.1 nM) added to SSc dermal fibroblasts in culture co-incubated with WIN55,212-2 and ZM-241385, as determined by EIA assay.

b. Effect on PIP supernatant levels of 1 nM AM-630 addition to SSc dermal fibroblasts in culture co-incubated with WIN55,212-2 and ZM-241385, as determined by EIA assay. Values are expressed as percent variation from the baseline (baseline=100%). SSc patients, n=5; RM-ANOVA: p=0.001; *p<0.05, **p<0.01, post-hoc Tukey-Kramer multiple comparison test.

SSc systemic sclerosis, *EIA* enzyme immunoassay, *PIP* procollagen type I carboxy-terminal peptide

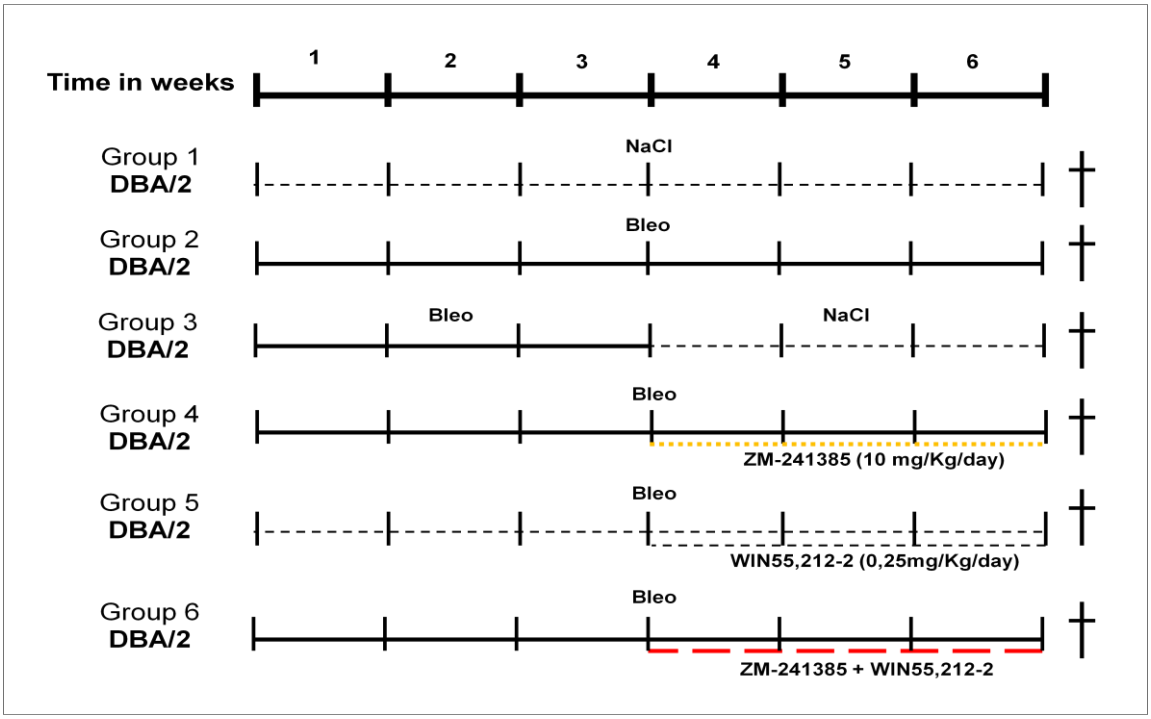

Fig. 8 Experimental design for WIN55,212-2 (0.25 mg/kg/day), ZM-241385 (10 mg/kg/day) and combined ZM-241385 + WIN55,212-2 treatment in DBA/2J mice with bleomycin-induced established dermal fibrosis.

Daggers indicate the time at which the mice were killed.

