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Nicolas Dumoitier

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Thèse de doctorat

de l'Université Sorbonne Paris Cité

Préparée à l'Université Paris Diderot

ED 273 Hématologie Oncogénèse et Biothérapie

UMRS 1016 – Equipe Neutrophile et Vascularites

Analysis of B lymphocytes in systemic autoimmune vascular diseases

Par Nicolas Dumoitier

Thèse de doctorat de Biologie

Dirigée par le Pr. Luc Mouthon et le Dr. Nadine Varin-Blank

Présentée et soutenue publiquement à l'hopital Bichat le 26 Avril 2017

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Abbreviations

ANA: anti-nuclear antibodies

ANCA: antineutrophil cytoplasm antibodies;

AAT: α1-antitrypsin;

AAV: ANCA associated vasculitis

BCR: B cell receptor

Breg: regulatory B cells

CYC: cyclophosphamide

dSSc: diffuse cutaneous systemic sclerosis

EGPA: eosinophilic granulomatosis with polyangiitis

ENT: ear nose throat;

GPA: granulomatosis with polyangiitis

GWAS: genome wide associated study

HLA: human leukocyte antigen;

IFN: interferon

IL: interleukin

IL-R: interleukin receptor

IVIG: intravenous immunoglobulin

lcSSc: limited cutaneous systemic sclerosis

LDL: low density lipid

mAb: monoclonal antibody

MCTD: mixed connective tissue disease

MPA: microscopic polyangiitis

MPO: myeloperoxidase;

MZ: marginal zone

PAH: pulmonary arterial hypertension

PDGF: Platelet-derived growth factor

PH: pulmonary hypertension

PR3: proteinase 3;

PRTN3: gene coding for PR3;

PTU: propylthiouracil

RA: rheumatoid arthritis

RTX: rituximab

SLE: systemic lupus erythematosous

SSc: systemic sclerosis

STAT: signal transducers and activators of transcription

Tfh: T follicular helper

Th: T helper cell

TGF: transforming growth factor

TLS: tertiary lymphoid structure

TNF: tumor necrosis factor

Treg: regulatory T cells

VSMC: vascular smooth muscle cells

Table des matières

Acknowledgments ***7***

Abbreviations ***8***

Introduction ***4***

1. Autoimmune vascular diseases **4**

A. General introduction 4

a) Atherosclerosis 6

b) Vasculitis: 7

c) Diseases associated with vascular remodelling 8

B. Systemic vasculitis 10

a) Classification 10

b) ANCA associated Vascultis 13

i) Epidemiology 13

ii) Clinical and immunological presentation 15

iii) Pathophysiology 18

iv) Prognosis 21

v) Non classical treatments targeting B cells 23

C. Systemic sclerosis 25

i) Epidemiology 25

ii) Pathophysiology 32

iii) Clinical and immunological presentation 37

iv) Prognosis 42

v) Treatment 42

D. Pulmonary arterial hypertension 44

2. B lymphocytes in autoimmunity **46**

A. Physiological B cell lymphopoïesis 46

a) Central B lymphopoiesis 48

b) Peripheral lymphopoiesis 51

B. Alterations in B lymphocytes lymphopoiesis 54

a) Pathological antibodies 54

b) Tertiary lymphoid structure 55

c) Autoantigens 56

d) Soluble factors associated with B cell activation 57

i) Multiple roles of Interleukin-6 in inflammation 57

vi) BAFF and APRIL: 61

e) B cell signalling abnormalities in autoimmunity 62

C. B lymphocytes: pro-inflammatory cells? 63

a) Pro-inflammatory secretions of B lymphocytes 63
b) Regulatory B cells 64

Aim of the Study ***73***

Results ***76***

1. Paper 1: Skewed peripheral B and T lymphocytes compartments in patients with ANCA associated vasculitis **77**

2. Paper 2: Scleroderma peripheral B lymphocytes secrete interleukin-6 and TGF-beta and activate fibroblasts **123**

3. Additional results: **192**

4. Paper 3: Basophils are activated and promote fibrosis through TGF-ß secretion in systemic sclerosis **198**

Discussion and perspectives ***252***

Annexe 1 ..... ***259***

Annexe 2 ..... ***272***

References ***283***

Résumé ***296***

Summary ***297***

INTRODUCTION

Introduction

Autoimmunity may be defined as a rupture of tolerance with a failure of the immune system to recognize as “self” its own cells and tissues. Consequently, immune cells react if those were foreign or invading bodies. Rose and Bona proposed three evidences at the origin of an autoimmune human disease (Rose and Bona, 1993). The first criteria for autoimmunity is a direct disease transfer from one human to another (or to animal) in which the immune system is competent. For instance, immune thrombocytopenic purpura (ITP) has been identified as an autoimmune disease since the serum transfer of patients with ITP into healthy subject induces the disease (Van Loghem Jj et al., 1959; van LOGHEM et al., 1962). Grave’s disease, myasthenia gravis or microscopic polyangiitis (MPA) present with similar disease transfers from mother to child (Scott, 1976; Bansal and Tobin, 2004). Hashimoto’s thyroidis or type 1 diabetes could also transfer during bone marrow allograft (Rossini et al., 1989; Karthaus et al., 1997). However, iwhen direct transfer is not identifying, the capacity to develop the disease in healthy animal upon transfer from a pathological generated model is also considered as autoimmune (Rose and Bona, 1993). For example, the collagen-induced arthritis mouse model of rheumatoid arthritis is a robust autoimmune disease easily transferable to healthy animals (Stuart and Dixon, 1983). Finally, evidences for the production of specific autoantibodies and activation of oligoclonal T cells against a defined autoantigen could also allowed definition an autoimmune disease. This is the case for granulomatosis with polyangiitis (GPA) in which we could detect anti-neutrophil cytoplasm antibodies (ANCA) and activation of T cells against membrane bound PR3 (Millet et al., 2015).

1. Autoimmune vascular diseases

A. General introduction

Clinicians and scientists have deciphered a number of mechanisms that pont out the importance of latent and persistent inflammation of vessels in disease progression. While a global classification of vascular diseases is not easy to set up, several specificities are used in this characterization. For instance, atherosclerosis, which corresponds to deposits of cholesterol in the lumen of aorta and other large arterial vessels, presents similarities with vasculitis, which is characterized by an inflammation of the walls of arteries and/or veins and finally, or other vasculopathies associated with vascular remodelling (**Figure 1**). The causes and consequences of the three types of inflammated diseases are multiple but are frequently associated with a dysregulation of the immune system.

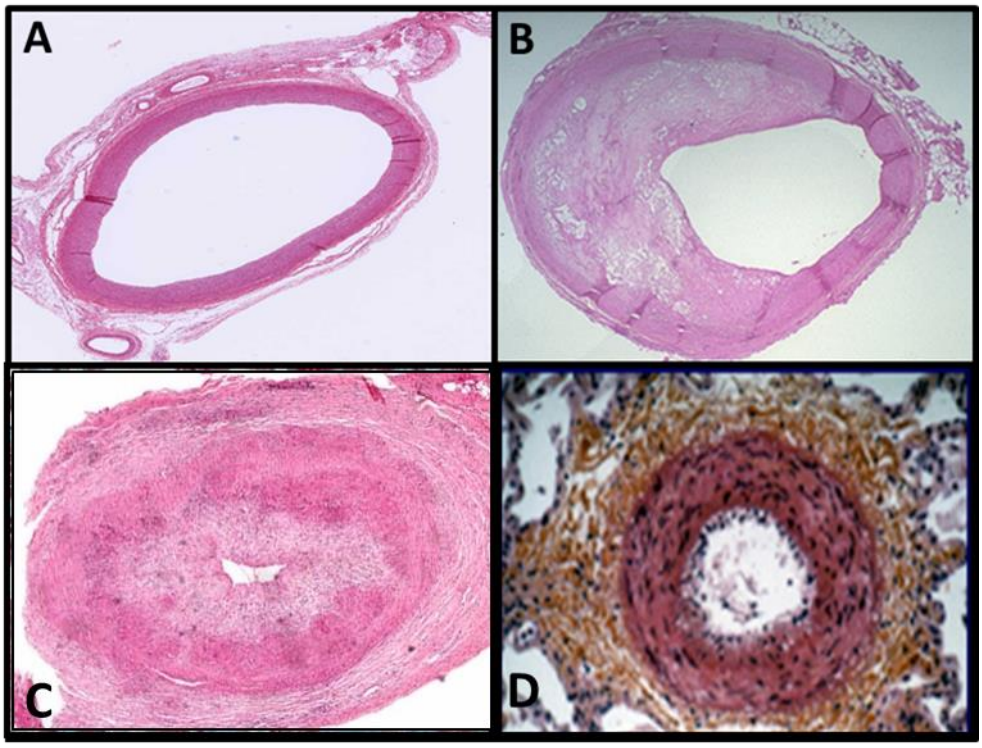


Figure 1. Histopathological pattern of various human vascular disorders: normal aorta (Panel A), atheromatous aorta (Panel B), temporal artery from giant cell arteritis (Panel C) and vascular remodelling of pulmonary arterioles (idiopathic pulmonary arterial hypertension) (Panel D).

a) Atherosclerosis

Atherosclerosis is characterized by an inflammation within the arterial wall leading to severe vascular events, including coronary artery disease, myocardial infraction, stroke and peripheral artery diseases (Insull, 2009; Taleb, 2016). The iniatial event is due to lipid retention, oxidation, and alteration, which provoke chronic inflammation, ultimately causing thrombosis or stenosis. Atherosclerosis develops progressively through the continuous evolution of arterial wall lesions caused by the accumulation of the cholesterol-rich lipids and the subsequent inflammatory response (Woolf, 1978).

Both innate and adaptive immunities play important roles in the development of atherosclerosis; notably implicating monocytes/macrophages, which are the surrogate cells of innate immunity. Also, on the other hand, T-helper cell type 1 (Th1) response has a potent proatherogenic effect, the pathogenic roles of other T cell subsets, such as the Th2 and Th17 dependant mechanisms, remain controversial. However, the anti-atherosclerotic protective roles of regulatory T cells and several Th2-related cytokines, such as interleukin-5, have been clearly established.

In addition, since a number of reports based on animal models argue for the important role of inflammatory cells in atherosclerosis and its complications, treatment of cardiovascular diseases with anti-inflammatory drugs is an attractive option to investigate.

However, atherosclerosis has mostly been described as resulting from the development and evolution of low density lipoprotein (LDL) particles deposits. These particules are enzymatic modified and are oxidized into proinflammatory particles, which trigger innate immune system reaction within the intima (Mayadas et al., 2009). Fat droplets may accumulate in the cytoplasm of vascular smooth muscle cells (VSMC), leading to mechanic and inflammatory stress responses into the cells (Schwartz et al., 1986).

Inflammation begins when endothelial cells become activated and express adhesion molecules, while smooth muscle cells secrete chemokines and chemoattractants, which induce the migration of neutrophils, monocytes and lymphocytes into the arterial wall. Intimal smooth muscle cells also secrete, within the extracellular matrix, proteoglycans, collagen, and elastic fibers triggering a complex remodelling of the whole vessel (Hansson, 2001).

As the disease develops with lipids accumulation and inflammation, the processes and histologic changes become increasingly complex and can vary considerably both between individuals and for any given individual (Ross, 1999). All of these changes may be significantly influenced by risk factors, notably stresses of local hemodynamics and blood flow patterns, hypertension, smoking, and diabetes, as well as genetically determined arterial susceptibility or resistance to atherosclerosis (Smithies and Maeda, 1995). The mechanisms involved for influencing atherosclerosis by these risk factors are the subject of an intensive investigation by molecular pathologists, along with proteomics and genomics, conducted to determine the exact molecular biological processes involved in their development.

In this work, we will not focus on atherosclerosis since this pathology is mainly influenced by hemodynamic and endothelium resistance (Borissoff et al., 2011).

b) Vasculitis:

Vasculitis are a group of rare diseases characterized by inflammation and infiltration of immune cells within vessel walls. If inflammation is not always observed during the course of vasculitis, it is still a common defining feature of all categories of vasculitis. Some categories of vasculitis also have a characteristic tissue injury unrelated to vasculitis. Features that vary among different forms of vasculitis and can be used for categorization include etiology, pathogenesis, the type of vessel affected, the inflammation process, the main organ affected, clinical manifestations,

genetic predispositions, and distinctive demographic characteristics (e.g., in regards to age, sex, ethnicity, and geographic distribution) (Jennette and Falk, 1997; Salvarani et al., 2002). Disease categorization based on etiology is often a preferred approach; however, this remain undoable for most vasculitis for which the etiology is unknown.

Jennette and colleagues (Jennette et al., 2013) have proposed to discriminate vasculitis between:

- infectious vasculitis, caused by direct invasion and proliferation of pathogens within the vessel wall with a resulting inflammation but also several vasculitis induced indirectly by the presence of a pathogen (ex: cryoglobulinemic vasculitis due to an autoimmune response initiated by a hepatitis C virus infection (Vassilopoulos and Calabrese, 2002) or HIV associated vasculitis). These vasculitis are commonly associated with the presence of circuliating immune complex. (Lidar et al., 2009)
- non-infectious vasculitis, not known to be caused by direct vessel wall invasion by pathogens

Many studies have shown the implication of both innate (monocytes in giant cell arteritis, neutrophils in granulomatosis with polyangiitis…) and adaptive immune cells (presence of auto-antibodies, T cell imbalance…) at the onset of the disease and along the entire course of vasculits (Savill, 1993; Barratt and Feehally, 2006; Ly et al., 2010). However, as observed for atherosclerosis, inflammation subsequent to vasculitis also induces important vascular damage and stress and could, upon long time, enhance vessel remodelling and atheroma rupture.

c) Diseases associated with vascular remodelling

Vascular dysfunction due to vascular remodelling, such as microvascular complications in both insulin-independent and insulin-dependent diabetes, are major contributors to morbidity, mortality and therapeutic costs (Emery, 1994; Spinetti et al., 2008). As previously mentioned, patients with diabetes have high risk to develop atherosclerotic disease (Ladeia et al., 2014).

Endothelial dysfunction has been identified as an early marker of vascular disease in type 1 diabetes. Alterations of the microcirculation involve small arteries, arterioles, capillaries and post capillary venules. Mechanisms involved in vascular alterations in diabetes are unclear and could be linked to hyper-glycosylation of both circulating proteins and collagen fibrins, metabolic disorders which induce endothelial cells activation (notably protein kinase C pathway) or to growth factors and probably insulin itself (Schena and Gesualdo, 2005; Porter and Riches, 2013). In these mechanisms of vascular remodelling, inflammation is pointed out in both type 1 and type 2 diabetes (Arroyo and Iruela-Arispe, 2010).

Pulmonary hypertension (PH) is a cardio-pulmonary disorder that may involve multiple clinical conditions and can upgrade the majority of cardiovascular and respiratory diseases (Ryan et al., 2012). The classification of PH is complex and discriminates groups of patients which share similar pathophysiological and hemodynamic characteristics upon treatments (Huertas et al., 2014). Pulmonary arterial hypertension (PAH) corresponds to the first group of PH and is characterized by an increase in mean pulmonary artery pressure over 25 mmHg at rest, leading to right heart failure and ultimately death while low capillary pressure is lower than 15mmHg. PAH may be associated with connective tissue diseases, notably systemic sclerosis (SSc), systemic lupus erythematosus (SLE), mixed connective tissue diseases (MCTD) (Sharp syndrome) and Sjögren's syndromes (Bussone et al., 2012; Kherbeck et al., 2013). In these different pathologies, PAH present one of the most severe complication showing the importance to study these patients subsets (Legendre and Mouthon, 2014). Clinical evolution of these patients is particularly constant; all patients present at early time a connective tissue disease preceeding PAH. These observations suggest that PAH could be associated with autoimmune and immunological events (Rabinovitch et al., 2014).

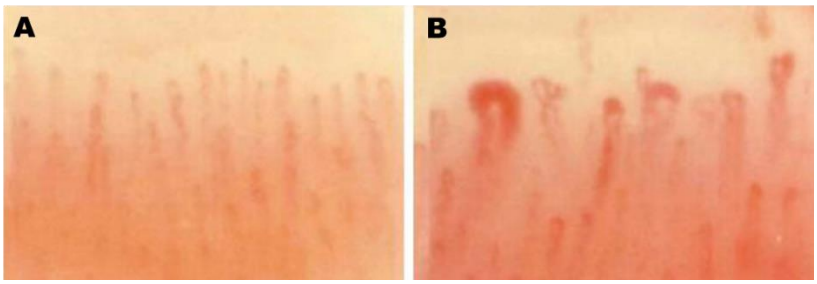


Figure 2. Nailfold capillaroscopy of a healthy individual (Panel A) and in a patient with systemic sclerosis with rarefaction of vascular loops and giant capillaries (Panel B) (Smith et al., 2012)

SSc is a rare connective tissue disease characterized by vascular involvement responsible for vascular hyperactivity and remodelling, together with fibroblasts activation and extra-cellular matrix synthesis (Dumoitier et al., 2014). SSc is often responsible for internal organ involvement as a consequence of remodelling of arterioles. In the lung, SSc is associated with the development of PAH and/or pulmonary fibrosis (**Figure 2**) (O'Callaghan et al., 2011; Wells and Denton, 2014). In recent years, significant progress has been made in the understanding of mechanisms contributing to the occurrence of vasculopathy and fibrosis during the course of SSc, both in human and animal models (Denton, 2015). However, despite these scientific achievements, no universal treatment has been found yet and the elucidating of the complex immune response involved in this disease could be a real breakthrough in the future.

Both SSc, PAH and PAH associated SSc have a poor prognosis and an interesting overlap of their respective immune context.

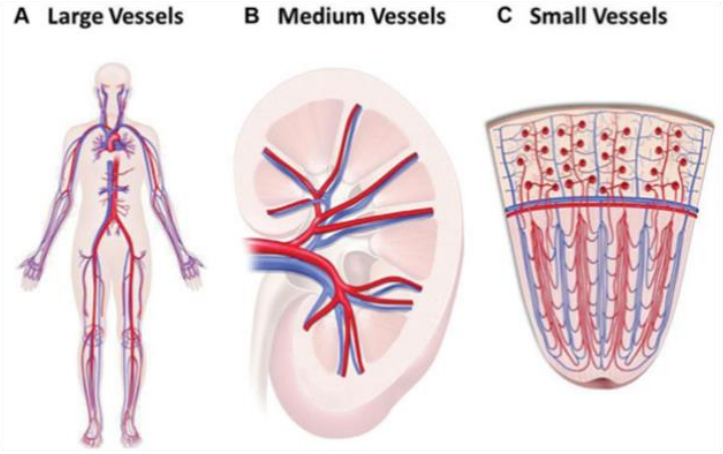
B. Systemic vasculitis

a) Classification

A first classification of systemic vascultis has been established by the American Society of Rheumatology in 1990 (Hunder et al., 1990). This important study has proposed to study 127

clinical manifestations associated to one or more of the 7 vasculitis: polyarteritis nodosa, Churg-Strauss syndrome, Wegener's granulomatosis, hypertensitivity vasculitis, Henoch-Schönlein purpura, giant cell arteritis and Takayasu arteritis. However, if this first classification has led to important improvements in the diagnosis of patients with vascultis, the characterization of microscopic polyangiitis (which was commonly and wrongly diagnosed as polyarteritis nodosa) and the development of etiology studies have allowed the scientific community to further increase the list to 10 vasculitis under the Chapel Hill Conference Consensus, first in 1994 and more recently in 2012, 11 vasculitis have been overviewed (Zashin et al., 1990; Jennette et al., 1994, 2013a). Chapel Hill Conference Consensus categorized non-infectious vasculitis by integrating knowledge about etiology, pathogenesis, pathology, demographics, and clinical manifestations. The first categorization level is based on the predominant type of vessels involved: large, medium and small-sized vessel vasculitis (**Figure 3**).

These terms refer to vessels that differ not only in size, but also in structural and functional attributes. There are further distinctions within each vessel type, for example, capillaries in different organs and different segments of the aorta have different biochemical and functional properties that make them susceptible to different pathogenic mechanisms (Jennette et al., 2013). On the other hand, large vessel vasculitis affects large arteries more often than medium vessels, medium vessel vasculitis affects predominantly medium arteries, and small vessel vasculitis affects predominantly small arteries, vessels and capillaries.



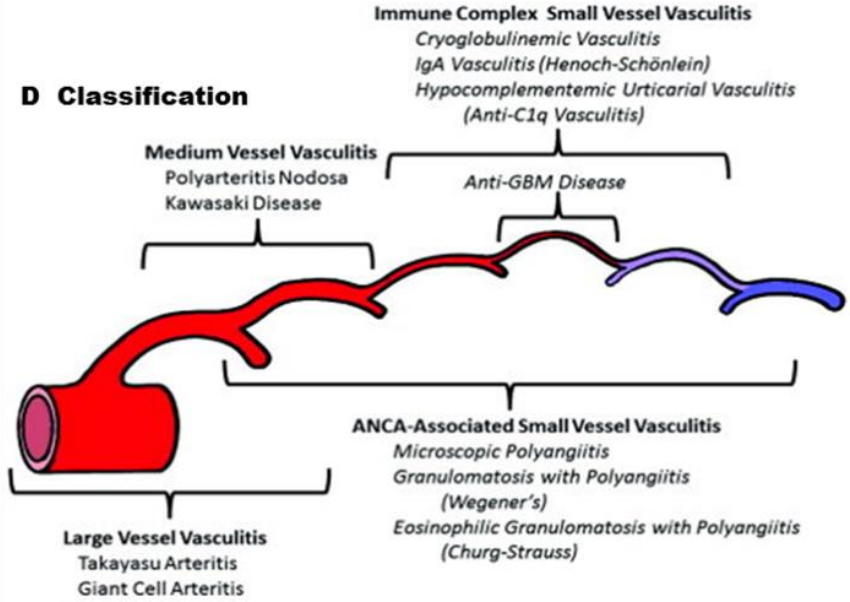


Figure 3. Revision of the Chapel Hill nomenclature of primary systemic vasculitis in 2012: systemic vasculitis may affect large vessels (Panel A), medium vessels (Panel B) or small vessels (Panel C); the revised Chapel Hill nomenclature proposed to discriminate between pauci-immune vasculitis (Anti-neutrophil cytoplasmic antibody (ANCA) associated vasculitis) and immune complex vasculitis (Panel D) - (Jennette et al., 2013)

In this classification, a subgroup of vascultis has emerged among others: ANCA-associated vascultis (AAV) which are the granulomatosis with poliangiitis (GPA), the microscopic polyangiitis (MPA) and the eosinophilic granulomatosis with polyangiitis (EGPA). These three vascultis are pauci-immune necrotizing vascultis which present with a wide range of clinical

manifestations from granuloma formation to renal damage or asthma. The detection of ANCA could be helpful for the diagnosis of GPA and microscopic polyangiitis MPA. The latter have been recently described as pathogenic, conversely to other vasculitis which present a wide broad of auto-antibodies or a more specific clinical presentation (Jennette et al., 2013b).

b) ANCA associated Vascultis

AAV are a heterogeneous group of diseases corresponding to necrotizing inflammation of small vessels with a wide range of clinical presentations (Millet et al., 2013). AAV are pauci-immune necrotizing vasculitis involving small-sized vessels which include GPA (formerly named Wegener's granulomatosis), EGPA (formerly named Churg–Strauss syndrome), and MPA. While granuloma presence seems to discriminate between GPA plus EGPA and MPA, only GPA and MPA seem to have a clear lymphocyte dysregulation as these two pathologies have been linked to the presence of anti-proteinase 3 (PR3) and anti-myeloperoxydase (MPO) respectively. The ANCA signature in EGPA is quite debated in the field and has lead the scientific community to separate EGPA from the GPA/MPA subgroup of vascultis which are mostly treated with the same immunosuppressive strategy (Chaigne et al., 2016a). However, multiple other aspects of vascultis allow us to differentiate GPA and MPA patients and to highlight the matter of a different immunological disorder in these patients.

i) Epidemiology

Epidemiological data have challenged the notion that GPA and MPA could be grouped under the so-called AAV entity (Millet et al., 2013) (**Figure 6**). Notably, marked geographical disparities have been found. For instance, GPA is infrequent in the Japanese population and rarely occurs in African Americans. This is not the case for MPA. In Europe, GPA is more frequent in the north countries, and a reverse tendency has been found for MPA. Also, a

periodical (7.5 years) non-random pattern of GPA occurrence has been recently described, but, it was not observed for MPA. Further implication of differential factors has been documented with fro instance the occurrence of anti-MPO-associated vasculitis upon propylthiouracil or hydralazine treatment while the treatment does not led to anti-PR3-associated vasculitis. In GPA, a link between infection and autoimmunity has been suggested since Staphylococcus aureus chronic carriage was found in patients exhibiting a higher relapse rate.

Moreover, genome wide associated studies (GWAS) have shown a differential pattern between GPA and MPA. GPA is associated with several single nucleotide polymorphisms in PR3 (PRTN3), SERPINA1 and HLA-DP genes.

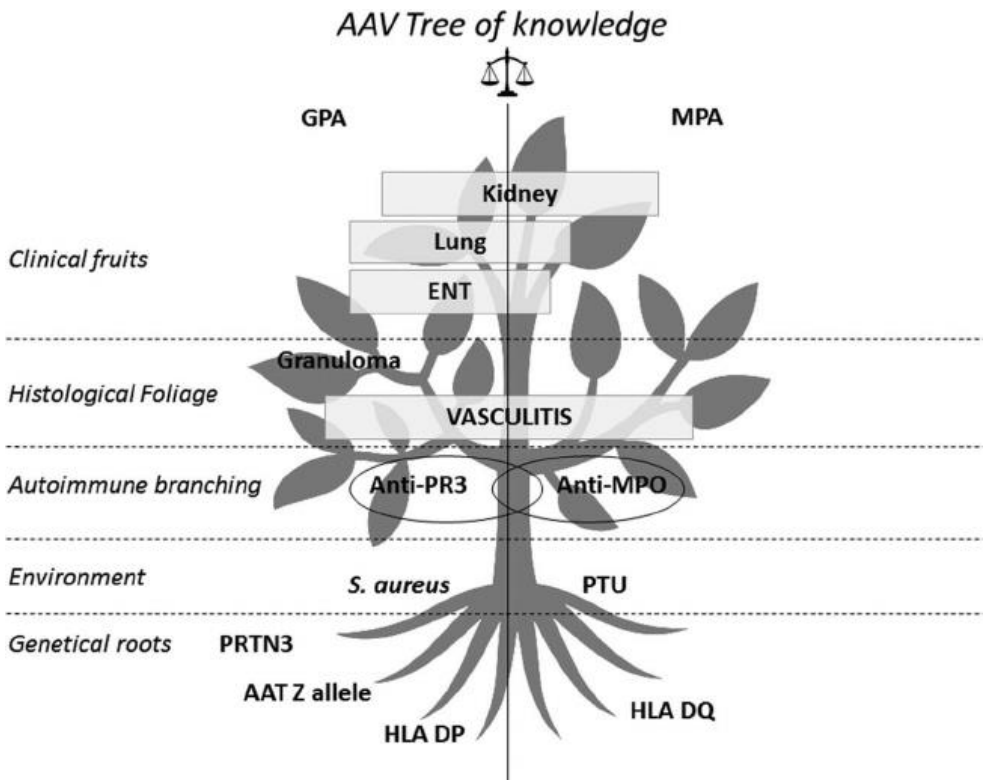


Figure 6. Compared features of granulomatosis with polyangiitis (GPA), and microscopic polyangiitis (MPA). The comparison between GPA and MPA includes different levels from genetic to pathophysiological characteristics and clinical manifestations illustrate the major differences between these two conditions – ANCA: antineutrophil cytoplasmic antibodies; AAT: α1-antitrypsin; AAV: ANCA-associated vasculitis; ENT: ear nose throat; HLA: human

leukocyte antigen; MPO: myeloperoxidase; PR3: proteinase 3; PRTN3: gene coding for PR3; PTU: propylthiouracil – adapted from (Millet *et al.*, *Ann Rheum Dis* 2013)

Finally, the third AAV, EGPA, has a prevalence of 11 cases per million of inhabitants which is lower than GPA (50 cases per million of inhabitants) or MPA (25 cases per million of inhabitants) (Chaigne et al., 2016b). Genetic susceptibility/predisposition has been suggested for EGPA with associations between EGPA and HLA. An association was found for HLA DRB1*04 and HLA-DRB1*07 twice, while a protective effect was reported with HLA-DRB3 and HLA-DRB1*13 (Vaglio et al., Secondary and Primary Vasculitis Study Group, 2007; Wieczorek et al., 2008a). Of note, a study on HLA-DRB1 locus in 403 patients with GPA and 103 with EGPA linked the extended interleukin 10 (IL-10)-3575/-1082/-592 TAC haplotypes with ANCA-negative EGPA (Wieczorek et al., 2008b). Even though IL-10 level was reported to be elevated in EGPA, we lack a post-transcription analysis of the haplotypes .Genes related to the costimulatory molecule CD226 (DNAX accessory molecule 1), the intracellular signaling-involved molecule PTPN22 and eotaxin-3 single nucleotide polymorphisms were investigated but were not associated with EGPA (Wieczorek et al., 2009).

While EGPA is commonly associated with asthma, no clear link was found between the frequency of asthma nor the seasonal cycle and the prevalence of EGPA (Bottero et al., 2007). Moreover, only several case reports and a limited number of studies have linked EGPA with infectious triggering, antigen inhalation or uses of drugs (Guillevin et al., 1999; MOUTHON et al., 2001).

ii) Clinical and immunological presentation

Inflammation of small-sized vessels in AAV is responsible for organ damage with granulomatous involvement of upper and lower respiratory tract in GPA and EGPA, and

glomerulonephritis, intra-alveolar hemorrhage, and/or peripheral neuropathy in both GPA, MPA, and EGPA (**Figure 7**) (Millet et al., 2013; Soussan et al., 2014; Chaigne et al., 2016b).

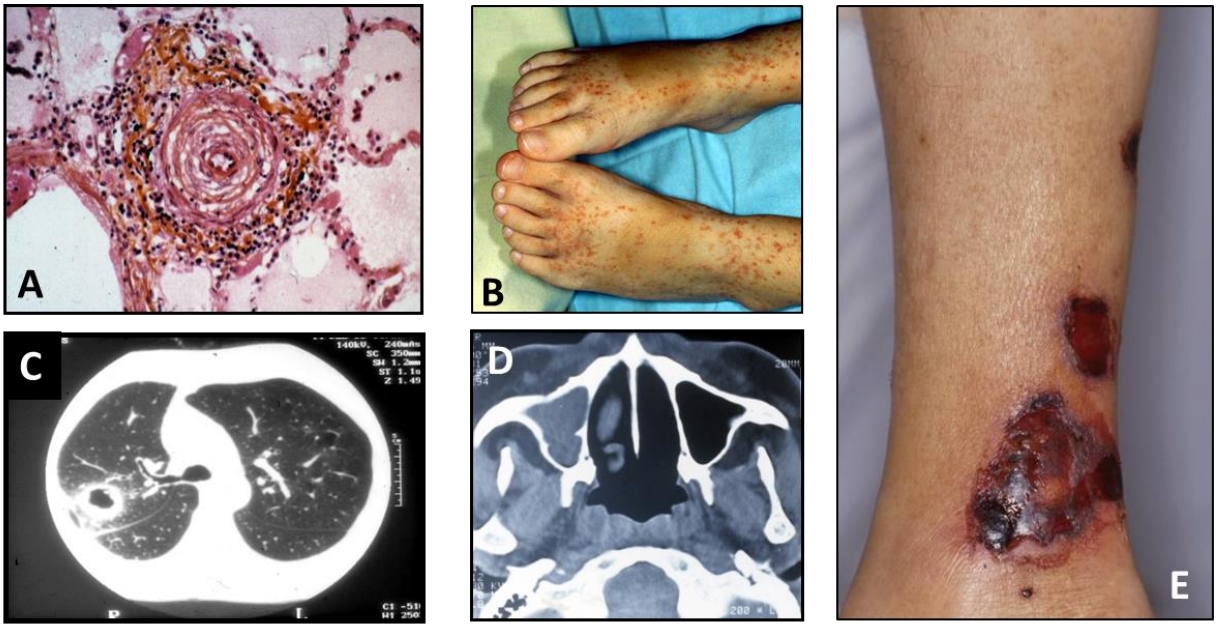


Figure 7. Clinical manifestations during Anti-neutrophil cytoplasm antibodies (ANCA) associated vasculitis: Lymphocyte invasion and destruction of blood vessels (Panel A), Henoch-Schönlein purpura (Panel B), chest X–ray of infiltrated lungs with cavities formation (Panel C), nasal X-ray of granuloma (Panel D), superinfected purpura (Panel E) – kindly provided by Pr. L. Mouthon

Detection and characterization of ANCA strongly support the implication of B-cells in the pathophysiology of AAV (**Figure 8**). The prevalence of ANCA among patients with AAV differs according to the type of vasculitis (Wiik, 2003; Millet et al., 2013). In GPA, PR3-ANCA are detected in 85% of patients while MPO-ANCA are identified in less than 10% of the cases (Van Der Woude, 1985; Hagen et al., 1993). MPO-ANCA are detected in 60% of the patients with MPA and in 31–38% of patients with EGPA (Wiik, 2003).

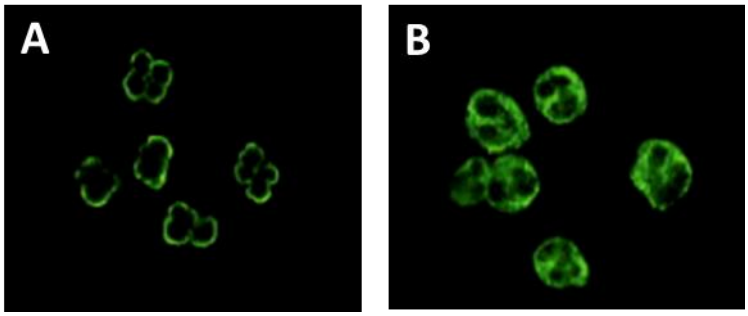


Figure 8. Immunofluorescence pattern of Anti-neutrophil cytoplasm antibodies (ANCA) against normal neutrophil. Panel A. Perinuclear ANCA pattern (p-ANCA); Panel B. Cytoplasmic ANCA pattern (c-ANCA) – kindly provided by Pr. L. Mouthon

Until recently, the pathogenesis of AAV was reported as involving predominantly neutrophils, eosinophils, T cells, and ANCA as antibodies. Importantly, data supporting B-cell implication in this setting was recently shown. One of the first reports on the implication of B cells in AAV focused on the analysis of B-cell infiltrations proximal to PR3-positive cells and on plasma cells within endonasal inflammatory lesions of patients with GPA (Voswinkel et al., 2006). Within these lesions, Voswinkel *et al.* reported on the activation and maturation of aggregated B-cells, arguing for a key role of B lymphocytes in the pathogenesis of AAV (**Figure 9**). This concept was reinforced by the results of two prospective randomized trials demonstrating the efficacy of rituximab, an anti-CD20 monoclonal antibody, in the induction of remission in patients with GPA or MPA, and more recently for the maintenance of remission in the same diseases, with the superiority of rituximab compared to azathioprine (Stone et al., RAVE-ITN Research Group, 2010; Specks et al., RAVE-ITN Research Group, 2013; Guillevin et al., 2014a; Dumoitier et al., 2015).

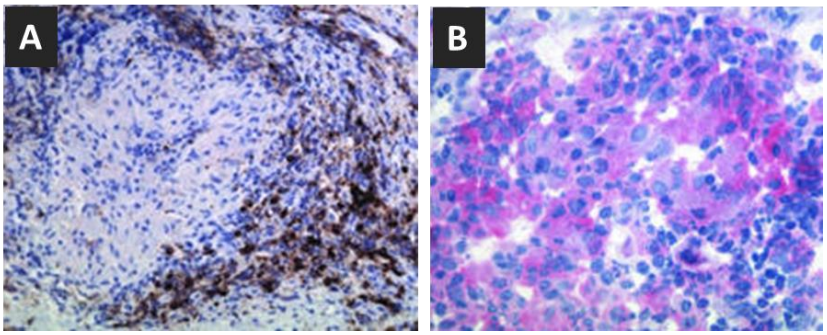


Figure 9. Immunohistochemistry of endonasal granuloma in a case of granulomatosis with polyangiitis (GPA): $CD20^+$ B cell (Panel A) infiltrate in close contact of neutrophils expressing PR3 (Panel B) - adapted from (Voswinkel et al., 2006)

Following these new insights due to clinical trials on rituximab in AAV, several studies on B lymphocytes subpopulations in patients with AAV have been reported. A correlation was found between the importance of B-cell infiltration within granulomatous lesions in GPA and the occurrence of relapses, despite peripheral B-cell depletion in few patients treated with rituximab (Ferraro et al., 2008).

iii) Pathophysiology

A lot of actors implicated in the pathophysiology of AAV have already been identified. The detection of ANCA underlined the importance of the neutrophils in AAV as well as the potential involvement of these antigens. However, the expression of both PR3 and MPO, as well as the modulation of the inflammatory process by their respective ANCA is still unclear (**Figure 10**).

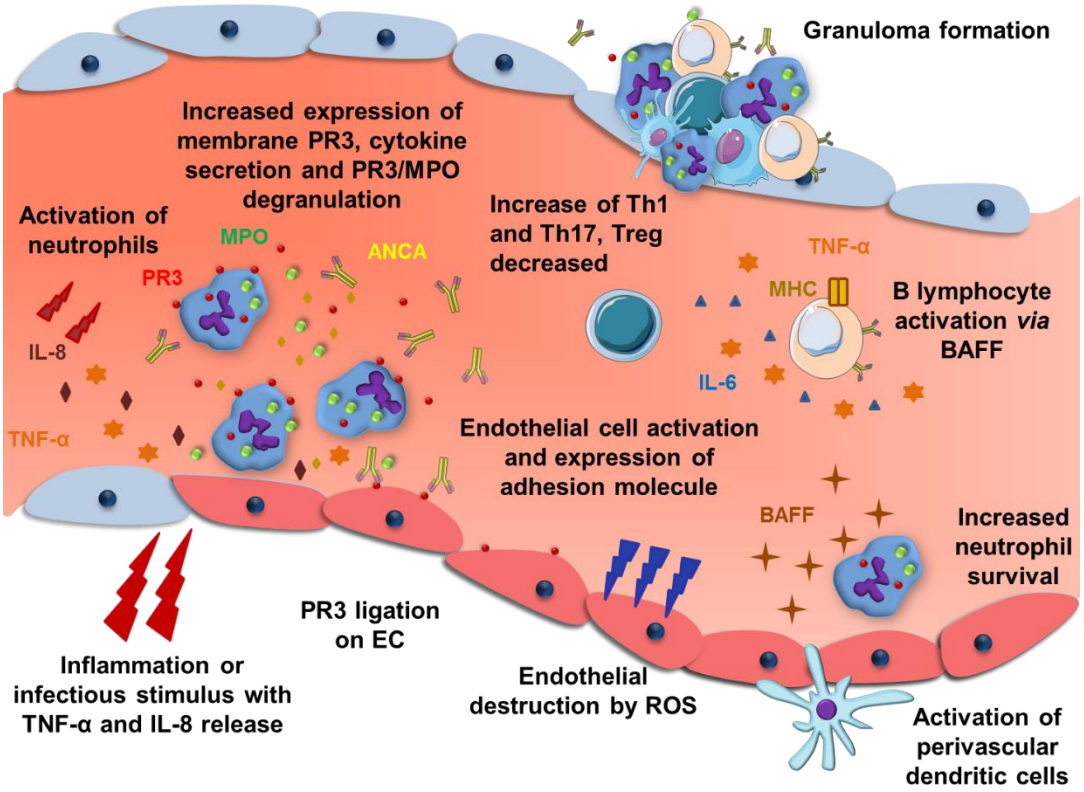


Figure 10. Pathophysiology of granulomatosis with polyangiitis (GPA) – ANCA: Anti-neutrophil cytoplasm antibodies; BAFF: B-cell activating factor;; EC: Endothelial cells; IL: Interleukin; MHC: Major histocompatibility complex; MPO: Myeloperoxydase; PR3: Proteinase 3; ROS: Reactive oxygen species; Th: T helper cell; TNF-α: Tumor necrosis factor-alpha; Treg: Regulatory T cells.

MPO and PR3 are two granule-stored microbicidal proteins expressed by neutrophils and monocytes and possess immunomodulatory properties (Witko-Sarsat et al., 2000). Recent studies have demonstrated the importance of neutrophils and monocytes through the expression of PR3 and MPO in both human and animal models (Roth et al., 2013; Millet et al., 2015). Besides the pathogenic role of ANCA, previous studies have investigated the role of genetic predisposition (Alberici et al., 2014), silica exposure (Gómez-Puerta et al., 2013) or autoimmune overlaps (Guellec et al., CRI (Club Rhumatismes et Inflammation) and the French Vasculitis Study Group, 2015) and have demonstrated the implication of T cells in AAV, showing a predominant Th1 polarization in the granuloma form of GPA contrasting with a

predominant Th2 polarization in the vasculitis form of EGPA (Abdulahad et al., 2011; Chaigne et al., 2016a).

Interestingly, in a cohort of patients with AAV, including GPA and MPA patients, tertiary lymphoid structures (TLS) have been identified in the tissues (Steinmetz et al., 2008). TLS were identified in glomerulonephritis lesions, composed of a germinal center containing follicular dendritic cells and memory as well aw non-memory B cells at the periphery of the germinal center, in contact with the T cells. Interestingly, highly proliferative B-cells have been found in the center of TLS, providing an important source of antigen-presenting cells enhancing T cells maturation at the periphery. However, there is no evidence has been obtained for antibody secretion or maturation of antibody-producing cells in renal lesions of patients with AAV. These results suggest an antibody-independent role of the B-cells in the pathological site and suggest a complex interplay between adaptive and innate immune cells in GPA and MPA (**Figure 9**).

Several B cell abnormalities have been found in patients with GPA and have been recently reviewed in our study (Dumoitier et al., 2015, **ANNEXE 2**). However, hardly any rich documentation has been published regarding the implication of B lymphocytes in MPA or EGPA while anti-MPO have been found clearly pathogenic *in vivo* (Tsurikisawa et al., 2013; Xiao et al., 2002).

In EGPA, the main immune dysfunction is linked to the presence of eosinophils as these cells present a dramatically increased proliferation, decreased apoptosis and increased tissue toxicity (**Figure 11**) (Chaigne et al., 2016b). Increased expression of activation markers have been described in both neutrophil in GPA and eosinophil in EGPA (Klion, 2015; Millet et al., 2015). Moreover, specific cytokine cascades have been found in the three AAV (Guilpain et al., 2005).

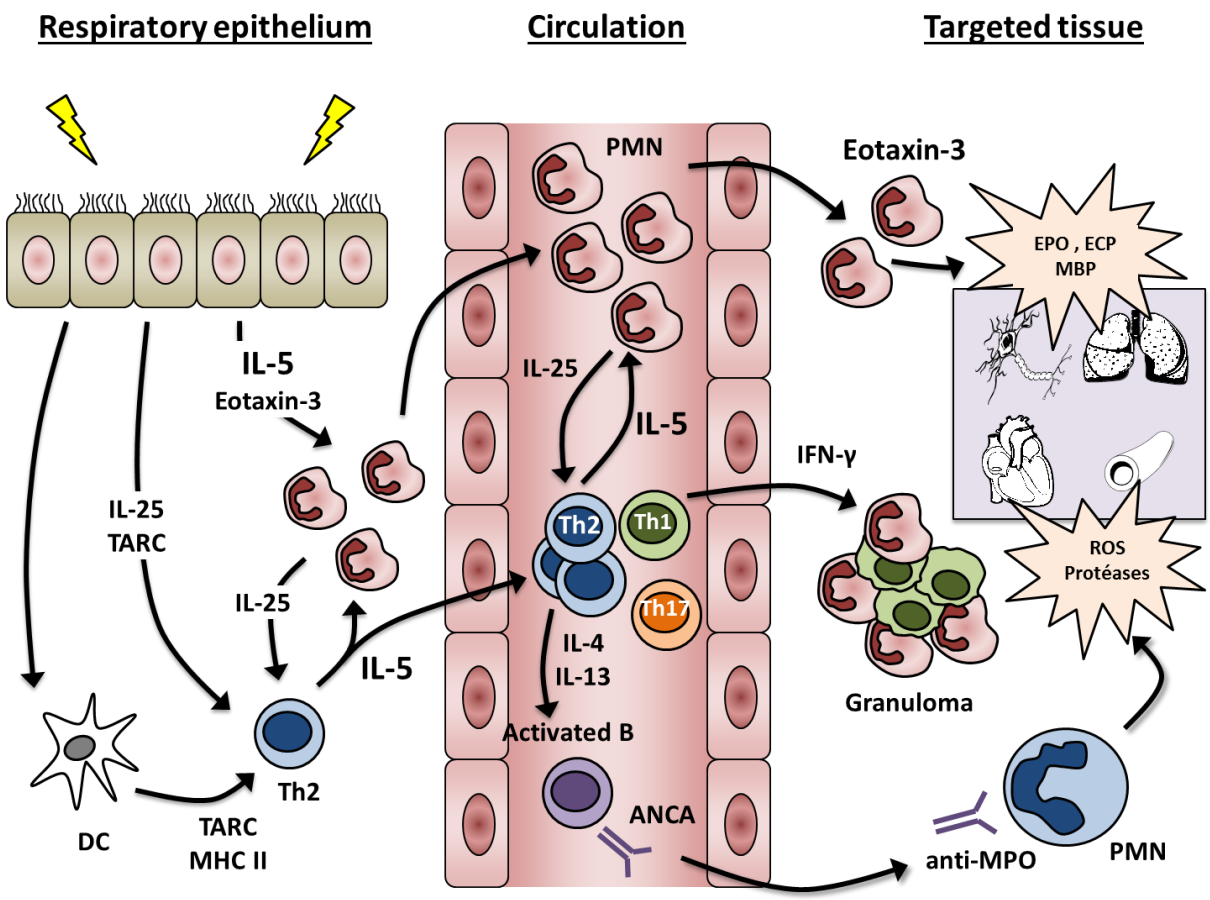


Figure 11. Pathophysiology of eosinophilic granulomatosis with polyangiitis (EGPA) – ANCA: Anti-neutrophil cytoplasm antibodies; DC: Dentritic cells; ECP: Eosinophil cationic protein; EPO: Eosinophil peroxidase; IFN: Interferon; IL: Interleukin; MBP: Major basic protein; MHC: Major histocompatibility complex; MPO: Myeloperoxydase; PMN: Polymorphonuclear cells; ROS: Reactive oxygen species; TARC: thymus and activation regulated chemokine; Th: T helper cells - adapted from (Chaigne et al., 2016b)

iv) Prognosis

AAV causes considerable morbidity and mortality, with end-stage renal failure developing in over 20% of the patients at five years (Booth et al., 2003). Prior to the introduction of steroids and cyclophosphamides, in the early 1970s, survival was uniformly dismal, with a median survival of five months for patients with GPA (Walton, 1958). Nowadays, use of immunosuppressive treatments leads to an induction of remission in 90% of the patients by six months (Jayne et al., 2003) and a five-year survival rate around 75% (Matteson et al., 1996).

However, although progress has been made in the management of patients with AAV, there are still refractory and frequently relapsing cases after treatment (Specks et al., RAVE-ITN Research Group, 2013).

Cyclophosphamide, an alkylating agent, is one of the major anti-cancer treatments (Cramblett, 1959). This drug is also associated with major immunosuppressive effects and has been reported to improve the outcome of patients with systemic autoimmune diseases (Taylor, 1963; Silva et al., 2011; Monach et al., 2013). Following these observations, cyclophosphamide was shown to improve the outcome of patients with GPA (Novack and Pearson, 1971), and cyclophosphamide has become the standard of care of AAV patients with severe disease. Although cyclophosphamide is known to deplete both B cells and T cells in peripheral blood, depletion is more pronounced in B cells than in T cells (Ziff et al., 1974). In vitro and in vivo experiments document the effect of cyclophosphamide on antibody production and secretion, in AAV and SLE, suggesting a possible impact on autoreactive B cells and autoantibody secretion (Casey, 1968; Buckley and Gills, 1969).

Anti-CD20 antibodies, and particularly rituximab (Rituxan® or Mabthera®), were designed to target B cells and treat B cell lymphoproliferative disorders, particularly B cell lymphomas and obtained an FDA agreement in 1997 in this indication (1994). Then, rituximab was tested in a number of autoimmune conditions such as cold agglutin disease (Lee and Kueck, 1998), immune thrombocytopenia (Shvidel et al., 2001) or thrombotic thrombocytopenic purpura (Faurschou et al., 2001). Specks *et al.*, reported in 2001 the first patient with GPA treated with rituximab in whom complete remission was documented at one year (Specks et al., 2001). Limited numbers of patients with refractory MPA and EGPA were also successfully treated with rituximab in the middle of the 2000's decade (Langford and Balow, 2003; Eriksson, 2005). The similar efficacy of rituximab and cyclophosphamide was documented in two prospective randomized trials, in the induction of remission for the treatment of GPA and MPA (Jones et

al., 2010; Stone et al., 2010; Specks et al., RAVE-ITN Research Group, 2013). More recently, the superiority of rituximab compared to azathioprine was demonstrated in the maintenance of remission in GPA and MPA patients (Guillevin et al., 2014b).

v) Non classical treatments targeting B cells

In autoimmune diseases, rituximab treatment leads to rapid and complete depletion of naïve B cells and nearly all peripheral B cell subsets (Szodoray et al., 2004). Interestingly, an *in vitro* nondepleting study also demonstrated a better efficacy of rituximab on $CD27^-$ naïve B cells compared to $CD27^+$ memory B regarding B cell activation, proliferation and viability (Kamburova et al., 2012). Few months after the first injection of rituximab, transitional B cells repopulation is observed and plasma cells recirculate in the peripheral blood of treated patients (Leandro et al., 2006; Teng et al., 2009). Moreover, notably in rheumatoid arthritis patients, a high proportion of circulating memory B cells is associated with increased risk of relapse in patients treated with rituximab (Leandro et al., 2006; Kamburova et al., 2012). In AAV, Bunch *et al.* suggested that after treatment by rituximab, the number of $CD5^+$ B lymphocytes, a B cell subset that seems to be poorly affected by anti-CD20 monoclonal antibody, could correlate with the risk of relapse and/or disease severity (Bunch et al., 2013).

Ten years ago, infliximab, a humanized anti-TNF-α monoclonal antibody was proposed for reduced the persistent inflammation and endothelial activation in AAV (Booth et al., 2004a; Wilkinson et al., 2006). Several studies have suggested interesting results for refractory patients (Josselin et al., 2008; Wilkinson et al., 2006). Recently, infliximab was evaluated in addition to classical treatment with cyclophosphamide for GPA patients with similar results when compared to cyclophosphamide treated patients in the first 12 months (Morgan et al., 2011). Targeting the bioactive form of TNF-α, secreted mostly by activated monocytes but also by activated B lymphocytes, is well known and benefits are described in other autoimmune

pathologies and especially in RA (Atzeni et al., 2013). However, treatments with TNF-a blockers seem to be associated with increased risk of solid malignancy in patients with AAV (Silva et al., 2011).

Increased levels of BAFF, TACI and IL-21, three major cytokines which participate to B lymphocytes activation, have been found in the serum of patient with active GPA and MPA (Krumbholz et al., 2005). As previously indicated, BAFF seems to increase autoreactive B cells selection and promote the activation and survival of pathogenic B cells (Mackay et al., 2010; Liu and Davidson, 2011). This pathogenic role of BAFF in AAV has been also suggested because of an increase secretion of BAFF by *in vitro* ANCA-stimulated neutrophils (Holden et al., 2011). Evidences of plasma cell selection by TACI have also been published in rheumatic diseases (Faurschou and Jayne, 2014). Belimumab, a humanized monoclonal antibody targeting BAFF, have been developed several years ago. BAFF-targeting therapies have been successfully tested in patients with SLE (Manzi et al., 2012; Strand et al., 2014) and patients with rheumatoid arthritis (Stohl et al., 2013), with good tolerance profile in both cases. FDA approved belimumab in treatment of active form of SLE in 2011. The BREVAS study was started in 2012 to evaluate belimumab for the maintenance of remission in patients with AAV, but the study will ne be completed because of an inappropriate design.

Based on IL-6 overexpression in patients with AAV (Catanoso et al., 2011), the role of this cytokine was also suggested in AAV pathophysiology. Stimulation of monocytes by anti-PR3 leads to increase secretion of IL-6 and other pro-inflammatory cytokines (Hattar et al., 2002). Similar results were obtained with endothelial cells stimulated with antibodies from patients with GPA and MPA, leading to an increased secretion of IL-6 and E-selectin (Muller Kobold et al., 1999). IL-6 is also involved in enhancing the maturation of B cells (Kishimoto, 2005a), particularly, in the induction of antibody secretion by B cells (Hirano et al., 1985; Suematsu et al., 1989). Tocilizumab, a humanized monoclonal antibody targeting the IL-6 receptor, have

been recently developed and tested effectively in rheumatoid arthritis (Maini et al., 2006). However, tocilizumab is also evaluated in other inflammatory conditions, such as giant cell arteritis, Behçet disease or Takayasu arteritis. Tocilizumab could also be an interesting alternative therapy in AAV.

Bortezomib, a proteasome inhibitor, have demonstrated promising results compared to cyclophosphamide for the treatment of glomerulonephritis in the anti-MPO mice model (Bontscho et al., 2011). Bortezomib significantly reduced plasma cells in bone marrow of treated mice, notably anti-MPO specific plasma cells, finding that was not observed in cyclophosphamide-treated group. Thus, bortezomib induced normalization of urinal abnormalities, crescent glomerulonephritis and finally neutrophils and macrophages (Bontscho et al., 2011).

C. Systemic sclerosis

i) Epidemiology

SSc mostly occurs in females (3 to 8 females for 1 male). Its prevalence varies between 30 and 240 per million inhabitants, being higher in North America and Australia than in Japan (Ranque and Mouthon, 2010) (**ANNEXE 1**). In Europe, the prevalence varies from 50 to more than 150, with 158 per million in France (Le Guern et al., 2004). Reports of sporadic clusters of higher prevalence suggest the existence of environmental risk factors.

A number of those environmental factors that may contribute to the occurrence of SSc has been investigated. Various activities, including manufacturing and rural activities have been associated with the occurrence of SSc, notably the exposure to silica, dust and/or hydrocarbons (Ranque and Mouthon, 2010). The first reports of the association between silica and SSc were made of limited numbers of patients followed by retrospective comparative studies which confirmed that exposure to silica conferred a high risk to develop SSc (Nietert et al., 1998).

Odds Ratio of 3.93 (1.84-8.54) and 5.57 (1.69-18.37) were calculated in studies conducted in Australia and France, respectively, leading a number of countries including France, Germany, Canada and South Africa to consider SSc as an occupational disease (Englert et al., 2000; Diot et al., 2002). In addition to silica, case control studies pointed out that past exposure to solvents was associated with SSc (Nietert et al., 1998), with discrepancies among studies regarding the types of solvents involved and/or patient gender. Thus, paint thinner or removers, mineral spirits, trichloroethylene, trichloroethane, perchloroethane, gasoline, aliphatic hydrocarbons, halogenated hydrocarbons, benzene, toluene or xylene-solvent shave been proposed as the most at risk solvents, although discrepancies were identified among studies. Nietert *et al*. found that SSc males were more frequently exposed to organic solvents (in particular trichloroethylene) than controls (OR 2.9 [1.1-7.6]), which was not the case for females (Nietert and Silver, 2000). In a recent meta-analysis of the literature, the occurrence of SSc was associated with increased ORs for silica, chlorinated solvents, trichloroethylene and welding fumes for male patients, aromatic solvents and ketones for female patients and white spirit for both (Marie et al., 2014). Several others toxic products including epoxy resins and pesticides, as well as paints, adhesive, dyeing hair, contact lenses and fabric dyes have been investigated in SSc case controls studies but were never found significantly at risk for SSc. Finally, although silicone breast implants were suspected as potentially responsible for SSc induction (Varga et al., 1989), two meta-analysis (Janowsky et al., 2000; Marie et al., 2014) concluded to the absence of association between SSc and breast implants.

In addition, outbreaks of SSc-like disorders have clearly been linked to chemical exposure, such as polyvinyl chloride intoxication (Garabrant et al., 2003) or toxic oil syndrome (Nietert and Silver, 2000).

Several viruses or certain chemicals, such as solvents, may also play a triggering role in the initiation and maintenance of the disease (**Table 1**). Notably, Lunardi *et al.* have reported the

presence of a "SSc peptide" recognized by serum immunoglobulin from 93% of patients with SSc. Interestingly, this peptide shows an important homology with UL94, a cytomegalovirus (CMV) late protein that can be recognized by purified IgG from patients (Lunardi et al., 2000). *In vitro*, patient antibodies targeting UL94 also seem to induce apoptosis of EC similarly to SSc specific antibodies suggesting a possible role of CMV in the triggering and the maintenance of SSc. Thus, it turns out that a cascade of extrinsic and intrinsic events seems necessary for the induction of the disease (Halenius and Hengel, 2014).

Table 1. Systemic sclerosis (SSc) and SSc-like disorders following specific environmental exposures – adapted from (Varga et al., 2016)

Exposure	Disease	Evidence
Crystalline silica/silica dust	SSc	Meta-analysis
Solvents	SSc	Meta-analysis
Vinyl chloride monomer	Vinyl chloride disease	Investigation of outbreak
Adulterated cooking oil	Toxic oil syndrome	Investigation of outbreak
Tryptophan	Eosinophilic myalgia syndrome	Investigation of outbreak
Gadolinium	Nephrogenic systemic fibrosis	Multiple case series
Drugs		
Bleomycin	Pulmonary fibrosis	Multiple case series
Pentazocine	Localized dermal fibrosis at injection site	Multiple case series

Many studies have been conducted in recent years in an attempt to identify genetic risk factors in SSc. From three U.S. cohorts, involving 703 families, including 11 multiplex SSc, first degree relatives were identified for an increased susceptibility to develop SSc, with a relative risk close to 13 (10-16 across cohorts), with an recurrence rate of 1.6% versus 0.026 % in the general population (Arnett et al., 2010a). For siblings, the relative risk in siblings (λs) is increased to 15 (10-27 across cohorts). For comparison the risk (λs) varies between 3 and 15 in rheumatoid arthritis (RA) and between 20 and 29 in systemic lupus erythematosus (SLE). Only one study has been performed in twins. Based on the analysis of 42 twin pairs (24 monozygotic) this study demonstrates a poor agreement with the clinical expression of the disease (4.7%) (Feghali-Bostwick et al., 2003). However, there is a higher concordance for the presence of anti-nuclear antibodies (ANA): 40% for dizygotic and 90% for monozygotic. These results suggest that genetic predisposition alone is not sufficient to develop SSc but might influence the autoantibody profile production.

Following the development of molecular biology, many data have been published on association studies. Unfortunately, a number of them were not repeated in large cohorts and/or could not be independently replicated by another group, which is essential for the validation of a genetic association signal. This was particularly the case for genes suspected to be involved in the regulation of fibrosis such as SPARC (encoding the secreted protein acid rich in cysteine), CTGF (encoding connective tissue growth factor), TGFB (encoding TGF-β) or FBN1 (encoding fibrillin 1) (Allanore et al., 2007) or vascular factors including KCNA5 (encoding the potassium voltage-gated channel 5) or uPAR (encoding the urokinase-type plasmogen activator receptor) (Wipff et al., 2008; Manetti et al., 2011). Interestingly, uPAR extinction in a mouse model led to dermal and pulmonary fibrosis together with microvasculopathy (Manetti et al., 2013). In parallel, a decrease of full-length uPAR protein expression in dermal biopsies of patients with SSc as compared to normal skin has been reported, suggesting a protective role

of this protein in SSc. In addition, the potential influence of KCNA5 SNP on SSc prevalence remains controversial (Bossini-Castillo et al., 2012).

The tumor necrosis factor ligand superfamily 4 gene (TNFSF4) encodes OX40L (CD252), the ligand of OX40 (CD134). OX40L, involved in antigen presentation in addition of T and B lymphocyte activation, is known as a susceptibility factor for SLE (Manku et al., 2013). Interestingly, a study performed in 1,059 patients with SSc and 698 controls showed an association between OX40L polymorphism and SSc and the major disease subgroups (Gourh et al., 2010). This result has been confirmed in replication and meta-analysis studies (Bossini-Castillo et al., 2011; Coustet et al., 2012).

Type I interferons (IFN) are key mediators of innate immunity and antimicrobial defense. IRF5 (interferon-regulatory factor 5 encoded by IRF5) is a transcription factor involved in the signaling of toll-like receptors (TLRs) and activation of target genes of interferon. Candidate genes studies have shown its association with the disease and its contribution to pulmonary fibrosis associated with SSc, independently of other risk factors identified (Dieudé et al., 2009a; Ito et al., 2009). These studies permanently identified IRF5 as a susceptibility factor to SSc. Moreover, recent updates highlighted the major role of IRF5 in the progression and in the occurrence of interstitial lung disease (ILD) in SSc (Sharif et al., 2012; Lindahl et al., 2013). Toward documented role of IRF5 in SSc patients, recent update have identified in IRF4 gene a common susceptibility locus for SSc and rheumatoid arthritis, reinforcing the interest in IFN regulator factor genes in SSc (López-Isac et al., 2016a).

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor of the STAT family that induces the expression of type I IFN, interleukin (IL)-12 and IL-23, and stimulates the synthesis of IFN-γ and IL-17. Thus, a variant of the gene located in the third intron (rs7574865), without specific biological function, has independently been found to be

associated with several autoimmune diseases in two European, one U.S. and a Japanese cohorts (Watford et al., 2004). However, STAT4 is probably not directly responsible for the autoimmune state, since the combination of IRF5 and STAT4 (Dieudé et al., 2009a) variants was associated with an increased risk of developing pulmonary fibrosis in patients with SSc (OR 2.72 [1.86-3.99], p=3.07×10-7 in presence of 3 or 4 at-risk alleles). In that study, STAT4 was found to be the second outside association major histocompatibility complex (MHC) signal (rs3821236 p=3.37×10-9, OR 1.30 [1.18-1.44]). Interestingly, Stat4$^{-/-}$ mice exposed to bleomycin developed reduced dermal fibrosis as compared to controls, as assessed by a 65 % decrease of dermis thickness, 68±5% decrease of hydroxyproline content and 71±6% decrease of myofibroblast numbers (Avouac et al., 2011). In addition, leukocyte infiltration was also reduced (63±5%) in skin lesions, as well as the pro-inflammatory cytokines production including TNF-α, IL-2 and IL-6. Thus, STAT4, which plays a major role in an inflammatory model of fibrosis, might represent a potential therapeutic target in SSc.

Two genes, PTPN22, encoding the protein tyrosine phosphatase called Lyp, and CSK encoding the c-src tyrosine kinase also called CSK, which modulates signaling through the T cell receptor (TCR), have been found to increase susceptibility to SSc. PTPN22 was one of the first susceptibility genes associated with several autoimmune diseases. Its main variant (rs2476601, 1858C>T, R620W) has been reported to be modestly associated with SSc (Gourh et al., 2006; Diaz-Gallo et al., 2011), particularly in patients with anti-centromere autoantibodies; however this association is attenuated by the fact that a significant proportion of patients with SSc also develop autoimmune thyroiditis, which is associated with polymorphisms of PTPN22. Furthermore, the genome wide association studies (GWAS) identified an association of a CSK variant (rs1378942) with SSc frequency which was unrelated to auto-antibody specificities (p=5.04 x 10-12; OR=1.20) (Martin et al., 2012). Recent study has also identified the TYK2 gene as a potential susceptibility gene in SSc through IL-12 pathway (López-Isac et al., 2016b)

Interestingly, in the 2 GWAS performed, CD247 (rs2056626; OR 0.86 [0.81 to 0.90] p=2.09×10−7), which encodes a subunit of the TCRζ (component of TCR/CD3 complex), was significantly associated with a risk of developing SSc (Radstake et al., 2010; Dieudé et al., 2011).

Several studies confirmed the major role of B cells in SSc. Thus, two variants (rs10516487 and rs3733197) of B cell-specific scaffold protein with ankyrin repeats (BANK1) gene, a promoter of the tyrosine kinase LYN phosphorylation, were found to be associated with dSSc (Dieudé et al., 2009b; Rueda et al., 2010), whereas another B lymphocytes specific gene, BLK (B lymphocyte kinase), which transduces the signal downstream of the BCR and is also involved in SLE (C8orf13-BLK region, rs13277113 and rs2736340) was associated with subgroups of lSSc and anti-centromere positive patients (Ito et al., 2010; Coustet et al., 2011).

The variant of TNFAIP3 (6q23) was also identified through the GWAS as a susceptibility factor for autoimmune diseases (Dieudé et al., 2010). TNFAIP3 is an intracellular protein that regulates negatively the NF-κB signaling pathway (by deubiquitination) downstream to the TNF-R super-family, TLRs, IL-1R and nucleotid-binding oligomerization domain protein 2 (NOD2) receptor. The rare G allele of rs5029939 was associated with SSc with an OR of 2.08 [1.59 to 2.72], p=1.16×10-7, with dSSc with an OR of 2.71 [1.94 to 3.79], p=5.2×10-9 and with pulmonary arterial hypertension with an OR of 3.11 [1.86 to 5.17], p=1.3×10-5.

Taken together, these data reinforce the autoimmune component in SSc, with evidence for involvement of different pathways contributing to autoimmunity in other diseases, although fine mechanisms contributing to the occurrence of SSc remain to be identified.

As for the vast majority of autoimmune disease, the association of human leukocyte antigen (HLA) locus with SSc has been identified many years ago. It has been clarified by working specifically on HLA class II from 1300 SSc patients from USA and 1000 controls (Arnett et

al., 2010b). The strongest associations with SSc in Caucasians and Hispanics were found for DRB1*1104, DQA1*0501, DQB1*0301, DQB1 alleles encoding a protein without leucine at position 26 (DQB126*epi). On the other hand, DRB1*0701, DQA1*0201, DQB1*0202 and DRB1*1501 alleles were protective. In a recent GWAS, several SNPs located in 6p21 in the MHC region were strongly associated with SSc, whereas the rs6457617 variant SNP located in the HLA DQB1 gene was strongly associated with SSc (p=2.31×10-18) (Allanore et al., 2011). These results are associated with a strong weight of ethnicity and heterogeneity of the disease.

In addition, many other alleles or haplotypes appear very specific to certain subgroups, particularly characterized by autoantibody status (Gorlova et al., 2011). Notably, DRB1*1101 and DPB1*1301 alleles have been associated with anti-topoisomerase I positivity when DRB1*0401-22 and DRB1*0801-11 alleles are associated with anti-centromere positivity (Gilchrist et al., 2001).

Genetics might help to identify new biomarkers that could allow better stratification of patients at risk of developing visceral manifestations and offering them targeted therapies before the occurrence of severe organ involvement. However, identified OR are often weak even if they combine several gene polymorphisms. Thus, new genetic approaches might probably be helpful in the near future to go further in this direction.

ii) Pathophysiology

Collagen accumulation in the dermis is one of the hallmarks of SSc. Extra-cellular matrix (ECM) accumulation is mostly the consequence of fibroblasts activation. *In vitro*, fibroblasts isolated from the involved skin of patient with SSc produce more collagen IV, proteoglycan and fibronectin than those obtained from the skin of healthy controls. Phenotypical changes of these fibroblasts have also been described, with the expression of alpha-smooth muscle actin (α-SMA), a marker of differentiation toward myofibroblast (Krieg et al., 2007). These

myofibroblasts are known to secrete more collagen and display vascular smooth muscle cell (SMVC) properties including migration phenotype. Distinctly from EC, which experience increased apoptosis, fibroblasts isolated from SSc patients have a defect in Fas/FasL induced apoptosis pathway which probably participates to the maintenance of the disease process (Santiago et al., 2001). Interestingly, a defect in metalloproteinase synthesis, which regulates formation of ECM, has also been reported (van der Slot et al., 2003).

In addition to intrinsic phenotype modifications of fibroblasts, extrinsic factors also contribute to explain excessive fibroblast activation in patients with SSc (**Figure 10**).

Transforming growth factor β (TGF-β) has been proposed as an utmost important factor in fibroblast activation (Denton and Abraham, 2001). Thus, fibroblasts phenotype modification in patients with SSc could be easily reproduced with incubation of normal fibroblasts with TGF-β (Smith and LeRoy, 1990). As in TGF-β-dependent experimental models, in patients with SSc, the expression of TGF-β receptors at the fibroblast surface has been found increased as compared to healthy control fibroblasts (Kawakami et al., 1998). This result, together with the observation of increased levels of endogenous TGF-β in lung and dermal SSc lesions suggests an over stimulation of this pathway, leading to fibroblast phenotypical modifications including over proliferation, ECM production and differentiation into myofibroblasts. In addition, the expression of endogenous inhibitors of TGF-β pathway, including Fli-1 and SMAD7, is also defective in SSc patient's fibroblasts (Chen et al., 1999; Bhattacharyya et al., 2005). However, the persistence of phenotypical modifications of fibroblasts from patients with SSc despite multiple passages, in vitro, and recent updates on epigenetic and DNA methylation mechanisms, suggest the existence of other mechanisms independent of TGF-β stimulation (Varga and Abraham, 2007).

In addition to TGF-β, IL-4, a cytokine secreted by T helper 2 (Th2) cells has been identified as a key cytokine in SSc. IL-4 express pro-fibrotic properties on fibroblasts and increased IL-4 production has been documented in the spleen of ROS-induced SSc mouse model as well as in the serum of patients with SSc (Postlethwaite et al., 1992). Interestingly, in bronchoalveolar fluid of SSc patients with interstitial lung disease, Luzina *et al.* have observed activated $CD8^+$ T lymphocytes that present an activated phenotype and expression of mRNA encoding IL-4 and latent TGF-β mRNA (Luzina et al., 2003). Another important local source of IL-4 could also be a subset of double positive CD4/CD8 T lymphocytes, present in dermal lesions of patients with SSc (Parel et al., 2007). In addition to IL-4, other growth factors have been identified in patients with SSc, including connective tissue growth factor (CTGF) or PDGF (Leask and Abraham, 2006).

Alternatively to these growth factors, autoantibodies may activate fibroblasts. Anti-fibroblast antibodies (AFA) are not specific to patients with SSc and could lead to fibroblasts activation by direct targeting surface adhesion molecules. Two studies notably reported that exposure of normal fibroblasts to AFA leads to the internalization of the antibody that could interact with caveolin pathway and induce the secretion of pro-fibrotic chemokines including CCL2, CXCL1, CXCL8, CKLF and ECGF1 (Chizzolini et al., 2002; Fineschi et al., 2008).

AFA directed at the PDGF receptor have been detected in patients with SSc. These autoantibodies have been reported to enhance in vitro the activation and pro-fibrotic phenotype of isolated fibroblast (Chizzolini et al., 2002; Svegliati et al., 2005). Unfortunately, these results remain controversial (Classen et al., 2009).

In patients with SSc, arterial and arteriolar hyperreactivity and myointimal proliferation can lead to vascular occlusion occurring in digital arteries, as well as in heart, lung and kidney arteries. The equilibrium of vascular endothelium in physiological conditions results from the

balance between vasodilator and vasoconstrictor molecules, as well as the expression of adhesion molecules at the surface of EC. In patients with SSc, the vascular tone is dysregulated, leading to perturbed interactions between EC, vascular smooth muscle cells (VSMC) and ECM components participating in vascular remodeling and occlusion (Bhattacharyya et al., 2012).

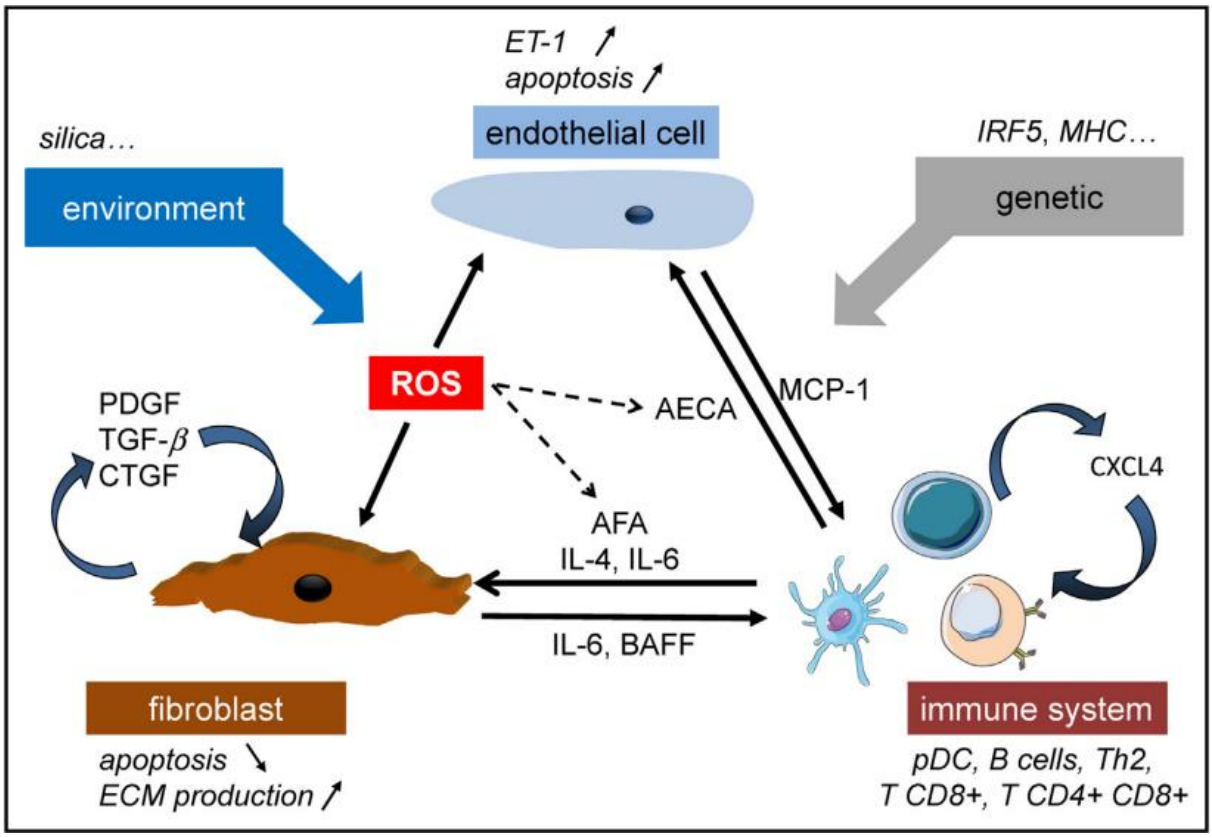


Figure 10. Physiopathology of systemic sclerosis – AECA: Anti-endothelial cell antibodies; AFA: Anti-fibroblast antibodies; BAFF: B-cell activating factor; CD: Cluster of differentiation; CTGF: Connective tissue growth factor; CXCL: Chemokine C-X-C motif ligand; ECM: Extra-cellular matrix; ET: Endothelin; IL: Interleukin; IRF: Interferon responsible factor; MCP: Monocyte chemotactic protein; MHC: Major histocompatibility complex; pDc: Plasmacytoid dendritic cell; PDGF: Platelet derived growth factor; ROS: Reactive oxygen species; TGF: Transforming growth factor; Th: T helper cell; (Dumoitier *et al.*, *Presse Med* 2014) **ANNEXE 1**

In patients with SSc, EC lesions are associated with increased plasma levels of factor VIII, decreased angiotensin converting enzyme production, increased activity of von Willebrand factor (vWf) and detection of circulating platelet aggregates (Kahaleh et al., 1981). At an early stage of the disease, EC involvement in SSc is characterized by increased apoptosis, loss of physiological barrier with permeabilisation of blood vessels and abnormal vascular tone regulation.

It has been proposed that EC activation and proliferation might occur as a consequence of the binding of anti-endothelial cell antibodies (AECA). Thus, in patients with SSc, AECA have been reported to induce EC apoptosis through antibody dependent cell cytotoxicity, involving NK cells and Fas/FasL pathway. However, although of interest, these in vitro data do not preclude the pathogenic role of these antibodies in vivo. In addition, AECA are not specific for SSc and can be found in various auto-immune diseases, binding to a relatively large number of EC antigens (Bordron et al., 1998). Finally, AECA failed to be identified in a number of experimental models of SSc including the Tight skin (Tsk)-1 mouse model, a genetic model of SSc. In the same line, the prevalence of antiphospholipid syndrome is very low in SSc, although anti-cardiolopin antibodies and increased vWf activity have been associated with the detection of PAH, suggesting that anti-cardiolipin antibodies could represent a marker of EC damage in PAH associated with SSc (Liberati et al., 2010).

Other mechanisms are involved in the perturbation of the vascular tone in patients with SSc. Thus, increased endothelin-1 (ET-1) levels, which are known to enhance vasoconstriction, inflammation, and mediate fibrosis and vascular remodeling, have been detected in the serum of SSc patients (Kahaleh, 1991). ET-1 is known to also activate fibroblasts and induce *in vitro* ECM production (Marsault et al., 1992). In patients with SSc, ET-1 levels have been shown to correlate with the severity of PAH. Moreover, ET-1 has been found to be increased in the lung at the mRNA and protein levels in patients with idiopathic PAH as compared to healthy

controls. In kidney biopsies from SSc patients who experienced scleroderma renal crisis, immunohistochemistry staining demonstrated the presence of ET-1 in glomeruli, arterioles and arcuate arteries, which was not the case in other vascular diseases involving the kidney (Mouthon et al., 2011). Interestingly, ET-1 receptors (ETR) inhibitors, including bosentan, ambrisentan and more recently macitentan, have demonstrated efficacy in the treatment of idiopathic PAH and PAH associated with SSc (Kovacs et al., 2012). In addition, defective prostacyclin synthesis and perturbed NO synthesis have also been reported to a possible key elements to explain EC dysfunction in patients with SSc (Schachna and Wigley, 2002).

Finally, vascular epidermal growth factor (VEGF) levels have been reported to be decreased in patients with SSc as compared to healthy control (Distler et al., 2004). Increased synthesis of MCP-1 and VCAM-1 by EC could also increase the recruitment of lymphocytes and promote the local and systemic inflammation in patients with SSc (Denton et al., 1998).

Thus, both fibroblasts and endothelial cells participate in the activation of innate and adaptive immune cells which could induce specific clinical and immunological presentation of SSc.

iii) Clinical and immunological presentation

First classification of SSc criteria in the 1980' were developed in patients with longstanding SSc (Masi and Subcommittee For Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980). However, according to the broader spectrum of SSc clinical manifestation, including skin thickening (measured using the modified Rodnan Skin Score (mRSS), puffy finger, digital ulcers, telangiectasia, abnormal naifold capillaroscopy, PAH, interstitial lung disease, Raynaud's phenomenon or auto-antibodies, a new table of criteria was published in 2013 that would enable identification of individuals with SSc (**Figure 11 and Table 2**) (van den Hoogen et al., 2013).

Moreover, common classification allow clinicians to discriminate between: limited cutaneous SSc (lcSSc) defined by skin thickening in areas solely below to the elbows and knees, with or without facial involvement and diffuse SSc (dSSc) defined by the presence of skin thickening proximal, as well as distal, to the elbows and knees, with or without facial or truncal involvement (LeRoy et al., 1988).

Both subtypes of SSc evolved differentially as patients with dSSc present rapid and dramatic succession of severe symptoms (pulmonary fibrosis, renal involvement) and increased mRSS at the early stage of the disease with a decrease of mRSS and improvement of patients after this early stage. On the other hand, lcSSc patients present a really progressive disease without a “peak” of disease activity. However, patients with lcSSc also showed severe presentations of the disease usually a decade after SSc diagnosis.

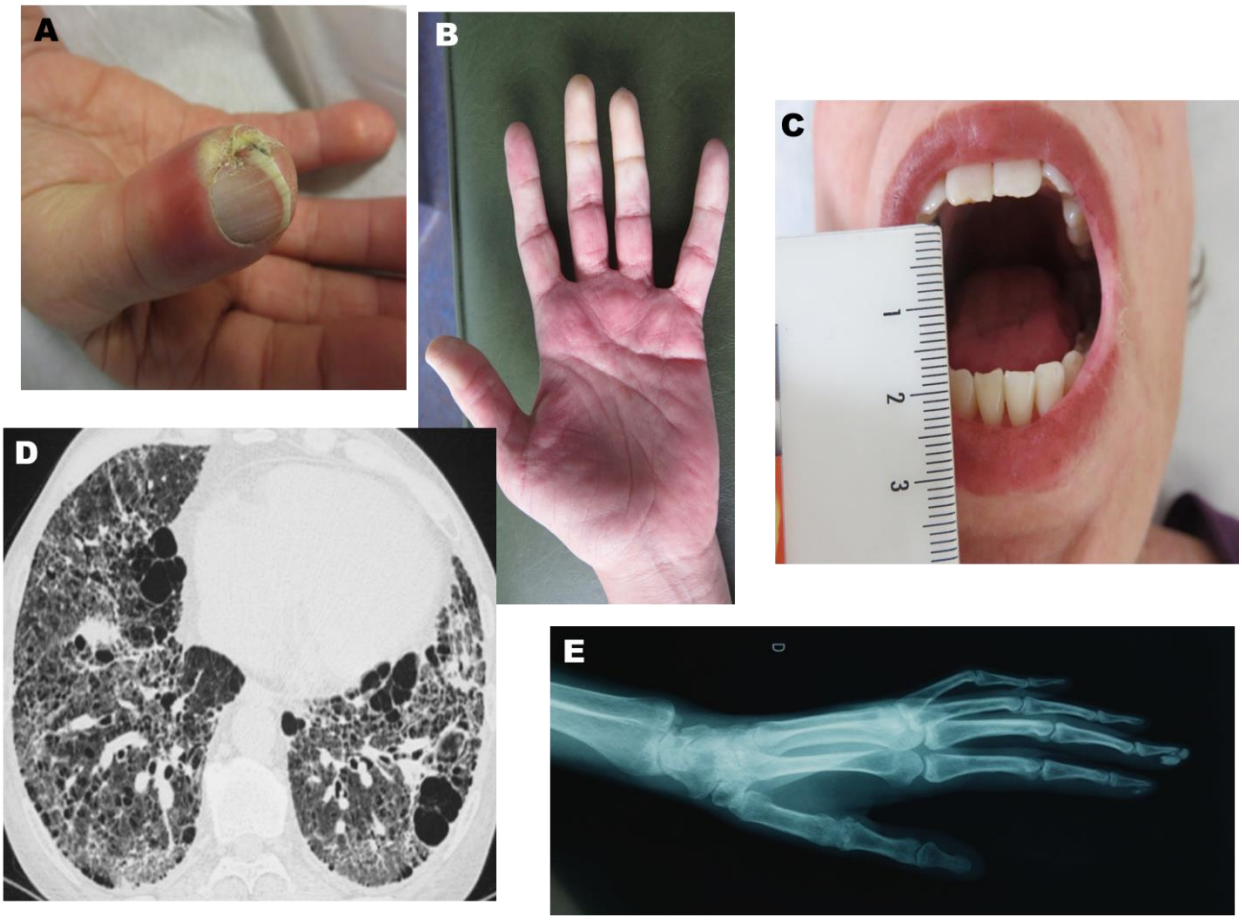


Figure 11. Clinical manifestations during systemic sclerosis: Superinfected digital ulcer (Panel A), Raynaud's phenomenon (Panel B), limitation of mouth opening (Panel C), computed tomography scanner showing pulmonary fibrosis (Panel D), left hand Xray with calcinosis of the pulp of the third phalange of the third finger (Panel E) – kindly provide by Pr. L. Mouthon

In addition to EC and fibroblasts, the immune system plays an important role in the pathogenesis of SSC. Thus, autoantibodies, imbalance in B and T lymphocytes subpopulations and perturbations of dendritic cells have been reported in SSc. Although the disease cannot be qualified as autoimmune, since autoantibodies have not been shown to induce/transfer the disease, the identification of specific autoantibodies is of major help in the diagnosis and/or the evaluation of the prognosis of SSc .

The distribution of autoantibodies in SSc patients correlates with phenotypes. Thus, anti-topoisomerase 1, anti-RNA polymerase III and anti-U3 RNP correlate with dSSc, while anti-centromere, anti-Pm/Scl, anti-Th/To and anti-U1 RNP are associated with lSSc. Various other antibodies have also been found in SSc patients, directed against several targets such as fibrillin-1, metalloproteinases or PDGF receptor.

Major criteria	Sub-criteria (only count the higher score)	Weight/Score
Skin thickening of the fingers of both hands extending proximal to metacarpophalangeal joints		9 (sufficient criterion)
Skin thickening of the fingers	Sclerodactyly	4
	Puffy fingers	2
Fingertip lesion	Fingertip pitting scars	3
	Digital tip ulcers	2
Telangiectasia		2
Abnormal capillaroscopy		2
PAH and/or ILD		2
Raynaud-s phenomenon		3
SSc-related autoantibodies		3

Table 2. ACR/EULAR new criteria classification for systemic sclerosis: Patients with a total score of ≥ 9 are classified as having definite systemic sclerosis (SSc) - ILD: Interstitial lung disease; PAH: Pulmonary arterial hypertension - adapted from (van den Hoogen et al., 2013a, 2013b)

These autoantibodies argue for a pathogenic role of B cells (Kraaij and van Laar, 2008). Thus, circulating B cells from SSc patients differ in their phenotype as compared to healthy controls, with increased proportions of naïve B cells and decreased numbers of memory B cells and plasma cells (Sato et al., 2001). CD19 and CD21, two activation co-receptors of the B-cell receptor (BCR), are overexpressed in these naïve and memory B cells. The activation receptors

CD80, CD86 and CD95 are upregulated on memory B cells, suggesting their participation to the pathogenetic process.

High levels of B cell activating factor (BAFF) have been measured in the serum of patients with SSc, together with an overexpression of BAFF-R at the surface of peripheral B cells from SSc patients (Hasegawa et al., 2006). BAFF activates the NF-κB pathway, promotes B cells survival and participates to the differentiation of autoreactive B cells. In addition, it has been documented that B cells infiltrate the dermis of SSc patients (Sunderkötter and Riemekasten, 2006; Chizzolini, 2008), that circulating levels of IL-6 are increased in SSc patients as compared to healthy controls and correlated with the extent of skin fibrosis. In Tsk-1 mouse, the depletion of B cells leads to a decrease of total IL-6 mRNA and improvement of fibrotic lesions (Sato et al., 2001). Finally, IL-6 is known to stimulate collagen secretion by fibroblasts and represent a potential “B cell link” to fibroblast activation.

T cells are also involved in the pathogenesis of SSc. Thus, a Th2 bias has been documented in SSc patients (Chizzolini, 2008). Increased levels of IL-4 and IL-13 have been measured in the serum of SSc patient. These two cytokines have been classically described as synergic Th2 polarization cytokines via STAT-6. Although the trigger contributing to the initiation of secretion of these cytokines is not identified, it has been documented that mature and activated Th2 secrete IL-4, IL-6 and IL-13, enhancing a positive loop of Th2 polarization and B cells stimulation. Interestingly enough, the treatment of Tsk-1 mice with an anti-IL-4 monoclonal antibody prevented the induction of skin lesions (Ong et al., 1998), underlining the major role this cytokine in SSc physiopathology.

In addition to B and T lymphocytes, dendritic cells appear of utmost importance in SSc. Thus, a recent proteome-wide analysis of the culture supernatant of plasmacytoid dendritic cells (pDC) isolated from patients with SSc allowed identification of increased levels of CXCL4,

with higher levels in diffuse than in limited forms, and increased levels in early diffuse vs late diffuse SSc patients (van Bon et al., 2014). Interestingly, CXCL4 was also detected in the skin of SSc patients and not in healthy control skin. Serum levels of CXCL4 correlated with clinical status of SSc patients. Notably, in patients with lung fibrosis and/or PAH, high levels of CXCL4 correlated with a poor prognosis. Finally, delivery of CXCL4 leads to increased leukocytes infiltration, skin thickening and CCL2 mRNA expression in the bleomycin-induced model of SSc. Taken together, these observations argue for a possible role of CXCL4 in the pathogenesis of SSc. Further studies will aim at confirming the interest of targeting CXCL4 in SSc patients.

iv) Prognosis

More than half patients with systemic sclerosis finally die from the disease (Denton, 2015). However, systemic sclerosis is one of the most heterogeneous disease with internal organ manifestations occurring in both dSSc and lcSSc while the kinetic of the two subforms is quite different. Thus, dSSc patients present a dramatic decrease of overall survival at the early stage of the disease with increased occurrence of interstitial lung diseases and renal crisis compared to patients with lcSSc. In contrast, patients with lcSSc will finally show pulmonary arterial hypertension and renal complications. Thus, prognosis of patients with SSc is highly correlated to clinical outcomes. For instance, patients developing renal crisis (5 to 10% of all) have a mortality of 30 to 40% while patients presenting associated PAH have a median survival inferior to 2 years (Denton et al., 2009; Le Pavec et al., 2010).

v) Treatment

Several approaches targeting respectively fibroblast activation pathway, endothelial cell activation and immune system have been tested in patients with SSc.

PDGF can be targeted by an inhibitor of the PDGF receptor, imatinib, a serine-proteine kinase receptor inhibitor, that has been developed in the treatment of chronic myeloid leukemia (Barst,

2005; Ghofrani et al., 2005). Imatinib binds to c-Abl, a signaling protein downstream of the TGF-β receptor, and blocks both TGF-β and PDGF receptor. Because of promising results in the bleomycin induced model of SSc (Aono et al., 2005), clinical trials investigated the efficacy of imatinib in patient with SSc (Sabnani et al., 2009; Spiera et al., 2011). However, the results were disappointing in terms of tolerance, with a large proportion of dropouts and poor efficacy (Mouthon, 2012).

Immune suppression using cyclophosphamide is also classically used in patients with active systemic sclerosis. However, novel therapeutic strategies have been proposed in experimental models of SSc and fibroblasts from patients with SSc in culture including inhibitors of Src kinase family, Rho associated kinases, Fos related antigen-2 (Fra2) or histone acetylases and DNA methyl transferases(Bagnato et al., 2013; Maurer et al., 2013). Several of these strategies will be investigated in the treatment of patients with SSc in the near future.

Direct targeting of TGF-β in SSC, using a monoclonal antibody anti-TGF-β (Frexolimumab) has been recently evaluated in a limited number of patients (Rice et al., 2015). Improvement of skin fibrosis, decreased myofibroblast infiltration together with decreased SERPINE1 and CTGF in the skin of treated patients suggested promising results of this therapeutic. However, the importance of the TGF-β in both immune and metabolic systems do not allow a larger clinical trial.

Taken together, targeting B cells (anti-CD20), IL-6, IL-4 and also T cells and mature B lymphocytes (anti-CD52) may leads to substantial clinical improvement in mouse models and in open series of patient with SSc (Isaacs et al., 1996; Ong et al., 1998; Stratton et al., 2001; Khanna et al., 2016). These findings argue for the important role of B and T cells in the pathogenesis of SSc.

Toward targeted therapies, autologous hematopoietic stem cell transplantation have been recently evaluated in patients with diffuse systemic sclerosis (Laar et al., 2014). Promising results have been published in a limited number of cases with important benefits for patients compared to cyclophosphamides treatment. However, the important issue of bone marrow transplantation impact significantly the development of this treatment in SSc.

D. Pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) constitutes a heterogeneous group of clinical entities sharing similar pathologies that have been subcategorized as idiopathic pulmonary arterial hypertension (IPAH), familial PAH, pulmonary hypertension (PH) associated with other diseases such as connective tissue diseases, (for instance systemic sclerosis), portopulmonary hypertension, and PH related to human immunodeficiency virus (HIV) infection, drugs, and toxins (Simonneau et al., 2004).

All subsets of patients received specific treatments regarding the origin of the disease. For instance, blood vessels dilatators, endothelin receptor antagonists and high dose calcium blockers are particularly used. However, if medical treatment is not efficient, an open-heart surgery might be an option for an atrial septostomy or, in several cases, a lung or heart-lung transplantation can be used.

Regarding inflammation processes, patients with PAH also present increased activation of both vascular endothelial cells and fibroblasts (Tuder et al., 1994). Similar secretion of collagen as observed in SSc was described in patients with connective tissue disease associated with PAH (Dorfmüller et al., 2007). Furthermore, increased releases of PDGF and EGF were also detected which participate to disease progression and severity (Humbert et al., 1998; Schermuly et al., 2005). Finally, endothelin pathway is critical in patients with PAH as it works as a powerful

vasoconstrictor and mitogen for smooth muscle cells which mediates the vasoconstriction of pulmonary artery (Ehrenreich et al., 1990; Galié et al., 2004).

In a subset of patients, particularly those with IPAH, PAH associated with connective tissue diseases or HIV infection, inflammation plays a pivotal role in the development and maintenance of the vascular remodelling. Thus, several autoantibodies, together with increased levels of pro-inflammatory cytokines have been detected in serum from patients with IPAH and connective tissue-PAH (Isern et al., 1992; Humbert et al., 1995; Le Pavec et al., 2010). Moreover, innate immune cells also secrete higher levels of chemokines such as CX3CL1 and RANTES which could participate in the recruitment of activated B and T cells (Balabanian et al., 2002; Dorfmüller et al., 2002).

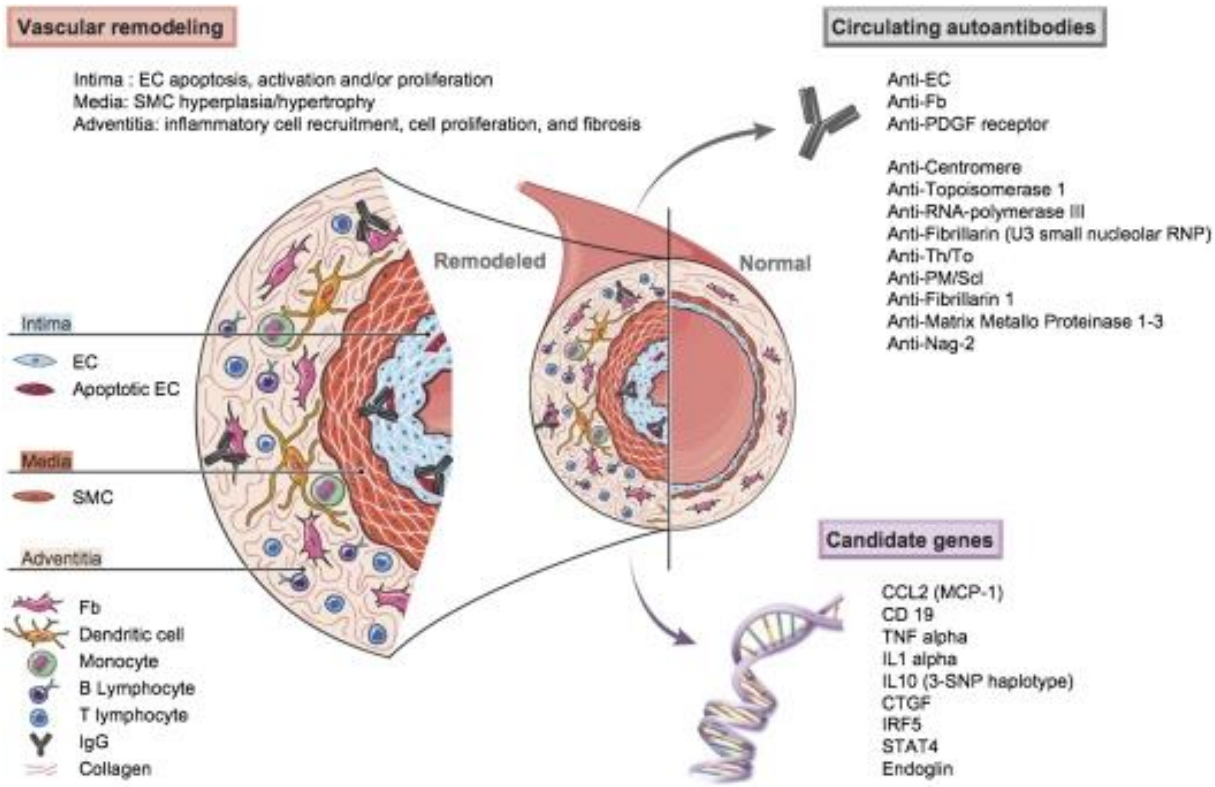


Figure 11. Summary of pulmonary arterial hypertension (PAH) pathophysiology associated with connective tissue diseases – CCL: CC chemokine ligand; CD: Cluster of differentiation; CTGF: Connective tissue growth factor; EC: Endothelial cell; Fb: Fibroblast; Ig:

Immunoglobulin; IL: Interleukin; IRF: Interferon responsible factor; MCP: Monocyte chemotactic protein; Nag: Novel antigen; PM/Scl: polymyosite/scleroderma; RNA: Ribonucleid acid; RNP: Ribonucleoprotein; SMC: smooth muscle cell; SNP: Single nucleotide polymorphism; STAT: Signal transducers and activators of transcription; TNF: Tumor necrosis factor; from (Le Pavec et al., 2010)

However, for IPAH and PAH associated with connective tissue diseases, the role of three cytokines have been particularly investigated recently: IL-1, IL-6 and TNF-α. Thus, both IL-6 and TNF-α levels in the serum of patients with PAH correlate with quality of life (Matura et al., 2015). Moreover, IL-6 and IL-1 have been found implicated in both endothelial cells and immune cells activations resulting in increased disease progression (Groth et al., 2014; Hashimoto-Kataoka et al., 2015). Concerning IL-6, recent updates have described a new trans-activation pathway of IL-6/IL-6R in endothelial cells in patients with SSc suggesting critical role of this cytokine in PAH (Jasiewicz et al., 2015). Moreover, IL-6 seems to work in synergy with endothelial-derived fibroblast growth factor 2 inducing pericyte activation and enhancement of smooth muscle-like cells in PAH (Ricard et al., 2014).