Bleo bleomycin

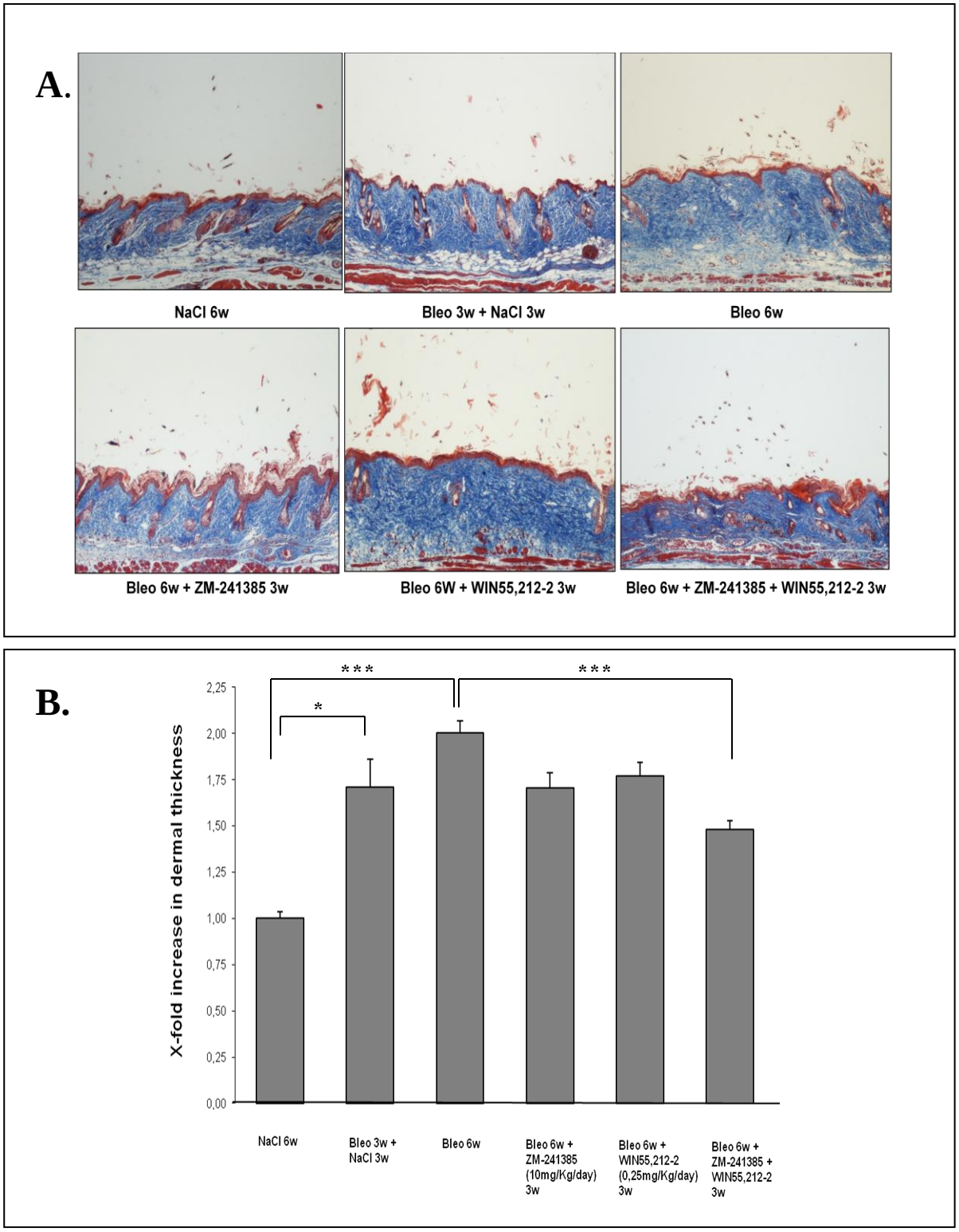

Fig. 9 Combined ZM-241385 + WIN55,212-2 treatment prevents dermal fibrosis progression in a murine model of established fibrosis induced by bleomycin.

a. Representative skin sections from each group stained with Masson's thricrome are shown, original magnification x100.

b. Histograms showing the effect of WIN55,212-2 (0.25 mg/kg/day), ZM-241385 (10 mg/kg/day) and ZM-241385 + WIN55,212-2 on dermal thickness (μm) in a murine model of scleroderma in which fibrosis is induced for 3 and 6 weeks of bleomycin injections.

Values are expressed as mean ± SD from the control group (NaCl 6 weeks=100%). Mice for each group, n=6; RM-ANOVA: p<0.0001; *p<0.05, ***p<0.001, post-hoc Dunn's multiple comparisons test.