2. B lymphocytes in autoimmunity

A. Physiological B cell lymphopoïesis

The incidence and diversity of chronic inflammatory diseases are increasing worldwide while the complexity of clinical symptoms and the need for new therapies that target inflammation, while not compromising the immune system, are improving. B lymphocytes are major actors of the adaptive immune system. These lymphoid cells expand from hematopoietic stem cells *via* a lineage specific and antigen independent differentiation process in the bone marrow. This maturation process is under the control of several growth factors and cytokines among which, IL-7 or Stem cell factor (SCF). The cells undergo the recombination of immunoglobulin gene

(Ig) variable regions and a selection process that allow finally the membrane expression of a B cell antigen receptor or BCR on immature naïve B cells. These cells are exposed to selection and to tolerance mechanisms. A second major step of activation takes place in secondary lymphoid organs through an antigen-dependent mechanism responsible for the selection and the maturation of mature B cells that finally undergo a final differentiation step in antibody secreting cells (or plasma cells) or in memory B cells. Finally, in several inflammatory or pathological contexts, structures responsible for such differentiation mechanisms take place in tissues and are so called tertiary lymphoid structures (TLS).

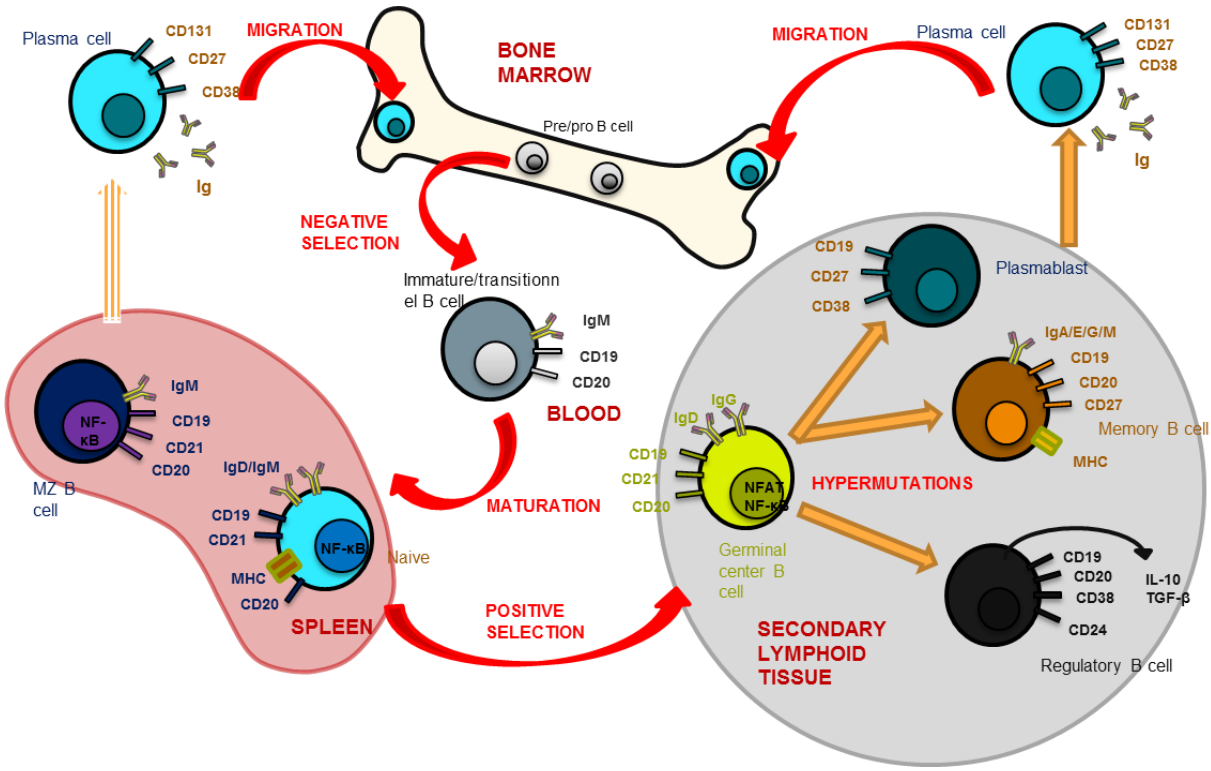


Figure 13. General scheme of B lymphopoiesis. The main organs and maturation steps of B-cell maturation. CD: cluster of differentiation; Ig: immunoglobulin; IL: interleukin; MHC: major histocompatibility complex; MZ B-cells: marginal zone B-cells; NFAT: nuclear factor of activated T cells; NF-κB: nuclear factor kappa B; TGF-β: transforming growth factor beta – adapted from (Dumoitier et al., 2015)

Since the differentiation of B lymphocytes can be divided into successive steps and results in several control checkpoints, the next chapters will describe the critical steps of B lymphocyte homeostasis. Indeed, a number of leukaemia and autoimmune diseases have been associated to alterations of such process of B cell maturation.

a) Central B lymphopoiesis

B lymphopoiesis corresponds to the early development phase of B lymphocytes derived from hematopoietic stem cells. It is achieved by the final expression of an immunoglobulin M at the membrane. Pro-B lymphocytes (also considered as B cell progenitors) maturation and survival is highly associated with stromal cell interplay. The bone marrow stromal cells express specific growth factors leading to the expression of successive transcription factors within hematopoietic stem cells: PU.1, Ikaros, E2A, EBF (early B cell factor) and PAX-5.

PU.1 is an important regulator of the development of hematopoietic stem cells and of the induction of Ikaros. Together, these two transcription factors trigger the transition into lymphoid precursors. Thus, E2A, EBF and PAX-5 regulate the development of pro-B lymphocytes by the extinction of Notch-1, the T cell progenitor transcription factor. Furthermore, E2A expression is associated to the expression of the Recombination-activating gene (RAG)-1 that plays a critical role in immunoglobulin gene rearrangements. Finally, Pax-5 that is essential for the initiation and the maintenance of pre-BCR and BCR signalling is constitutively expressed through the following steps of maturation toward final plasma cells differentiation.

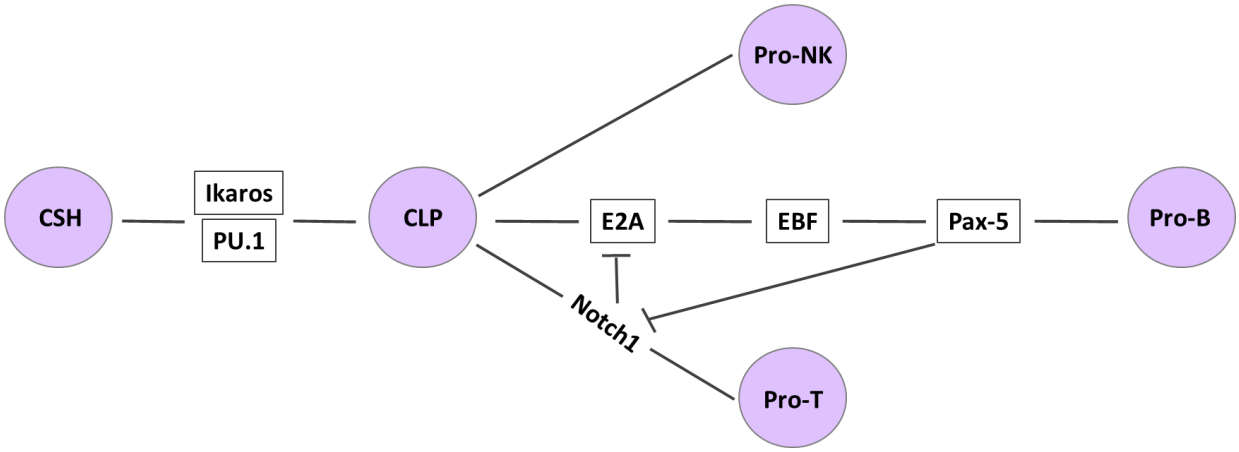


Figure 14. Early development of human B lymphocytes in the bone marrow – CLP: common Lymphoid progenitor cell; CSH: Hematopoietic stem cell; EBF: Early B-cell factor; E2A: E2A immunoglobulin enhancer-binding factors E12/E47; Pax-5: Paired box protein-5 - adapted from (Busslinger *et al.*,2004.)

In parallel to the expression of the previously described transcription factors, pro-B lymphocytes undergo the rearrangement process of heavy (IgH) chain immunoglobulin genes, first between D and J segments followed by V-DJ recombination. When the recombination is functional one specific μ heavy chain is expressed and the other recombination are blocked. Bone marrow niches express various cytokines and growth factors such as IL-7 and induce the expression of the Terminal deoxynucleotidyl Transferase (TdT) together with RAG-1 and RAG-2. Both enzymes are required for the recombination process of IGHV and, TdT adds a random number of nucleotides increasing the variability of the immunoglobulin expressed before the entry into the pre-B lymphocyte stage.

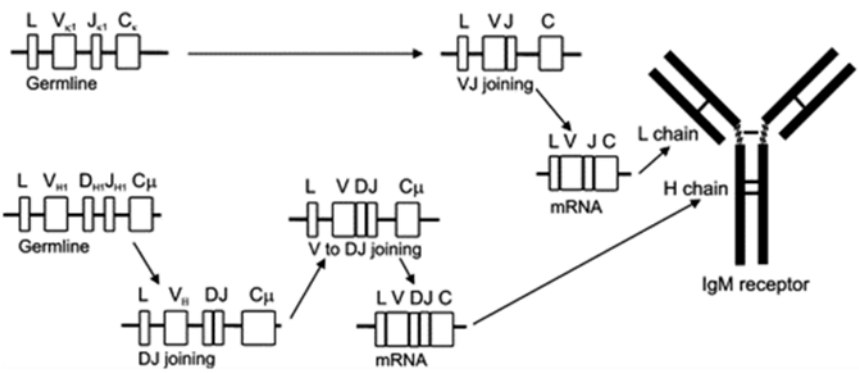


Figure 15. V(D)J rearrangement of immunoglobulin genes; Ig: Immunoglobulin; mRNA: Messenger ribonucleic acid; (adapted from Ollila *et al*., 2004)

Intracellular μ heavy chain is associated with the surrogate light chain composed of λ5 light and VpreB chains and with two signalling subunits Igα and Igβ chains (CD79a and b), already present since the pro-B cell differentiation stage, to form the pré-BCR complex. This pre-BCR complex is expressed at the membrane and can interact with bone marrow stromal cells, notably through galectins, leading to the stimulation of the signalosome, essential for the maintenance of B lymphocyte survival. Differentiation into pre-B cell is also characterized by the expression of CD19 and CD20 an important co-activator of the antigen receptor and a ionic canal for calcium in B cells, respectively. The pre-BCR expressed in pre-B cells undergoes further recombination process at the κ or λ □□□□□ that will form the definitive light chain and replace the surrogate light chain in immature B cells. If the first rearrangement leads to an efficient BCR, all the Ig genes present on the second chromosome undergo an allelic exclusion to eliminate the possibility to express a second Ig. At this step again, BCR ligation induces upregulation of RAG genes involved in the light chain rearrangement toward the formation of immature naïve B cells (Hertz and Nemazee, 1997). Therefore a fully recombined and active antigenic receptor (IgM) is expressed at the membrane and new surface markers are expressed such as CD23, CD22 or CD40. This stage of differentiation is also characterized by an

important negative selection mechanism which takes place to control normal B lymphocyte development and acquire a self-tolerant repertoire. The mechanism of receptor editing allows rearrangement at the V locus that modifies the specificity of the BCR when a self ligand is recognized. When cells still recognize self antigens they get inactivated via the mechanisms of anergy or enter the apoptotic process.

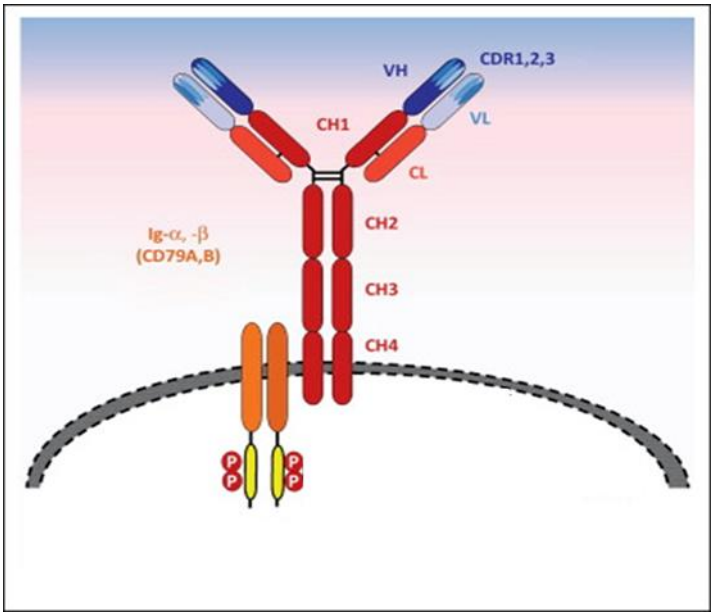


Figure 16. Structure of the B-cell receptor (BCR)–CD: Cluster of differentiation; CDR: Complementary determined region; CH: Constant heavy chain domain; CL: Constant light chain domain; Ig: Immunoglobulin; VH: Variable heavy chain domain; VL: Variable light chain domain - adapted from (Burger and Chiorazzi, 2013)

Several autoimmune diseases characterized by the presence of autoreactive B cells and autoantibodies are associated to the loss of regulation during these late steps of B lymphocyte ontogeny (Eisenberg, 2012). However, *de novo* peripheral editions of the BCR can also induce the generation of autoreactive B cells after lymphocytes have left the bone marrow and enter the peripheral circulation.

b) Peripheral lymphopoiesis

Following their exit from the bone marrow, immature naïve B lymphocytes enter the peripheral circulation, in both blood and lymph. The cells are highly responsive to B cell activating factor (BAFF) and TLR ligand binding both of them inducing survival and activation of the cells (Rowland et al., 2010; Hua and Hou, 2013).

Via these circulations, B lymphocytes enter into secondary lymphoid organs, particularly lymphoid follicles of spleen and lymph nodes, and recognize non-degraded antigens presented by specific antigen-presenting cells (Harwood and Batista, 2010). Follicular dendritic cells present within these follicles secrete high amounts of CXCL13 able to attract B lymphocytes expressing their receptor CXCR5 and present the non-degraded antigens. In presence of several costimulatory signals, notably complement opsonisation, CD154 ligation and cytokines such as IL-4 and IL-21, antigenic recognition by the BCR induces the migration of B lymphocytes into the germinal center (Garside et al., 1998; T et al., 1AD). Interaction with T follicular helper (Tfh) cells results in proliferation and differentiation of immature B cells into germinal center B cells expressing high levels of CD40 and IL-21R (Spolski and Leonard, 2010). During this activation process, two major mechanisms are initiated: somatic hypermutations and isotype class-switching.

- Generation of B lymphocytes able to produce high-affinity antibodies occurs through the accumulation of mutations in the variable (V) regions of rearranged immunoglobulin (Ig) genes (Weigert et al., 1970). Reactivation of TdT and other enzymes has been shown to play a pivotal role in somatic hypermutation in the germinal center (Bross et al., 2000; Papavasiliou and Schatz, 2000; Diaz and Casali, 2002). The alternative cycles of mutations and combined increased proliferation of resulting B cells, induce the production of a number of hypereactive clones of B lymphocytes while non-specific clones or those with a weaker affinity are eliminated as they cannot survive without a positive BCR signalling selection. No clear link has been made between somatic

hypermutation and autoimmunity while this step is clearly implicated in the development of lymphoma (Dunn-Walters et al., 2001). However secondary organs are still sites for a negative selection process: B lymphocytes that recognize self Ag present in the periphery but absent in the BM are eliminated.

Isotype switching occurs rapidly after activation of B lymphocytes and corresponds to the modification of the heavy chain of the BCR, primarily IgD and IgM transform into IgA, IgG or IgE (Stavnezer and Schrader, 2014). The switch improves the ability and the efficiency of an antibody to remove the pathogens that induce the humoral response. Without this recognition, B cells, which have not encounter any antigen, are eliminated progressively. Briefly, IgE are particularly efficient against parasites and activate both basophils and mast cells; IgA are efficient for mucosal immunity; IgM induce an important complement activation and IgG present higher affinity and avidity to their antigens. The switch is initiated by the activation-induced cytidine deaminase (AID) which promotes deletional recombination.

Following generation of hypermutated clones and isotype class-switched mature B lymphocytes, B cells evolve into two distinct subsets: plasmablasts and then plasma cells which produce secreted antibodies or, memory B cells which participate in the maintenance of a secondary response to previously recognized antigens. Phenotypically, plasmablasts express high levels of CD27 and CD38 and become plasma cells when they acquire CD138 expression and lose CD20 expression which explain the maintenance of autoantibody titter in patients treated with an anti-CD20 monoclonal antibody. Memory B cells acquire CD27 expression but remain mostly in the spleen, bone marrow and lymph nodes where they can encounter their antigen.

Unfortunately, if allelic exclusion, negative selection in bone marrow and BCR editing in bone marrow and in secondary lymphoid structures eliminate most of autoreactive B cells, loss of tolerance mechanisms might lead to evasion of some clones that become pathological.

B. Alterations in B lymphocytes lymphopoiesis

a) Pathological antibodies

As mentioned before, autoimmunity is often associated to the expression of autoantibodies. B lymphocytes repertoire allows the theoretical recognition of a wide range of antigens. However, this astonishing diversity also leads to a possible recognition of self-antigens inducing therefore autoimmunity. In fact, in a physiological context, 55 to 75% of immature B cells are polyreactive and recognize, at least partially, one self-antigen (Wardemann et al., 2003). However, only 5% of the population presents with an autoimmune pathology, which demonstrate the existence of regulatory mechanisms to eliminate or control autoreactive B cells production. Thus, one of the previously described checkpoints, has been bypassed inducing a rupture of tolerance.

In the bone marrow, mice deficient for the Bcl-2 interacting mediator of cell death (BIM) produce spontaneously increased levels of anti-dsDNA in a SLE-like model (Enders et al., 2003). Indeed, BIM is engaged during the V(D)J recombination process and allows the elimination of cells with higher affinity for auto-antigens present at the central system.

In the germinal center, an absence of Fas/FasL signalling is associated with increased autoimmune symptoms in both human and mouse suggesting that this pathway is highly implicated in the elimination of autoreactive clones upon somatic hypermutation process (Wang et al., 1996; Strasser et al., 2009). Moreover, dysregulation of Tfh cells might also be associated with loss of tolerance and increased activation of Tfh has been observed in patients with SLE

(Craft, 2012)(Sawaf et al., 2016). Thus, the Roquin$^{san/san}$ mouse model has shown that excess of activated Tfh induces the formation of spontaneous germinal center and SLE-like syndrom with production of anti-DNA antibodies together with a glomerulonephritis and immune complex deposits (Linterman et al., 2009). Also, at the germinal center level, aberrant activation of AID has been found in patients with autoantibodies suggesting an important role of this enzyme in the control of normal germinal center response and humoral immunity (Zaheen and Martin, 2011).

The B-cell activation factor (BAFF) and A proliferation-inducing ligand (APRIL) are two pro-B cells cytokines that play critical role in several models of SLE and notably the NZB/NZW F1 and the MRL/lpr mice models. This specific point will be discussed bellow. If the presence of auto-reactive B cells is a key event in the manifestation of autoimmune diseases, it is not the exclusive event. Indeed, a major part of the auto-antibodies is not pathogenic such as the anti-SSA in the Sjogren syndrom. However, several antibodies, like anti-platelets in immune thrombocytopenic purpura or anti-dsDNA in SLE or the ANCA in ANCA associated vasculitis, are clearly linked to disease pathogenesis and progression. These differences suggest a differential interplay between B-cells and their environment regarding the specificity of the autoantigens and the immunological context. This could be explained by both *in situ* differentiation and by the antigens specificity.

b) Tertiary lymphoid structure

Autoreactive plasmocytes producing autoantibodies are localized in both bone marrow or in secondary lymphoid organs like the spleen but also in tertiary lymphoid structures (TLS).

In systemic autoimmune diseases, such as rheumatoid arthritis and Sjögren's syndrome, target tissue infiltration by B cells correlates with disease activity and progression (Guellec et

al., 2015; Mo et al., 2011). In GPA patients, B cells infiltrating granulomatous lesions might be activated. Voswinkel and colleagues performed the first BCR sequencing for biopsies of endonasal lesions of active GPA patients (Voswinkel et al., 2008). These authors and others suggested an *in situ* activation and selection of B cells in contact with PR3 at the surface of neutrophils. In another study, a correlation was found between the importance of B cell infiltrates within granulomatous lesions in GPA and the occurrence of relapses, in spite of the peripheral B cell depletion in some patients treated with rituximab (Ferraro et al., 2008). Interestingly, in a cohort of patients with AAV, including GPA and MPA patients, TLS have been identified in the tissues involved (Steinmetz et al., 2008); notably, in glomerulonephritis lesions. They were composed of a germinal center containing follicular dendritic cells and memory and non-memory B cells at the periphery of the germinal center, in contact with T cells.

CXCL13, a B cell specific chemokine, was detected in TLS and CXCR5, the CXCL13 receptor, was expressed by infiltrated B lymphocytes (Mueller et al., 2014). Similar results were found regarding CCL21, expressed by perivascular stromal cells, and CCR7, expressed by B and T lymphocytes infiltrating ectopic lymphoid structure (Mueller et al., 2014). .Zhao et al. also investigated the expression of CCR10 at the surface of circulating and tissue infiltrating B cells. However, they found no difference of expression, suggesting a chemokine specific mechanism for the migration of B cells within nasal granulomatous lesions in GPA.

c) Autoantigens

In SLE, autoreactive plasmocytes have been observed in bone marrow, spleen and kidney (Hiepe and Radbruch, 2016). Moreover, specific anti-dsDNA plasmocytes have been found preferentially in the kidney of NZB/W mice model of SLE (Hoyer et al., 2004; Hutloff et al., 2004). Furthermore, the level of IgG anti-dsDNA is positively correlated with the amount of

plasmocytes in kidney of these mice and disease severity suggesting a critical role for these plasmocytes *in situ*. These results also suggest a possible differentiation directly in the inflamed tissue under propitious microenvironment.

These data are supported by the previous detection of increased levels of BAFF in the serum of active GPA patients as compared to healthy donors (Krumbholz et al., 2005). Detection of B-cell Activating Factor of the Tumor Necrosis Factor (TNF) Family (BAFF) has also been reported in endonasal lesion of GPA patients. These results support the potential activation of B cells both in peripheral blood and tissue lesions, possibly through BAFF-BAFF-R interaction resulting in increased survival and activation of B cells and emergence of autoreactive B-cells. Morevover, ANCA activated neutrophils secrete increased amounts of BAFF *in vitro*, suggesting a potential crosstalk between neutrophils and B lymphocytes in patients (Holden et al., 2011a).

d) Soluble factors associated with B cell activation

i) Multiple roles of Interleukin-6 in inflammation

Interleukin (IL)-6, one of the first cytokines identified, has recently been recognized as a potential actor in inflammatory diseases (Rincon, 2012; Tanaka and Kishimoto, 2012). This cytokine originally described as a marker of inflammation is now considered as an essential agent controlling inflammation.

IL-6, initially designated as a B cell differentiation factor, is representative of the redundancy and pleiotropic activities of cytokines, particularly in inflammation (Hirano et al., 1986). First reports have shown that IL-6 is produced by monocytes and macrophages immediately after stimulation of Toll-like receptors (TLRs) with distinct pathogen-associated molecular patterns (PAMPs), in the early phase of infection-linked inflammation (Kishimoto, 2005b). Then, IL-6

was found to be produced by a number of immune and non-immune effector cells, including T and B cells, fibroblasts, keratinocytes, mesangial, glial, endothelial cells as well as many tumour cells (**Figure 17**). Moreover, IL-6 can induce strong release of a broad spectrum of acute-inflammation proteins, such as C-reactive protein (CRP), serum amyloid A (SAA), fibrinogen or hepcidin (Heinrich et al., 1990).

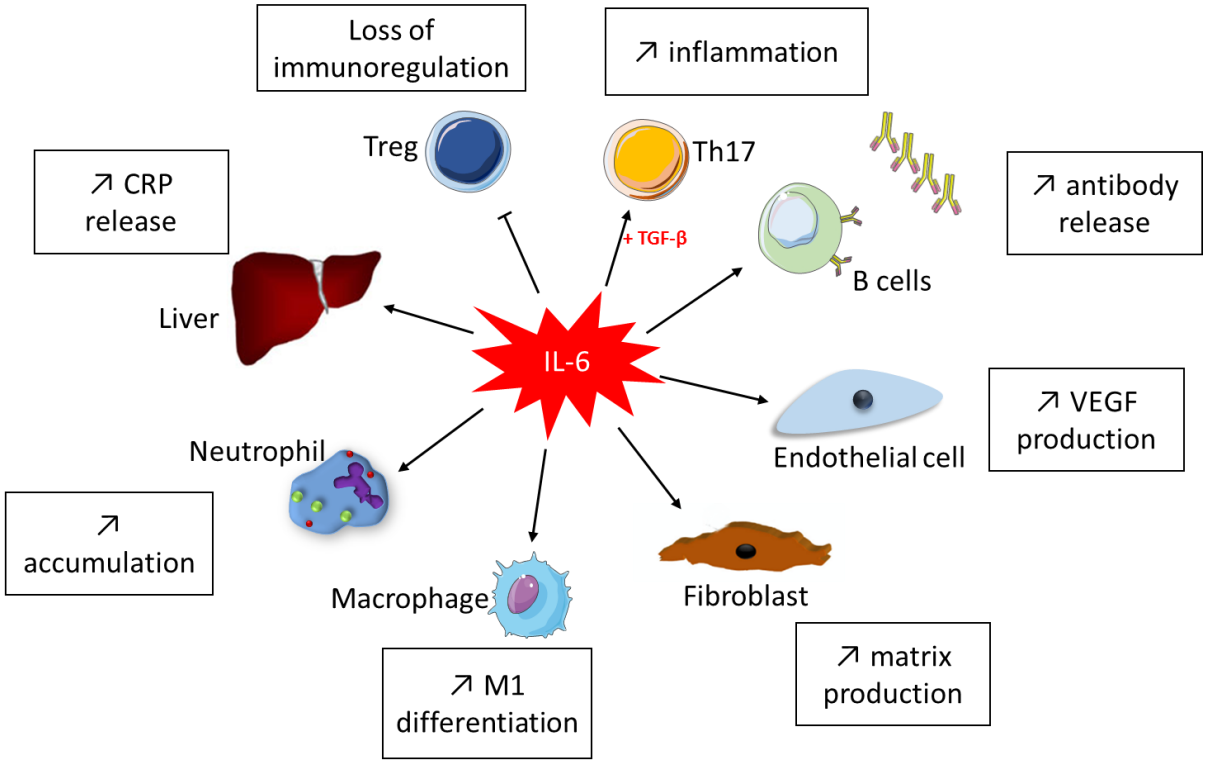


Figure 17. Overview of Interleukin (IL)-6 impacts in the inflammation context – CRP: C-reactive protein; M1: Macrophage 1; TGF-β: Transforming growth factor beta. Th: T helper cell; Treg: regulatory T cell; VEGF: Vascular endothelial growth factor – adapted from (Rincon, 2012)

In addition to this stress and the transient acute response to infection or aggression of the host, IL-6 plays a pivotal and deleterious role in the development of chronic inflammation diseases (Akira et al., 1993; Kishimoto, 2005b). Excessive or persistent production of IL-6 is clearly associated with the development of arthritis, Castleman disease or hyper γ-globulinemia (Yoshizaki et al., 1989; Hirano et al., 1986; Ohshima et al., 1998). Nowadays, numerous IL-6

impacts on immune cells have been reported demonstrating the broad spectrum of modifications that could be induced by IL-6 dysregulation (**Table 2**)

Table 2. Summary of immunological and clinical impacts of Interleukin (IL)-6 *in vivo* - adapted from (Ho et al., 2015)

Major targets	Main effects
T-helper cells	Negatively regulates Th2 response
	Inhibition of TGF-β-induced Treg differentiation
	Together with TGF-β promotes differentiation of Th17 cells from naïve T cells
	In combination with IL-1β, TGF-β, and IL-23 allows polarization of naive Vγ9Vδ2 T cells to produce IL-17
Hematopoietic system	Controls proliferation and differentiation of the progenitor cells in many hematopoietic lineages
	Controls leukemic multipotent progenitor cell fate and regulates the development of chronic myelogenous leukemia
	Promotes or prevents lymphoma development by acting at distinct stages of hematopoietic development
	Induces activation of T and B cellsas well as hematopoiesis and lupus-like autoimmune manifestations in Lyn-deficient mice
Nervous system	Is required for myelin oligodendrocyte glycoprotein-induced experimental autoimmune encephalomyelitis
	B cell-specific IL-6 regulated Th17 responses in experimental autoimmune encephalomyelitis
	Maintains plasmablast-like B cells survival and increases autoantibody production in patients with neuromyelitis optica
	Induces $CD4^+$ T follicular helper cell-differentiation program and exacerbates autoimmune reaction
Cardiovascular system	Is constitutively produced in cardiac myxomas
	Aggravates the severity of autoimmune myocarditis induced by cardiac α-myosin peptide immunization
	Increases the severity of angiotensin II-induced cardiac fibrosis
	Is protective in viral-induced autoimmune myocarditis
Respiratory system	Exacerbates pleural exudation and polymorphonuclear cell infiltration in immune-mediated lung damage
	Increases death rate in acute pancreatitis-associated acute lung injury in mice
	Induces Th17 cell differentiation within the lung and causes lung damage
Liver	Induces production of a variety of acute phase proteins

Major targets	Main effects
	Protects liver from damage in concanavalin A-induced T cell-mediated hepatitis
	Is protective in liver injury induced by bacterial hepatotoxin stimulation or partial hepatectomy
	Down-regulates expression of liver cytochrome P450 3A4 enzyme
Gastrointestinal system	Prevents apoptosis of intestinal mucosal T cells in Crohn's disease
	Protects wound healing from colon injury by inducing epithelial proliferation
Kidney	Increases severity and inflammatory cell infiltration in kidney in lupus-prone mice
	Activates trans-signaling pathway to exacerbate immune-mediated kidney damage in Lyn-deficient mice
	Upregulates proliferation of macrophages that accumulate and cause damage in the kidney

CD: Cluster of differentiation; TGF-β: Transforming growth factor beta; Th: T helper cell; Treg: regulatory T cells

A major role of IL-6 was demonstrated in the T cell polarization toward Th9 or Th17 depending on the cytokines present in the microenvironment (Omenetti and Pizarro, 2015; Schütze et al., 2016). Furthermore, IL-6 inhibited the polarization of T cells into Treg, bypassing the regulation by TGF-β and resulting in a loss of immunoregulation. IL-6 production has also been associated to the microenvironment remodelling with an increase of matrix production by fibroblast together with an increase proliferation and migration, particularly during chronic inflammation (**Figure 18**). Endothelial cells also can be stimulated by IL-6secretion resulting in an increased VEGF release which induces hyper-vascular activation *in situ*.

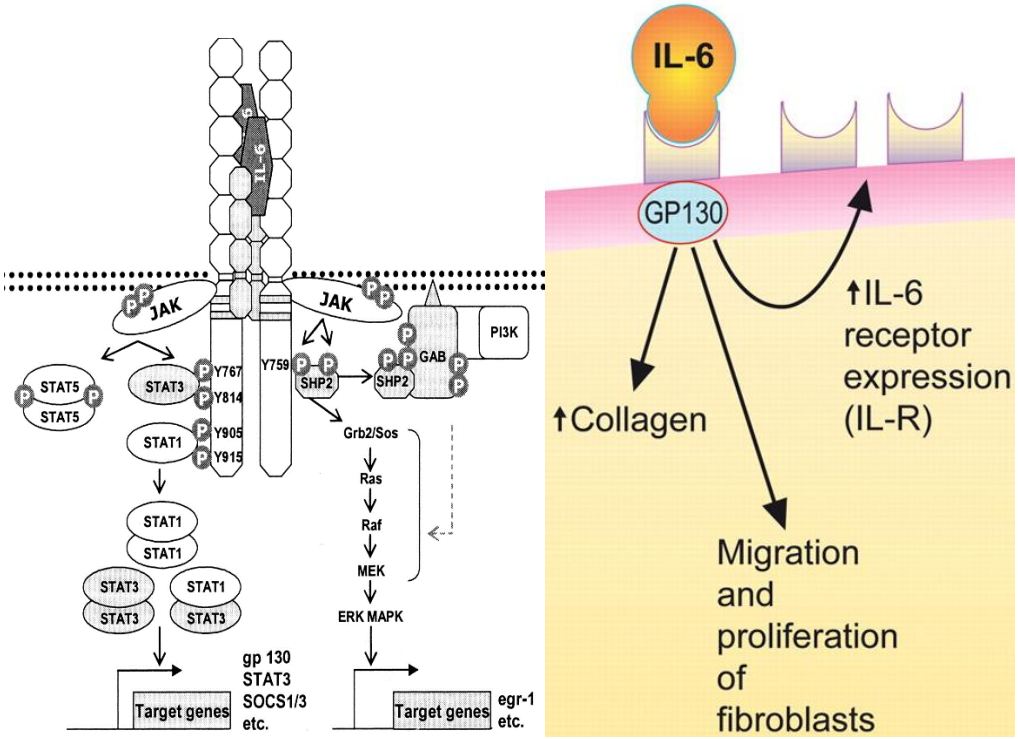


Figure 18. IL-6 activation pathways in fibroblasts – JAK: Janus activation kinase; SHP: small heterodimer partner; STAT: Signal transductor and activator of transcription - adapted from (Kosmidou et al., 2002)

vi) BAFF and APRIL:

The B-cell activation factor (BAFF) and A proliferation-inducing ligand (APRIL), are two member of the TNF cytokine family. These cytokines are commonly secreted by neutrophil, dendritic cells and other innate immune cells (Scapini et al., 2008). The first function of BAFF and APRIL is to induce the survival of B lymphocytes during the maturation process of B cells through BAFF-R and TACI, respectively (Schneider, 2005). Moreover, several studies have demonstrated the role of BAFF and APRIL in the induction of antibody production at the late stage of differentiation by B lymphocytes (Matsushita and Sato, 2005; Bosello et al., 2007). Also, stimulation by these two cytokines triggers increased secretion of pro-inflammatory cytokines by B cells together with decreased apoptosis level (Mackay and Schneider, 2009). Since a number of autoimmune diseases presents with a wide range of auto-antibodies and

dysregulation of B lymphocytes, many studies have addressed the role of BAFF and APRIL in autoimmunity.

In SLE, many studies have evaluated BAFF and APRIL roles in the stimulation of B cells and the induction of autoantibody secretion (Groom et al., 2007; Treml et al., 2007). High serum levels of BAFF preferentially support the survival of B cells and induce class switching of autoreactive cells that recognize nucleic acids (Stohl et al., 2003; Groom et al., 2007). Moreover, basophils, cells with an emergent role in SLE, have been described as a potential important booster of lupus through the secretion of IL-4 and BAFF (Davidson and Diamond, 2010). Thus, two phase III clinical trials have been conducted in 2010-2011 and showed that addition of anti-BAFF monoclonal antibody (belimumab), results in better outcome for patients with moderately active SLE (Merrill et al., 2010; Navarra et al., 2011). These results led to the US Food and Drug Administration (FDA) approval for belimumab in SLE.

In AAV, increased production of BAFF has been reported in neutrophils from patients with GPA, which could contribute to the maturation of B cells particularly *in situ* where, in close contact with neutrophils, B cells could be educated to link the PR3 (Scapini et al., 2008).

e) B cell signalling abnormalities in autoimmunity

Following BCR stimulation, SRC family kinases are activated, leading to the phosphorylation of immune receptor tyrosine activation motifs (ITAMs) of the CD79ab (Ig-α, Ig-β) complex (Vargas et al., 2013). Similarly, PI3K is activated to catalyse the conversion of membrane-associated PIP2 to PIP3 leading to BTK recruitment to the plasma membrane through the interaction of its PH domain with PIP3 (Tomlinson et al., 2004). Concomitantly, the phosphorylation of Ig-α and Ig-β ITAMs leads to the recruitment of SYK kinase via SYK SH2 domains. Following activation, BTK initiates multiple downstream pathways generating

pleiotropic effects: PLCγ activation, generation of second messengers, such as inositol-triphosphate (IP3), diacylglycerol (DAG) and calcium flux, cell proliferation, differentiation, apoptosis or survival (Reth and Brummer, 2004).

To date, almost only B cell-derived tumour cells have been treated with the newly developed inhibitor of BTK, ibrutinib. The rationale for this, is that lymphoma cells, similarly to non-transformed B cells, may be dependent on BCR signalling for their survival. Although animal models of autoimmunity indicate very promising outcomes after ibrutinib treatment, the drug has not yet been used in important clinical trials in human (Chang et al., 2011). However, one study has shown promising results for ibrutinib in Waldenström's macroglobulinemia (Treon et al., 2015).

Several other studies have described signalling abnormalities of various kinases such as increased activation of SYK in SLE, rheumatoid arthritis or immune thrombocytopenic purpura (Iwata et al., 2015a, 2015b). Interestingly, fostamatinib, an inhibitor of SYK has also been effective in a phase III clinical trial with active rheumatoid arthritis patients showing an improvement of ACR20 response over placebo (Weinblatt et al., 2014).

C. B lymphocytes: pro-inflammatory cells?

a) Pro-inflammatory secretions of B lymphocytes

In autoimmune conditions, B cells can produce IL-6, IL-21 and TNF-α, notably under the influence of type I interferon (IFN-α and IFN-β) or TLR ligands (Rieckmann et al., 1997; Yehudai et al., 2012). These cytokines exert deleterious effects within inflammatory lesions, especially in rheumatoid arthritis for which patients have large improvement upon anti-TNF-α blocking agents. Interestingly, IL-6 and TNF-α have been reported as increased in the serum of patients with GPA and MPA (Muller Kobold et al., 1999; Jónasdóttir et al., 2001).

In AAV, TNF-α is known to increase the expression of PR3 at the surface of neutrophils (Csernok et al., 1994). Thus, Reumaux *et al.* reported the increased binding of ANCA to PR3 expressed on neutrophil surface after priming with TNF-α (Reumaux et al., 2006). Interestingly, triggering by TNF-α also activates NADPH oxydase in neutrophils, suggesting a possible amplification of the immune response against neutrophil and maintenance of inflammation in AAV patients (Kettritz et al., 2002). Two open studies suggested a beneficial effect of infliximab, a chimeric monoclonal anti-TNF-α antibody in GPA patients refractory to cyclophosphamide (Bartolucci et al., 2002; Booth et al., 2004). However, this finding was not confirmed in the maintenance of remission in a prospective randomized study using etanercept, a soluble dimerized TNF-α receptor (Silva et al., 2011).

B-cell depletion therapy using rituximab demonstrated decreased IL-6 and TNF-α serum levels in rituximab-treated patients compared to glucocorticoid-treated patients in AAV (Monach et al., 2013), suggesting that B cells could be an important source of pro-inflammatory cytokines in AAV. However, evidences are still lacking whether these inflammatory cytokines are directly produced by B cells.

b) Regulatory B cells

Following the peak of inflammation, the hallmark of an effective immune response is the recruitment or the expansion of immune cells with immunosuppressive functions that leads to a correct resolution of inflammation. If unresolved, this inflammatory response causes injury to the host tissues leading to the development of autoimmune pathologies (Medzhitov, 2008). In order to keep a healthy balance, the immune system needs to control the activity of these numerous components in a very precise way.

For years, only T lymphocytes have been proposed to correctly suppress hyperactivation of the immune system notably in the context of infection, allergy or transplantation. Thus, one of the first immunosuppressive functions reported for T cells was the homeostatic control of antibody responses by B cells (Moore et al., 2001). Regulatory T cells (Treg) exert notably their suppressive effects by producing immunoregulatory cytokines such as transforming growth factor beta (TGF-β), Interleukin (IL)-10 or IL-35. These cytokines are able to increase T lymphocyte anergy, induce Treg expansion and on the other hand to decrease T helper (Th)1 and Th17 differentiation and downregulate the expression of costimulatory molecules including CD80, CD86 and CMH class II, which affect the ability of antigen presenting cells (APCs) to activate T cells (Moore et al., 2001).

Secretion of these cytokines has been associated with the expression of the transcription factor Forkhead box P3 (Foxp3) in Treg together with higher expression of CD25 (Ohkura et al., 2013). Recent reports highlighted the relationship between expression of Foxp3 and IL-10 signaling as FoxP3 acts as a cotranscription factor of STAT3 in association with epigenetic modifications of IL-10 promoter leading to a ‘‘conditional’’ gain of function of FoxP3 (Hossain et al., 2013). Also, Foxp3+ and Foxp3- precursor cells might allow the development of peripheral IL-10-expressing Treg cells by a mechanism dependent on TGF-β but independent of IL-10 showing a significant contextual cross-talk between IL-10 and TGF-β (Maynard et al., 2007). These findings argue for a complex interplay between IL-10, TGF-β and Foxp3 in Tregs.

Recent updates have highlighted the emergent role of B cells in the secretion of both IL-10 and TGF-β and thus in Treg expansion in several autoimmune models. Thus, due to our better understanding of the mechanisms of Treg emergence, maintenance and suppressive effects, the unilateral suppressive effect of T cells over B cells has been eluded and replaced by the notion of a complex modulation interplay (Rosser and Mauri, 2015).

A first report on impairment of T cell response by regulatory B cells (Bregs) was described in 1974 where a delayed hypersensitivity response was observed (Katz et al., 1974). However, only the study on absence of B cells in experimental autoimmune encephalomyelitis (EAE) mouse model in 1996 has proved the necessity of B cells to negatively regulate hyperactivation of the immune system, responsible for chronic diseases (Wolf et al., 1996). Following this report, various teams have identified what is now characterized as Bregs, a heterogeneous population of B lymphocytes that suppress effector T cell responses (Rosser and Mauri, 2015).

The absence of a common phenotype for Bregs led to multiple debates in the scientific community with a lack of reproducible data in all inflammatory models or diseases. This lack of constant phenotype focused new researches on the immunosuppressive mechanisms of these cells. **Table 3**.

Unfortunately, phenotypes of mouse and human Bregs are still not fully defined and very heterogeneous regarding the pathological background or the tissue. Blair *et al.* reported the identification of $CD24^{hi}$ $CD38^{hi}$ B cells with regulatory functions and IL-10 production in SLE patients (Blair et al., 2010), this phenotype was also found in lymphoma patients and in artherosclerosis (Ait-Oufella et al., 2010). Furthermore, in mice, other markers have also been associated with Bregs phenotype, such as upregulation of CD1d, CD5 or CD22 (Yanaba et al., 2008).

Multiple studies have demonstrated the ability of Bregs to produce and secrete IL-10 that leads to suppress inflammatory responses.

Table 3. Phenotypes of human Interleukin (IL)-10 producing regulatory B cells (Breg) subsets

Subtype	**Mechanism of suppression**	**Phenotype**	**References**
B10 cells	IL-10 production	$CD24^{hi}CD27^{+}$	Horikawa et al., 2013 Yanaba et al., 2008
Plasmablasts	IL-10 production	$CD19^{+}CD24^{hi}CD27^{int}$	Matsumoto et al.,2014
Immature cells	IL-10 production	$CD19^{+}CD24^{hi}CD38^{hi}$	Blair et al.,2010
Br1 cells	IL-10 production	$CD19^{+}CD25^{hi}CD71^{hi}$	Van de Veen et al., 2013
B10 : IL-10 producing B cells ; Br1 : regulatory B cell 1 ; CD : Cluster of differentiation			

If IL-10 was the only commonly used marker of Bregs, the presence in normal amounts of these cells in most of autoimmune or inflammatory models suggest also IL-10 independent pathways for the regulation by Bregs that could be impaired in these contexts. Recent reports demonstrated novel immunoregulatory function for unclassical Bregs that secrete or express other immunosuppressive molecules.

The TGF-β1 is another immunosuppressive cytokine, known to promote T cell survival by inhibiting activation-induced cell death and to block their proliferation by inhibiting IL-2 production via Smad3 (Li et al., 2006). This factor also promotes Treg while inhibiting Th1 and Th2 development via the blockage of the transcription factors required for expression of IFN-γ (T-bet) and IL-4 (GATA-3) (Heath et al., 2000; Horwitz et al., 2003).

Several studies have shown that IL-10 and TGF-β1 cooperate to downregulate immune responses especially within regulatory T cells (Levings et al., 2002). The production and action of these two cytokines are interrelated and involve a positive feedback loop in which each cytokine enhances the expression of the other. Indeed, lymphocytes from mice deficient for IL-10 produce less TGF-β1 and exogenous IL-10 was shown to stimulate TGF- β1 production in vitro (Seder et al., 1998; De Winter et al., 2002). IL-10 not only enhances the production of TGF- β1, but can also control the ability of the cells to respond to the cytokine (Cottrez and Groux, 2001). These findings therefore revealed an interrelation between TGF-β1 and IL-10 in immune tolerance.

A subset of B-cells secreting TGF-β1 named Br3, has been described for the first time by Lotz*et al.* in 1994. TGF-β1 is produced by both CD5+ and CD5- B cells from autoimmune-prone NZB mice (Douglas et al., 1997) and is also expressed by normal human B cells. High levels of TGF-β1 secretion by ex vivo LPS stimulated splenocytes has been associated with their ability to down-regulate pathogenic responses in autoimmune diabetes through an induction of CD4+ apoptosis (Tian et al., 2001). Thus, this TGF-β1 production has been correlated with the Bregs suppressive function. Indeed, TGF-β1 is critical for the induction of Tregs (Chen et al., 2003). Singh *et al.* demonstrated *in vitro* that Bregs could induce Foxp3 expression by activated T cells in a TGF-β1-dependent manner (Singh et al., 2008) .Taking together, TGF-β1 may have an important role in Bregs control of autoimmunity (Mauri and Ehrenstein, 2008).

There are also descriptions of human IL-10 and TGF-β1 producing $CD25^{hi}CD27^{hi}CD86^{hi}CD1d^{hi}$ B lymphocytes able to suppress autologous T cell proliferation and induce the generation of $FoxP3^{+}CTLA4^{+}$ Tregs in a cell-to-cell contact-mediated and TGF-β1 dependent mechanism (Kessel et al., 2012). In contrast to IL-10, TGF-β1 promotes apoptosis of $CD5^{+}$ B cells (Vitale et al., 2010) and could act as a negative regulator of B10 cells.

Interestingly, TGF-β1 is well known to transform naive T-cells into inducible Tregs (Chen et al., 2003; Marie et al., 2005) enhancing the regulatory environment, but, in combination with IL-6, TGF-β1 could skew naive T-cells toward IL-17-secreting cells and push the balance towards effector T-cells (San Segundo et al., 2013). These similarities between Treg and Bregs led the researchers to investigate other T cells cytokines with regulatory functions that could be secreted by B cells.

Besides TGF-β1 and IL-10, which have been described for years, new regulatory cytokines have emerged in the B and T cells interface. Conversely to IL-12 and IL-23, which have been associated with chronic inflammatory diseases, IL-35, a member of the same cytokine family, was recently discovered and linked to autoimmune diseases (Vignali and Kuchroo, 2012). Similarly to other signals described in this review, IL-35 biological activity was first observed in Treg with decreased expression of pro-inflammatory cytokines by activated T cells in an infectious context (Collison et al., 2007) while, IL-35 producing B cells seem critical cells in autoimmune diseases (Shen et al., 2014). This specific subpopulation of Bregs represents an interesting overlapping population which secrete both IL-10, TGF-β1 but also Granzyme B (GrzB) (Shen et al., 2014; Wang et al., 2014). Morevover, this population presents with a plasmocyte-like phenotype like several other Bregs subpopulation already described (Rosser and Mauri, 2015).

Table 4. Phenotypes of B cells associated with regulatory function

Mechanism of regulation/suppression	**Subtypes**	**Species**	**Models**	**Phenotypes within CD19$^+$**	**References**
IL-10 secretion	B10 cells Br1 cells Plasmablasts Immature B cells Fas-L$^+$Breg GrzB$^+$Breg IL-35$^+$Breg	Human, mouse	EAE, RA, CHS, islet allograft, solid cancer, SLE, MS, AAV, HIV, healthy controls	CD1d^{hi}CD5$^+$ CD24^{hi}CD38^{hi} CD25^{hi}CD71^{hi} CD24^{hi}CD27^{int} CD24^{hi}CD38^{hi}*PD-L1*$^+$ CD5$^+$*Fas-L*$^+$ CD5$^+$CD38$^+$CD1d$^+$IgM$^+$CD147$^+$CD86$^+$ CD154$^+$CD25$^+$IDO$^+$*GrzB*$^+$ B220^{low}CD5$^+$CD1d^{hi}*IL-35*$^+$	Horikawa et al.,2013; Van de Veen et al.,2013 Matsumoto et al.,2014 Blair et al.,2010;
TGFβ1 secretion	Br3 iBreg	Human, mouse Human	Islet transplant CLL Healthy controls	TIM1$^+$ CCR6$^+$CXCR3$^+$*IL10*$^+$ IDO$^+$ -	Lee K.M et al.,2014 Lotz M et al.,1994 Lee JH et al., 2011
IL-35 secretion	IL-35$^+$Breg	Mouse	EAE	B220^{low}CD5$^+$CD1d^{hi}*IL-10*$^+$	Shen, Nature, 2014 Cai, Lupus 2015
Granzyme B secretion	GrzB$^+$Breg	Human	SLE, MS, HIV, solid cancer, kidney transplantation	CD5$^+$CD43$^+$CD86$^+$CD147$^-$IL-10$^-$ CD5$^+$CD27$^+$CD138$^+$CD38$^+$IgD$^-$ CD5$^+$CD38$^+$CD1d$^+$IgM$^+$CD147$^+$CD86$^+$ CD154$^+$CD25$^+$IDO$^+$*IL-10*$^+$	Blood 2006 JI 2009
Fas-L expression	Fas-L$^+$Breg	Human Mouse	DLBL, CLL, MM SLE, RA, diabetes, allergy, transplantation	CD5$^+$*IL-10*$^+$	
TRAIL expression	TRAIL$^+$Breg	Human	DLBL, CLL	*Fas-L*$^+$*?*	Sedger LM 2010 Blood
PD-L1 expression	Immature B cells	Human	Kidney transplantation	CD24^{hi}CD38^{hi}*IL-10*$^+$	Khan 2015, Nature Comm
CTLA-4 expression	CTLA-4$^+$ B cells	Human	AAV	-	

AAV:ANCA associated vasculitis; Breg: regulatory B cells; CD : Cluster of differntiation ; CHS: Contact hypersensitivity response; CLL: Chronic lymphocytic leukemia; CTLA-4: Cytotoxic T-lymphocyte-associated protein 4; DLBL: Diffuse large B cell lymphoma, EAE, Experimental autoimmune encephalomyelitis, GrzB: Granzyme B; HIV: Human immunodeficiency virus; IDO: Indoleamine-pyrrole 2,3-dioxygenase; Ig: Immunoglobulin; IL: Interleukin; MM: Multiple myeloma, MS, Multiple sclerosis, PD-L1: Programmed death ligand 1 ; RA: Rheumatoid arthritis; SLE: Systemic lupus erythematosus; TGF : Transforming growth factor ; TRAIL: TNF-related apoptosis-inducing ligand

IL-35 is able by its own to induce differentiation of both B and T cells in Bregs and Tregs respectively, through Stat-1 and Stat-4 activation (Choi et al., 2015; Egwuagu et al., 2015).

Furthermore, secretion of IL-35 by Tregs and Bregs has been found highly correlated to EAE, multiple sclerosis, SLE and IBD activity and to the response to treatment (Cai et al., 2015; Fonseca-Camarillo et al., 2015). Interestingly, recent publications in animal models have shown that depletion of IL-35 does not result in loss of tolerance (Choi et al., 2015). These findings argue for a powerful balance in B cells between IL-6/TNF-α on one side and IL-10/IL-35 on the other, which could play a key role in both chronic inflammation and autoimmune diseases (Shen and Fillatreau, 2015).

Toward cytokine secretions, regulatory functions of B cells have also been found associated with the expression of several membrane markers inducing cell-death like PD-L1, CTLA-4 or Fas-L, together with Granzyme B and TRAIL expression. These findings argue for multiple suppressor mechanisms notably in autoimmunity (**Table 4)**.

AIM OF THE STUDY

Aim of the Study

Autoimmune vascular diseases are characterized by the production of autoantibodies by activated B cells. A number of studies has addressed the characterization of this specific production and therapies, targeting B cells, have demonstrated promising strategies in several diseases such as ANCA associated vasculitis. In contrast, several other pathologies indicate resistance to these treatments while the activation of B cells is demonstrated by its implication in vascular remodelling, inflammatory mechanisms and fibrosis related to the disease. In AAV patients, activation of innate immune cells is accompanied by important amounts of different B cells in granulomatous lesions. These B cells are activated and their BCR have been rearranged. Clinical trials have demonstrated that, in AAV patients, Rituximab (anti-CD20) is as efficient as cyclophosphamide in the primary attack treatment of AAV. Despite treatment efficacy, no consistent study has analysed the phenotype of these B lymphocytes and characterized their promising role in vascular and systemic inflammation.

In systemic sclerosis (SSc) patients, recent studies have also highlighted the potential pathogenic role of B lymphocytes that can activate fibroblasts by increasing collagen production (François et al., 2013). Up to now, the deleterious effect of B lymphocytes from patients with SSc has not been has not been. Moreover, one could hypothesize that B lymphocytes might be differentially activated depending on the heterogeneity of the disease and the severity of clinical manifestation as pulmonary arterial hypertension (PAH) or interstitial lung disease.

Therefore, the aim of this study was to provide comprehensive and comparative analysis of both phenotype and function of features B lymphocytes in three diseases characterized by an

important inflammation and vascular remodelling: ANCA associated vasculitis, systemic sclerosis and pulmonary arterial hypertension associated with systemic sclerosis.

RESULTS

Results

Paper 1: Phenotypical and functional analysis of peripheral B and T lymphocytes in granulomatosis with polyangiitis and microscopic polyangiitis

Paper 2: Peripheral B lymphocytes secrete both interleukin 6 and transforming growth factor-beta and potentiate fibroblasts activation in systemic sclerosis

Préliminary results: Analysis of B cell activation in idiopathic pulmonary arterial hypertension

Paper 3: Basophils are activated and promote fibrosis through TGF-ß secretion in systemic sclerosis

1. Paper 1: Skewed peripheral B and T lymphocytes compartments in patients with ANCA associated vasculitis

Will be submitted before thesis defense

Skewed peripheral B- and T-cell compartments in patients with ANCA-associated vasculitis

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Conflicts of interest:

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Abstract

Objective: To characterize lymphocytes dysregulation in patients with granulomatosis with polyangiitis (GPA) or microscopic polyangiitis (MPA).

Methods: Using flow cytometry, we analyzed B- and T- cell subsets in peripheral blood from 37 patients with active disease (29 GPA and 8 MPA) and 22 healthy controls (HC).

Results: GPA patients had increased Th2 (1.8*vs.*1.0%, p=0.02), Th9 (1.5*vs*.0.3%, p=0.0007) and Th17 (1.4*vs.*0.9%, p=0.03) cells compared to HC. $FoxP3^{low}/CD25^{+}$ nonregulatory T cells were significantly higher in GPA (5.8*vs*.4.0%, p=0.04) and MPA (17.9*vs*.4.0%, p=0.04) *vs.* HC, whereas Tregs did not differ between patients and HC.

Patients with anti-MPO anti-neutrophil cytoplasm antibodies (ANCA) had significantly more $CD21^{-}$ B cells than HC or PR3-ANCA patients (6.9*vs*.3.3% and 4.4%, p=0.01). CD69 expressing B-cells were significantly higher in GPA and MPA (3.0 and 5.9 *vs*.1.4%, p=0.02 and p=0.03, respectively) compared to HC, whereas BAFF-R expression was decreased in GPA and MPA (MFI ratio 11.8 and 13.7 *vs.*45.1 in HC, p<0.0001 and p=0.003, respectively). Finally, IL-6-producing B cells were increased in GPA *vs.* HC (25.8*vs.*14.9%, p<0.0001) and decreased in MPA *vs*. HC (4.6 *vs*.14.9%, p=0.005), whereas TNF-α-producing B cells were lower in both GPA and MPA patients compared to controls (15 and 8.4 *vs*.30%, p=0.01 and p=0.006, respectively).

Conclusion: Skewed T-cell polarization toward Th2, Th9 and Th17 responses characterizes GPA, whereas B cell populations are dysregulated in both GPA and MPA with an activated phenotype and decreased BAFF-R expression. Finally, inflammatory B cells producing IL-6 are dramatically increased in GPA providing an additional mechanism by which rituximab could be effective.

Abbreviations

AAV: ANCA-associated vasculitis

ANCA: anti-neutrophil cytoplasm antibodies

BAFF: B-cell activating factor

BAFF-R: BAFF receptor

BCR: B-cell receptor

c-ANCA: cytoplasmic ANCA

EGPA: eosinophilic granulomatosis with polyangiitis

ENT: ear nose and throat

GPA: granulomatosis with polyangiitis

HC: healthy control

IL: interleukin

IFN: interferon

MFI: median fluorescence intensity

MPA: microscopic polyangiitis

MPO: myeloperoxidase

p-ANCA: perinuclear ANCA

PR3: proteinase 3

SLE: systemic lupus erythematosus

Th: T helper

TNF-α: tumor necrosis factor-alpha

Treg: regulatory T cells

Introduction

Primary systemic vasculitis constitute a heterogeneous group of rare diseases characterized by inflammation of blood vessels and classified according to the size of involved vessels [1,2]. Anti-neutrophil cytoplasm antibodies (ANCA)-associated vasculitis (AAV) are pauciimmune necrotizing vasculitis involving small-sized vessels which include granulomatosis with polyangiitis (GPA), eosinophilic granulomatosis with polyangiitis (EGPA) and microscopic polyangiitis (MPA) [3]. The immunofluorescence pattern of ANCA distinguishes between cytoplasmic ANCA (c-ANCA), targeting proteinase 3 (PR3) in most of the cases, and perinuclear ANCA (p-ANCA), usually targeting myeloperoxidase (MPO).

Major advances have been done during the last fifteen years in the understanding of the pathophysiology of the disease. *In vitro* studies have demonstrated induction of respiratory burst in TNF-α primed neutrophils and monocytes after incubation with either PR3- or MPO-ANCA [4,5]. Xiao and colleagues published in 2002 an experimental model of MPO-ANCA-associated vasculitis, demonstrating the pathogenic role of MPO-ANCA *in vivo* [6]. For PR3-ANCA, although a recent model of humanized severe combined immunodeficiency (SCID) mice suggested that transfer of human PR3-ANCA could induce vasculitis, the direct pathogenic effect of these antibodies has not been clearly established *in vivo* [7]. In contrast, other components of the immune system could be involved, in particular neutrophils expressing increased amounts of PR3 at their membrane that could play an important role in the pathogenesis of GPA [8]

Although GPA and MPA share a number of common clinical features, the clinical presentation varies, with prominent granulomatous involvement of upper and lower respiratory tracts in GPA [9], and glomerulonephritis, intra-alveolar haemorrhage and/or peripheral neuropathy in

MPA [2]. Both conditions are associated with relapses, although they are more frequent in patients with PR3-ANCA than in those with MPO-ANCA [10]**.**

The treatment of systemic vasculitis relies on glucocorticoids and cyclophosphamide in severe forms of MPA and in systemic GPA. Interestingly, rituximab, a monoclonal anti-CD20 antibody has been found to be effective in both conditions in the induction of remission and [10:11:12] more recently in its maintenance [13].

Several studies reported on the role of T cells or B cells in the pathogenesis of AAV [14,15], whereas only few studies analyzed the potential role of both B and T lymphocytes subpopulations in active GPA and MPA patients [16]. Herein, we report disturbed B- and T-cell phenotypes in untreated active GPA or MPA patients with dramatic increase of Th2, Th9 and Th17 cells in GPA patients, perturbed B cell populations in both GPA and MPA and dramatic increase of IL-6 producing B cells in GPA.

Patients and Methods

Patients

Peripheral blood was obtained from patients with active GPA and MPA fulfilling the 2012 Revised International Chapel Hill Consensus Conference Nomenclature of Vasculitides criteria for the diagnosis of GPA and MPA [1], included in the multicenter and prospective Neutrovasc study (AOM 10055 - NI 10017). Active patients were defined as having either GPA or MPA and a Birmingham vasculitis activity score (BVAS) above 3. None of them received glucocorticoids and/or immunosuppressants at the time of blood sampling. In parallel, peripheral blood was obtained from 22 healthy controls (blood donors) (HC) who were

recruited among blood donors at the “Etablissement Français du Sang”, Saint Antoine Hospital, Paris, France.

All patients and HC gave written informed consent. Sera and peripheral blood mononuclear cells (PBMC) were collected with approval of the ethics committee of the “Groupe hospitalier Pitié-Salpêtrière” and the study conforms to the principles outlined in the Declaration of Helsinki.

Lymphocyte culture

PBMC were isolated from peripheral blood on Ethylenediamine tetraacetic acid (EDTA)-anticoagulated tubes using a Ficoll-Hypaque density gradient centrifugation (Eurobio, Courtaboeuf, France). PBMC were cultured in Roswell Park Memorial Institute medium 1640 (RPMI) obtained from Gibco (Life Technologies, Saint-Aubin, France), with 10% heat-inactivated fetal calf serum (FCS) (Eurobio) at 37°C in 5% CO_2 atmosphere.

Quantification of cytokine expression and production

Expression of interferon-gamma (IFN-γ), interleukin (IL)-4, IL-9, IL-17 and IL-21 from T cells and IL-6 and Tumor necrosis factor alpha (TNF-α) from B cells were assessed after PBMC stimulation for 4 hours with 50 ng/mM of PMA and 1 µg/ml of ionomycin, with 1 µl/million of cells of GolgiStop solution. Cells were then washed and stained.

Flow cytometry analysis

We performed flow cytometry analysis on PBMC after staining with: anti-CD19-Brillant Violet 605 (BV605), anti-CD27-AlexaFluor700 (BD Biosciences), anti-CD21-Peridin-chlorophyll protein-Cyanin5.5 (PerCP-Cy5.5) (Biolegend, London, UK). For B lymphocyte subpopulations, we further investigated membrane expressions of markers within $CD19^+$ B cells as previously described [17].

Anti-CD3-AlexaFluor 700, anti-CD4-BV605 from BD Biosciences and anti-CD8-PERCP-Cy5.5 from Biolegend were used to define T lymphocyte subpopulations. In combination with these markers, various antibodies were used to stain extracellular or intracellular proteins of B and T lymphocytes and are listed in Supplemental Table 1.

In longitudinal experiments, cytometer settings were standardized using BD Cytometer setup, SPHERO Rainbow Calibration Particles (BD) and BD Combeads. Flow cytometry acquisition was assessed using a BD LSR Fortessa at the cytometry plateform of Cochin Institute (Cybio) and data were analyzed using FlowJo analysis software (Treestar, Ashland, OR, USA).

Statistical analysis

All analysis were carried out using Prism 6 (GraphPad, La Jolla, CA, USA) with statistical significance defined as: * p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001. Non parametric tests including Mann-Whitney U-test were used to compare median values. Correlations were performed using Spearman test.

Results

Differential activation of T cells in patients with AAV

Clinical and immunological characteristics of patients with active GPA and MPA are depicted in Supplemental table 2. Number of total lymphocytes and of B- and T-cells were similar between GPA and MPA patients and HC (data not shown). Expression of IFN-γ from T helper (Th)1, IL-4 from Th2, IL-9 from Th9 and IL-17A from Th17 cells are presented in **Figure 1** (Panel A). Increased proportions of Th2 and Th9 cells were observed in AAV patients compared to HC (1.8 *vs*.1% and 1.1 vs. 0.2%; p=0.02 and p<0.001 respectively) (**Figure 1**, Panel B), whereas a decreased proportion of Th1 cells was observed in AAV patients (8.5 *vs*. 13.2% in HC; p=0.03) and Th17 cells were comparable between groups. Consequently, Th2/Th1 (0.16 *vs.* 0.07; p=0.006) and Th17/Th1 (0.14 *vs.* 0.06; p<0.001) ratios were increased in patients with AAV as compared to HC (**Figure 1**, Panel C). Patients with active GPA showed increased percentage of peripheral Th9 (1.1 *vs.* 0.2; p<0.001), but both GPA and MPA expressed an increased Th9/Th1 ratio compared to HC (0.12 and 0.13 *vs.* 0.01; p<0.0001 and p=0.05 respectively).

A remarkable heterogeneity was observed regarding the proportion of $CD25^{hi}$ $Foxp3^{+}$ regulatory T cells (Treg) in AAV patients, particularly in those with active MPA (**Figure 2**, Panels A and B). However, increased percentages of activated T cells (Tact) ($Foxp3^{low}$ $CD25^{+}$) were found in both GPA and MPA (5.8 and 17.9 *vs.* 4%; p=0.08 and p=0.03, respectively). As a consequence, Treg/Tact ratio was decreased in both AAV subtypes (0.12 *vs.* 0.21; p=0.03), although it failed to reach significance in the case of MPA patients (**Figure 2**, Panel B).

A decreased proportion of $CXCR5^{+}$ circulating follicular T helper (Tfh) cells was found in patients with AAV *vs*. HC (10.3% *vs.* 15%; p=0.006). Interestingly, only patients with GPA showed a decreased proportion of PD-1^{+} $CXCR5^{+}$ competent Tfh cells (3.1% *vs.* 4.5; p=0.03).

However, we observed a decreased proportion of PD-1^{+} cells within CXCR5^{+} T cells in patients with MPA compared to HC (50.7% *vs.* 66.7%; p=0.03), suggesting Tfh dysregulation in both MPA and GPA patients.

We further discriminated patients according to ANCA specificity or clinical manifestations, especially for the presence of granuloma in GPA, and failed to identify differences that had not been previously underlined using GPA/MPA comparison (Supplemental figure 1 and 2).

Perturbation B cell maturation in patients with active MPA

We further analyzed B cell maturation using the expression of CD21, CD27, CD38 and IgD (Figure 3 and Supplemental figure 3). Patients with active MPA showed increased proportions of naïve B lymphocytes (CD19^{+} IgD^{+} CD27^{-}) (59.8% *vs.* 50.7%; p=0.01) and decreased proportions of memory B cells (CD19^{+} IgD^{-} CD27^{+}) *vs.* HC (9.3% *vs.* 31.1%; p=0.003) (Figure 3, Panel C). Similar results were obtained when we analyzed the mature B cell classification (Bm) as we found increased proportions of Bm2 (CD19^{+} IgD^{+} CD38^{+}) (60.8 *vs.* 42.5%; p=0.02) and decreased early and late Bm5 (CD19^{+} IgD^{-} CD38^{+} and CD19^{+} IgD^{-} CD38^{-} respectively) (11.3 *vs.* 24 and 12.1 *vs.* 4.2; p=0.01 and p=0.02 respectively) in patients with active MPA compared to HC (Supplemental figure 3). Notably, a slight decrease in percentage of early Bm5 was also observed in patients with active GPA (13.6% *vs.* 24%; p=0.01). More interestingly, according to ANCA specificity, patients with MPO-ANCA showed increased CD21^{-} B lymphocytes (24.1% *vs.* 13.4%; p=0.01) and decreased peripheral memory B cells (14.5% *vs.* 31.1%; p=0.005) which was not the case for patients with PR3-ANCA.

Peripheral B cell activation in patients with active AAV

We further phenotyped B cells from patients with AAV and found increased expression of CD40 in patients with active GPA compared to HC using MFI ratio analysis (46.2 *vs.* 20.6; p=0.05) which was not the case for patients with active MPA (**Figure 4**, Panel A). Expression

of CD69 on B cells was increased in patients with both GPA and MPA *vs.* HC (3 and 5.9 *vs.* 1.5%; p=0.03 and p=0.03 respectively), but no significant change of expression of other activation markers (CD20, CD86, CD95) and inhibitory receptors (CD22, CD32, CD72) was noted in AAV patients as compared to HC (Figure 4 and Supplemental figure 5), as well as for the expression of IL-6R or IL-21R. In contrast, a dramatic decrease of B-cell activating factor (BAFF) receptor expression was observed in both GPA and MPA patients compared to HC (MFI ratio 11.8 and 13.7 *vs.* 45.2; p<0.0001 and p=0.003 respectively) (Figure 4, Panel B). Similar results were obtained when AAV patients were compared according to MPO or PR3 specificity (Supplemental figure 6).

B cells from patients with active GPA produce increased amounts of IL-6

We finally analyzed the expression of two proinflammatory cytokines, i.e. IL-6 and TNF-α, by B cells (Figure 5, Panel A). B cells from GPA patients showed a strikingly increased expression of IL-6 compared to HC (25.8 *vs.* 14.9%; p<0.0001), whereas B cells from MPA patients had dramatically decreased expression of IL-6 (4.6 *vs.* 14.9%; p=0.005) (Figure 5, Panel A). In contrast, similar decrease in TNF-α expression by B cells was noted in both AAV compared to HC (14.1% *vs.* 30%; p=0.001). Similar results were obtained when AAV patients were compared according to anti-MPO or anti-PR3 ANCA specificity (Supplemental Figure 7, panel A).

Furthermore, we analyzed the percentage of IL-6$^+$ B cells in parallel with Th/Treg subsets in patients with GPA. A positive correlation was found between IL-6$^+$ B cells and Th2 (r=0.63; p=0.03) and Th9 (r=0.54; p=0.05) cells, but not with Th1, Th17 or Treg cells (Figure 5, Panel B and Supplemental figure 7, Panel B). Using similar analysis, we found only one negative correlation between the proportion of TNF-α$^+$ B cells and the proportion of Th1 within CD4$^+$ T

cells in patients with GPA (r=-0.68; p=0.01) (Figure 5, Panel C and Supplemental figure 7, Panel B).

Discussion

In the present work, we provide evidence for a dysregulation of lymphocytes compartments in patients with active untreated AAV. We observed skewed T-cell polarization toward Th2, Th9 and Th17 responses in GPA, whereas B cell populations were dysregulated in both GPA and MPA with an activated phenotype and decreased BAFF-R expression. We also established that IL-6-producing B cells were dramatically increased in GPA patients whereas TNF-α-producing B cells were decreased in both GPA and MPA patients.

First, we observed specific T-cell polarization toward Th2, Th9 and Th17 responses in GPA patients which is in agreement with previous studies [18–20], as well as an increased percentage of peripheral activated T cells (Tact) ($CD25^{+}$ $Foxp3^{low}$) conversely to previous report [24]. These findings in human are similar to what was observed when naive mouse T cells were co-cultured with apoptotic neutrophils expressing PR3 [21]. In addition, in the same mouse model, PR3-ANCA induced an increased expansion of Th17 cell in agreement with what we observed in GPA patients. Moreover, we confirmed our previous report showing that patients with active GPA displayed Th9 and Th17 expansions whereas no change was observed for Th1 lymphocytes [8]. Since IL-17 is commonly detected in vasculitis lesions in the kidney, these findings suggest the potential interest of targeting Th17 and IL-17A in GPA patients [20]. Unlike what has been published in the literature [22], we failed to identify any significant difference according to clinical manifestations, particularly for the presence of granuloma in GPA patients**.**

In parallel, we observed a decreased proportion of Th1 cells in patients with MPA or MPO-ANCA resulting in similar Th2/Th1, Th9/Th1 and Th17/Th1 ratios to what was observed in GPA patients, whereas no expansion of Th2, Th9 or Th17 cells was observed in MPA. These data are important since very few studies were dedicated to the analysis of T cells in patients with MPA, even if these data should be interpreted with caution because of the small number of MPA patients. Overall, our data suggests that T helper cells are particularly disrupted in GPA patients and not in the limited number of MPA patients, supporting the existence of differences in the activation of T helper cells between GPA and MPA patients as previously suggested [23]. We did not identify any diminution of Tregs in our cohort of AAV patients, although other groups did, suggesting it could be used as a possible disease activity marker [27]. However, we found a significantly decreased Treg/Tact ratio in patients with AAV which could contribute to explain the functional limitation of Treg previously observed in AAV patients [27]. In addition, Tregs from GPA patients may convert into IL-17A-secreting cells as it was observed in other systemic autoimmune diseases and suggested in a recent review [28,29]. Finally, Zhao and colleagues recently reported an increased proportion of Tfh in patients with either active or inactive GPA under cyclophosphamide treatment [30]. In our study, we found a decreased percentage of Tfh in AAV patients, particularly in patients with MPA or patients with anti-MPO ANCA who also presented decreased PD-1 expression which could participate in the B-T cell cooperation and the expansion of autoreactive B cells [31–33].

Second, we observed that B-cell compartment was dysregulated in MPA with increased proportion of naïve B cells and decreased circulating memory B cells, whereas no significant difference was found in GPA patients. This result is in agreement with previous studies [16] [34]. Besides B-cell maturation perturbation, we identified for the first time that MPA patients had an increased proportion of $CD21^{-/lo}$ B lymphocytes, which have previously been shown to be enriched in autoreactive B cells in Sjögren's syndrome, hepatitis C virus-related

cryoglobulinemia vasculitis (mettre ma ref SVP), systemic lupus erythematosus and rheumatoid arthritis [35,36]. This difference between GPA and MPA patient reinforced the paradigm of GPA and MPA toward autoimmunity. B cells from MPA patients are thus potentially enriched in autoreactive B cells while B cells from GPA patients displayed classical maturation subpopulations contrasting with markedly deregulated neutrophils and innate immunity [37]. In addition, we observed in both GPA and MPA a B-cell activated phenotype with increased expression of CD69 and CD40. Both of these activation markers are known to play a role in B- and T-cell cooperation and were found to be increased in B cells from patients with SLE or rheumatoid arthritis [38,39]. These receptors also influence the development of autoreactive B cells in several experimental models [40–42]. We also found that BAFF receptor expression on B cells was dramatically decreased in both GPA and MPA which could be related to the increased activation of the BAFF pathway in AAV [43]. This finding also revealed a potential interplay between neutrophils and B cells, as neutrophils highly express BAFF in patients with GPA, which could participate in B cell activation [44,45].

Then, we analysed the ability of B cells to produce proinflammatory cytokines, in particular IL-6 and TNF- α. Similarly to previous data in monocytes, B cells from active GPA patients produced less TNF-α than HC [50]. In the present work, we also provide similar results in patients with MPA. In contrast, B cells producing IL-6 were strikingly increased in GPA, whereas the opposite was found in MPA patients. Recently, Wright and colleagues have shown that *in vivo* and *in vitro* stimulations of neutrophils by IL-6 enhanced neutrophil migration through IL-8, one of the well described neutrophil activation pathway in GPA [51,52]. Furthermore, increased IL-6 production by B cells could induce Th2, Th9 and Th17, which is consistent with our findings in patients with GPA [53–55]. Thus, we found a positive correlation between the percentage of IL-6 producing B cells and the proportions of Th2 and Th9 cells in patients with GPA which was not the case for Th1, Th17 or Treg. These data highlight the

interest of using anti-IL-6R monoclonal antibody in patients with AAV [56–58]. Moreover, in the RAVE study, patients with PR3-ANCA (mostly GPA) responded very well to rituximab, which could suggest that the depletion of IL-6 producing B-cells could participate in the induction of remission [10].

Finally, our study has some limitations. The number of patients with MPA was low, therefore findings in this group should be interpreted with caution. The NeutroVasc study was a prospective study in which consecutive patients with active AAV who did not receive glucocorticoid or immunosuppressive treatments were included. These very restrictive inclusion criteria explain the low number of MPA patients. However, because of these restrictive inclusion criteria, the data from our analysis provide new insights in B and T cells dysregulations in untreated and active AAV.

In conclusion, skewed T-cell polarization toward Th2, Th9 and Th17 responses characterizes GPA but not MPA, whereas B cell populations are dysregulated in both GPA and MPA with an activated phenotype and decreased BAFF-R expression. B cells cytokine production disturbances were identified with a dramatic increase of IL-6-producing B cells in GPA and decreased TNF-α producing B cells in both GPA and MPA, providing an additional mechanism by which rituximab could be effective in GPA and raising the question of the potential interest of anti-IL-6 in GPA.

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Legend to Figures

Figure 1. Analysis of T lymphocytes distribution in patients with active ANCA associated vasculitis (AAV) and healthy controls (HC). Flow cytometry analysis of peripheral T helper (Th) cells was assessed using the gating strategy depicted in Panel A. Percentages of Th1 (IFN-γ^+), Th2 (IL-4$^+$), Th9 (IL-9$^+$) and Th17 (IL-17A$^+$) were measured in total CD3$^+$ CD4$^+$ lived cells in 25 patients with active granulomatosis with polyangiitis (GPA) (□), 5 patients with active microscopic polyangiitis (MPA) (▽) and 17 HC (●) (Panel B). Th2/Th1, Th9/Th1 and Th17/Th1 T cell ratios were also calculated (Panel C). Comparisons between HC and either GPA, MPA or total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

Figure 2. Analysis of regulatory T cell and follicular T helper cell distributions in patients with active ANCA associated vasculitis (AAV) and healthy controls (HC). Flow cytometry analysis of peripheral T cells subsets was assessed using the gating strategy depicted in Panel A. Percentages of Foxp3$^+$, Treg (Foxp3$^+$ CD25^{hi}) and activated T cells (Foxp3^{low} CD25$^+$) were measured within 25 patients with active granulomatosis with polyangiitis (GPA) (□), 5 patients with active microscopic polyangiitis (MPA) (▽) and 17 HC (●) (Panel B). Treg/activated T cells ratio within CD3$^+$ CD4$^+$ T cells was also calculated (Panel B). Follicular T helper cells (CXCR5$^+$) and competent Tfh (PD-1$^+$ CXCR5$^+$) were assessed as shown in gating strategy and represented in proportion of CD3$^+$ CD4$^+$ T cells and total Tfh cells (Panel C). Comparisons between HC and either GPA, MPA or total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01.

Figure 3. Analysis of B lymphocytes distributions in patients with active ANCA associated vasculitis (AAV) and healthy controls (HC). Flow cytometry analysis of peripheral B cells subsets was assessed using the gating strategy depicted in Panel A. Percentages of total B lymphocytes ($CD19^+$), $CD21^-$ B cells, $CD27^+$ and plasmablasts ($CD27^{hi}$ $CD38^{hi}$) were measured in total lymphocytes and in total B lymphocytes in 28 patients with active granulomatosis with polyangiitis (GPA) (□), 8 patients with active microscopic polyangiitis (MPA) (▽) and 21 HC (●) (Panel B). Percentages of B lymphocyte subpopulations were quantified as depicted: i) naive B lymphocytes as $CD19^+$ $CD27^-$ IgD^+ cells; ii) memory cells as $CD19^+$ $CD27^+$ IgD^- ; iii) marginal zone B cells as $CD19^+$ IgD^+ $CD27^{+;}$ iv) double negative B cells as $CD19^+$ $CD27^-$ IgD^- cells. Comparisons between HC and either GPA, MPA or total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01.

Figure 4. Analysis of B lymphocytes activation in patients with active ANCA associated vasculitis (AAV) and healthy controls (HC). Medians of fluorescence intensity (MFI) of CD40 vs isotype control (MFI ratio) and percentages of $CD69^+$, $CD86^+$ and $CD95^+$ peripheral B lymphocytes ($CD19^+$) were measured within 28 patients with active granulomatosis with polyangiitis (GPA) (□), 8 patients with active microscopic polyangiitis (MPA) (▽) and 21 HC (●) (Panel A). Percentages of IL-$6R^+$ B cells, expression of BAFF-receptor (BR3) and IL-21R using MFI ratio were quantified in peripheral B cells (Panel B). Comparisons between HC and either GPA, MPA and total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

Figure 5. Analysis of cytokine productions by B lymphocytes in patients with active ANCA associated vasculitis (AAV) and healthy controls (HC). Flow cytometry analysis of cytokine producing peripheral B cells was assessed using the gating strategy depicted in panel A. Percentages of IL-6^{+} and TNF-α^{+} B cells in 15 patients with active granulomatosis with polyangiitis (GPA) (□), 5 patients with active microscopic polyangiitis (MPA) (▽) and 20 HC (●), were quantified (Panel A). Percentages of IL-6^{+} and TNF-α^{+} B cells were correlated to percentage of T helper (Th)1, Th2, Th9 and Th17 within total T cells in patients with active GPA (Panel B). Comparisons between HC and either GPA, MPA and total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Correlations of IL-6 and TNF-α expressions with T helper subpopulations were performed using Spearman test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

Legend to supplemental Figures

Supplemental Figure 1. Analysis of T lymphocytes distribution in patients with active anti-proteinase 3 (PR3) and anti-myeloperoxydase (MPO) ANCA associated vasculitis (AAV) and healthy controls (HC). Percentages of Th1 (IFN-γ^+), Th2 (IL-4^+), Th9 (IL-9^+) and Th17 (IL-$17A^+$) were measured in total $CD3^+$ $CD4^+$ lived cells in 25 patients with PR3-ANCA (⬡), 5 patients with MPO-ANCA (△) and 17 HC (●) (Panel A). Th2/Th1, Th9/Th1 and Th17/Th1 T cells ratios were also calculated (Panel B). Comparisons between HC and either PR3-ANCA, MPO-ANCA or total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

Supplemental Figure 2. Analysis of regulatory T cell and follicular T helper cell distributions in patients with active anti-proteinase 3 (PR3) and anti-myeloperoxydase (MPO) ANCA associated vasculitis (AAV) and healthy controls (HC). Percentages of $Foxp3^+$, Treg ($Foxp3^+$ $CD25^{hi}$) and activated T cells ($Foxp3^{low}$ $CD25^+$) were quantified in 25 patients with PR3-ANCA (⬡), 5 patients with MPO-ANCA (△) and 17 HC (●) (Panel A). Treg/activated T cells ratio within $CD3^+$ $CD4^+$ T cells was also calculated (Panel A). Follicular T helper cells ($CXCR5^+$) and competent Tfh (PD-1^+ $CXCR5^+$) were assessed as depicted in gating strategy and represented in percentages of $CD3^+$ $CD4^+$ T cells and total Tfh cells (Panel B). Comparisons between HC and either PR3-ANCA, MPO-ANCA or total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01.

Supplemental Figure 3. Analysis of mature B lymphocyte (Bm) subpopulations in patients with active ANCA associated vasculitis (AAV) and healthy controls (HC). Subpopulations

of mature B lymphocyte (Bm) were quantified in 28 patients with active granulomatosis with polyangiitis (GPA) (□), 8 patients with active microscopic polyangiitis (MPA) (▽) and 18 HC (●). Percentages of Bm subpopulations were quantified in Panel A as depicted: i) Bm1 cells as $CD19^{+}$ $CD38^{-}$ IgD^{+} cells (upper left); ii) Bm2 cells as $CD19^{+}$ $CD38^{+}$ IgD^{+} cells (upper right); iii) Bm2' cells as $CD19^{+}$ $CD38^{hi}$ IgD^{+} cells (middle left); iv) Bm3/Bm4 cells as $CD19^{+}$ $CD38^{hi}$ IgD^{-} cells (middle right); v) early Bm5 as $CD19^{+}$ $CD38^{+}$ IgD^{-} (lower left); vi) Bm5 cells as $CD19^{+}$ $CD38^{-}$ IgD^{-} cells (lower right). Subpopulations of B cell maturation regarding expression of CD21 and CD38 were also assessed in Panel B. Comparisons between HC and either GPA, MPA and total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01.

Supplemental Figure 4. Analysis of B lymphocytes distributions in patients with active anti-proteinase 3 (PR3) and anti-myeloperoxydase (MPO) ANCA associated vasculitis (AAV) and healthy controls (HC). Percentages of total B lymphocytes ($CD19^{+}$), $CD21^{-}$ B cells, $CD27^{+}$ and plasmablasts ($CD27^{hi}$ $CD38^{hi}$) were measured in total lymphocytes and in total B lymphocytes in 27 patients with PR3-ANCA (⬡), 9 patients with MPO-ANCA (△) and 21 HC (●) (Panel A). Percentages of B lymphocyte subpopulations were quantified as depicted: i) naive B lymphocytes as $CD19^{+}$ $CD27^{-}$ IgD^{+} cells; ii) memory cells as $CD19^{+}$ $CD27^{+}$ IgD^{-} ; iii) marginal zone B cells as $CD19^{+}$ IgD^{+} $CD27^{+;}$ iv) double negative B cells as $CD19^{+}$ $CD27^{-}$ IgD^{-} cells. Comparisons between HC and either PR3-ANCA, MPO-ANCA or total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01.

Supplemental Figure 5. Receptors expression at the surface of peripheral B lymphocytes from patients with active ANCA associated vasculitis (AAV) and healthy controls (HC). Expression of CD20 (upper left) and inhibitory receptors $CD22^+$ (upper right), $CD32^+$ (lower left) and $CD72^+$ (lower right) were analyzed at the surface of B lymphocytes from 28 patients with active granulomatosis with polyangiitis (GPA) (white histogram), 8 patients with active microscopic polyangiitis (MPA) (grey histogram) and 18 HC (black histogram). Mean of fluorescence intensity were represented by histograms (mean + standard deviation). Comparisons between HC and either GPA, MPA and total AAV patients (dotted histogram) were performed using Mann-Whitney U-test.

Supplemental Figure 6. Analysis of B lymphocytes activation in patients with active anti-proteinase 3 (PR3) and anti-myeloperoxydase (MPO) ANCA associated vasculitis (AAV) and healthy controls (HC). Medians of fluorescence intensity (MFI) of CD40 vs isotype control and percentages of $CD69^+$, $CD86^+$ and $CD95^+$ peripheral B lymphocytes ($CD19^+$) were measured in 27 patients with PR3-ANCA (⬡), 9 patients with MPO-ANCA (△) and 21 HC (●) (Panel A). Percentage of IL-$6R^+$ B cells, expression of BAFF-receptor (BR3) and IL-21R using MFI ratio were quantified in peripheral B cells (Panel B). Comparisons between HC and either PR3-ANCA, MPO-ANCA and total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

Supplemental Figure 7. Analysis of cytokine productions by B lymphocytes in patients with active anti-proteinase 3 (PR3) and anti-myeloperoxydase (MPO) ANCA associated vasculitis (AAV) and healthy controls (HC). Percentages of IL-6$^+$ and TNF-α$^+$ B cells within 14 patients with PR3-ANCA (⬡), 6 patients with MPO-ANCA (△) and 20 HC (●) (Panel A). Percentages of IL-6$^+$ and TNF-α$^+$ B cells correlated with the percentage of Tregs within total T cells in patients with active GPA (Panel B). Comparisons between HC and either PR3-ANCA, MPO-ANCA and total AAV patients (■) were assessed using non-parametric Mann-Whitney test. Correlations of IL-6 and TNF-α expressions with Tregs subpopulations were performed using Spearman test. Statistical significance was defined as: * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001.

Supplemental Table 1. Antibodies used for flow cytometry experiments.

Target	Fluorochrome	Clone	Society
BR3	Fluorescein isothiocyanate (FITC)	11C1	BD biosciences
CCR6	Peridin-Chlorophyll protein-Cyanin5.5 (PerCP-Cy5.5)	G034E3	Biolegend
CD3	AlexaFluor700	UCHT-1	BD biosciences
CD4	Brillant Violet 605 (BV605)	RPA-T4	BD biosciences
CD5	BV421	UCHT2	BD biosciences

CD8	PerCP-Cy5.5	RPA-T8	BD biosciences
CD19	BV605	SJ25C1	BD biosciences
CD20	BV421	2H7	BD biosciences
CD21	PerCP-Cy5.5	Bu32	Biolegend
CD22	Phycoerythrin (PE)	HIB22	Biolegend
CD24	PE	ML5	BD biosciences
CD25	BV421	M-A251	BD biosciences
CD27	AlexaFluor700	M-T271	BD biosciences
CD32	Allophycocyanin (APC)	Fli8.26	BD biosciences
CD38	PE-Cy7	HIT2	BD biosciences
CD40	APC-H7	5C3	BD biosciences
CD45	APC	H130	BD biosciences
CD45RA	FITC	HI-100	BD biosciences
CD62L	APC	DREG-56	BD biosciences
CD69	APC	FN50	BD biosciences
CD72	FITC	J4-117	BD biosciences
CD86	BV421	2331 (PUN-1)	BD biosciences
CD95	PE	DX2	BD biosciences
CXCR5 (CD185)	PE	J252D4	Biolegend
FoxP3	PE	259D/C7	BD biosciences
HLA-DR	PerCP-Cy5.5	L243	Biolegend
ICOS (CD278)	FITC	C398.4A	Biolegend
IgD	APC	IA6-2	BD biosciences
IgG	PE	G18-145	BD biosciences

IgM	BV421	G20-127	BD biosciences
IFN-γ	FITC	B27	BD biosciences
IL-4	APC	8D4-8	Ebioscience
IL-6	PE	MQ2-6A3	BD biosciences
IL-6Rα	Biotin	UV4	Biolegend
IL-9	PE	MH9D1	Ebioscience
IL-10	APC	JES3-9D7	BD Biosciences
IL-17A	PE	N49-653	BD biosciences
IL-21	APC	3A3-N2	Biolegend
IL-21R	APC	17A12	BD biosciences
PD-1	BV421	EH12.1	BD biosciences
TGF-β1	PERCP-Cy5.5	TW4-9E7	BD Biosciences
Livedead	BV421	-	LifeTechnologies
Biotin-Streptavidin	BV421	-	Biolegend

Supplemental Table 2. Clinical characteristics of ANCA-associated vasculitis (AAV) patients at the time of blood sampling (n=37)

Patient ID	**Age (years)**	**Disease duration (days)**	**Previous Treatment**	**BVAS**	**Previous features during the course of AAVs**	**ANCA**	**CRP (mg/l)**
GPA 1	27	0	None	25	ENT, L, K	PR3	66
GPA 2	24	2048	AZA, CYC	27	ENT, L, K, S	PR3	72
GPA 3	48	0	None	17	ENT, L, K	PR3	185

GPA 4	48	126	CYC	9	ENT, L	PR3	63
GPA 5	69	1	None	19	ENT, L, K	PR3	166
GPA 6	51	7263	CYC, MTX	9	ENT,L	PR3	60
GPA 7	19	0	None	26	E, ENT,L, K	PR3	187
GPA 8	71	1114	AZA, CYC	6	L	PR3	21
GPA 9	84	0	None	3	ENT, L	PR3	31
GPA 10	64	2378	AZA, CYC, MTX	3	L	PR3	7
GPA 11	67	2	None	29	ENT, L, K, PN	PR3	221
GPA 12	72	1560	AZA, CYC, Ritux	14	ENT, L, K,PN	PR3	352
GPA 13	34	2475	AZA, CYC	8	E, ENT	PR3	1
GPA 14	66	4317	AZA, CYC MMF, Ritux	3	L, S	PR3	47
GPA 15	51	1961	AZA, Ritux	16	B, ENT, L, S	neg	25
GPA 16	88	2108	None	9	ENT, L, K, PN	PR3	132
GPA 17	32	0	None	20	E, L, K	PR3	33
GPA 18	27	106	None	13	ENT, L	PR3	2
GPA 19	35	3	None	13	L, K, PN	PR3	89
GPA 20	36	2412	AZA	12	L, K	MPO	6
GPA 21	59	4	None	26	ENT, L, S,PN	PR3	136
GPA 22	81	2	None	27	ENT, L, K	PR3	28
GPA 23	41	0	None	11	E, ENT, L, S	PR3	83

GPA 24	79	0	None	27	ENT, L, K, PN	PR3	131
GPA 25	72	1	None	30	ENT, L, K, PN	MPO	244
GPA 26	73	1892	AZA, CYC	17	ENT, L	PR3	135
GPA 27	27	2423	AZA, CYC Ritux	9	ENT, L	PR3	1
GPA 28	52	8644	MTX, CYC	13	L,K	PR3	4
GPA 29	77	581	Ritux	21	ENT, L, S, PN	PR3	10
MPA 1	60	2542	CYC	12	PN	MPO	21
MPA 2	71	0	None	12	K	MPO	16
MPA 3	73	1226	AZA, CYC	6	E	MPO	4
MPA 4	44	13	None	15	K, L	MPO	3
MPA 5	75	3911	AZA, CYC	6	ENT, K, L	MPO	2
MPA 6	75	1	None	18	K, L	MPO	70
MPA 7	58	2	None	15	K, L	MPO	97
MPA 8	58	1	None	20	ENT	MPO	124

AAV: ANCA-associated vasculitides, GPA: granulomatosis with polyangiitis, MPA: microscopic polyangiitis

B: bowel; E: eye; ENT: ear, nose, throat; K: kidney; L: lung; PN: peripheral neuropathy; S: skin

AZA: azathioprine; CYC: cyclophosphamide; MMF: mycofenolate mofetil; MTX: methotrexate; Ritux: rituximab

ANCA: anti-neutrophil cytoplasmic antibody; Neg: ANCA negative; MPO: anti-myeloperoxydase; PR3: anti-proteinase 3

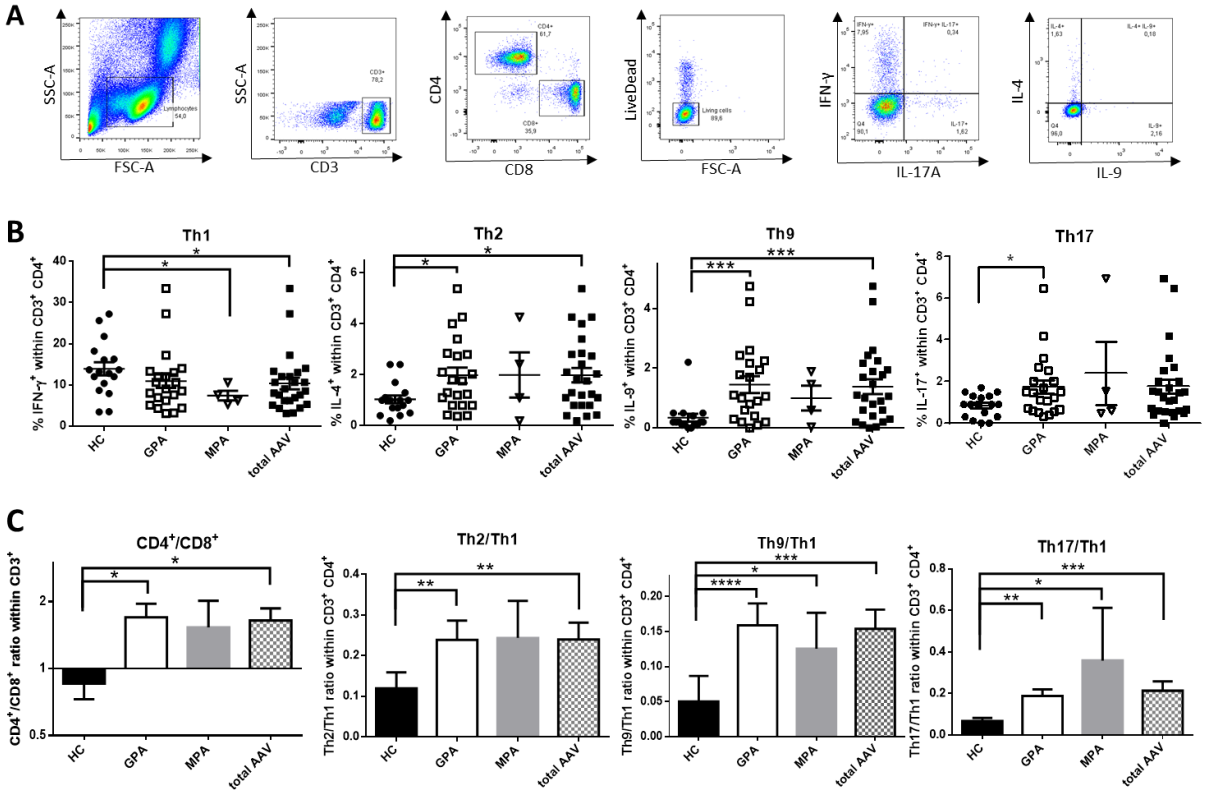
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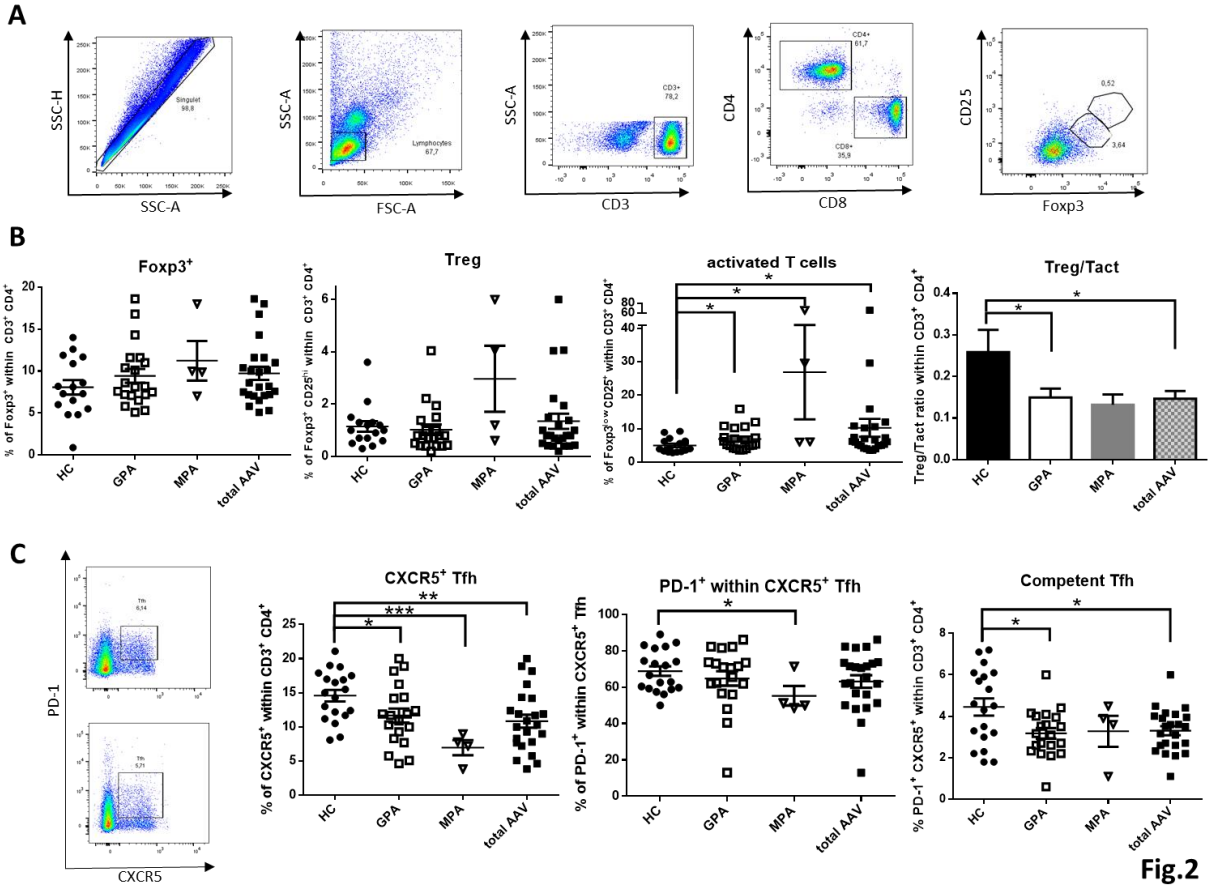
Fig.1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Lymphocytes
67,7
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CD3
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79,2
CD4
CD8
CD4+
61,7
CD8+
35,9
CD2.5
Foxp3
0,52
3,64
B
Foxp3+
% of Foxp3+ within CD3+ CD4+
HC
GPA
MPA
total AAV
Treg
% of Foxp3+ CD25hi within CD3+ CD4+
activated T cells
% of Foxp3low CD25+ within CD3+ CD4+
Treg/Tact
Treg/Tact ratio within CD3+ CD4+
C
PD-1
CXCR5
Tfh
6,14
Tfh
0,71
CXCR5+ Tfh
% of CXCR5+ within CD3+ CD4+
PD-1+ within CXCR5+ Tfh
% of PD-1+ within CXCR5+ Tfh
Competent Tfh
% PD-1+ CXCR5+ within CD3+ CD4+