Bleo bleomycin, *W* weeks

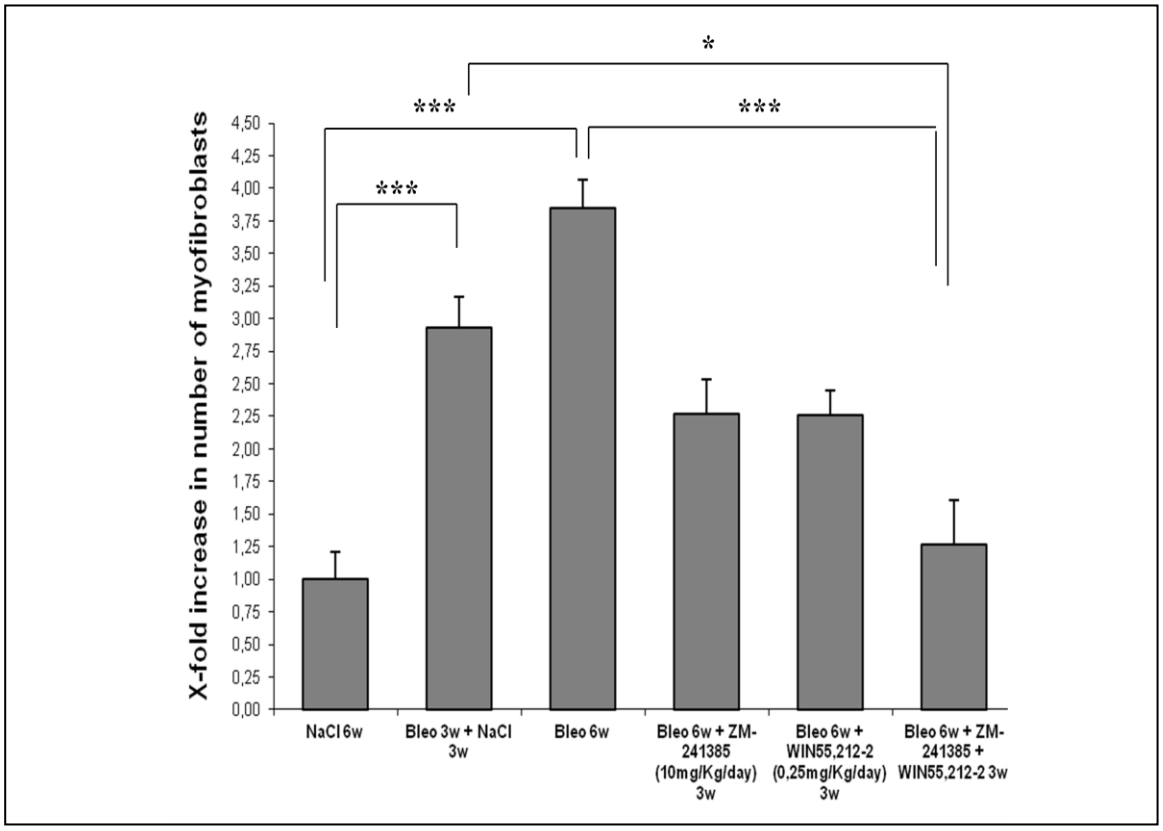

Fig. 10 Combinated treatment ZM-241385 + WIN55,212-2 prevents myofibroblast trans-differentiation in a murine model of established fibrosis induced by bleomycin.

Effect of WIN55,212-2 (0.25 mg/kg/day), ZM-241385 (10 mg/kg/day) and ZM-241385 + WIN55,212-2 on α-SMA expression in a modified model of scleroderma in which fibrosis is induced for 3 and 6 weeks of bleomycin injections, as determined by α-SMA positive fibroblastic cells per microscopic field. Values are expressed as mean ± SD from the control group (NaCl 6 weeks=100%). Mice for each group, n=6; RM-ANOVA: p<0.0001; *p<0.05, ***p<0.001, post-hoc Dunn's multiple comparisons test.

Bleo bleomycin, *W* weeks

PUBLICATION ARISING FROM THIS THESIS:

Lazzerini PE*, Natale M*, Gianchecchi E*, Capecchi PL, Montilli C, Zimbone S, Castrichini M, Balistreri E, Ricci G, Selvi E, Garcia-Gonzalez E, Galeazzi M, Laghi-Pasini F (2012) Adenosine A2A receptor activation stimulates collagen production in sclerodermic dermal fibroblasts either directly and through a cross-talk with the cannabinoid system. *J Mol Med* 90:331-342.

***These authors contributed equally to this work.**