Fig.2

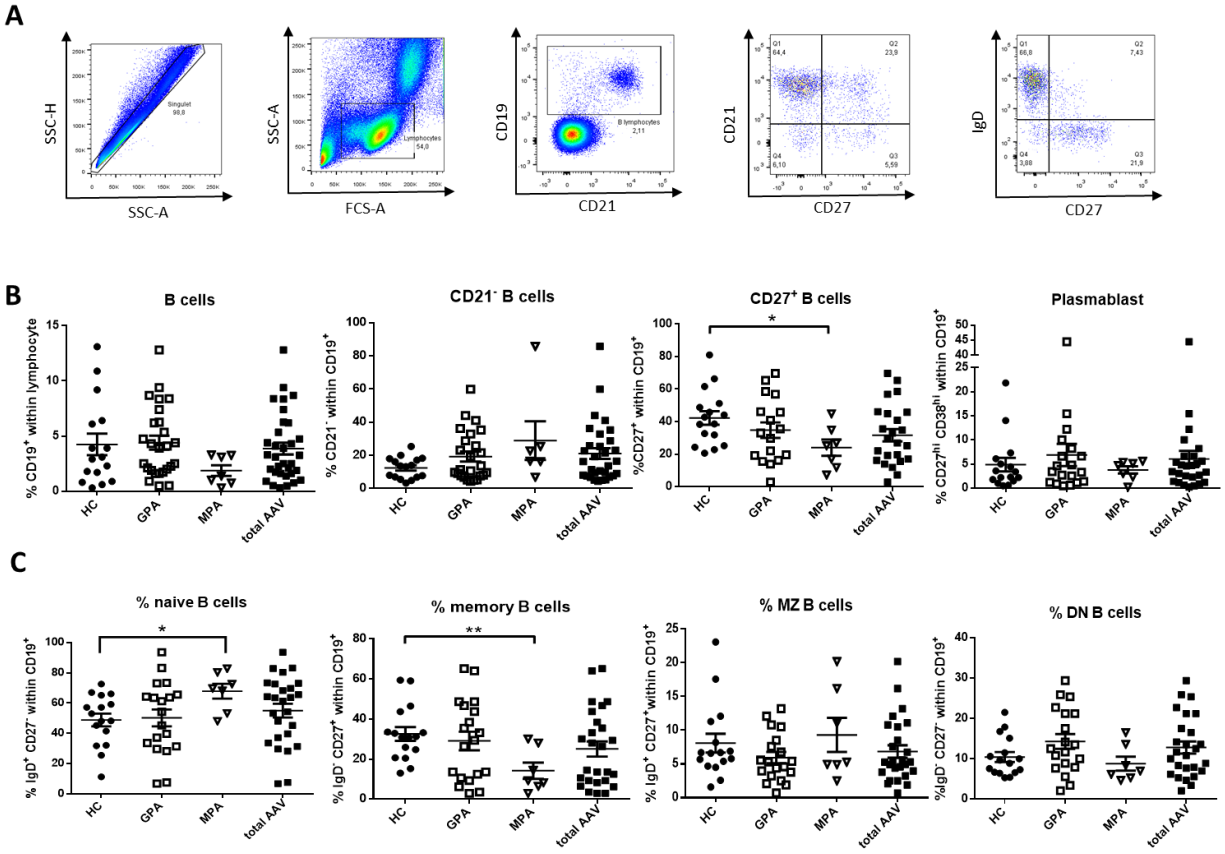

A
SSC-H
SSC-A
SSC-A
FCS-A
CD19
CD21
CD21
CD27
IgD
CD27
B
B cells
% CD19+ within lymphocyte
CD21- B cells
% CD21- within CD19+
CD27+ B cells
%CD27+ within CD19+
Plasmablast
% CD27hi CD38hi within CD19+
HC
GPA
MPA
total AAV
C
% naive B cells
% IgD+ CD27- within CD19+
% memory B cells
% IgD- CD27+ within CD19+
% MZ B cells
% IgD+ CD27+ within CD19+
% DN B cells
%IgD- CD27- within CD19+

Fig.3

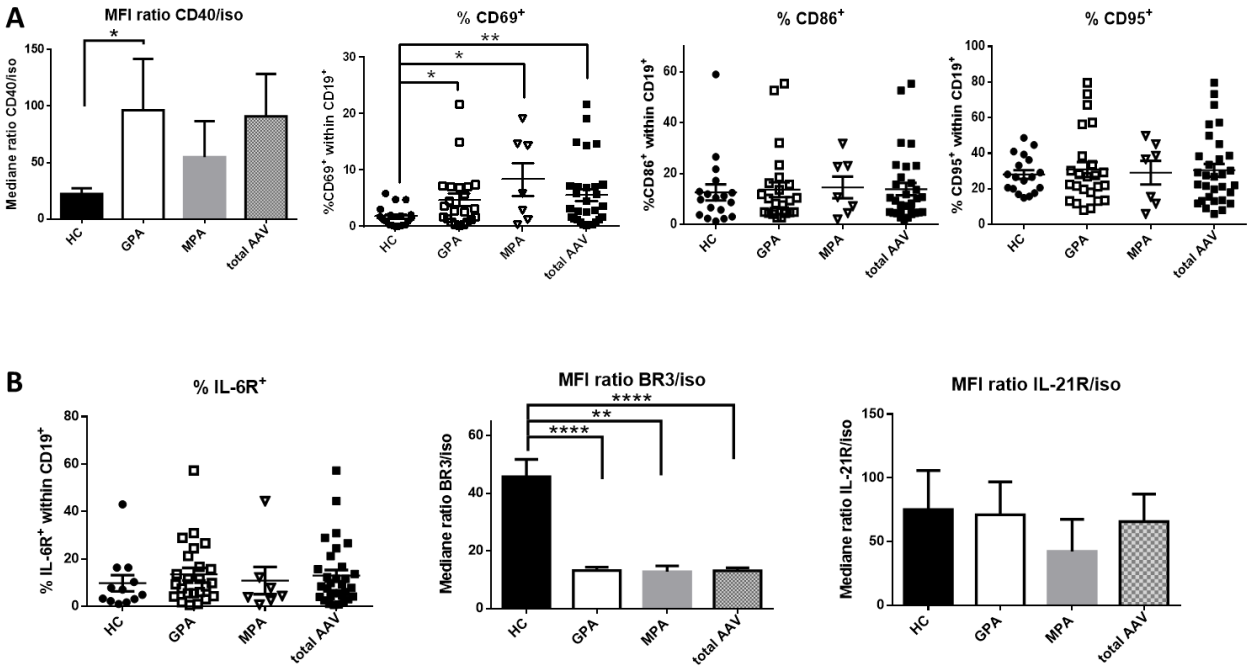

A
MFI ratio CD40/iso
Mediane ratio CD40/iso
% CD69+
%CD69+ within CD19+
% CD86+
%CD86+ within CD19+
% CD95+
% CD95+ within CD19+
HC
GPA
MPA
total AAV
B
% IL-6R+
% IL-6R+ within CD19+
MFI ratio BR3/iso
Mediane ratio BR3/iso
MFI ratio IL-21R/iso
Mediane ratio IL-21R/iso

Fig.4

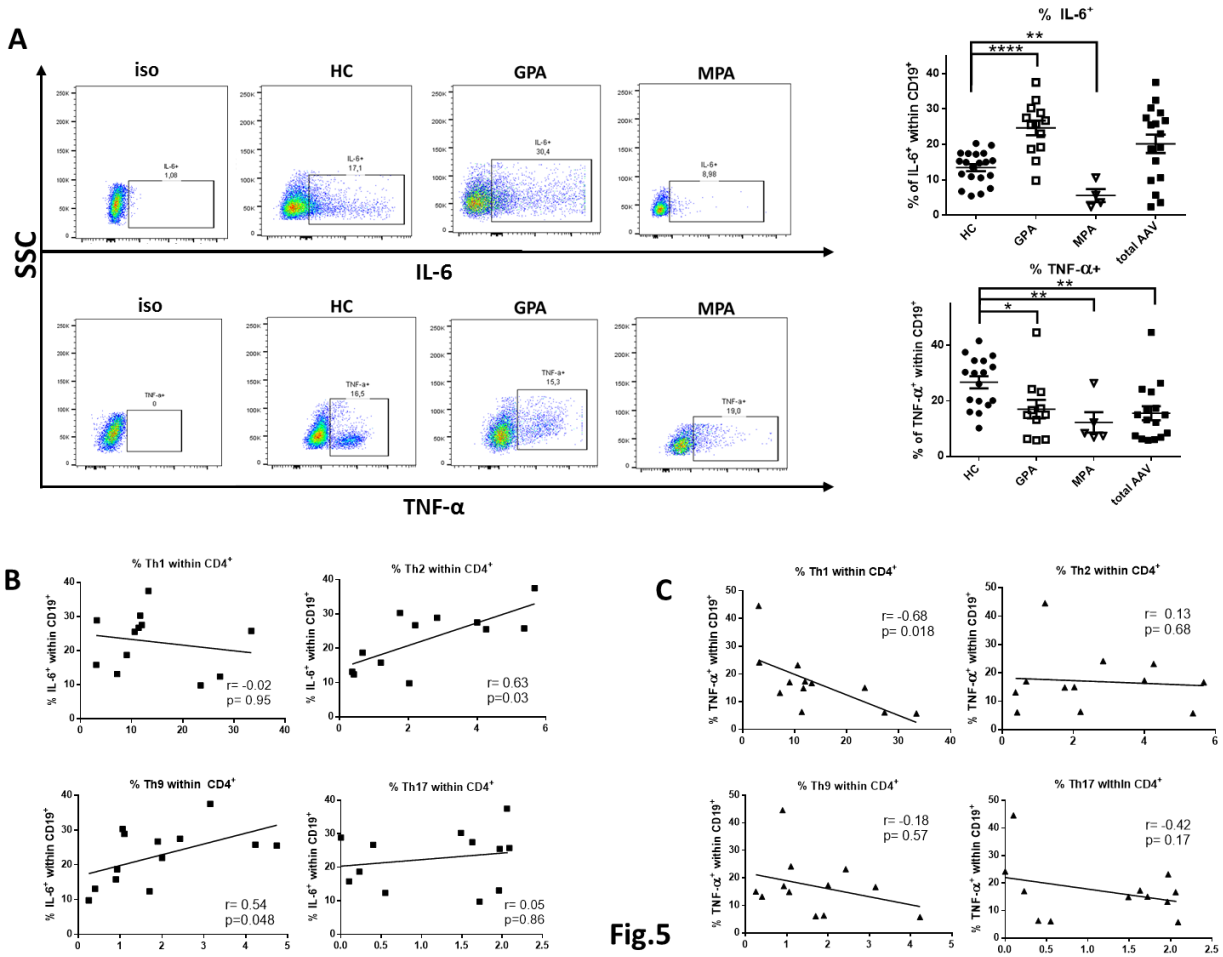
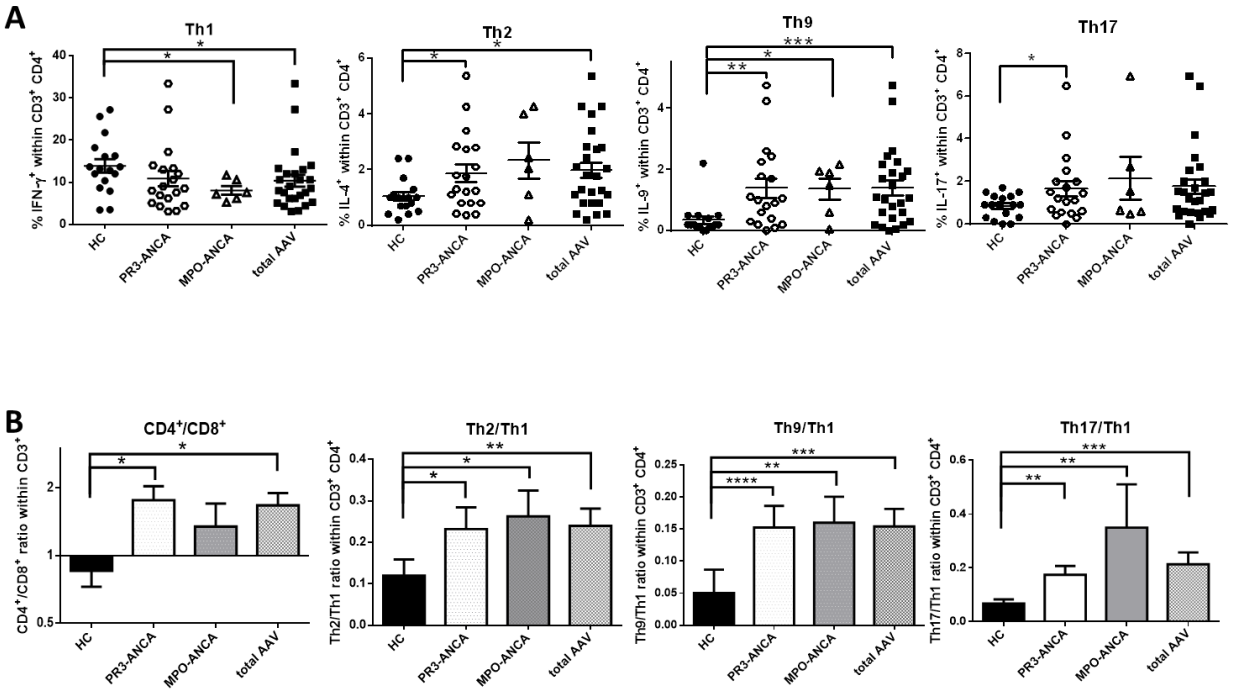
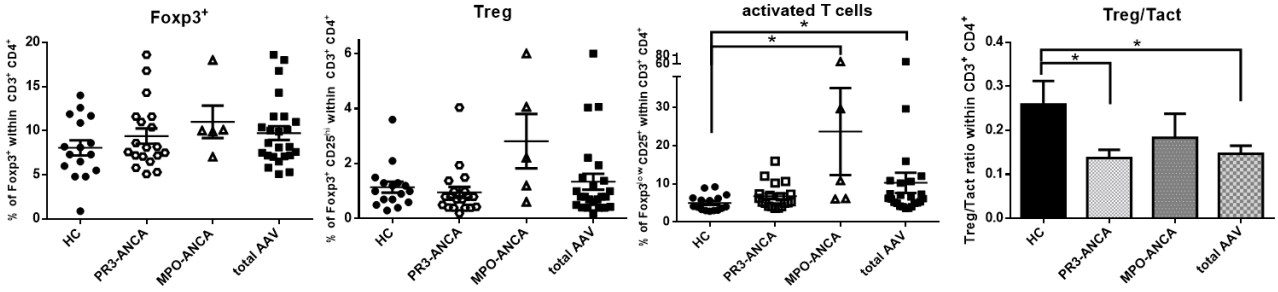

A
iso
HC
GPA
MPA
SSC
IL-6
TNF-α
% IL-6+
% of IL-6+ within CD19+
% TNF-α+
% of TNF-α+ within CD19+
HC
GPA
MPA
total AAV
B
% Th1 within CD4+
% Th2 within CD4+
% Th9 within CD4+
% Th17 within CD4+
% IL-6+ within CD19+
r= -0.02
p= 0.95
r= 0.63
p=0.03
r= 0.54
p=0.048
r= 0.05
p=0.86
C
% TNF-α+ within CD19+
r= -0.68
p= 0.018
r= 0.13
p= 0.68
r= -0.18
p= 0.57
r= -0.42
p= 0.17

Fig.5

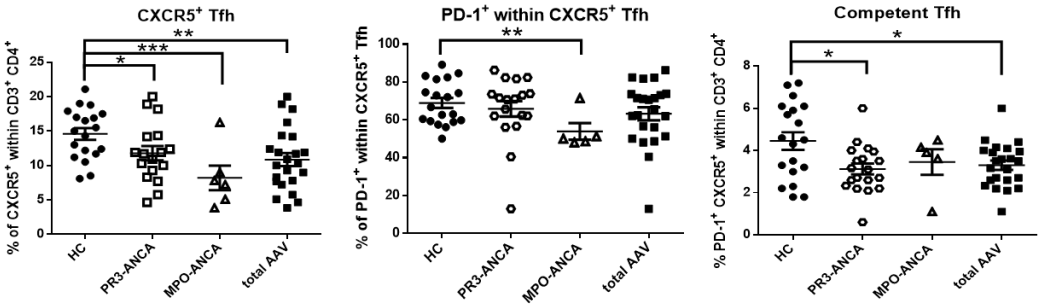


Supplemental fig.1

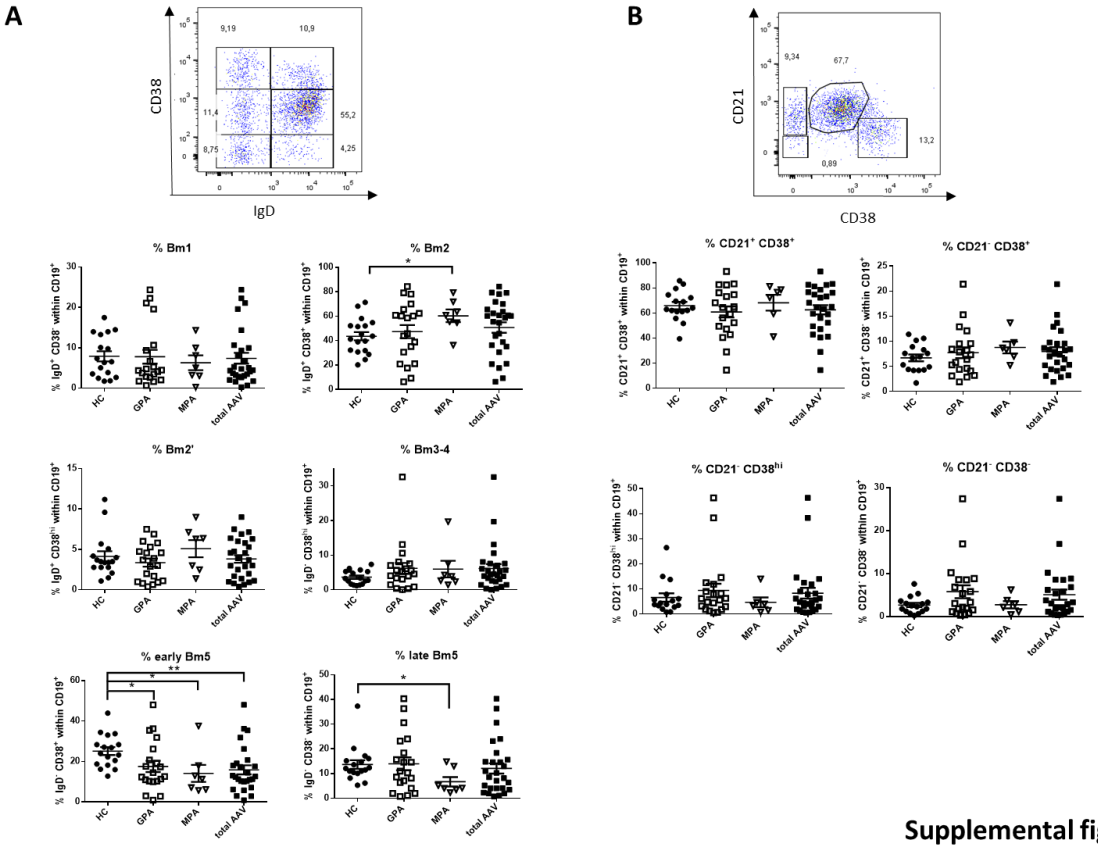
A



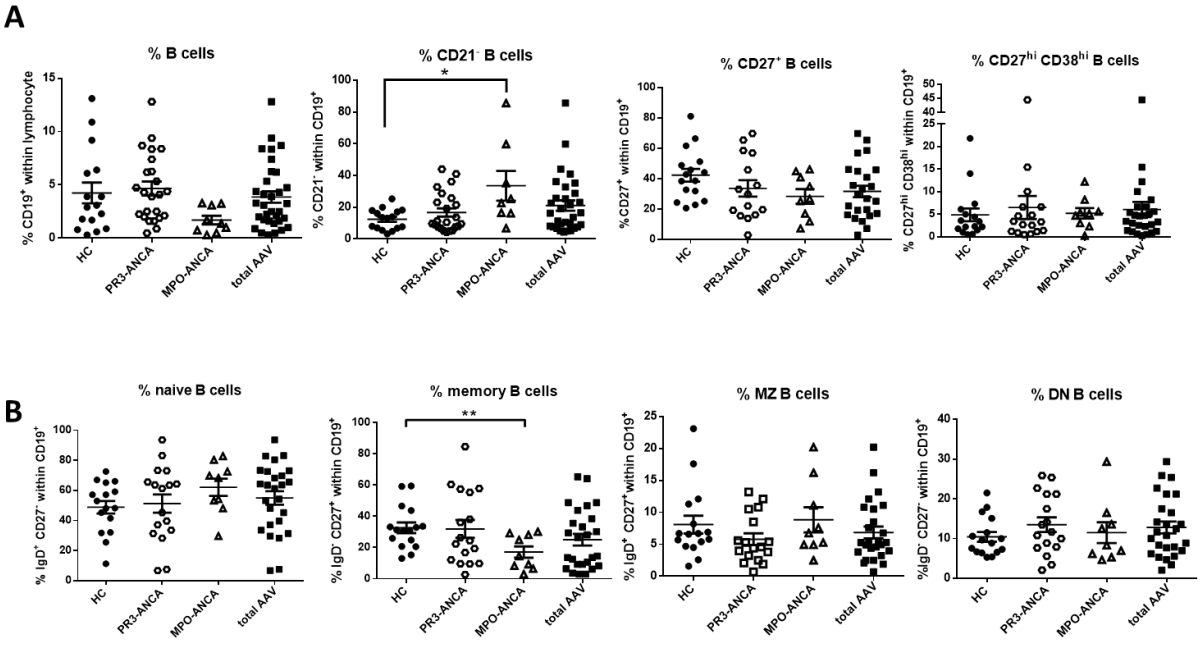
B



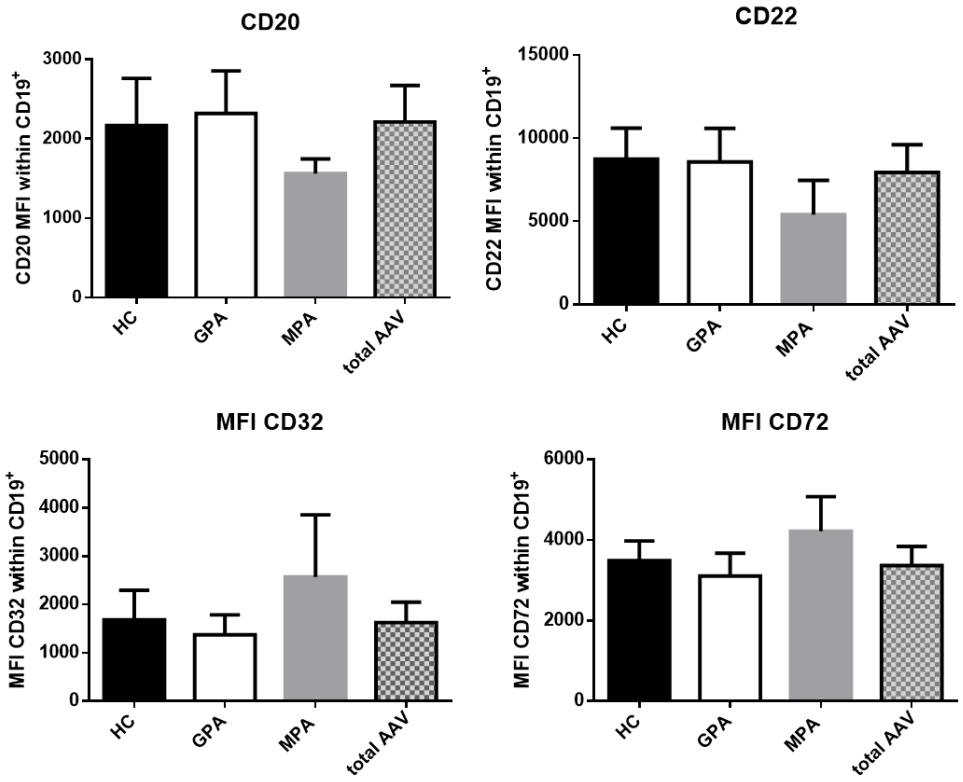
Supplemental fig.2



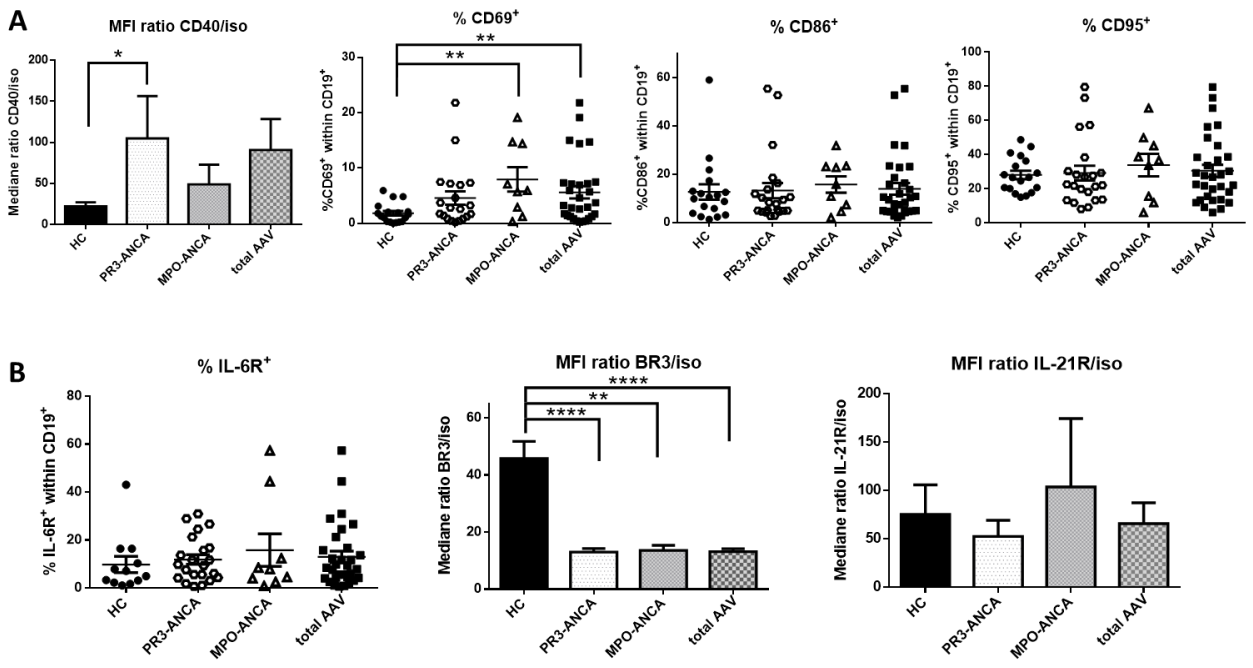
Supplemental fig.3



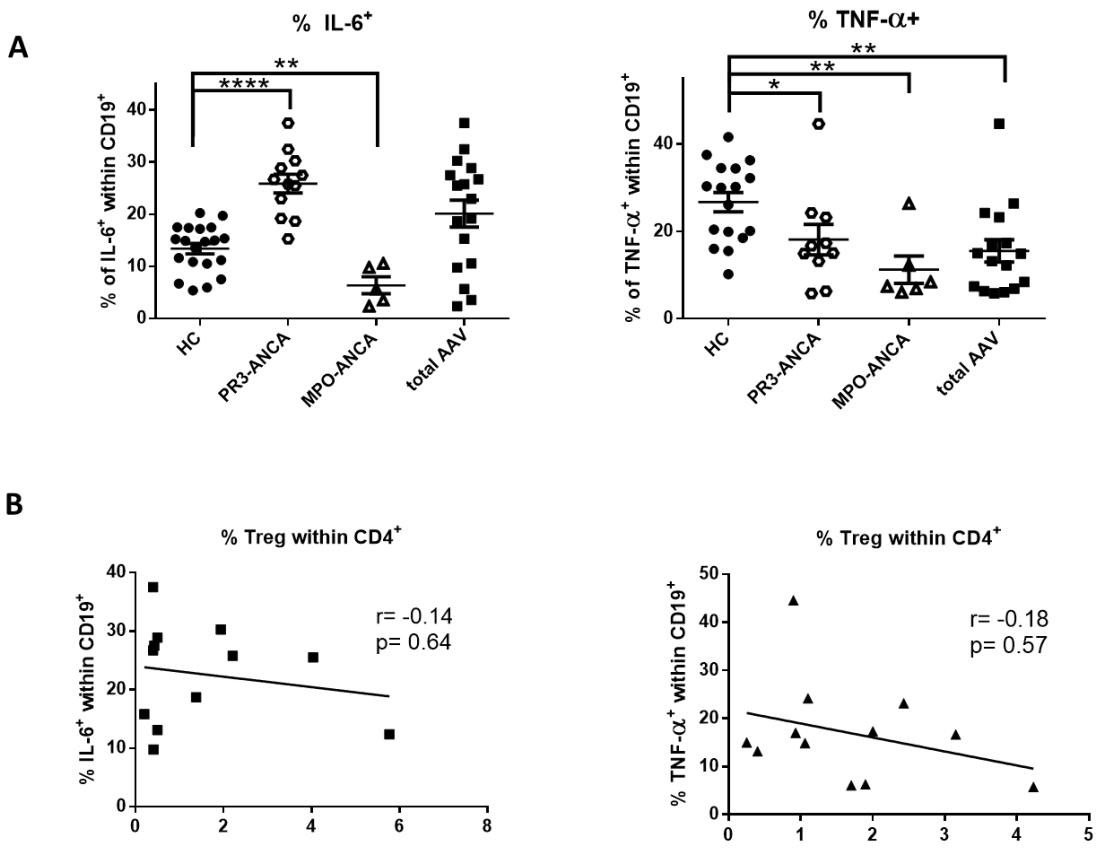
Supplemental fig.4



Supplemental fig.5



Supplemental fig.6



Supplemental fig.7

2. Paper 2: Scleroderma peripheral B lymphocytes secrete interleukin-6 and TGF-beta and activate fibroblasts

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Scleroderma peripheral B lymphocytes secrete interleukin-6 and TGF-β and activate fibroblasts

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Abstract word count: 247

Abstract

Objective: To study the role of B lymphocytes in systemic sclerosis (SSc).

Patients and Methods: Peripheral B cell subpopulations, interleukin 6 (IL-6) and transforming growth factor-beta (TGF-β) productions were analyzed using flow cytometry and multiplex assay. Fibroblasts proliferation rate upon incubation with supernatants of B cells isolated from SSc patients or healthy controls (HC) was assessed using XTT, BrdU and Ki67. Collagen production was assessed using a collagen assay.

Results: Ninety untreated patients (12 males) fulfilling the ACR/EULAR criteria for SSc (23 diffuse SSc (dSSc) and 67 limited cutaneous SSc (lcSSc)) and 30 HC were recruited. Increased proportions of B cells expressing CD69 and CD95 were identified among SSc patients. B lymphocytes from dSSc patients vs lcSSc patients and HC expressed increased proportion of CD5 (24.12±7.93% vs 14.09±6.58% (p=0.03) and 14.21±5.34% (p=0.01)), CD86 (39.89±22.11% vs 17.72±13.98% (p=0.0007) and 11.68±11.09% (p<0.0001)), IL-6 receptor (IL-6R) (33.64±23.12% vs 17.91±13.62% (p<0.0001) and 12.08±8.68% (p=0.0009)) and IL-21 receptor (IL-21R) (32.55±20.19% vs 5.76±4.40% (p<0.0001) and 5.93±3.29% (p<0.0001)) positive cells. IL-6 (314.3±317.8pg/ml vs 6.10±2.58pg/ml (p=0.0007)) and TGF-β (1020±569pg/ml vs 163.8±98.69pg/ml (p=0.0011)) secreted by B lymphocytes were increased in SSc patients vs HC. Fibroblast proliferation and collagen production were also significantly increased in the presence of B cell supernatant from SSc patients as compared to HC.

Conclusions: Activated B cells were increased in SSc patients and the upregulation of CD5, CD86, IL-6R and IL-21R discriminated between dSSc and lcSSc patients. Peripheral B lymphocytes from SSc patients secreted IL-6 and TGF-β, and activated fibroblasts *in vitro*.

Introduction

Systemic sclerosis (SSc) is a connective tissue disease characterized by fibroblasts activation, increased extra-cellular matrix synthesis and vascular remodeling (Dumoitier et al., 2014a). Activation of fibroblasts and endothelial cells, together with increased oxidative stress represent the hallmarks of SSc pathogenesis. Patients with SSc are classified depending on the extent of skin involvement: limited cutaneous SSc (lcSSc), with alterations essentially limited to the hands and face, and diffuse SSc (dSSc), with skin lesions proximal to the elbows and knees and frequent visceral involvement. Although autoantibodies have been detected in the serum of patients with SSc and are helpful for the diagnosis and evaluation of prognosis, B lymphocyte implication in the pathogenesis of SSc is still poorly defined [2; 3].

Interestingly, circulating B lymphocytes from SSc patients differ from those of HC, with increased proportions of naïve B cells and decreased numbers of memory B cells and plasma cells (Sato et al., 2004). In addition, signaling alterations, including overexpression of both CD19 and CD21, two activating co-receptors of the B-cell antigen receptor (BCR), have been detected both in the Tsk-1 mouse model of SSc and in naive and memory B lymphocytes from patients with SSc. Moreover, the activation receptors CD80, CD86 and CD95 are upregulated in memory B cells from SSc patients, suggesting their participation to the pathogenic process (Sato et al., 2004). High levels of B cell activating factor (BAFF) have been measured in the serum of SSc patients, together with an overexpression of BAFF-R at the surface of peripheral B cells from those patients (Matsushita and Sato, 2005). BAFF through the NF-κB pathway, promotes B cells survival and participates to autoreactive B cells differentiation (Liu and Davidson, 2011). In addition, B cells infiltrates have been detected in the dermis of SSc patients [7; 8] and serum levels of IL-6 are increased and correlate with the extent of skin fibrosis both for SSc patients and Tsk-1 mouse model [9; 10]. IL-6 stimulates collagen secretion by fibroblasts and therefore might be an intermediate for fibroblast activation (Chizzolini, 2008).

More recently, depletion of B cells using a chimeric anti-CD20 monoclonal antibody allowed improving skin fibrosis and prevented loss of pulmonary function in patients with SSc (Bosello et al., 2015). Moreover, targeting IL-6 receptor (IL-6R) with tocilizumab showed promising effect on skin involvement in a prospective randomized trial [13;14].

Herein, we describe abnormal phenotypic patterns of peripheral B lymphocytes in patients with SSc, notably between lcSSc and dSSc, together with increased cytokine production leading to fibroblast activation.

Patients and Methods

Patients

Peripheral blood was obtained from 90 patients with SSc fulfilling the American College of Rheumatism (ACR) / European League Against Rheumatism (EULAR) criteria for the diagnosis of SSc (van den Hoogen et al., 2013d), followed at Cochin Hospital in the department of Internal Medicine. Patient's clinical characteristics are summarized in Supplemental Table 1. LcSSc was defined by skin thickening in areas solely distal to the elbows and knees, with or without facial involvement. Diffuse SSc was defined by the presence of skin thickening proximal, as well as distal, to the elbows and knees, with or without facial or truncal involvement (LeRoy 1988). Early systemic sclerosis was defined as the first twelve months after the first non-Raynaud's symptom of systemic sclerosis. Peripheral blood was obtained from 30 healthy controls (blood donors) (HC) who were included at the "Etablissement Français du Sang", Saint Antoine Hospital, Paris, France.

All patients and HC gave a written informed consent. Sera and peripheral blood mononuclear cells (PBMC) were collected with approval of the ethics committee of the "Groupe hospitalier Pitié-Salpêtrière" and the study conforms to the principles outlined in the Declaration of Helsinki.

Fibroblast culture and functional studies

Skin biopsies were performed in involved skin from 4 patients with SSc. Normal human dermal fibroblasts were cultured from skin biopsies obtained from 4 HC. Biopsy specimen were cut into small pieces and seeded into Petri dishes and then into 175cm^2 plastic flasks. Patients and HC fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) obtained from Gibco, with 10% heat-inactivated FCS (Eurobio) at 37°C in 5% CO2.

At the third passage, fibroblasts from HC or SSc patients were plated in a 96 wells plate at a concentration of 5.10^4 cells/ml. After 24 hours, fibroblasts were synchronized by replacing supplemented DMEM by DMEM without FCS. After 24 h of synchronization, media were replaced by supplemented DMEM with or without 8 representative supernatants of B lymphocytes from 4 HC or 4 patients with dSSc, in triplicate. After three days of culture, viability of fibroblasts was measured using the Colorimetric cell viability kit III (XTT) from Promokine (Promocell GmBH, Heidelberg, Germany), proliferation was quantified using the Cell proliferation ELISA BrdU from Roche (Boulogne-Billancourt, France) and indirect immunofluorescence of Ki67 evaluated using an anti-Ki67 (Abcam, Cambridge, UK) and an anti-rabbit fluorescein isothiocyanate-coupled secondary antibody (Jackson Immunoresearch). Collagen quantification was assessed after five days of culture using Sircol assay kit from Biocolor (Carrickfergus, UK) according to manufacturer protocol. Real Time-PCR (RT-PCR) were assessed using 2.5 10^4 fibroblasts cultured in a 24 well plate at the same concentration as described above. RNA extracted using RNeasy mini kit from Qiagen (Hilden, Germany) according to the manufacturer protocol, were quantified using NanoVue (GE Healthcare Life Science, Little Chalfont, UK). Four hundred nanograms of total RNA was retrotranscripted in cDNA using iScriptTMcDNA Synthesis kit from Biorad (Marnes-la-Coquette, France)

following the manufacturer protocol. Alpha-Smooth muscle actin (α-SMA), Collagen type I, Snail and Vimentin mRNA expressions were quantified compared to β2-microglobulin as housekeeping gene. Real-time PCR was performed using Power SYBR®Greenq-PCR kit on a StepOnePlus Real Time PCR System™ (Applied Biosystem, Life Technology, Saint-Aubin, France). Primers obtained from Eurogentec (Liège, Belgium) were referenced in Supplemental Table 3**.**

Immunohistochemical study

Immunohistochemistry was performed on paraffin embedded lung samples: 4 µm sections were dewaxed and rehydrated progressively. EDTA buffer (pH 9.0) or Citrate buffer (pH 6.0) were used for antigen retrieval and endogenous peroxidase was quenched with hydrogen peroxide. Sections were blocked with 5% bovine serum albumin and incubated for 32 minutes with polyclonal antibody against CD20 (reference BSB5195, as recommended by the manufacturer; Bio SB, Santa Barbara, USA) or CD69 (reference ab202909, as recommended by the manufacturer; Abcam, Cambridge, USA). After incubation with a biotinylated secondary antibody, its detection was performed with HRP-streptavidin and DAB-solution.

Statistical analysis

All analysis were carried out using Prism 6 (GraphPad, La Jolla, CA, USA) with statistical significance defined as:* p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001. Non parametric tests including Mann-Whitney U-test were used for two group independent comparisons. Correlations of protein expressions were performed using Spearman test. Comparisons between fibroblast responses to B lymphocyte stimulation were analyzed using paired t-tests.

Results

Differential peripheral B lymphocytes maturation in patients with SSc

The overall number of B and T cells was similar between HC and SSc patients, despite a larger dispersal for the later, mainly observed in patients with lcSSc (Supplemental figure 2). Similarly, B lymphocyte subpopulations counts including naïve, effector memory, Breg cells and plasmablasts were equivalent among the cohort between HC, total SSc, dSSc and lcSSc patients (Figure 1). In contrast, the proportion of IgD^- $CD27^+$ memory B lymphocytes was significantly decreased in patients with SSc as compared to HC (p=0.04) (Figure 1), with similar distribution between dSSc and lcSSc. An additional differential proportion of marginal zone B cells was found between patients with dSSc and lcSSc that was compensated when global SSc was compared to HC. Specific analysis of mature B cells (Bm) classification identified the $CD38^+IgD^-$early Bm5 subpopulation as the predominantly decreased memory B cell subset, irrespective to lcSSc or dSSc disease form (p=0.001) (Supplemental figure 3).

Peripheral B lymphocytes from patients with SSc are activated

Patients with SSc exhibited increased proportions of B lymphocytes expressing activation markers CD95 (p=0.0004), CD69 (p=0.002) and CD86 (p=0.001) as compared to HC (Figure 2A). Similar results were obtained using mean fluorescence intensity (MFI) quantification (Supplemental Figure 4A). We also identified a B cell subpopulation expressing both CD69 and CD95 in SSc patients that was absent in HC (p=0.0002) (Figure 2B). Interestingly, subpopulations expressing CD69 were correlated to those expressing CD95 in SSc patients, both in dSSc and lcSSc forms (r=0.76; p<0.0001) (Figure 2C). Interestingly, percentages of $CD95^+$ B cells were higher in patients with early lcSSc compared to patients with non-early lcSSc (p=0.0027) (Figure 2D). In contrast, the proportion of peripheral $CD69^+$ B lymphocytes was higher in patients with early dSSc as compared to those with non-early dSSc (p=0.048).

Furthermore, when we combine these two activation markers, the percentage of $CD69^+$ $CD95^+$ B cells was higher in early SSc patients than in non-early SSc patients ($p<0.0001$). Association between other activation markers were not found for both SSc patients and HC.

Additionally, the proportions of cells expressing CD86 differed significantly between dSSc and lcSSc ($p=0.0007$). Similar difference was also observed for CD5 ($p=0.03$) (Figure 2A), whereas CD86 and CD5 B cell subsets were not associated with autoantibody status (data not shown). Since these two receptors play a critical role within B/T lymphocyte synapse, we wanted to investigate the potential correlations between the proportion of $CD5^+$ and $CD86^+$ B lymphocytes with the proportions of T helper (Th)1 ($CD3^+$ $CD4^+$ IFN- γ^+), Th2, ($CD3^+$ $CD4^+$ IL-4^+), Th9 ($CD3^+$ $CD4^+$ IL-9^+) and Th17 ($CD3^+$ $CD4^+$ IL-$17A^+$) (Supplemental Figure 5). No correlation of the percentage of CD5 or CD86 with these T cell subsets was found except a correlation between CD86 and the percentage of Th2. Similar results were obtained when we analyzed separately patients with dSSc and lcSSc.

In contrast, the proportion of cells expressing the inhibitory receptors CD22, CD32 and CD72 did not differ significantly between HC and SSc patients, except for CD32 between dSSc and lcSSc ($p=0.02$) (Figure 3A). Regarding membrane expression, B lymphocytes from SSc patients, showed increased levels of the constitutively expressed activating co-receptor CD20 as compared to HC cells ($p=0.001$), whereas no significant variation was found for the expression of the inhibitory receptors CD22, CD32, CD72 or of HLA-DR (Supplemental figures 4B and 6A). Finally, concerning the crosstalk of B cells with the microenvironment, a slight decrease of BAFF-receptor (BR3) expressing cells was detected for SSc patients compared to HC ($p=0.04$); however, this difference was not significant at the cellular level (Supplemental figure 6B). Noteworthy, a remarkable increase of the populations expressing either IL-6R ($p=0.01$) or IL-21R ($p=0.02$) was observed in SSc patients with major differences

for IL-21R expression between dSSc and lcSSc ($p<0.0001$) regarding both subpopulation percentages and MFI analysis (Figure 3B and Supplemental figure 6C).

In order to identify clinical manifestations of SSc that could be associated with B cell subsets, expression of activation markers and receptors were evaluated in the presence or not of pulmonary arterial hypertension (PAH), interstitial lung disease (ILD), arthralgia (joint) and digital ulcers (DU) (Supplemental Table 2). An increased proportion of B cells expressing CD86 was slightly associated with PAH and digital ulcers in patients with SSc while increased subpopulation of $CD95^+$ B cells was associated with arthralgias. More interestingly, patients with either arthralgias, PAH and/or ILD had increased proportions of B cells expressing the IL-6R. For these two late manifestations, similar proportions of B lymphocytes expressing IL-6R were observed which allowed discriminating between patients with PAH and/or ILD, and those without documented pulmonary involvement with lower proportion of peripheral IL-$6R^+$ B cells (Supplemental figure 7).

Overall, no significant difference was found in B cell phenotypes on the basis of autoantibody specificity (data not shown).

B lymphocytes are detected in the lungs from patients with SSc-associated PAH

We performed immunostaining in lung samples from three patients with SSc-associated PAH and three controls (normal lung tissue from patients with non-small cell lung carcinoma). In SSc-patients we observed a number of lymphoid follicles, mostly in the vicinity of bronchioles/small airways, whereas only scattered B-lymphocytes are perceived in the lung parenchyma of the control patient, as assessed by anti-CD20 immunostaining. In lymphocyte-rich areas, scattered $CD69^+$ cells can be observed, whereas only faint staining of sparse cells is observed in control patients (Supplemental figure 8).

Phenotypical characteristics of peripheral B cells are consistent with higher production of IL-6 and TGF-β in SSc patients

A major increase in IL-6 producing cells was evidenced for SSc patients as compared to HC ($p<0.0001$) (Figure 4A). This difference was also found for both IL-6^+ $CD27^-$ IgD^+ naïve B cells (ratio 1:2 $p<0.0001$) and IL-6^+ $CD27^+$ IgD^- memory B cells (ratio 1:1.8 $p=0.0003$) but not for marginal zone or double negative effector B cells (data not shown). We further analyzed the intracellular staining of IL-6 in both T lymphocytes (defined as $CD27^+$ $CD19^-$ cells) and monocytes (using FCS/SSC parameters in $CD19^-$ $CD27^-$ cells) as shown in Supplemental figure 9 (Panel A). No difference was observed for percentages of IL-6^+ T cells between patients with SSc and HC (Supplemental figure 9 Panel B), whereas a decreased proportion of monocytes expressed IL-6 in SSc patients as compared to HC (Panel C). Thus, in patients with SSc, we identified the B cells as the main source of IL-6 compared to T lymphocytes and monocytes, which was not the case in HC (Supplemental figure 9 Panel D). We also investigated IL-10 and TGF-β (Figure 4B and 4C) production by purified B lymphocytes after three days of culture upon stimulation or not with CpG. Number of IL-10 producing B cells did not differ significantly between our various samples in such conditions and their phenotype corresponded to $CD19^+$ $CD24^{hi}$ $CD27^{+/-}$ $CD38^{hi}$ predominantly $CD5^-$ (Supplemental figure 10A). More interestingly, peripheral B cells isolated from patients with SSc expressed increased proportion of TGF-β^+ $CD19^+$ B lymphocytes as compared to HC, even in the absence of further stimulation of TLR9 through CpG incubation ($p=0.0002$) (Figure 4C). TGF-β expressing B lymphocytes were $CD19^+$ $CD5^-$ $CD24^{low}$ $CD27^+$ $CD38^{hi}$ both in HC and patients with SSc and did not overlap with IL-10^+ B lymphocytes (Supplemental figure 10B).

B cells from SSc patients secrete higher amounts of IL-6 and TGF-β and lower amounts of IL-10 upon stimulation

We detected higher levels of IL-6 in the serum of SSc patients as compared to HC (p=0.01) (Figure 5A, left panel). Conversely, no significant difference was found for IL-10 or TGF-β (Figure 5A, middle and right panel). Interestingly, purified B cells from SSc patients, incubated3 days with CpG or for 4 hours with a mix of PMA/ionomycin secreted increased amounts of IL-6 whereas those from HC remained low (p=0.0007) (Figure 5B).We also detected slightly increased IL-10 concentration in B cell supernatant from SSc patients as compared to HC after stimulation with PMA/ionomycin (p=0.01) (Figure 5C). Unexpectedly, while CpG stimulation promoted a work increase of IL-10 secretion for SSc patients a strong increase was observed for HC in such condition. According to the increased proportion of TGF-β producing cells, we evidenced increased amount of TGF-β in the supernatant of CpG stimulated SSc B cells as compared to HC (p=0.001) while PMA/ionomycin treatment did not influence significantly the release as compared to unstimulated cells (Figure 5D and Supplemental figure 11).

A correlation was found between modified Rodnan Skin Score (mRSS) and the concentration of TGF-β in culture supernatant from B cell stimulated with CpG (r=0.60; p=0.04) (Figure 5E). Similar correlation was between mRSS and the concentration of IL-6 within supernatants of B cells stimulated with PMA/ionomycin cocktail for 4 hours (r=0.71; p=0.01), suggesting a potential pro-fibrotic role of B lymphocytes in SSc patients.

In vitro stimulation of HC and SSc dermal fibroblasts by B lymphocyte supernatant increase proliferative and fibrotic phenotype

Culture of fibroblasts from HC or fibroblasts isolated from involved skin of SSc patients for 3 days in the presence of supernatants of B cells from HC did not influence significantly their metabolic activity (Figure 6A) and their proliferation rate as assessed by immunofluorescence using Ki67 labeling (Supplemental figure 12). A randomly increased viability was observed for

all fibroblasts when cultured in the presence of supernatant from isolated B cell from SSc patients from without reaching significance (Figure 6A). Previous stimulation of HC or SSc B cells did not provide additional effect on viability. However, supernatant from CpG or PMA stimulated SSc B cells significantly enhanced to variable extents the proliferation of both HC and SSc fibroblasts (Figure 6B). In agreement with their increased proliferation rate, both HC and SSc fibroblasts showed poor production of collagen in the presence of PMA stimulated B cell supernatant from SSc patients compared to HC (Figure 6C). When supernatants of CpG stimulated purified B cells from HC and SSc patients were used to stimulate fibroblasts in culture, collagen production was increased differentially in both HC and SSc fibroblasts (Figure 6C).

Furthermore, we analyzed by q-PCR, expression levels of α-Smooth muscle actin (SMA), Snail and vimentin mRNA within HC and SSc stimulated fibroblasts (Supplemental figure 13). Unfortunately, no consistent detection of α-SMA was obtained with either HC or SSc fibroblasts using both q-PCR and immunofluorescence (data not shown). However, when both HC and SSc fibroblasts were stimulated with either CpG stimulated or PMA/ionomycin stimulated purified B cell supernatant from SSc patients, an increased expression of vimentin was observed. Increased expression of the transcription factor Snail and of collagen mRNA levels were also detected when fibroblasts from either HC or SSc patients were stimulated with CpG stimulated B cell supernatant from SSc patients. These increases were abrogated when supernatants were pre-incubated with anti-TGF-β antibody for CpG supernatant, or anti-IL-6R antibody for PMA supernatant. Furthermore, incubation with anti-TGF-β antibody or anti-IL-6R antibody induced a drastic decrease in fibroblast proliferation without any difference in viability assay, reinforcing the potential pathophysiological role of B lymphocytes in SSc (Supplemental figure 13).

Discussion

In the present work, we provide evidence for an increase in SSc patients as compared to HC of a peripheral B cell subpopulation that express both activation markers and cytokine receptors as well as high levels of intracellular IL-6 and TGF-β. We also establish that B lymphocytes supernatants from SSc patients activate at higher levels than those from HC both normal and scleroderma human fibroblasts.

In SSc patients, we observed an increased expression of CD86 and CD95 on B cells altogether with a lower proportion of peripheral memory B cells, in agreement with results obtained by Sato et al. (Sato et al., 2004). We further restricted this decrease to the early Bm5 memory peripheral B cell subpopulation as in peripheral blood of patients with Sjögren's syndrome [16]. Interestingly, we also found in patients with SSc, an increased expression of CD69, IL-6R and IL-21R on peripheral B lymphocytes, and further correlated the increased proportions of $CD69^+$cells within decreased $CD95^+$ B lymphocytes. Furthermore, we showed a major differential proportion of $CD5^+$, $CD86^+$, IL-$6R^+$ or IL-$21R^+$ B cells between dSSc and lcSSc patients. Indeed patients with dSSc had higher proportion of these B cells compared to both lcSSc patients and HC. In contrast, both lcSSc and dSSc patients showed increased proportions of $CD69^+$ and $CD95^+$ B cells compared to HC. Moreover, we found several correlations between the activation of B lymphocytes and disease duration in SSc patients, with higher expression of CD95 within the subgroup of early lcSSc patients, increased percentage of $CD69^+$ for patients with early dSSc. In addition, we detected the double positive $CD69^+$ $CD95^+$ peripheral B cells subpopulation in patients with early SSc, suggesting a possible link between B cells activation at an early stage of the disease and disease severity. Of note, IL-6R expression is strongly associated with arthralgia, PAH and/or ILD. Similar proportions of B lymphocytes expressing IL-6R were observed for PAH and ILD. This finding allowed discriminating patients with or without lung involvement, the later exhibiting lower proportions of peripheral IL-$6R^+$

B cells. In lung tissue from patients with SSc-PAH, we observed important numbers of B cells, with a number of lymphoid follicles, mostly in the vicinity of bronchioles/small airways, whereas only scattered B-lymphocytes were perceived in the lung parenchyma of control patients, providing further evidence in the implication of B cells in lung involvement in patients with SSc. Altogether, our data argue for an activation profile of B lymphocytes in SSc patients, probably due to BCR engagement via autoantigens for both lSSc and dSSc [17; 18]. But, it also suggests a differential activation between dSSc and lSSc B lymphocytes as for patients with PAH and/or ILD vs absence of lung involvement; this differential activation could potentially occur through cytokine modulation as suggested in a recent clinical study [19]. No correlation was found between B lymphocytes activation and autoantibody status, probably arguing that they represent additional markers of disease activity/severity. Since B cells from patients with dSSc, with PAH and/or ILD are activated, these cells could be proposed as biomarkers.

We also provide novel evidences regarding the production and secretion of cytokines. We identified both naïve and memory B lymphocytes derived from SSc patients as major IL-6 secreting cells. However, a direct correlation between IL-6 levels in the supernatant and IL-6R expression at the membrane of B lymphocytes was not observed. This result does not argue for an autocrine activating loop in SSc B lymphocytes, similar to the autostimulatory IL-6-driven B cell differentiation in patients with systemic lupus erythematosus [20]. The mechanisms leading to B lymphocytes overactivation might rather be due to the increased concentration of serum IL-6 in the serum of SSc patients vs HC as previously reported by Needleman et al.[21]. Moreover, we shown that IL-6 levels in the supernatant of purified B cells isolated from SSc patients also correlate with mRSS, suggesting a critical role of B lymphocyte in the induction of fibrosis. Monocytes have been proposed as the unique immune cells producing IL-6 in patients with SSc [22;23]. Herein, we provide new evidences for the characterization of IL-6 producing B lymphocytes in patients with SSc. These B lymphocytes might represent

interesting targets for SSc treatment since the elevated levels of IL-6 in patients sera correlate with disease severity (Sato et al., 2001),

Additionally, in contrast with a previous study, we report elevated TGF-β production and release by peripheral B lymphocytes from SSc patients as compared to HC both at steady state and upon specific TLR stimulation [24]. These high levels of TGF-β in supernatants of sorted B cells also correlate with mRSS, suggesting a role of B cells in fibrosis development. Indeed Hasegawa *et al.* reported a lack of substantial increase in the release of TGF-β by regulatory B cells, as well as activated T cells [25] or monocytes [26] in SSc patients, in line with a non-immune production of TGF-β in those patients. Herein, we identified a specific B lymphocyte subpopulation that secretes high level of TGF-β and exhibits a phenotype similar to those recently described for GVHD [27]. The absence of IL-10 secretion by SSc B lymphocytes might also contribute to the loss of immunoregulation observed in SSc [28; 29]. Unfortunately, we were not able to perform co-culture experiments using B and T cells in order to evaluate these immunomodulatory roles or potential interplay with T cells due to the limitation in the amount of biological samples available.

Pathogenic effect of the B lymphocytes in SSc remains poorly understood (Arnett, 2006).Recent reports have addressed interesting therapeutic targeting both in mouse models and in open studies on limited populations of patients with SSc [30; 31; 32; 33]. Notably, François and colleagues reported a direct implication of normal B cells in increased proliferation and extracellular matrix production by HC and SSc fibroblasts [34]. In the present study, we demonstrate that supernatants of B lymphocytes purified from SSc patients induce fibroblast activation even in absence of direct cell-to-cell interaction or autoantibodies secretion, but more as a consequence of an increased secretion of IL-6 and TGF-β. We also found that supernatants of purified B lymphocyte with the higher level of TGF-β (CpG

supernatant) induce an increased expression of vimentin and the transcription factor Snail, two proteins that have been associated with the differentiation of fibroblasts into myofibroblasts [35; 36; 37]. Moreover, incubation with either anti-TGF-β or anti-IL-6R antibodies induce a dramatic decrease in fibroblast proliferation without any difference in viability assay, suggesting an implication of these pathways in B cell/fibroblast crosstalk in SSc. These data further underline a possible interplay between B lymphocytes and fibroblasts through new secretory pathways that need to be further investigated in SSc pathogenesis. Furthermore, the recent results obtained with IL-6 targeted therapy in the bleomycin induced-scleroderma mouse model and in patients with SSc highlight the prevalent role of this cytokine in SSc pathophysiology and reinforce the interest for evaluating IL-6 secretion capacity of peripheral B cells [21; 33;38; 39]. Finally, these data also argue for the interest of investigating the effects of IL-6 targeted therapy in patient with SSc as the faSScinate study has shown interesting results using a monoclonal anti-IL-6R antibody (tocilizumab) (Khanna et al., 2016). Our study, based on a consistent number of phenotypically characterized SSc patients who did not receive glucocorticoid or immunosuppressive treatments, provide new insights in the functional implication of B lymphocytes in SSc.

Conclusion

Clinical studies have shown promising results for anti-CD20 and anti-IL-6R based therapies in patients with SSc [21; 39; 40]. We observed an increased activation of peripheral B cells in patients with dSSc as compared to lcSSc patients. *In vitro*, B lymphocytes from SSc patients secrete high amounts of IL-6 and TGF-β which induce fibroblast proliferation, collagen secretion and differentiation into myofibroblasts in both healthy individuals and SSc fibroblasts. This report highlights for the first time differential B lymphocyte subpopulations between the two different SSc subtypes.

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N.D, B.C, A.R, S.L, M.M, P.B, A.B, N.T and N.V-B declare that they have no conflicts of interest concerning this article. B.T received Honoria and consulting fees from Roche. L.M received traveling grants and consulting fees from Roche.

Legend to Figures

Figure 1. Maturation status of B lymphocytes populations from healthy controls (HC) and patients with systemic sclerosis (SSc). B lymphocyte subsets were analyzed in 21 HC (●) and 34 patients with SSc (□), including 9 patients with diffuse SSc (dSSc) (▲) and 25 with limited SSc (lcSSc) (▽). Percentages of B lymphocyte subpopulations were quantified as depicted: i) memory/plasmablast cells as $CD19^{+}$ $CD27^{+}$ IgD^{-} cells (upper left); ii) naive B lymphocytes as $CD19^{+}$ $CD27^{-}IgD^{+}$cells (upper middle); iii) plasmablasts as $CD19^{+}$ $CD27^{++}$ $CD38^{hi}$ cells (upper right); iv) effector memory B cells as $CD19^{+}$ $CD27^{-}IgD^{-}$ cells (lower left); v) regulatory B cells (Breg) as $CD19^{+}$ $CD24^{hi}$ $CD38^{hi}$ cells (lower middle); vi) marginal zone B cells as $CD19^{+}IgD^{+}$ $CD27^{+}$(lower right). Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05

Figure 2. Activation markers of peripheral B lymphocytes from healthy controls (HC) and patients with systemic sclerosis (SSc). B lymphocytes activation markers were analyzed in 21 HC (●) and 43 patients with SSc (□), including 11 with diffuse SSc (dSSc) (▲) and 32 with limited SSc (lcSSc) (▽). Percentages of $CD95^{+}$ (upper left), $CD69^{+}$ (upper right), $CD86^{+}$ (lower left) and $CD5^{+}$ (lower right) were quantified in total $CD19^{+}$B lymphocytes (Panel A). Percentages of double positive $CD69^{+}$ $CD95^{+}$ B $CD19^{+}$ lymphocytes were quantified (Panel B). Correlation of CD69 and CD95 expression on peripheral $CD19^{+}$B lymphocytes from patients with dSSc (dotted line) or with lcSSc (full line) was analysed using a Spearman's test (Panel C). Percentages of $CD69^{+}$, $CD95^{+}$ and double positive $CD69^{+}$ $CD95^{+}$ B cells were compared between early onset SSc (included less than 1 year after the first non-Raynaud's SSc symptom) of both SSc, and non-early onset SSc (included more than 1 year after the first non-Raynaud's SSc symptom) (Panel D). Comparisons between groups were performed using Mann-Whitney

U-test. Statistical significance was defined as:* p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001.

Figure 3. Receptor expression at the membrane of peripheral $CD19^+$ B lymphocytes from healthy controls (HC) and patients with systemic sclerosis (SSc). Inhibitory ($CD22^+$ (left), $CD32^+$ (middle) and $CD72^+$ (right)) (Panel A), IL-$6R^+$ (lower left) and IL-$21R^+$ (lower right)) (Panel B) receptors was analyzed at the surface of $CD19^+$B lymphocytes from 20 HC (●) and 43 patients with SSc (□), including 11 patients with diffuse SSc (dSSc) (▲) and 32 patients with limited SSc (lcSSc) (▽). A typical dot plot of IL-6R and IL-21R expression is shown in B upper panel. Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05; ***p-value < 0.001 ****p-value < 0.0001.

Figure 4. Intracellular cytokine staining of peripheral B lymphocytes from healthy controls (HC) and patients with systemic sclerosis (SSc). Detection of intracellular IL-6 was analyzed in 18 HC (●) and 32 patients with SSc (□), including 10 with diffuse SSc (dSSc) (▲) and 22 with limited cutaneous SSc (lcSSc) (▽). Percentages of IL-6 expression were quantified in B lymphocyte subsets in Panel A: i) total B lymphocytes ($CD19^+$) (left); ii) naïve B cells ($CD19^+$ $CD27^-$ IgD^+) (middle); iii) memory B cells ($CD19^+$ $CD27^+$ IgD^-) (right). Detection of intracellular IL-10 and TGF-β were analyzed in 11 HC (●) and 12 patients with SSc (□), including 4 patients with dSSc (▲) and 8 patients with lcSSc (▽). Percentages of IL-10^+ cells were quantified in: i) unstimulated total B lymphocytes (lower left); ii) CpG ODN stimulated B cells (lower right) (Panel B). Percentages of TGF-β were quantified in: i) unstimulated total B lymphocytes (lower left); ii) CpG ODN stimulated B cells (lower right) (Panel C). Typical dot plots of IL-6 (A right), IL-10 (upper B) and TGF-β (upper C) staining are shown.

Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001 ****p-value < 0.0001.

Figure 5. Cytokine concentrations in sera and supernatants of peripheral stimulated B cells from healthy controls (HC) and systemic sclerosis (SSc) patients. IL-6, IL-10 and TGF-β were quantified in the serum of 10 HC (●) and 22 patients with SSc (□), including 7 patients with diffuse SSc (dSSc) (▲) and 15 patients with limited cutaneous SSc (lcSSc) (▽) and expressed in pg/mL (Panel A). IL-6 (Panel B), IL-10 (Panel C) and TGF-β (Panel D) were quantified in the supernatants of purified B cells after 4 hours of stimulation with PMA/ionomycinin 6 HC (●) and 11 patients with SSc (□), including 4 patients with dSSc (▲) and 7 patients with lcSSc (▽) and expressed in pg/mL.IL-6 (B), IL-10 (C) and TGF-β (D) were quantified in the supernatant of purified B cells after 4 hours stimulation with CpG in 4 HC (●) and 12 patients with SSc (□), including 4 patients with dSSc (▲) and 8 patients with lcSSc (▽). Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05; ***p-value < 0.001. Correlations between modified Rodnan skin score (mRSS) and concentrations of IL-6 within purified PMA stimulated B lymphocytes supernatants (left) and TGF-β within purified CpG stimulated B lymphocytes supernatants (right) from 11 patients with SSc were assessed using Spearman test.

Figure 6. Impact of purified B lymphocyte supernatants from healthy controls (HC) and patients with systemic sclerosis (SSc) on fibroblast proliferation and collagen production. Fibroblasts isolated from 4 HC (left column) and 4 patients with SSc (right column) were cultured three days in the presence of supernatants from: i) 3 days cultured unstimulated

purified B lymphocytes (UNS); ii) 3 days cultured CpG stimulated purified B lymphocytes (CpG); iii) 4 hours cultured PMA/ionomycin stimulated purified B lymphocytes (PMA), from 4 HC (white histogram) and 4 non-autologous patients with diffuse SSc (black histogram). Viability of fibroblasts was quantified using XTT reduction assay kit and results were expressed as percentage of XTT reduction in stimulated vs unstimulated fibroblasts (Panel A). Proliferation of fibroblasts was quantified using BrdU incorporation dosage (Panel B). Results were expressed as percentage of BrdU incorporation by stimulated vs unstimulated fibroblast. Collagen production by fibroblasts was quantified using Sircol Biocolor assay and expressed as percentage of collagen production of stimulated vs unstimulated fibroblasts (Panel C). All experiments were performed twice with similar results. Comparisons between groups were performed using paired t-test and represented in histogram (mean ± standard deviation (SD). Statistical significance was defined as:* p-value < 0.05; ***p-value < 0.001.

Supplemental methods

Lymphocyte culture

PBMC were isolated from peripheral blood on Ethylenediaminetetraacetic acid (EDTA)-anticoagulated tubes from HC or patients with SSc using a Ficoll-Hypaque density gradient centrifugation (Eurobio, Courtaboeuf, France). B lymphocytes were negatively sorted from PBMC using the magnetic pan B-cells isolation kit II from Milteny Biotec (Ref 130-101-638, Paris, France). PBMC and purified B lymphocytes were cultured in Roswell Park Memorial Institute medium 1640 (RPMI) obtained from Gibco (LifeTechnologies, Saint-Aubin, France), with 10% heat-inactivated fetal calf serum (FCS) (Eurobio) at 37°C in 5% CO_2 atmosphere.

Lymphocyte stimulation

Purified B lymphocytes (5.10^5; purity>95%) were stimulated in a 48 well plate at a concentration of 1.10^6 cells/ml using 50 ng/ml of phorbolmyristate acetate (PMA), 1 µg/ml of

ionomycin and, for generation of regulatory B cells, additional 10 µg/ml of CpG Oligodeoxynucleotide (ODN) 2006 (Invivogen, Toulouse, France). After three days of culture, supernatants were collected and 5.10^5 B lymphocytes were stimulated with PMA/ionomycine with 1 µl/million of cells of GolgiStop solution (BD Biosciences, Le Pont de Claix, France) for four hours and then stained for flow cytometry analysis. Cells were then washed, stained and then fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences). Finally, cells were stained with intracellular antibodies.

Interferon-gamma (IFN-γ), IL-4, IL-6, IL-9, IL-17, IL-21 and Tumor necrosis factor alpha (TNF-α) production by B or T lymphocytes were assessed after PBMC stimulation for 4 hours with 50 ng/ml of PMA, 1 µg/ml of ionomycin and 1 µl/million of cells of GolgiStop solution. Cells were then washed and stained.

Quantification of cytokine production in cell culture supernatants, HC or SSc serum was assessed using a multiplex assay from MesoScaleDiscovery (MSD, Rockville, MD, USA).

Flow cytometry analysis

We performed flow cytometry analysis with peripheral blood of SSc patients and healthy controls after total PBMC staining with an anti-CD19-Brillant Violet 605 (BV605), an anti-CD27-AlexaFluor700 obtained from BD Biosciences and an anti-CD21-Peridin-chlorophyll protein-Cyanin5.5 (PERCP-Cy5.5) obtained from Biolegend (London, UK). For B lymphocyte subpopulations, we further investigated membrane IgD, CD21, CD27 and CD38 expressions within $CD19^+$B cells (Supplemental Figure 2) as previously described [41].

Anti-CD3-AlexaFluor 700, anti-CD4-BV 421 from BD Biosciences and anti-CD8-PERCP-Cy5.5 from Biolegend were used to define T lymphocyte subpopulations. In combination with these markers, various antibodies were used to stain extracellular or intracellular proteins of B and T lymphocytes and are listed in Supplemental Table 4.

In longitudinal experiments, cytometer settings were standardized using BD Cytometer setup, SPHERO Rainbow Calibration Particles (BD) and BD Combeads. Flow cytometry acquisition was assessed using a BD LSR Fortessa at the cytometry plateform of Cochin Institute (Cybio) and data were analyzed using FlowJo analysis software (Treestar, Ashland, OR, USA).

Legend to supplemental Figures

Supplemental Figure 1. Flow cytometry gating strategy of peripheral B and T lymphocytes. Lymphocytes were identified using the side scatter (SSC) and forward scatter (FSC) parameters. B lymphocytes were identified as $CD19^{+}$ $CD21^{+/-}$ lymphocytes. Dot plots representative of the various markers used to characterize B lymphocytes are shown. T lymphocytes were defined as $CD3^{+}$ cells expressing either $CD4^{+}$ or $CD8^{+}$ (panel B). T lymphocyte subpopulations were identified as follows: i) T helper (Th) 1 as viable $CD3^{+}$ $CD4^{+}$ IFN-γ^{+} cells, ii) Th2 as viable $CD3^{+}$ $CD4^{+}$ IL-4^{+} cells, iii) Th9 as viable cells $CD3^{+}$ $CD4^{+}$ IL-9^{+}, iv) Th17 as viable $CD3^{+}$ $CD4^{+}$ IL-17^{+} cells and v) T CD8 effector cells as viable $CD3^{+}$ $CD8^{+}$ IFN-γ^{+} cells.

Supplemental Figure 2. Absolute B and T cell counts in peripheral blood from healthy controls (HC) and patients with systemic sclerosis (SSc). B cell (left panel) and T cell (right panel)

absolute numbers were quantified as respectively $CD3^-$ $CD19^+$ $CD45^+$ $CD56^-$ and $CD3^+$ $CD19^-$ $CD45^+$ $CD56^-$ in total peripheral blood using the Trucount technology from 12 HC (black box) and 22 patients with SSc (white box), including 8 patients with diffuse SSc (dSSc) (dotted box) and 14 patients with limited SSc (lcSSc) (stripped box) and represented by boxes and whiskers (mean + Min/Max). Comparisons between groups were performed using Mann-Whitney U-test.

Supplemental Figure 3. Analysis of mature B lymphocyte (Bm) subpopulations in healthy controls (HC) and patients with systemic sclerosis (SSc). Subpopulations of mature B lymphocyte (Bm) were quantified in 21 HC (●) and 34 patients with SSc (□), including 9 patients with diffuse SSc (dSSc) (▲) and 25 patients with limited cutaneous SSc (lcSSc) (▽). Percentages of Bm subpopulations were quantified as depicted: i) Bm1 cells as $CD19^+$ $CD38^-$ IgD^+ cells (upper left); ii) Bm2 cells as $CD19^+$ $CD38^+$ IgD^+ cells (upper middle); iii) Bm2' cells as $CD19^+$ $CD38^{hi}$ IgD^+ cells (upper right); iv) Bm3/Bm4 cells as $CD19^+$ $CD38^{hi}$ IgD^- cells (lower left); v) early Bm5 as $CD19^+$ $CD38^+$ IgD^- (lower middle); vi) Bm5 cells as $CD19^+$ $CD38^-$ IgD^- cells (lower right). Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05.

Supplemental Figure 4. Activation marker expression on peripheral B lymphocytes from healthy controls (HC) and patients with systemic sclerosis (SSc). Activation marker on B lymphocytes were analyzed in 21 HC (white histogram) and 43 patients with SSc (black histogram), including 11 patients with diffuse SSc (dSSc) (dotted histogram) and 32 patients with limited cutaneous SSc (lcSSc) (stripped histogram) and represented by histograms (mean ± standard deviation). Mean of fluorescence intensity of CD69 (left), CD86 (middle) and CD95 (right) were quantified in total B lymphocytes (Panel A). Mean of fluorescence intensity of

HLA-DR (left) and CD20 (right) were quantified in total B lymphocytes (Panel B). Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001.

Supplemental Figure 5. Correlations between T lymphocytes activation and $CD5^+$ or $CD86^+$ B lymphocytes from patients with SSc. Proportions of T helper (Th)1 (upper left), Th2 (upper right), Th9 (lower left) and Th17 (lower right) in peripheral blood from 31 patients with SSc including 25 patients with lcSSc and 6 patients with dSSc correlated with percentages of $CD5^+$ B lymphocytes (Panel A) or $CD86^+$ B lymphocytes (Panel B). Correlations were performed using Spearman correlation.

Supplemental Figure 6. Receptors expression at the surface of peripheral B lymphocytes from healthy controls (HC) and patients with systemic sclerosis (SSc). Inhibitory ($CD22^+$ (left), $CD32^+$ (middle) and $CD72^+$ (right)) (Panel A) and activation (BAFF-receptor ($BR3^+$) (Panel B), IL-$6R^+$ (left) and IL-$21R^+$ (right)) (Panel C) receptors were analyzed at the surface of B lymphocytes from 20 HC (white histogram) and 43 patients with SSc (black histogram), including 11 patients with diffuse SSc (dSSc) (dotted histogram) and 32 patients with limited SSc (lcSSc) (stripped histogram). Mean of fluorescence intensity were represented by histograms (mean + standard deviation). Proportion of $BR3^+$ cells among total B lymphocytes is also presented (Panel B left). Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001 ****p-value < 0.0001.

Supplemental Figure 7. Association of B lymphocytes activation markers with clinical manifestations of systemic sclerosis (SSc). Activation markers and receptors on B lymphocytes were analyzed in 21 HC (●) and 43 patients with SSc. Patients with SSc presenting nonexclusive clinical manifestations including 5 patients with pulmonary arterial hypertension (PAH) (■), 7 patients with interstitial lung disease (ILD) (▼), 14 patients with joint involvement (joint) (♦) and 9 patients with digital ulcers (DU) (■) were compared with patients without dedicated symptom (⊖). $CD86^+$ B lymphocytes (Panel A), $CD95^+$ B lymphocytes (Panel B), IL-$6R^+$ B lymphocytes (Panels C and D) were quantified in total B lymphocytes from the different subgroups. Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001 ****p-value < 0.0001.

Supplemental Figure 8. Immunostaining in lung samples from a patient with SSc-associated PAH and a normal lung tissue from a patient with non-small cell lung carcinoma. The individual patient and control depicted are representative of others patients and controls tested. B-lymphocytes are highlighted by anti-CD20 staining in the lung of a SSc patient (panel A) and a control patient (panel B); $CD69^+$ cells are depicted in the lung of a SSc patient (panel C) and a control patient (panel D).

Supplemental Figure 9. Intracellular IL-6 staining within peripheral blood mononuclear cells (PBMC) from healthy controls (HC) and patients with systemic sclerosis (SSc). Intracellular staining of IL-6 was analysis in PBMC from 18 HC and 23 patients with SSc, including 5 with diffuse SSc (dSSc) and 18 with limited cutaneous SSc (lcSSc) (Panel A). Percentages of IL-6 were quantified in T lymphocytes ($CD19^-$ $CD21^-$ $CD27^+$) (panel B) and in monocytes ($CD19^-$ $CD21^-$ $CD27^-$) (panel C). Percentages of each subpopulation of cells were quantified in total

IL-6 positive viable cells (Panel D). Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as: * p-value < 0.05; ***p-value < 0.001 ****p-value < 0.0001.

Supplemental Figure 10. Phenotypic characterization of IL-10$^+$ and TGF-β$^+$ peripheral B lymphocytes. CD5, CD24, CD27 and CD38 expression were evaluated in IL-10$^+$ CD19$^+$ viable B cells (Panel A) and in TGF-β$^+$ CD19$^+$ viable B cells (Panel B).

Supplemental Figure 11. Cytokine concentrations in supernatants of unstimulated peripheral B cells from healthy controls (HC) and systemic sclerosis (SSc) patients. IL-6, IL-10 and TGF-β were quantified in the supernatant of purified B cells after 3 days of culture without any stimulation in 4 HC (●) and 11 patients with SSc (□), including 3 patients with dSSc (▲) and 8 patients with lcSSc (▽). Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05.

Supplemental Figure 12. Impact of B lymphocyte supernatants from healthy controls (HC) and patients with systemic sclerosis (SSc) on proliferation of non-autologous HC and SSc fibroblasts. Fibroblasts isolated from 4 HC (left column) and 4 patients with SSc (right column) were cultured three days in the presence of supernatants from: i) 3 days culture unstimulated purified B lymphocytes (UNS); ii) 3 days culture CpG stimulated purified B lymphocytes (CpG); iii) 4 hours culture PMA/ionomycin stimulated purified B lymphocytes (PMA), from 4 HC (white histogram) and 4 non-autologous patients with diffuse SSc (black histogram). Proliferation of fibroblasts was quantified using immunofluorescence staining of Ki67 (Upper

Panel). Representative fluorescence patterns (20X) of Ki67 immunostaining were represented for one fibroblast from one HC and one fibroblast from one SSc patient after three days of culture with different stimulated B cell supernatants.

Supplemental Figure 13. Impact of purified B lymphocyte supernatants from patients with systemic sclerosis (SSc) on proliferation and phenotype of non-autologous healthy control (HC) and SSc fibroblasts. Fibroblasts isolated from 4 HC and from involved skin of 4 patients with dSSc were cultured in the presence of supernatants from: i) 3 days culture CpG stimulated purified B lymphocytes (CpG); ii) 4 hours culture PMA/ionomycin stimulated purified B lymphocytes (PMA), from 4 non-autologous patients with diffuse SSc. For each condition, supernatant was supplemented with either an anti-TGF-β Ab (within CpG supernatant) or an anti-IL-6R Ab (within PMA supernatant). Proliferation (Panel A) and mRNA level of Collagen, Vimentin and Snail (Panel B) were quantified after three days of culture. Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as:* p-value < 0.05

Supplemental Table 1. Clinical characteristics of SSc patients at the time of blood sampling (n=90)

Gender (female): n (%)	78 (86.67)
Age, yr: median (SD)	56.66 (13.42)
Disease duration, month: median (SD)	92 (88)
Limited cutaneous SSc: n (%)	67 (74.44)
Skin sclerosis: n (%)	39 (58.21)
Modified Rodnan Skin Score: median (SD)	2 (3.73)
Mouth opening (mm): median (SD)	40 (13.61)
Digital ulcers: n (%)	16 (23.88)
Calcinosis: n (%)	19 (28.39)
Raynaud's phenomenon: n (%)	58 (86.57)
Telangiectasia: n (%)	27 (40.3)
Sclerodactyly: n (%)	21 (31.34)
Interstitial lung disease: n (%)	18 (26.86)
DLCO, %: median (SD)	87 (23.96)
Pulmonary arterial hypertension: n (%)	4 (5.97)
Renal involvement: n (%)	4 (5.97)
Gastro-intestinal involvement: n (%)	37 (55.22)
Arthralgia: n (%)	23 (34.33)
Muscle involvement: n (%)	11 (16.42)
Diffuse SSc: n (%)	23 (25.56)
Skin sclerosis: n (%)	23 (100)
- Modified Rodnan Skin Score: median (SD)	14 (8.77)
- Mouth opening (mm): median (SD)	30 (8.13)
Digital ulcers: n (%)	16 (69.56)
Calcinosis: n (%)	5 (21.74)
Raynaud's phenomenon: n (%)	21 (91.3)
Telangiectasia: n (%)	12 (52.17)
Sclerodactyly: n (%)	5 (21.74)
Interstitial lung disease: n (%)	13 (56.22)
DLCO, %: median (SD)	51 (19.23)

Pulmonary arterial hypertension: n (%)	3 (13.04)
Renal involvement: n (%)	3 (13.04)
Gastro-intestinal involvement: n (%)	17 (73.91)
Arthralgia: n (%)	13 (56.22)
Muscle involvement: n (%)	4 (17.39)
Immunological features n (%)	
- Anti-nuclear antibodies	83 (92.22)
- Anti-centromere antibodies	38 (42.22)
- Anti-topoisomerase 1 antibodies	30 (33.33)
- Anti-RNP antibodies	5 (5.55)
- Anti-PM/Scl antibodies	5 (5.55)
- Anti-RNA Pol III	3 (3.33)
- Other antibodies (anti-SSA, rheumatoid factor, anti-cardiolipin …)	34 (37.77)

Variables are expressed as number and percentages or median (SD) as appropriate.

Renal involvement was defined as elevated serum creatinin level (>150mL/min) or renal crisis.

Abbreviations: DLCO, diffusing capacity of the lung for carbon monoxide; PM/Scl: polymyositis/scleroderma; RNP: ribonucleoprotein; SD, standard deviation; SSc, systemic sclerosis; yr, years

Supplemental Table 2. Association of B lymphocytes activation markers with clinical manifestations in systemic sclerosis patients†

Marker/Clinical manifestation	PAH (n=7) vs no PAH	ILD (n=31) vs no ILD	PAH or ILD (n=38) vs no pulmonary involvement	Arthralgia (n=36) vs no arthralgia	Digital ulcers (n=32) vs no digital ulcers
% $CD5^+$within $CD19^+$	14.12 vs 16.09 (p=0.82)	17.18 vs 13.50 (p=0.68)	16.21 vs 10.78 (p=0.35)	14.20 vs 14.80 (p=0.97)	14.21 vs 16.75 (p=0.64)
% $CD86^+$within $CD19^+$	47.82 vs 19.26 (p=0.03)	34.20 vs 21.92 (p=0.49)	34.84 vs 19.42 (p=0.13)	29.75 vs 19.76 (p=0.36)	36.17 vs 18.72 (p=0.04)
% $CD95^+$within $CD19^+$	49.78 vs 45.41 (p=0.62)	49.46 vs 45.31 (p=0.93)	50.95 vs 43.24 (p=0.47)	55.23 vs 38.16 (p=0.01)	44.79 vs 46.91 (p=0.70)
% IL-$6R^+$within $CD19^+$	46.92 vs 22.24 (p=0.01)	40.80 vs 20.99 (p=0.03)	42.12 vs 13.24 (p<0.0001)	32.47 vs 14.35 (p=0.007)	26.59 vs 24.35 (p=0.81)
% IL-$21R^+$within $CD19^+$	15.77 vs 8.46 (p=0.31)	8.46 vs 13.27 (p=0.63)	11.90 vs 8.31 (p=0.92)	14.38 vs 7.77 (p=0.22)	15.77 vs 11.9 (p=0.99)

† % of B lymphocytes subpopulations in each clinical manifestation subgroups were compared using Mann-Whitney U-test.

Significance was characterized by p-value<0.05 (light grey); p-value<0.01 (grey); p-value<0.001 (dark grey).

ILD: interstitial lung disease; PAH: pulmonary arterial hypertension.

Supplemental Table 3. Primer sequences used for amplification

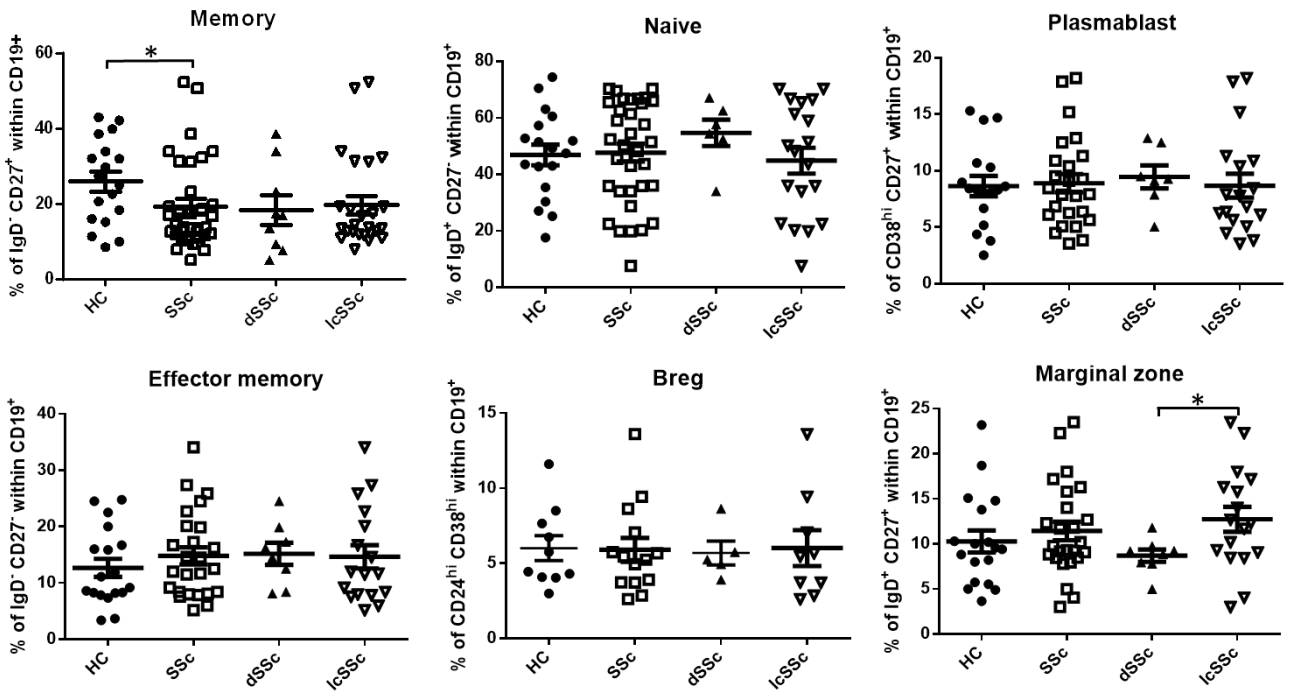
Gene	Primer Forward	Primer Reverse
Alpha-Smooth muscle actin	5'-CAATGGCTCTGGGCTCTGTAAG-3'	5'-TGTTCTATCGGGTACTTCAGGGTC-3'
Collagene I	5'-CACACGTCTCGGTCATGGTA-3'	5'-CGGCTCCTGCTCCTCTTAG-3'
Vimentin	5'-GGCTCGTCACCTTCGTGAAT-3'	5'-GCAGAGAAATCCTGCTCTCCT-3'
Snail1	5'-CCAGTGCCTCGACCACTATG-3'	5'-CTGCTGGAAGGTAAACTCTGGA-3'
Slug	5'-AAGGCGTTTTCCAGACCCTG-3'	5'-AAGAAAAAGGCTTCTCCCCCGT-3'
β2microglobulin	5'-CTCCGTGGCCTTAGCTGTG-3'	5'-TTTGGAGTACGCTGGATAGCCT-3'

Supplemental Table 4. Antibodies used for flow cytometry experiments.

Target	**Fluorochrome**	**Clone**	**Society**
BR3	Fluorescein isothiocyanate (FITC)	11C1	BD biosciences
CCR6	Peridin-Chlorophyll protein-Cyanin5.5 (PerCP-Cy5.5)	G034E3	Biolegend
CD3	AlexaFluor700	UCHT-1	BD biosciences
CD4	Brillant Violet 605 (BV605)	RPA-T4	BD biosciences
CD5	BV421	UCHT2	BD biosciences
CD8	PerCP-Cy5.5	RPA-T8	BD biosciences
CD19	BV605	SJ25C1	BD biosciences
CD20	BV421	2H7	BD biosciences
CD21	PerCP-Cy5.5	Bu32	Biolegend
CD22	Phycoerythrin (PE)	HIB22	Biolegend
CD24	PE	ML5	BD biosciences
CD25	BV421	M-A251	BD biosciences

CD27	AlexaFluor700	M-T271	BD biosciences
CD32	Allophycocyanin (APC)	Fli8.26	BD biosciences
CD38	PE-Cy7	HIT2	BD biosciences
CD40	APC-H7	5C3	BD biosciences
CD45	APC	H130	BD biosciences
CD45RA	FITC	HI-100	BD biosciences
CD62L	APC	DREG-56	BD biosciences
CD69	APC	FN50	BD biosciences
CD72	FITC	J4-117	BD biosciences
CD86	BV421	2331 (PUN-1)	BD biosciences
CD95	PE	DX2	BD biosciences
CXCR5 (CD185)	PE	J252D4	Biolegend
FoxP3	PE	259D/C7	BD biosciences
HLA-DR	PerCP-Cy5.5	L243	Biolegend
ICOS (CD278)	FITC	C398.4A	Biolegend
IgD	APC	IA6-2	BD biosciences
IgG	PE	G18-145	BD biosciences
IgM	BV421	G20-127	BD biosciences
IFN-γ	FITC	B27	BD biosciences
IL-4	APC	8D4-8	Ebioscience
IL-6	PE	MQ2-6A3	BD biosciences
IL-6Rα	Biotin	UV4	Biolegend
IL-9	PE	MH9D1	Ebioscience
IL-10	APC	JES3-9D7	BD Biosciences

IL-17A	PE	N49-653	BD biosciences
IL-21	APC	3A3-N2	Biolegend
IL-21R	APC	17A12	BD biosciences
PD-1	BV421	EH12.1	BD biosciences
TGF-β1	PERCP-Cy5.5	TW4-9E7	BD Biosciences
Livedead	BV421	-	LifeTechnologies
Biotin-Streptavidin	BV421	-	Biolegend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*
HC
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dSSc
lcSSc
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80
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20
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HC
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lcSSc
Plasmablast
% of CD38hi CD27+ within CD19+
20
15
10
5
0
HC
SSc
dSSc
lcSSc
Effector memory
% of IgD- CD27- within CD19+
40
30
20
10
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HC
SSc
dSSc
lcSSc
Breg
% of CD24hi CD38hi within CD19+
15
10
5
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HC
SSc
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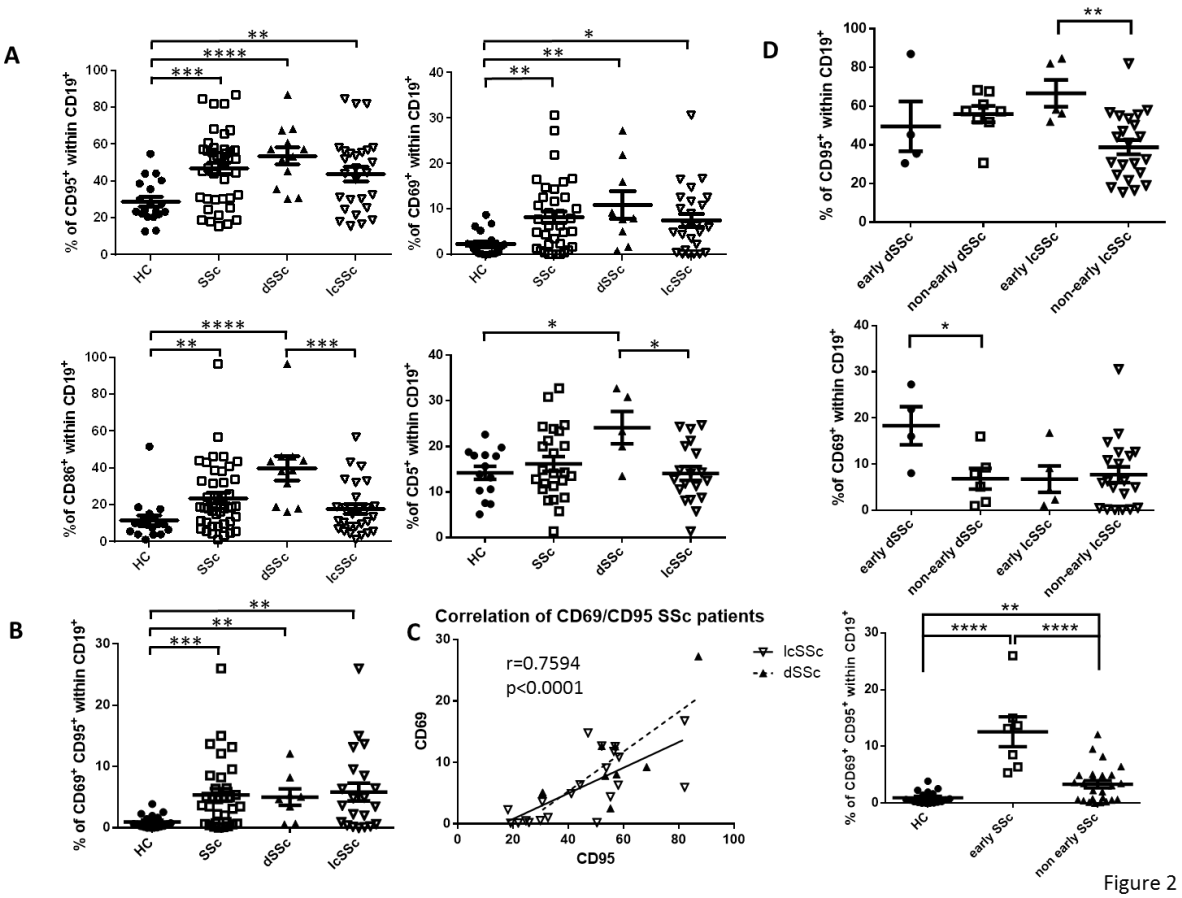
Figure 1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B
% of CD69+ CD95+ within CD19+
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Correlation of CD69/CD95 SSc patients
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dSSc
CD69
CD95
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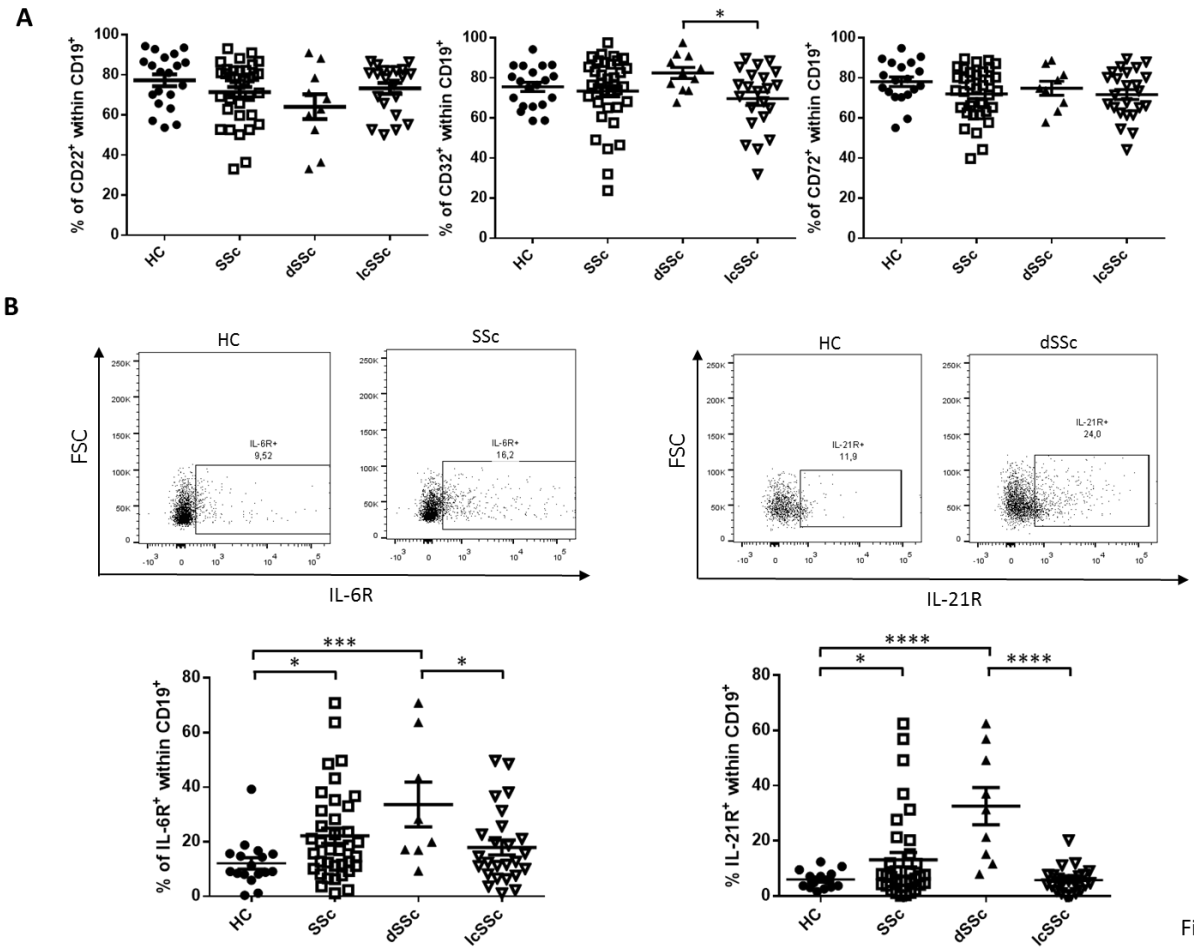
early SSc
non early SSc

Figure 2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% of CD22+ within CD19+
% of CD32+ within CD19+
%of CD72+ within CD19+
HC
SSc
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SSc
dSSc
FSC
IL-6R
IL-21R
IL-6R+
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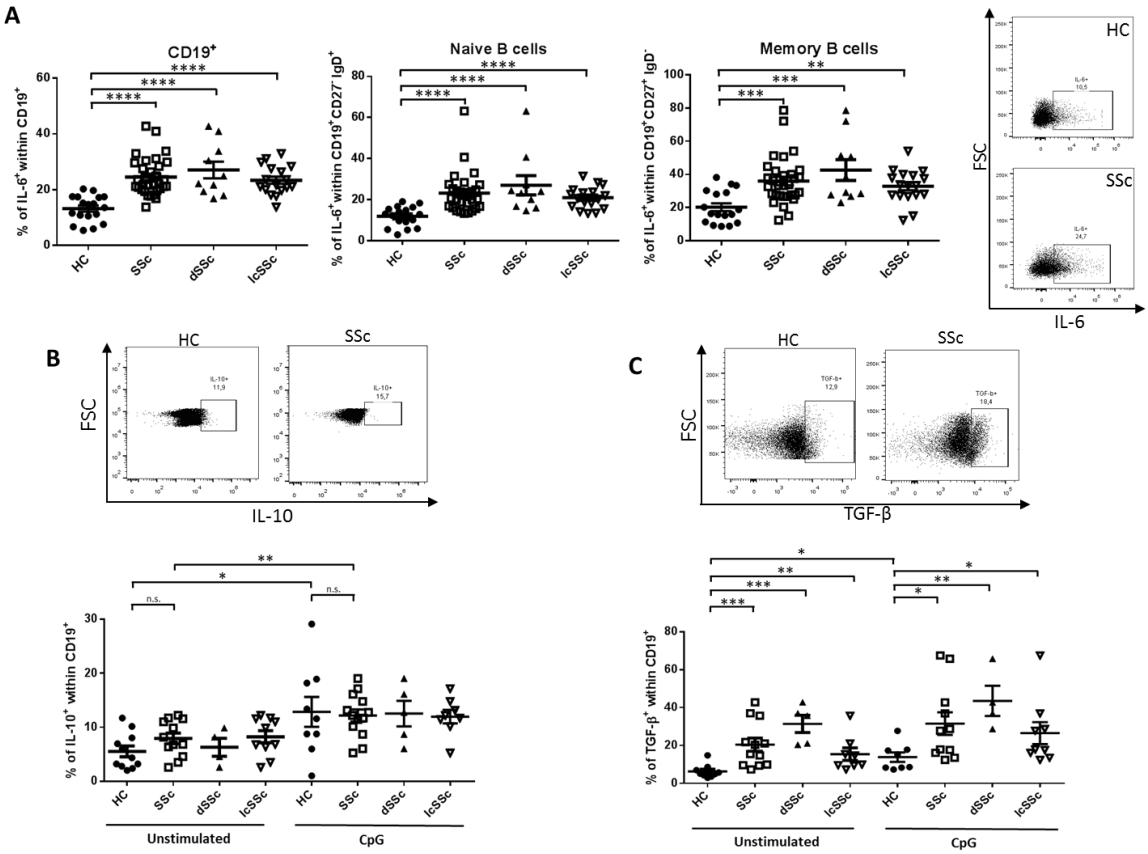
% IL-21R+ within CD19+

Figure 3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CD19+
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Memory B cells
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% of IL-6+ within CD19+CD27+ IgD-
HC
SSc
dSSc
lcSSc
FSC
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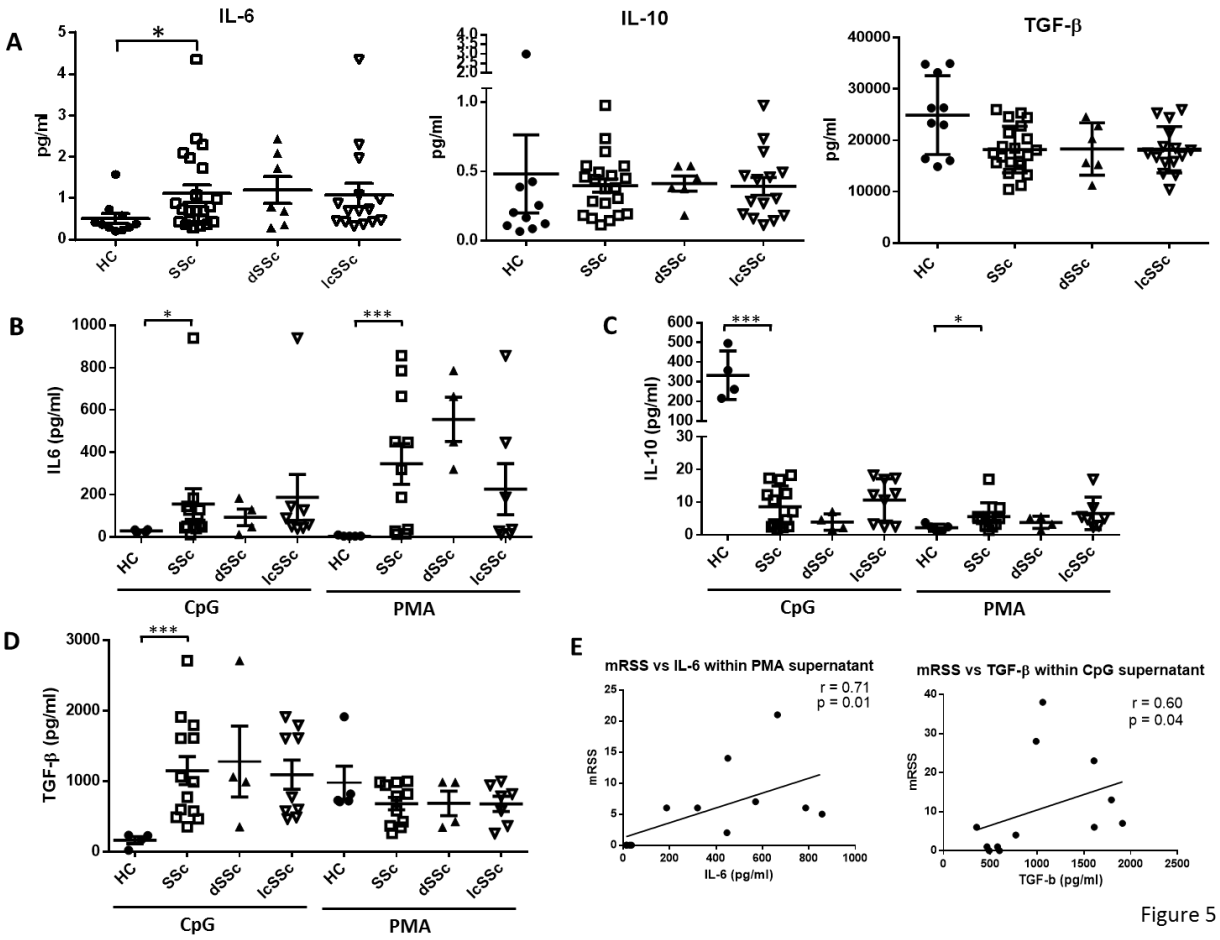
CpG
C
TGF-β
% of TGF-β+ within CD19+

Figure 4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IL-6
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IL-10 (pg/ml)
D
TGF-β (pg/ml)
E
mRSS vs IL-6 within PMA supernatant
r = 0.71
p = 0.01
mRSS
IL-6 (pg/ml)
mRSS vs TGF-β within CpG supernatant
r = 0.60
p = 0.04
TGF-b (pg/ml)

Figure 5

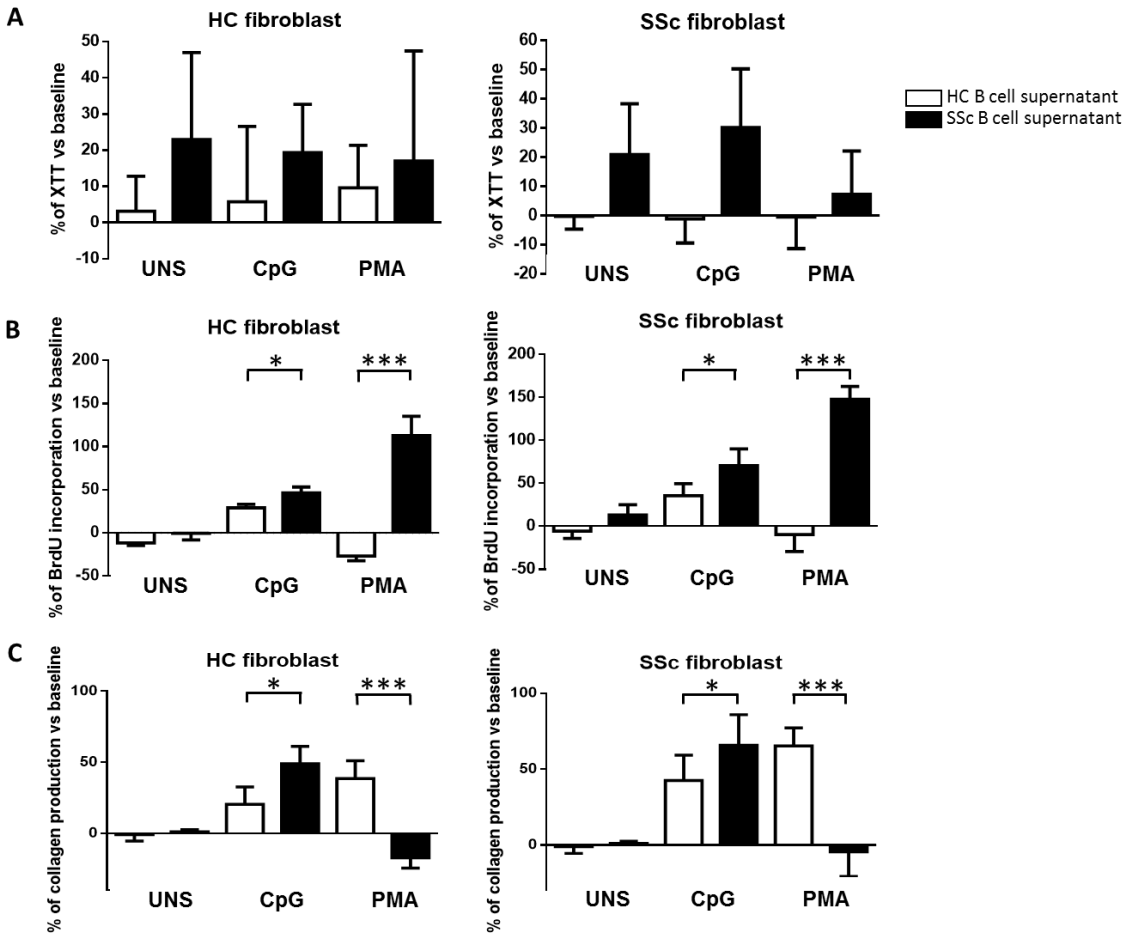
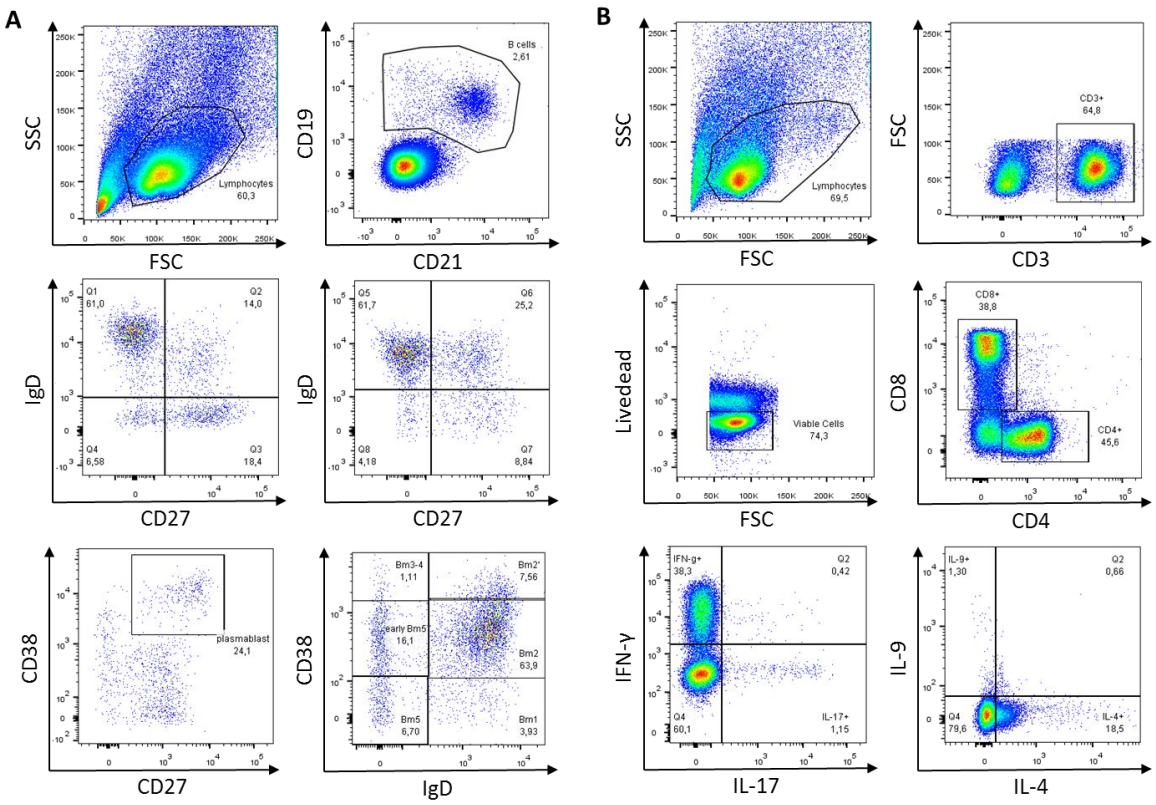
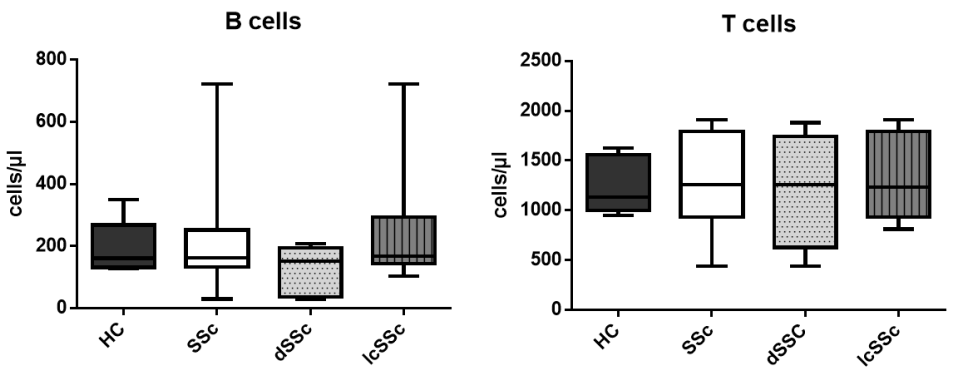

A
HC fibroblast
SSc fibroblast
% of XTT vs baseline
HC B cell supernatant
SSc B cell supernatant
UNS
CpG
PMA
B
% of BrdU incorporation vs baseline
*
***
C
% of collagen production vs baseline

Figure 6



Supplemental figure 1



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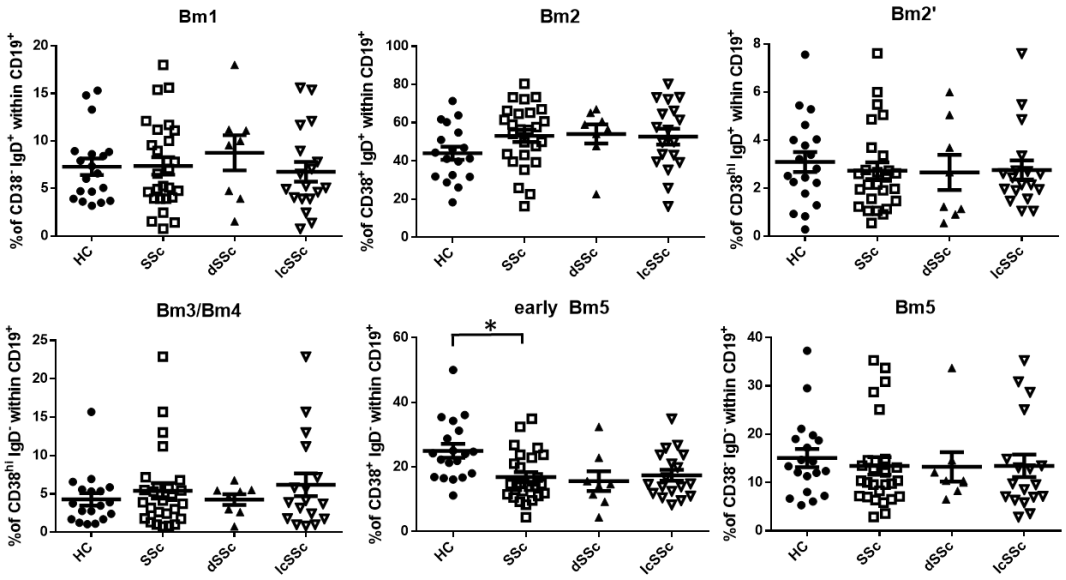
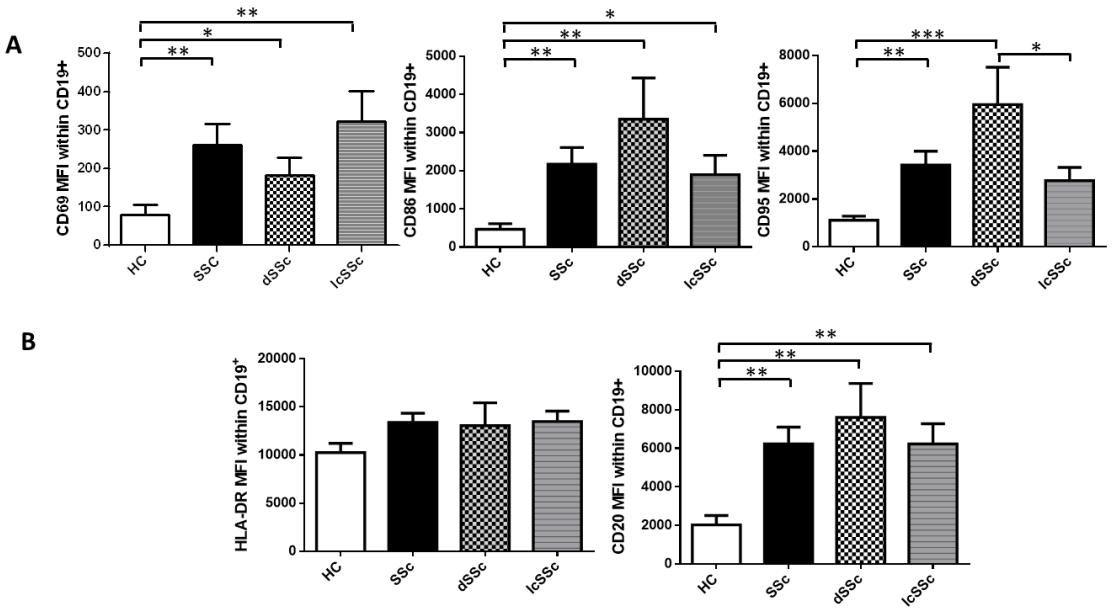


Supplemental figure 3



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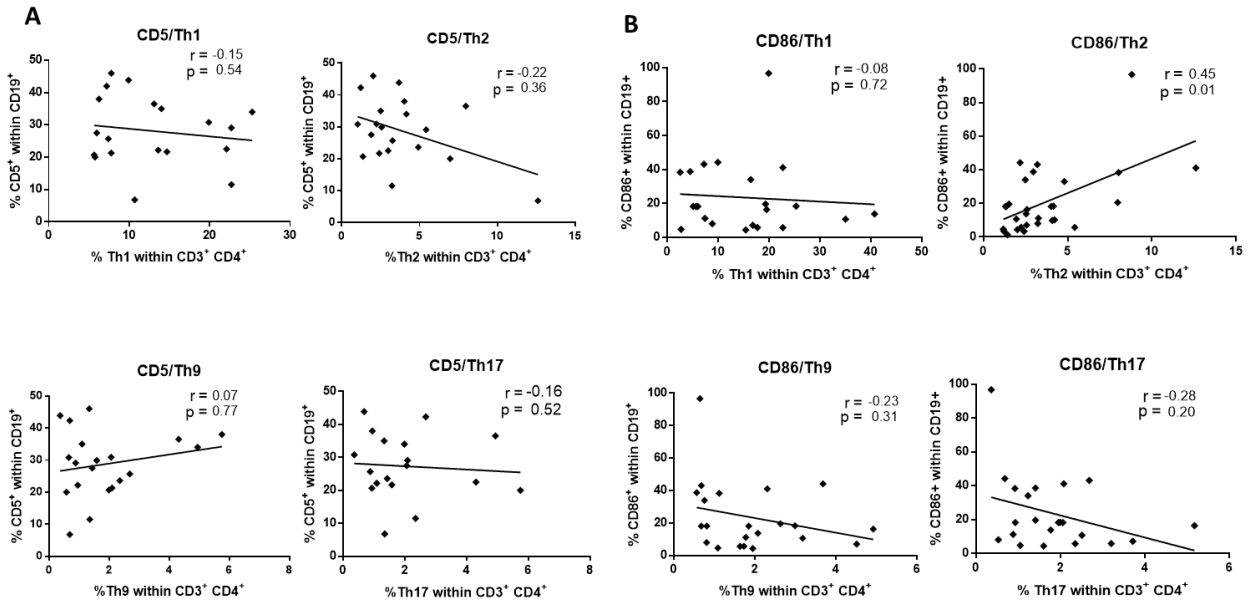

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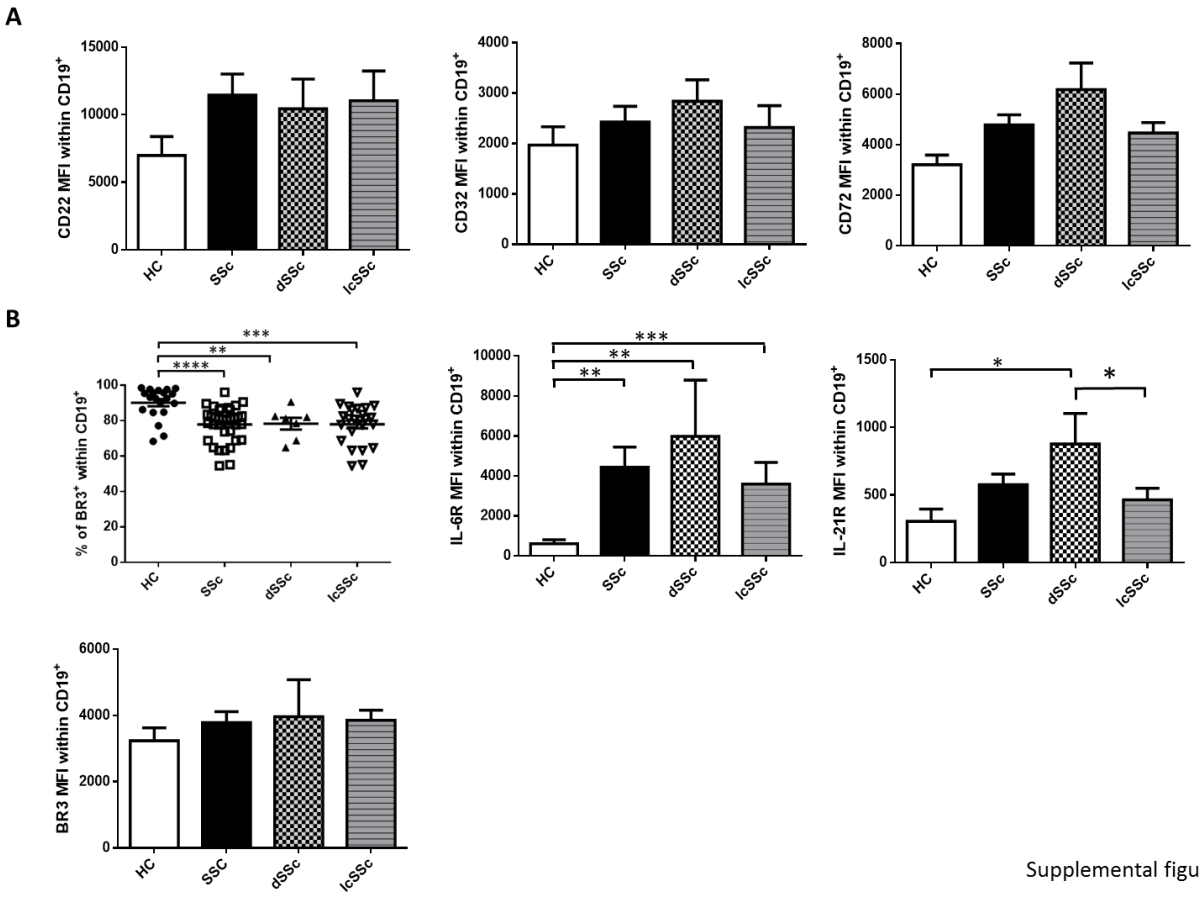
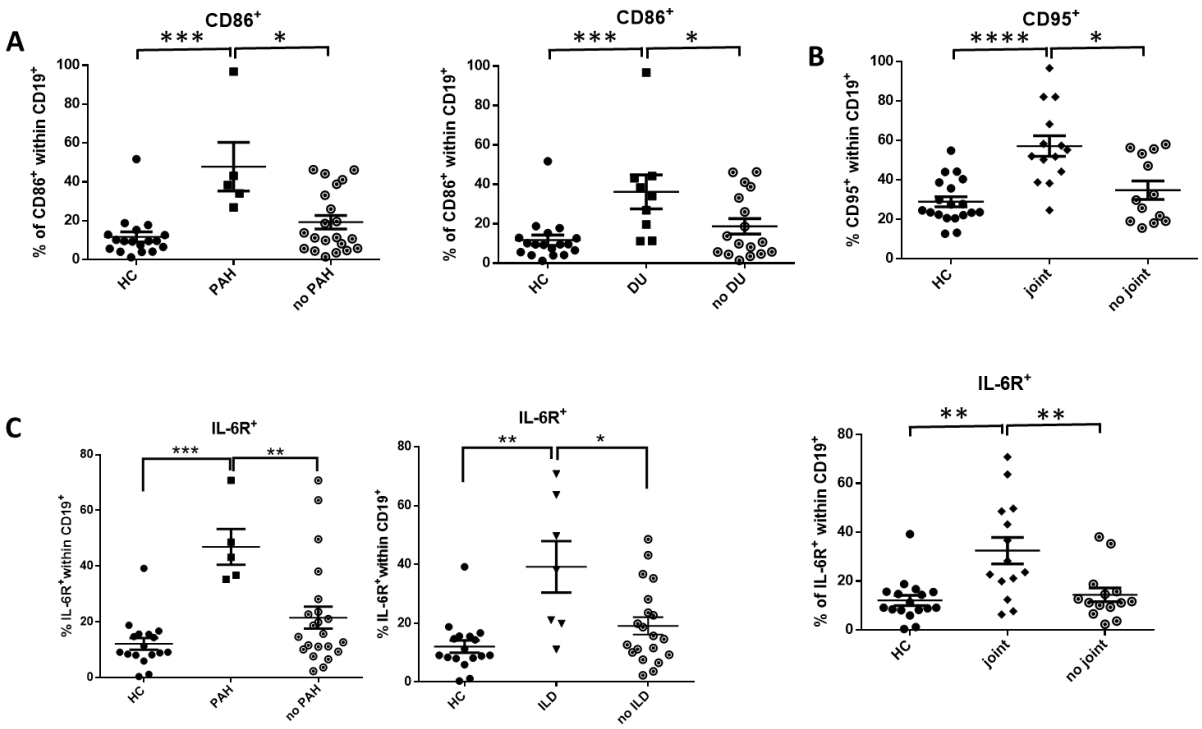


Supplemental figure 6



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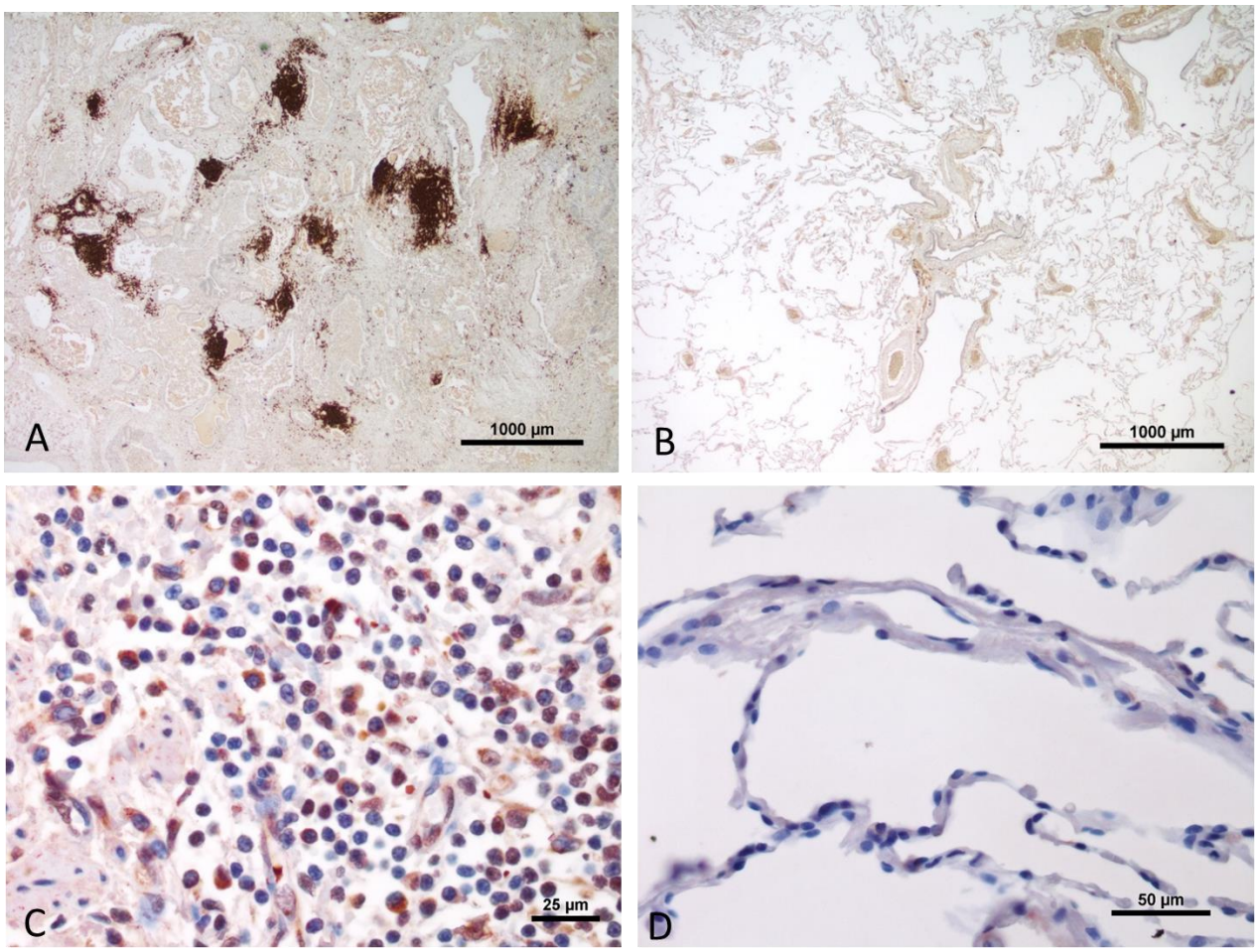

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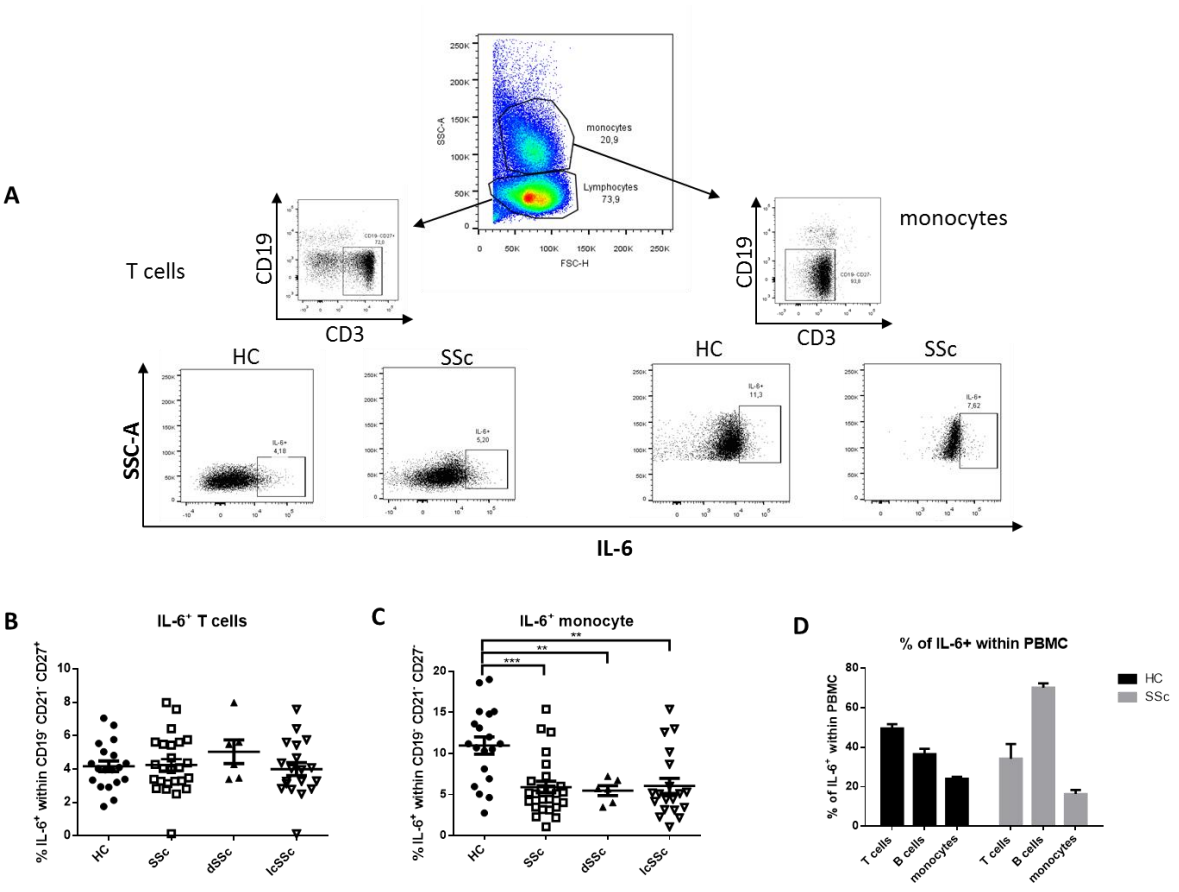
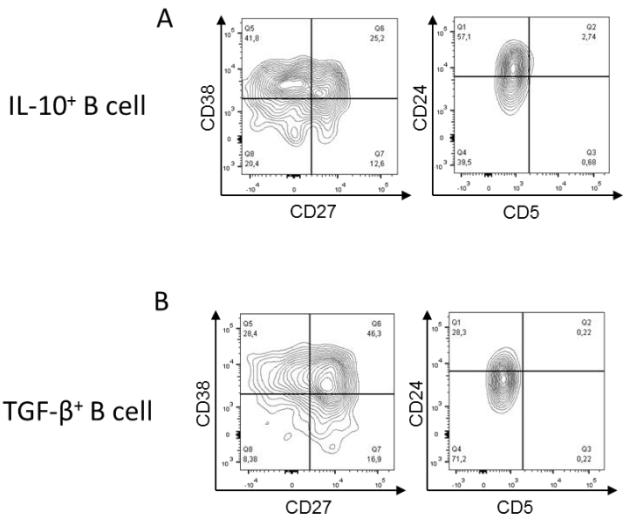
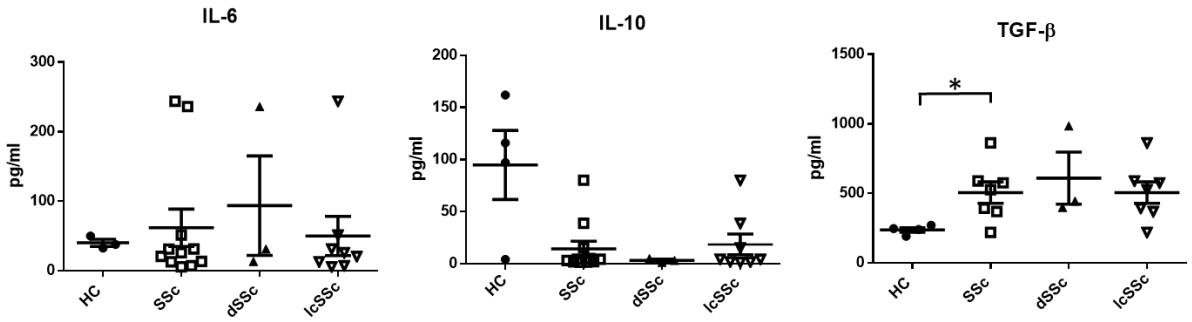


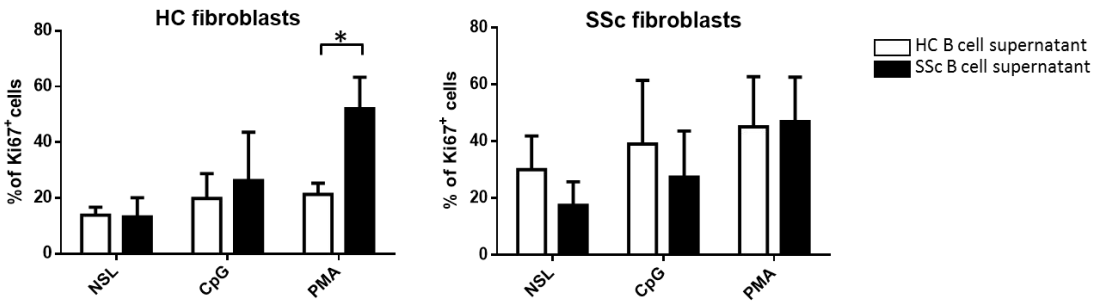
Supplemental figure 9

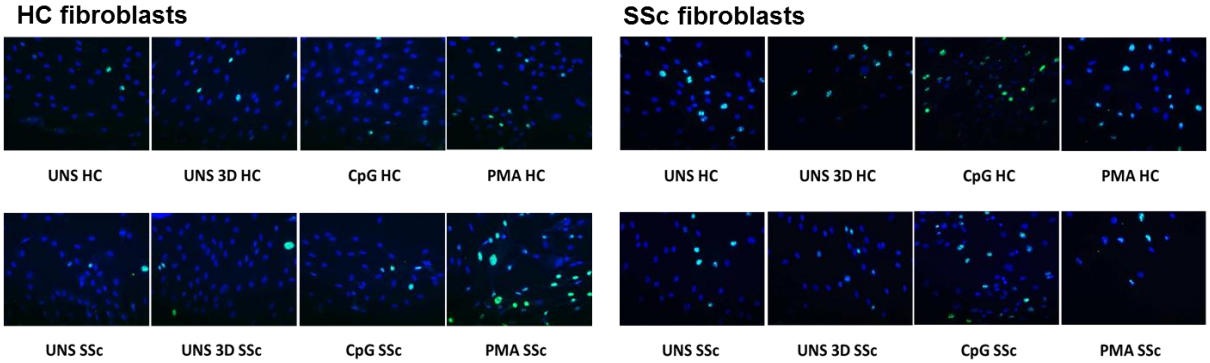


Supplemental figure 10

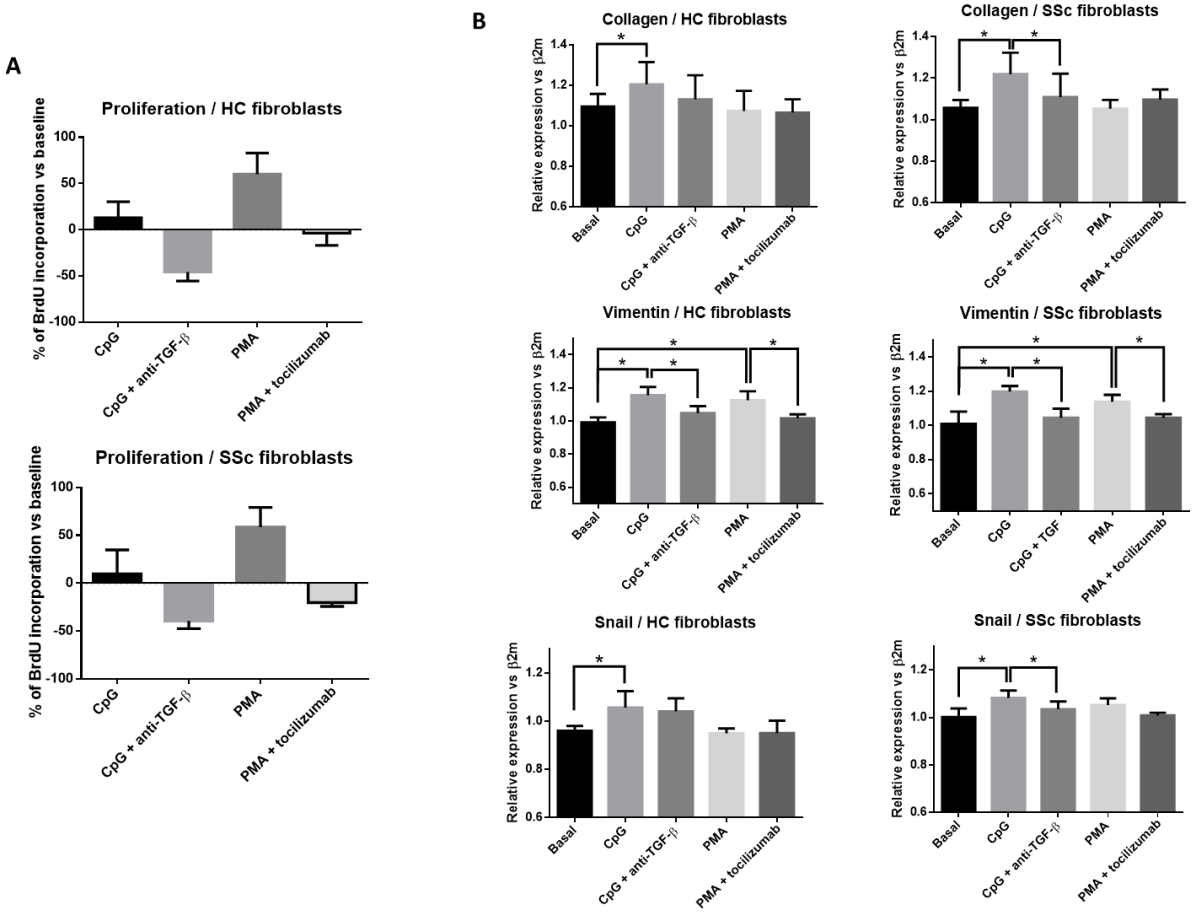


Supplemental figure 11





Supplemental figure 12



Supplemental figure 13

3. Preliminary results:

Introduction:

In addition to our study on patients with systemic sclerosis (SSc) which develop pulmonary arterial hypertension (PAH), we intended to evaluate the impact of PAH on B cell phenotype independently of SSc. Thus, we selected only patients with IPAH only which present similar inflammatory phenotype to what those observed in patients with PAH-SSc.

Patients and methods

Patients

In collaboration with the team of the Pr. Marc Humbert at Kremlin Bicètre Hospital, we performed phenotypic analysis of B lymphocytes in patients with idiopathic pulmonary arterial hypertension (IPAH) (n=8) which did not receive any immunosuppressant. PAH was defined as an increase in the mean pulmonary artery pressure superior to 25 mmHg at rest evaluated at Kremlin Bicètre Hospital. All patients gave a written consent. We particularly focused on phenotypic dysregulations that have been underlined in patients with PAH associated with SSc (Paper 1).

Lymphocytes culture

PBMC were isolated from peripheral blood on Ethylenediamine tetraacetic acid (EDTA)-anticoagulated tubes from HC or patients with IPAH using a Ficoll-Hypaque density gradient centrifugation (Eurobio, Courtaboeuf, France).

Interferon-gamma (IFN-γ), IL-4, IL-6, IL-9, IL-17, IL-21 and Tumor necrosis factor alpha (TNF-α) production by B or T lymphocytes was assessed after PBMC stimulation for 4 hours with 50 ng/ml of PMA, 1 µg/ml of ionomycin and 1 µl/million of cells of GolgiStop solution. Cells were then washed and stained as described (Jung et al., 1993).

Flow cytometry analysis

We performed flow cytometry analysis with peripheral blood of SSc patients and healthy controls after total PBMC staining with an anti-CD19-Brillant Violet 605 (BV605), an anti-CD27-AlexaFluor700 obtained from BD Biosciences and an anti-CD21-Peridin-chlorophyll protein-Cyanin5.5 (PERCP-Cy5.5) obtained from Biolegend (London, UK). For B lymphocyte subpopulations, were analyzed for the expression of membrane IgD, CD21, CD27 and CD38 expressions within $CD19^{+}$ B cells (Figure 1) as previously described (Kaminski et al., 2012).

Anti-CD3-AlexaFluor 700, anti-CD4-BV 421 from BD Biosciences and anti-CD8-PERCP-Cy5.5 from Biolegend were used to define T lymphocyte subpopulations. In combination with these markers, other antibodies were used to stain extracellular or intracellular proteins of B and T lymphocytes.

In longitudinal experiments, cytometer settings were standardized using BD Cytometer setup, SPHERO Rainbow Calibration Particles (BD) and BD Combeads. Flow cytometry acquisition was assessed using a BD LSR Fortessa at the cytometry plateform of Cochin Institute (Cybio) and data were analysed using FlowJo analysis software (Treestar, Ashland, OR, USA).

Statistical analysis

All analysis were carried out using Prism 6 (GraphPad, La Jolla, CA, USA) with statistical significance defined as:* p-value < 0.05; ** p-value < 0.01; ***p-value < 0.001; ****p-value < 0.0001. Non parametric tests including Mann-Whitney U-test were used for two group independent comparisons between these two groups.

Results

Peripheral B lymphocytes from patients with IPAH are moderately activated

The proportion and amounts of B or T lymphocyte weren't significantly modified in patients with IPAH compared to patients with PAH-SSc, SSc only or healthy controls (HC) (Figure 1). Increased proportions of B lymphocytes expressing CD95 were observed in patients with IPAH compared to HC (43.8% vs. 24.5%; p=0.007) which is similar to what was observed in patients with either SSc or PAH-SSc (Figure 2). CD5, CD69 or CD86 percentages were not significantly different in patients with IPAH which is consistent to what was observed for patients with PAH-SSc for CD69 and CD86 while the proportion of CD5 was slightly increased in PAH-SSc patients without reaching significance in this small cohort.

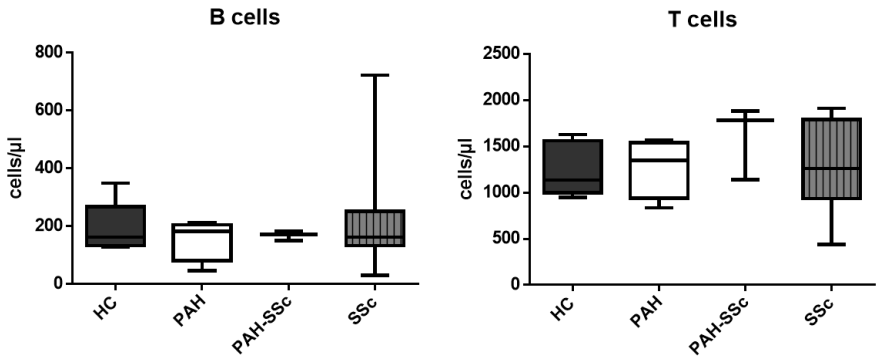


Figure 1

Figure 1: Analysis of B and T lymphocytes amounts per milliliter of blood

Decreased proportions of B cell activating factor (BAFF)-receptor expressing B cells were found in patients with IPAH (75.7% vs. 93%; p=0.02), consistent with the levels for patients with PAH-SSc or SSc only (Figure 3). We did not evidence differences for expression of IL-21R or CD72 within the various groups of patients and HC.

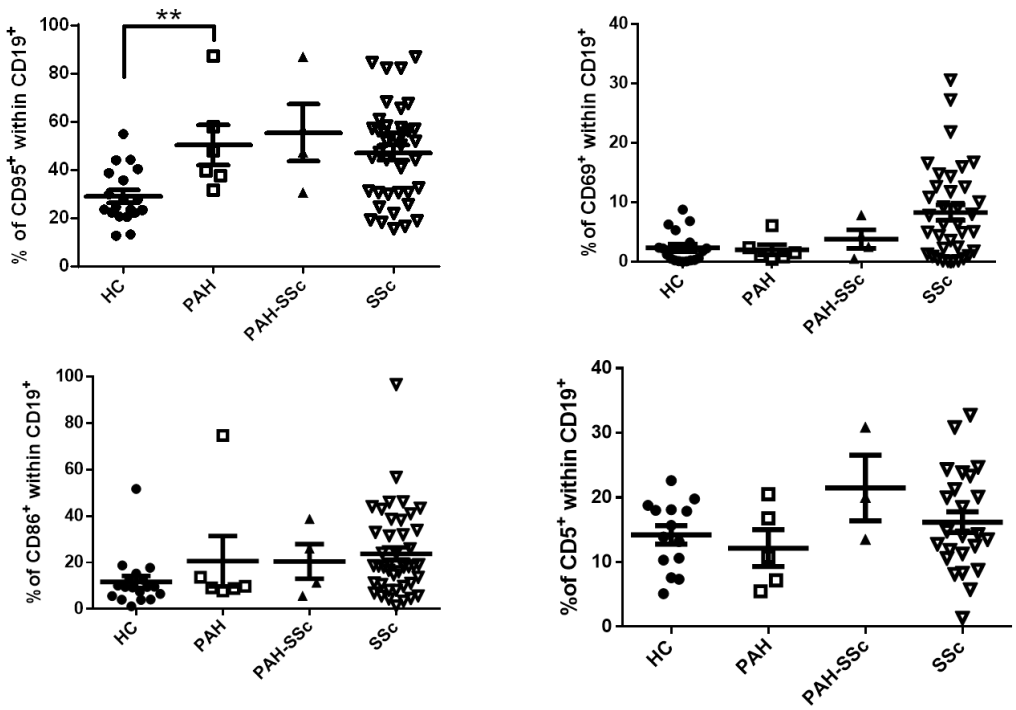


Figure 2

Figure 2: Analysis of activation marker expression within CD19^{+} B lymphocytes

B lymphocytes from IPAH patients expressed decreased IL-6R but increased IL-6 secretion

B lymphocytes from patients with IPAH expressed similar levels of IL-6R compare to B cells from HC. This resul is very different from patients with either SSc or PAH-SSc (Figure 3). However, B lymphocytes from patients with IPAH produced higher levels of IL-6 than HC, PAH-SSc and SSc patients (Figure 4).

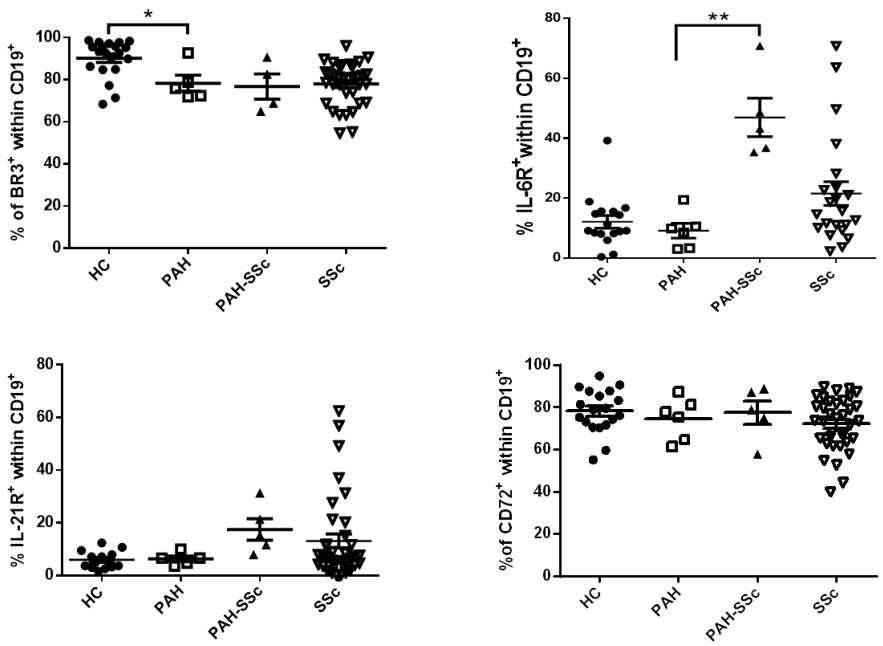


Figure 3

Figure 3: Analysis of cytokine receptor expressions within $CD19^{+}$ B lymphocytes

Conclusion

We showed that B lymphocytes from patients with IPAH expressed an activated phenotype but in a moderate form as compared to what we observed in patients with PAH-SSc or SSc. Moreover, B cells from patients with IPAH did not express high levels of IL-6R in contrast with the clear different identified in patients with PAH-SSc. However, we observed dramatic increase of IL-6 production in B cells from patients with IPAH compared to PAH-SSc patients. These data reinforce the interest in IL-6/IL-6R pathway in PAH pathophysiology and suggest different role of B lymphocytes between IPAH and PAH associated with connective tissue disease.

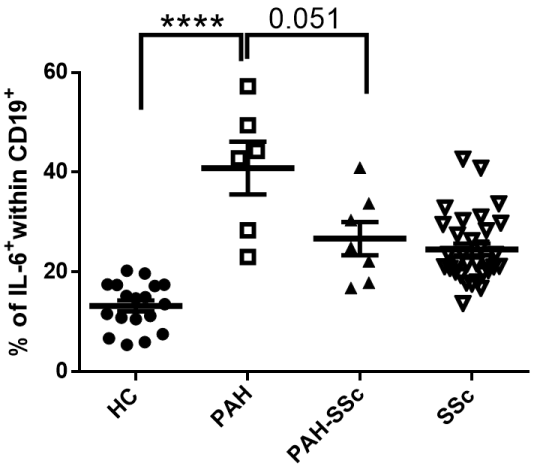


Figure 4

Figure 4: Analysis of IL-6 secretion within $CD19^+$ B lymphocytes

4. Paper 3: Basophils are activated and promote fibrosis through TGF-ß secretion in systemic sclerosis

Submitted in Journal of Immunology

Basophils are activated and promote fibrosis through TGF-ß secretion in systemic sclerosis

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Abstract

Background: Recent studies have emphasized the fibrotic and immunological roles of basophils.

Objective: To study the role of basophils in patients with systemic sclerosis (SSc).

Methods: Surface expressions of CD203c, type 3 C-C chemokine receptor (CCR3), chemoattractant receptor-homologous molecule expressed on T helper 2 lymphocytes (CRTH2), and intracellular synthesis of B-cell activating factor (BAFF) and transforming growth factor-β (TGF-β) were measured in blood basophils from SSc patients and healthy controls (HC). The ability of basophils to stimulate B cells or activate fibroblasts isolated from SSc patients was assessed using flow cytometry and xCelligence assay.

Results: 65 previously untreated patients with SSc and 38 HC were included. Spontaneous expression levels of CD203c and CCR3 on basophils were significantly higher and lower in SSc patients than in HC, respectively. Patients with SSc had a lower proportion of $CRTH2^{+}$ basophils than HC and CRTH2 expression on basophils negatively correlated with disease characteristics including pulmonary hypertension and modified Rodnan skin score. Activated basophils produced more BAFF, and increased IL-6 and TGF-β production was noted within B cells. Lastly, we demonstrated basophil profibrotic function in SSc by showing higher proportion of TGF-ß-secreting basophils and enhanced proliferation of fibroblasts induced by activated basophils in a TGF-ß-dependent manner in SSc.

Conclusion: Peripheral basophils from SSc patients are activated, are able to simulate B cells, and enhance fibroblast proliferation through the production of TGF-ß, supporting a key role of basophils in the pathophysiology of SSc.

Key Messages

- Basophils from SSc patients are activated, overexpress CD203c and express low levels of CCR3.
- CRTH2 expression at the surface of basophils is a potential marker of disease severity.
- Basophils participate in fibrosis by overproducing TGF-ß and enhancing fibroblasts proliferation in SSc.

Capsule summary (35 words / 35 words)

This study highlights basophils' implication in SSc by showing their contribution to fibrosis and immunoregulation. CRTH2's expression being correlated with clinical parameters, this study also suggests that basophils could serve as a potential prognostic marker.

Key words: systemic sclerosis; basophils; B lymphocytes; fibroblasts; CRTH2; TGF-ß.

Total word count: 3215 words

Abstract word count: 237 words

Abbreviations

Ab: Antibody

ACR: American College of Rheumatology

AF700: AlexaFluor700

BAFF: B-cell activating factor

BD: BD Biosciences®

BV605 : Brillant Violet 605

CCR: C-C chemokine receptor

CD: Cluster of differentiation

DMEM: Dulbecco's modified Eagle's medium

dSSc: Diffuse systemic sclerosis

EDTA: Ethylenediamine tetraacetic acid

EULAR: European League Against Rheumatism

FC: Fragment crystallizable

FCS: Fetal calf serum

FCƐRI: Immunoglobulin E receptor FC

FITC: Fluorescein isothiocyanate

GvH: Graft versus host

HC: Healthy controls

HLA: Human leucocyte antigen

IgE: Immunoglobulin E

IL: Interleukine

ILC2s: Type 2 innate lymphoid cells

ILD: Interstitial lung disease

LcSSc: Limited cutaneous systemic sclerosis

Lin: lineage

mAb: Monoclonal antibody

MFI: Mean fluorescence intensity

mRSS: Modified Rodnan skin score

PAH: Pulmonary arterial hypertension

PB: Pacific Blue

PBMC: Peripheral blood mononuclear cells

PE-Cy7: Phycoerythrin Cyanin 7

PERCP-Cy5.5: Peridin-chlorophyll protein-Cyanin5.5

PMA: Phorbol myristate acetate

RPMI: Roswell Park Memorial Institute medium 1640

SLE: Systemic lupus erythematous

SSc: Systemic sclerosis

TGF-ß: Transforming growth factor-β

Introduction

Systemic sclerosis (SSc) is a rare and severe multisystem connective tissue disease characterized by skin and internal organs fibrosis and vascular abnormalities, along with the presence of autoantibodies (Denton, 2015; Dumoitier et al., 2014b). Patients' prognosis remains poor, mostly depending on lung involvement caused by both fibrotic mechanisms leading to interstitial lung disease (ILD) and/or vascular abnormalities leading to pulmonary arterial hypertension (PAH) (Launay et al., 2013; Steen and Medsger, 2007). Pathophysiological mechanisms need to be unraveled in order to identify biomarkers that could help both to detect and treat these complications at an early stage.

Significant progress has been made in understanding this condition's pathophysiology. The "ménage à trois" between immune cells (mainly Th2 cells and B cells), endothelial cells and fibroblasts is currently the main hypothesis leading to the disease's onset, which also requires exposure to environmental factors and genetic predisposition (Denton, 2015; Dumoitier et al., 2014b). Th2 cells are increased in the blood of patients with SSc and overrepresented in SSc tissues (Scaletti et al., 2002; Truchetet et al., 2011). Th2-polarized T cells release profibrotic mediators such as interleukine-4 (IL-4), IL-5, IL-6, IL-10, IL-13 which lead to fibrosis (O'Reilly et al., 2012). High levels of IL-4 and IL-13 were detected in serum and tissues from SSc patients (Hasegawa et al., 1997; Vettori et al., 2014). In SSc, B cells are characterized by polyclonal B cell activation and production of both specific autoantibodies and profibrotic cytokines such as IL-6 (Dumoitier et al., 2014b). Autoantibodies targeting endothelial cells and fibroblasts were also suggested to play a key role in the onset of vascular and fibrotic damages during SSc (Dib et al., 2012; Terrier et al., 2010). One of the key players involved in the genesis of SSc's fibrotic process is transforming growth factor-β (TGF-β), i.e. a potent growth factor which also promotes cell migration and differentiation of fibroblasts into myofibroblasts. Targeting TGF- β seems to be a promising treatment option (Lafyatis, 2014; Rice et al., 2015).

Basophils are rare granulocytes that link innate and adaptive immune responses. They have long been associated with parasitic infections and allergy, and a strong level of evidence has been accumulated to support their ability to influence a Th2 oriented immune response (Karasuyama and Yamanishi, 2014). During the past decade, substantial efforts have been made to better understand their role in the pathophysiology of various immunological disorders. Noteworthy, they play an important role in innate immune responses by producing key mediators (e.g. histamine and IL-4) which can promote alternatively activated macrophages (Egawa et al., 2013) and stimulate B cells. Thus, they contribute to enhance antibody production in physiological conditions (Chen et al., 2009; Denzel et al., 2008) and autoimmune diseases (Charles et al., 2010). Recently, murine studies of basophils in graft-versus-host (GvH) disease have also emphasized basophils' possible profibrotic functions. In a cardiac GvH model, Schiechl G *et al.* reported that basophils trigger the development of fibrosis (Schiechl et al., 2016). Interestingly, it has also been shown that TGF-ß promote basophils' differentiation (Sillaber et al., 1992). Thus, we anticipated that basophils could be involved in SSc's pathophysiology.

Methods

Patients

Peripheral blood was obtained from 65 untreated patients with SSc fulfilling the American College of Rheumatology (ACR) / European League Against Rheumatism (EULAR) criteria for the diagnosis of SSc (van den Hoogen et al., 2013c), followed at Cochin Hospital in the Internal Medicine department. Limited cutaneous SSc (LcSSc) was defined by skin thickening in distal areas (i.e. lower than elbows and knees), with or without facial involvement. Diffuse SSc (dSSc) was defined by the presence of proximal (i.e. upper than elbows and knees) and/or distal skin thickening, with or without facial or truncal involvement (LeRoy et al., 1988). Peripheral blood was obtained from 38 healthy controls (HC) who were included at the "Etablissement Français du Sang", Saint Antoine Hospital, Paris, France.

All patients and HC gave a written informed consent. Blood was collected with approval of the ethics committee of the "Groupe hospitalier Pitié-Salpêtrière" and the study conforms to the principles outlined in the Declaration of Helsinki.

Basophil analysis and basophil activation test

For basophil baseline analysis and basophil activation test, 1 mL of whole blood was harvested in ethylenediamine tetraacetic acid (EDTA)-anticoagulated tubes from HC or patients with SSc. Five hundred microliters of whole blood was used for baseline basophil analysis and 500 µL of whole blood was used for basophil activation test. Basophil activation test was performed using 50 ng/ml of phorbol myristate acetate (PMA), 1 µg/ml of ionomycin, 1 µg/ml of IL-3 (Sigma-Aldrich, Saint-Quentin Fallavier, France) and 1 µg/ml of immunoglobulin E (IgE) (Abcam, Paris, France) for 2 hours before staining for flow cytometry analysis.

Human basophilic KU812F cells

The basophilic cell line KU812F (Kishi, 1985; Okamura et al., 1989) was cultured in Roswell Park Memorial Institute medium 1640 (RPMI) obtained from Gibco (Life Technologies, Saint-Aubin, France), with 10% heat-inactivated fetal calf serum (FCS) (Eurobio) at 37°C in 5% CO_2 atmosphere. Stimulation of 10^6 cells was performed in 1.5 ml micro tubes (Eppendorf, Montesson, France) with 50 ng/ml of PMA, 1 µg/ml of ionomycin, 1 µg/ml of IL-3 and 1 µg/ml of IgE for two hours.

B lymphocyte functional study

B lymphocytes were sorted from peripheral blood using a Ficoll-Hypaque density gradient centrifugation (Eurobio) and a magnetic negative isolation with a pan B-cell isolation kit II from Milteny Biotec® (Ref 130-101-638, Paris, France). We cultured 5.10^5 B lymphocytes from either HC or SSc patients at the concentration of 10^6 cells/mL in a 24-well plate for three days in presence or absence of KU812F cells at a concentration of 10^4 cells/ml. After three days, we performed flow cytometry analysis.

Fibroblasts functional study

Skin biopsies were performed in involved skin from patients with SSc. Normal human dermal fibroblasts were cultured from skin biopsies obtained from HC. Biopsy specimen were cut into small pieces and seeded into Petri dishes and then into 175 cm^2 plastic flasks. Patients and HC fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) obtained from Gibco, with 10% heat-inactivated FCS at 37°C in 5% CO2.

At the third passage, fibroblasts from HC or SSc patients were plated in a 96-well plate at a concentration of 2500 cells/well in the presence or absence of human basophilic KU812F cells, with or without anti-TGF-ß monoclonal antibody (mAb) (Sigma-Aldrich). Triplicate wells were used for each concentration of KU812F including 625 cells/well 1250 cells/well, 2500cells/well

and 5000 cells/well. Proliferation and viability of fibroblasts were measured using xCelligence real-time cell analyzer (ACEA Biosciences Inc., San Diego, CA, USA).

Flow cytometry analysis

We performed flow cytometry analysis with peripheral blood of SSc patients and healthy controls. We stained basophils with an anti-CD45-AlexaFluor700 (AF700) antibody (Ab), an anti-lineage (Lin) cocktail-Pacific Blue (PB) Ab, an anti-CD11c-PB Ab obtained from Biolegend (London, UK) an anti- FCƐRI-Fluorescein isothiocyanate (FITC) Ab obtained from eBioscience (Paris, France) and an anti-CD123-Phycoerythrin Cyanin 7 (PE-Cy7) Ab obtained from BD Biosciences (Le Pont-de-Claix, France). Basophils were identified in peripheral blood mononuclear cells (PBMC) as Lin^- $CD11c^-$ $CD45^+$ $FCƐRI^+$ $CD123^+$ cells (Fig E1). We stained sorted B lymphocytes with an anti-CD19-Brillant Violet 605 (BV605) Ab and an anti-CD21-Peridin-chlorophyll protein-Cyanin5.5 (PERCP-Cy5.5) obtained from Biolegend (London, UK). B lymphocytes were identified as $CD19^+$ cells. To perform intracellular staining cells were washed, stained, then fixed and permeabilized using Cytofix/Cytoperm kit (BD Biosciences). In combination with these markers, various Abs were used to stain extracellular or intracellular proteins of basophils and B lymphocytes, as listed in Table E1.

In longitudinal experiments, cytometer settings were standardized using BD Cytometer setup, SPHERO Rainbow Calibration Particles (BD) and BD Combeads. Flow cytometry acquisition was assessed using a BD LSR Fortessa at the cytometry plateform of Cochin Institute (Cybio) and data were analyzed using FlowJo analysis software vX.0.7 (Treestar, Ashland, OR, USA).

Statistical analysis

Non-parametric tests including Mann-Whitney U-test were used for independent two group comparisons. Comparisons between fibroblast and B-lymphocyte responses to basophils were analyzed using paired t-tests. Correlations of protein expressions were performed using

Spearman's rank correlation coefficient. All analyses were carried out using Prism 6 (GraphPad, La Jolla, CA, USA) with statistical significance defined as: * $P < 0.05$; ** $P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Results

Increased proportion of activated peripheral basophils in patients with systemic sclerosis

Sixty-five consecutive untreated patients with SSc, 53 lcSSc and 12 dSSc, were included in this study (Table E2). The proportion of peripheral basophils was higher in patients with SSc than HC as evaluated by the percentage of basophils among $CD45^+$ Lin^- $CD11c^-$ PMBCs (median [interquartile range] 3.5% [2.0 – 6.4] *versus* (*vs*) 2.6% [1.2 – 4.1]; $P < 0.05$) without any difference between patients with lcSSc or dSSc. There was no difference in the absolute count of basophils between patients and HC (Fig E1). Interestingly, basophils of patients with SSc exhibited more activation markers than those of HC (Fig 1). Compared to HC, we found in patients with SSc a higher proportion of $CD203c^+$ cells among basophils (37.5 % [24.3 – 59.0] *vs* 25.6% [13.6 – 41.7]; $P < 0.001$) (Fig 1, *A*), an increased quantification of this marker's mean fluorescence intensity (MFI) (624.5 [462.3 – 897.3] *vs* 440 [324.3 – 650]; $P < 0.001$) (Fig1, *C*) and a lower proportion of C-C chemokine receptor type 3 $(CCR3)^+$ cells (70.2% [35.3 – 88.2] *vs* 92.8% [77.3 – 97.5]; $P < 0.001$) (Fig 1, *B*). Expression of activation markers did not differ between patients with lcSSc or dSSc. Upon activation, the proportion of basophils between patients with SSc and HC was similar for both $CD203c^+$ cells and $CCR3^+$ cells (Fig E2). As for HC, other identification markers of basophils including CD22 and CD38 were also expressed by basophils of patients with SSc without any difference upon activation (Fig E3).

Basophils' functional markers in patients with systemic sclerosis

In order to analyze the potential role of basophils in patients with SSc, we checked for functional markers. We did not see any difference in the proportions of cells expressing CD178, CD44, CD62L, CD178, CD196, or HLA-DR. However, we found that the MFI quantification of CD154 (CD40 ligand) within basophils was significantly higher in patients with SSc than in HC (854 [541 – 1334] *vs* 485 [147 – 871]; $P < 0.05$) (Fig 2 and Fig E4). Upon stimulation, the

proportion of $CD54^+$ basophils was significantly lower in patients with SSc as compared to HC (68.3% [32.9 – 77.1] *vs* 77.8% [71.1 – 87.9]; $P < 0.05$) (Fig E4).

Interestingly, patients with SSc had a lower proportion of $CRTH2^+$ basophils than HC (99.8% [98.9 – 100] *vs* 100% [99.7– 100]; $P < 0.05$) and this difference was maintained after basophil activation (99.8% [98.8 – 100] *vs* 100 [100 – 100]; $P < 0.05$) (Fig 3, *A*). We found that SSc patients with PAH had lower MFI quantification of CRTH2 among basophils (31476 [30213 – 31633] *vs* 59576 [41312 – 67788]; $P < 0.001$) (Fig 3, *B*). Similarly, SSc patient with gastro-intestinal tract involvement had a lower MFI quantification of CRTH2 among basophils (52046 [36576 – 62276] *vs* 69391 [57853 – 73191]; $P < 0.05$) (Fig 3, *E*). Finally, MFI quantification of CRTH2 among basophils positively correlated with patients' diffusing lung capacity of carbon monoxide (r = 0.60, $P < 0.05$) (Fig 3, *C*) and negatively correlated with patients' modified Rodnan skin score (r = - 0.77, $P < 0.01$) (Fig 3, *F*).

Basophils stimulate B cells of patients with systemic sclerosis

We further checked for cytokine production by basophils in patients with SSc. We found that basophils produced B-cell activating factor (BAFF) (Fig 4), since the MFI quantification of BAFF within basophils was higher in SSc patients than in HC (1551 [1001 – 4373] *vs* 857 [722 – 1498]; $P < 0.05$) (Fig 4, *D*).

To further decipher the impact of basophils on B lymphocytes from SSc patients, we looked at the functional properties of B cells isolated from the peripheral blood of patients with SSc or HC when cultured with KU812F basophils (Fig 5). After three days of culture, we found that activated KU812F basophils were able to decrease the proportions of $CD95^+$ cells (9.4% [5.4 – 14.1] *vs* 10.4% [6.0 – 14.8]; $P < 0.05$) (Fig 5, *D*) and to increase the proportion of IL-6 producing cells (21.2% [16.5 – 29.9] *vs* 14.9 [12.3– 16.7]; $P < 0.05$) (Figure 5E) and TGF-β

(5.6% [4.7 – 7.2] *vs* 4.1 [2.6– 5.4]; $P < 0.05$) (Fig 5, *F*) among B cells isolated from patients with SSc. Such differences were only seen when KU812F basophils were activated (Fig 5).

On the contrary, we failed to observe similar discrepancies when we compared the proportion of $CD40^+$ (Fig 5, *A*), $CD86^+$ (Fig 5, *C*) or BAFF-receptor$^+$ (Fig 5, *B*) B cells of patients with SSc *vs* HC.

Basophils enhance the proliferation of fibroblasts of patients with systemic sclerosis in a TGF-ß dependent manner.

We wondered whether basophils from SSc patients produce TGF-ß. Strikingly, we found that the proportion of TGF-ß-secreting basophils was higher in SSc patients as compared to HC (21.9% [15.1 – 35.1] *vs* 9.7% [5.0 – 17.5]; $P < 0.05$) (Fig 4, *C*). To further analyze the role of this secretion, we next tested whether KU812F basophils were able to influence fibroblasts' proliferation (Fig 6).

We found that the proliferation of fibroblasts from SSc patients was higher when cultured with activated 1250 KU812F basophils than with unstimulated KU812F basophils (Fig 6, *B*). Moreover, this proliferation was higher than that of fibroblasts from HC also cultured with 1250 KU812F activated basophils (Fig 6, *A*). Conversely, compared to their proliferation when cultured with unstimulated KU812F basophils, the proliferation of HC's fibroblasts was not increased when these were cultured with activated KU812F basophils (Fig E5, *B*). No difference between HC and SSc patients' fibroblast proliferation was found when KU812F basophils were not activated (Fig E5, *A*).

To confirm that this difference of proliferation was TGF-ß-mediated, we first confirmed that KU812F basophils were able to secrete TGF-ß (Fig 6, *C*). We then tested TGF-ß's inhibition on the proliferation of patients' dermal fibroblasts cultured with activated KU812F basophils and observed that the inhibition of TGF-ß decreased the proliferation rate of patients'

fibroblasts to baseline values (Fig 6, *D*). Altogether these results demonstrate that activated basophils enhance the proliferation of fibroblasts of patients with SSc in a TGF-ß-dependent manner.

Discussion

In this study, we have identified in patients with SSc that peripheral basophils are activated and promote fibrosis through a TGF-ß-dependent manner compelling evidence for a key role of these cells in the pathophysiology of the disease.

To date, to our knowledge a very limited number of studies focusing on the role of basophils in SSc have been published. Previous works highlighted this cell's ability to release histamine which is increased in patients with SSc (de Paulis et al., 1991). An impairment of the inhibitory effect of sodium on basophil-related histamine release (Tedeschi et al., 1996) and a reduced priming effect of IL-3 on IgE-mediated histamine release (Tedeschi et al., 1998) have also been reported. These results suggest that the histamine release of PBMC's from SSc patients is altered. However, this is to our knowledge the first study to decipher basophils' activation status and functions in patients with SSc. Indeed, we herein provide a large description of basophils' biological functions in SSc patients and investigated basophils-lymphocytes' and basophils-fibroblasts interactions. Overall, our study demonstrates that basophils are activated in SSc patients, as characterized phenotypically by an increased expression of CD203c and, a downregulation of CCR3, and functionally by an increased production of both BAFF and TGF-ß.

Next, this is also the first report showing that there is a correlation between basophils' characteristics and SSc patients' clinical manifestations. Interestingly, we found that the prostaglandin D_2 receptor CRTH2 was associated with disease features. CRTH2 is an identification marker of both basophils (Eberlein et al., 2015) and type 2 innate lymphoid cells (ILC2s) (Wohlfahrt et al., 2016), which have already been reported to be associated with disease fibrosis. Wohlfahrt et al. recently reported that ILC2 counts correlate with the extent of fibrosis as assessed by the mRSS (Wohlfahrt et al., 2016). Moreover, Boin et al reported that SSc patients with ILD had lower CCR5/CRTH2 T cell ratios than SSc patients without ILD (Boin

et al., 2008). Our results may seem to be in contradiction with these studies. However, in our study, we found a correlation between disease manifestations and MFI, whereas disease manifestations correlated with CRTH2^{+} cell counts in the two previous studies. Altogether these two studies advocate for a Th2-cytokine environment favored by CRTH2 on T cells and ILC2s, which enhances fibrotic manifestations. Additionally, we showed that the expression of CRTH2 at the surface of basophils negatively correlates with vascular involvement of the disease, notably PAH, which is of interest as stable synthetic analogues of prostaglandin are used for the treatment of SSc associated-PAH (Badesch et al., 2000). Thus, the expression of CRTH2 at the surface of basophil appears to be a potential marker of disease severity.

Another original finding of our work is the evidence for a participation of basophils in the fibrotic mechanisms of SSc. Firstly, we showed that basophils stimulate B cells from SSc patients to induce IL-6 and TGF-β secretion. This result is in agreement with a recent report demonstrating that B cells secrete both IL-6 and TGF-β in SSc patients resulting in activation of normal and SSc fibroblasts (*Dumoitier et al. unpublished data*). We suggest that basophils interact with B cells through higher BAFF secretion, leading to increased B cell's survival and IL-6 and TGF-ß secretion. Basophils are already known to stimulate B cells in autoimmune and inflammatory diseases such as systemic lupus erythematosus (Meyer-Bahlburg and Dijkstra, 2016) or IgG4-related disease (Watanabe et al., 2013). Basophils' ability to stimulate B cells relies on the secretion of BAFF and IL-4 (Merluzzi et al., 2015). Although we failed to detect an increased secretion of IL-4, we provided evidence for an overexpression of CD154 that could increase B-cell activation in patients with SSc. Interestingly, an increased expression of CD154 at the surface of basophils has already been reported in asthma (Devouassoux, 2000) and allergy (Siegmund et al., 2000). We observed that B lymphocytes do not overexpress CD40 at their surface when cultured with KU812F basophils, which express CD154 (Hirano et al., 2006).

Still, the engagement of CD40 at the surface of B cells by CD154 is required for an optimal B-cell response (Lee et al., 2003) and auto-antibody production (Xu et al., 2015).

Here, we also demonstrate that basophils closely interact with fibroblasts. Interaction between fibroblasts and basophils has been previously discussed in a mouse model of atopic dermatitis (Jiao et al., 2015) and it has been shown that basophils express epidermal growth factor, which can induce proliferation and differentiation of fibroblasts (Qi et al., 2010; Shoyab et al., 1989). The secretion of IL-4 by basophils, a well-known fibrogenic cytokine, also enhances this interaction. In response to this cytokine, human fibroblasts are able to synthesize elevated levels of extracellular matrix proteins which concur to fibrosis (Postlethwaite et al., 1992). In line with these findings, Schiechl et al. have recently reported that both basophils and IL-4 play crucial roles for the activation of fibroblasts and the development of organ fibrosis in a mouse model of cardiac GvH disease (Schiechl et al., 2016). Interestingly, we bring evidence that basophils' TGF-ß secretion is involved in organ fibrogenesis in patients with SSc (Fig 6). It is well established that TGF-ß is one of the key players in SSc pathophysiology and targeting TGF-ß is considered as a promising strategy (Rice et al., 2015). The ability of basophils to produce this cytokine has not been previously reported. The TGF-ß gene being expressed by the KU812F basophil cell line (Okamura et al., 1989), we believe that the model used in the present study is robust and able to explore the functionality of basophil activation and TGF-ß secretion on fibroblasts from patients with SSc. Previous studies have suggested that monocytes, macrophages, mast cells and fibroblasts produce TGF-ß in SSc (Hasegawa et al., 2004b; Stevenson et al., 2008; Hügle et al., 2011, 2012). Identifying additional cells that produce an excess of TGF-ß is therefore of major relevance in the context of this connective tissue disease.

In conclusion, this study provides evidence that basophils, through interactions with both B cells and fibroblasts exert a pro-fibrotic role in SSc's pathogenesis. Further studies are warranted in order to assess whether targeting these cells could be of interest in daily practice.

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Conflicts of interest:

None

Figure legends

FIG 1. Expression of activation markers of peripheral basophils from patients with SSc

Basophil activation markers CD203c **(A, C)** and CCR3 **(B, D)** were analyzed in 38 healthy controls (HC) (●) and 65 SSc patients (□), including 12 with diffuse SSc (dSSc) (Δ) and 53 with limited SSc (lcSSc) (∇). Percentages and mean fluorescence intensities (MFI) are shown. Comparisons were performed using Mann-Whitney U-test. Statistical significance was defined as: *** $P < 0.001$.

FIG 2. Expression of functional receptors of peripheral basophils from patients with SSc

Basophil functional markers CD154 **(A, D)**, human leucocyte antigen-DR (HLA-DR) **(B, E)** and CD178 **(C, F)** were analyzed in 13 healthy controls (HC) (●) and 20 patients with SSc (□). Percentages and mean fluorescence intensities (MFI) are shown. Comparisons were performed using Mann-Whitney U-test. Statistical significance was defined as: * $P < 0.05$.

FIG 3. Decreased basophil CRTH2 expression in patients with SSc correlates with distinct features of the disease.

Basophil CRTH2 expression **(A, D)** was analyzed in 10 HC (●) and in 20 patients with SSc (□). Its mean fluorescence intensity (MFI) was studied with regards to patients' characteristics including pulmonary hypertension (PAH) **(B)**, gastro-intestinal involvement (GI) **(E)**, diffusing lung capacity of carbon monoxide (DLCO) **(C)**, and modified Rodnan skin score (mRSS) **(F)**. Comparisons and correlations were performed using Mann-Whitney U-test and Spearman coefficient test, respectively. Statistical significance was defined as: * $P < 0.05$; **** $P < 0.0001$.

FIG 4. Intracellular cytokine staining of peripheral basophils from patients with SSc.

Detection of intracellular B cell activating factor (BAFF) **(A, D)**, interleukin-4 (IL-4) **(B, E)** and transforming growth factor-ß (TGF-ß) **(C, F)** were analyzed in basophils in 20 healthy controls (HC) (●) and 20 patients with SSc (□). Percentages and mean fluorescence intensities (MFI) are shown. Comparisons were performed using Mann-Whitney U-test. Statistical significance was defined as: * $P < 0.05$.

FIG 5. Impact of basophils on isolated B cells from HC and patients with SSc.

Five hundred thousand B cells from 7 HC or 7 SSc were cultured either alone (black), with 50,000 unstimulated KU812F basophils (grey), or with 50,000 stimulated KU812F basophils (white). After 3 days, CD40 **(A)**, BAFF receptor **(B)**, CD86 **(C)**, CD95 **(D)**, IL-6 **(E),** and TGF-β **(F)**, were evaluated in B cells. Comparisons were performed using paired t-test. Statistical significance was defined as: * $P < 0.05$. MFI: mean fluorescence intensity.

FIG 6. Impact of basophils on the proliferation of dermal fibroblasts isolated from HC and from patients with SSc

Dermal fibroblasts (2500/well) isolated from 5 HC and from 5 SSc were cultured with KU812F basophils at 4 different concentrations. Comparisons of proliferation ratio were made between HC and SSc in presence of activated KU812F basophils **(A)**, between SSc fibroblasts with stimulated or unstimulated KU812F basophils **(B)**, and between SSc fibroblasts with stimulated KU812F with or without anti-TGF-ß **(D)**. **(C)** shows the production of TGF-ß by KU812F at 24 hours after stimulation. Comparisons were performed using t-test. Statistical significance was defined as: * $P < 0.05$.

Legends to supplemental figures

FIG E1. Gating strategy and frequency of peripheral basophils in SSc.

Peripheral blood mononuclear cells were identified using the forward scatter (FSC) and the side scatter (SSC) parameters. Basophils were then identified as FCƐRI$^+$ CD123$^+$ among CD45$^+$ Lin$^-$ CD11c$^-$ cells **(A)**. The percentage of basophils among CD45$^+$ Lin$^-$ CD11c$^-$cells was established for 38 healthy controls (HC) (●) and 65 patients with SSc (□), including 12 with diffuse SSc (dSSc) (Δ) and 53 with limited cutaneous SSc (lcSSc) (∇) **(B)**. Absolute basophil count was established for 20 HC (●), and 65 patients with SSc (□), including 12 with dSSc (Δ) and 53 with lcSSc (∇) **(C)**. Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as: * $P < 0.05$.

FIG E2. Frequency and activation markers of basophils upon stimulation in HC and in patients SSc

Percentages and MFI of CD203c (**A, C**) and CCR3 **(B, D)** were quantified in basophils from HC (●) and SSc patients (□) before and after stimulation. Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as: *** $P < 0.001$.

Fig E3. Additional identification markers of peripheral basophils from HC and patients with SSc

Percentages and MFI-of CD22 **(A, C)** and CD38 **(B, D)** were quantified within basophils from HC (●) and SSc patients (□) before and after 2h-stimulation. Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as: * $P < 0.05$.

FIG E4. Additional functional markers of peripheral basophils from HC and patients with systemic sclerosis SSc

Percentages and MFI of CD44 **(A, E)**, CD62L **(B, F),** CD54 (**C, F**) and CD196 (**D, G**) were quantified within basophils from HC (●) and SSc patients (□) before and after 2h-stimulation. Comparisons between groups were performed using Mann-Whitney U-test. Statistical significance was defined as: * $P < 0.05$.

FIG E5. Impact of basophils on the proliferation of dermal fibroblasts isolated from HC and from patients with SSc

(A) Proliferation of dermal fibroblasts (2500/well) isolated from HC (dark grey) and from patients (grey) cultured with unstimulated basophils at 4 different concentrations. **(B)** Proliferation of fibroblasts isolated from HC cultured with unstimulated (dark grey) or activated basophils (black) after 4 days of culture. Comparisons between groups were performed using t-tests and means ± standard deviations are represented. Statistical significance was defined as: * $P < 0.05$.

Table E1. Antibodies used for flow cytometry experiments.

Target	Fluorochrome	Clone	Society
BAFF	PE	1D6	ebioscience
BAFF-R (BR3)	FITC	11C1	BD
CD11c	PB	3.9	Biolegend
CD22	PE	HIB22	Biolegend
CD38	PerCP-Cy5.5	HIT2	BD
CD40	APC-Cy 7	5C3	BD
CD44	BV 605	G44-26	BD
CD45	AF 700	30-F11	Biolegend
CD54	PE-Cy5	HA58	BD
CD62L	PE-Cy5	DREG-56	BD
CD86	BV 421	2331 (PUN-1)	BD
CD95	PE	DX2	BD
CD123	PE-Cy7	7G3	BD
CD154	PE	TRAP1	BD
CD178	APC	NOK-1	BD
CD193	AF 647	5E8	BD
CD196	PerCP-Cy5.5	G034E3	Biolegend
CD203c	BV 605	NP4D6	Biolegend
CD294 (CRTH2)	PE	BM16	Biolegend

FCεRI	FITC	AER-37	eBioscience
HLA-DR	PerCP-Cy5.5	Tu36	Biolegend
IL-4	APC	8D4-8	eBioscience
IL-6	PE	MQ2-6A3	BD
Lineage	PB	-	Biolegend
Livedead	BV421	-	LifeTechnologies
TGF-β	APC	TW7-16B4	Biolegend

Abbreviations: AF: alexa fluor; APC: allophycocyanin; APC-Cy 7: allophycocyanin-cyanin 7; BAFF: B-cell activating factor; BD: BD biosciences; BV: brilliant violet; CD: cluster of differentiation; FCεRI: receptor for the Fc region of immunoglobulin E; FITC: fluorescein isothiocyanate; HLA: human leucocyte antigen; IL: interleukine; PB: pacific blue; PE: phycoerythrin; PE-Cy5: PE-Cyanin 5; PE-Cy7: PE-cyanin 7; PerCP-Cy5.5: peridin-chlorophyll protein-cyanin5.5; TGF-β: transforming growth factor-ß.

Table E2. Clinical characteristics of SSc patients at the time of blood sampling

Characteristics	**SSc (n=65)**
Clinical Features	
Gender (female),	54 (83)
Age, median [IQR] years	58 [49-67]
Disease duration, median [IQR] years	8 [4-13]
Limited cutaneous SSc:	53 (82)
Diffuse SSc:	12 (18)
Skin sclerosis:	
- Modified Rodnan Skin Score: median [IQR]	2 [0-5]
- Mouth opening (mm): median [IQR]	40 [33-47]
Digital ulcers:	20 (31)
Calcinosis:	14 (22)
Raynaud's phenomenon:	59 (91)
Telangiectasia:	34 (52)
Sclerodactyly:	25 (38)
Interstitial lung disease:	29 (45)
DLCO, %: median [IQR]	66 [51-74]
Pulmonary arterial hypertension:	7 (11)
Renal involvement*:	1 (2)
Gastro-intestinal involvement:	36 (55)
Arthralgia:	19 (29)
Muscle involvement:	10 (15)
Anti-nuclear antibodies	60 (92)
- Anti-centromere antibodies	27 (42)
- Anti-topoisomerase 1 antibodies	19 (29)
- Anti-RNP antibodies	4 (6)
- Anti-PM/Scl antibodies	6 (9)
- Anti-RNA Pol III	2 (3)

Data are represented as n (%) unless indicated differently.
** Renal involvement was defined as elevated serum creatinin level (>150µmoL/L) or renal crisis*
SSc, systemic sclerosis; IQR, interquartile range, DLCO, diffusing capacity of the lung for carbon monoxide; RNP: ribonucleoprotein; PM/Scl: polymyositis/scleroderma.

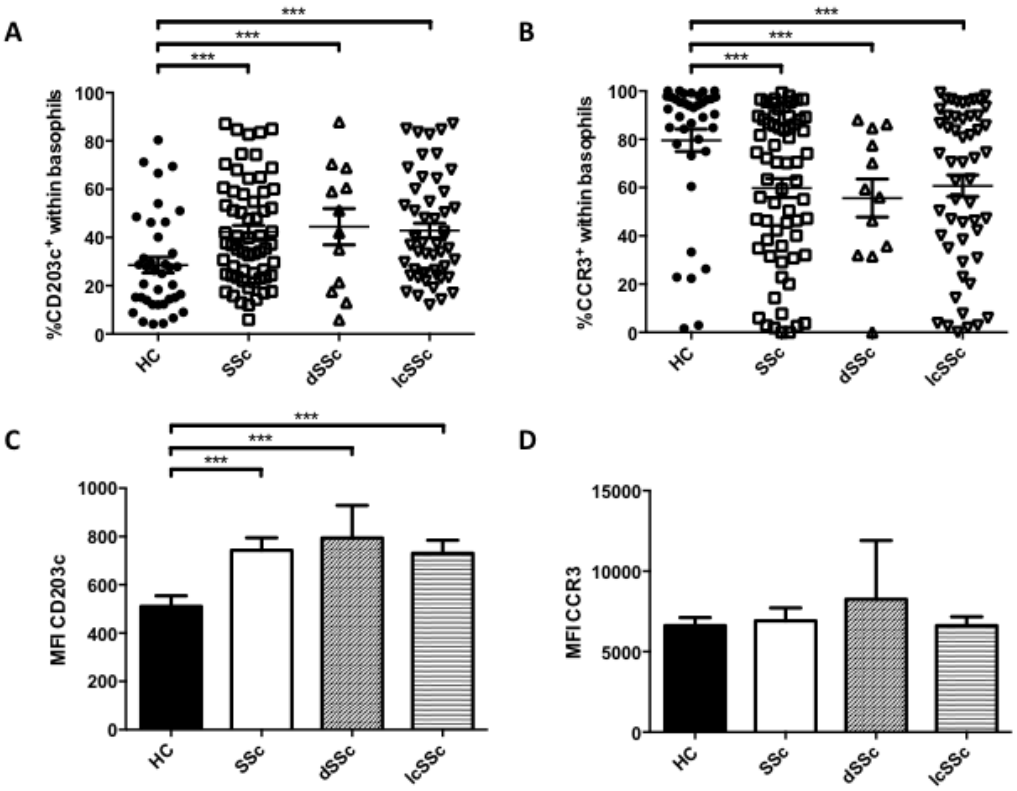

A
B
C
D
***
%CD203c+ within basophils
%CCR3+ within basophils
MFI CD203c
MFI CCR3
HC
SSc
dSSc
lcSSc

Figure 1

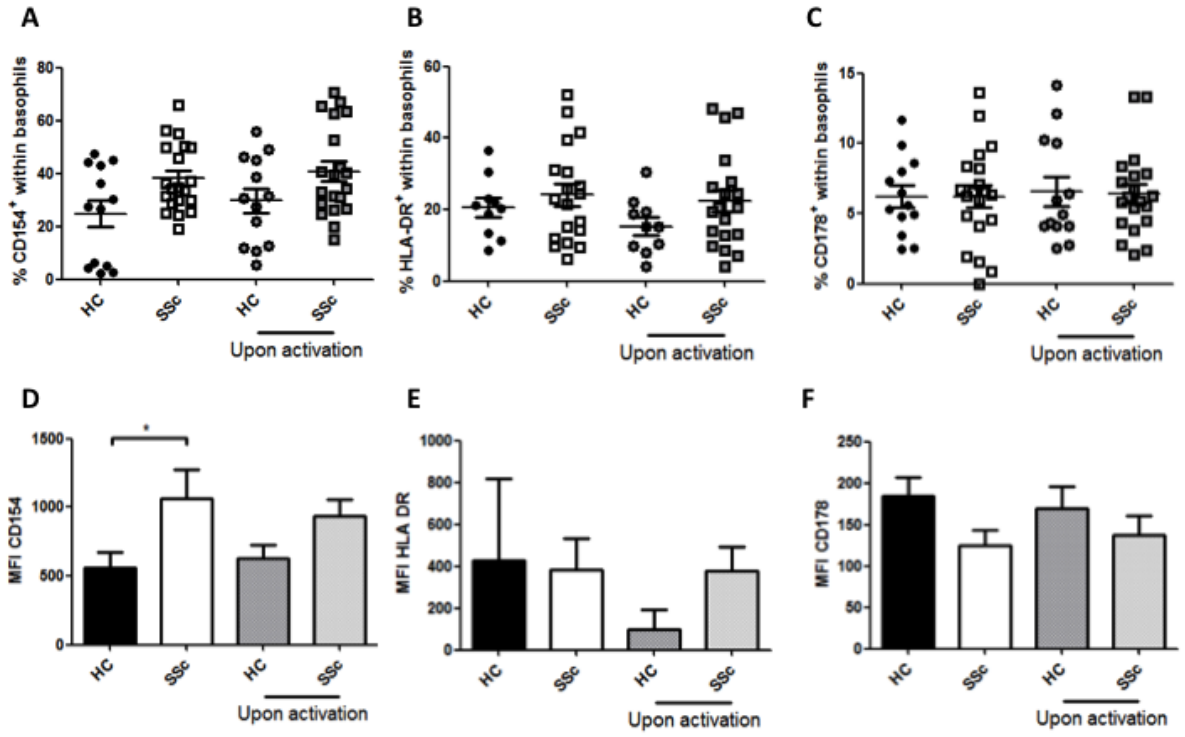

A
% CD154+ within basophils
80
60
40
20
0
HC
SSc
HC
SSc
Upon activation
B
% HLA-DR+ within basophils
60
40
20
0
HC
SSc
HC
SSc
Upon activation
C
% CD178+ within basophils
15
10
5
0
HC
SSc
HC
SSc
Upon activation
D
MFI CD154
1500
1000
500
0
*
HC
SSc
HC
SSc
Upon activation
E
MFI HLA DR
1000
800
600
400
200
0
HC
SSc
HC
SSc
Upon activation
F
MFI CD178
250
200
150
100
50
0
HC
SSc
HC
SSc
Upon activation

Figure 2

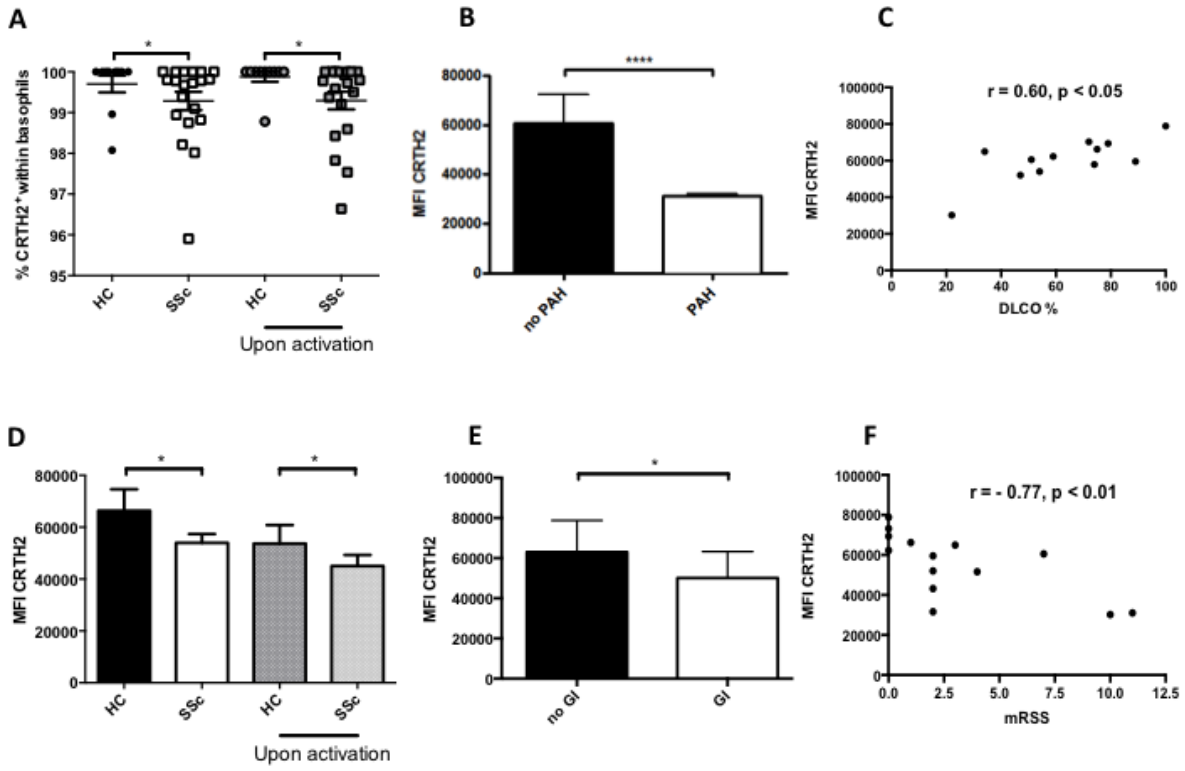

A
% CRTH2+ within basophils
HC
SSc
HC
SSc
Upon activation
B
MFI CRTH2
no PAH
PAH
C
r = 0.60, p < 0.05
MFI CRTH2
DLCO %
D
MFI CRTH2
HC
SSc
HC
SSc
Upon activation
E
MFI CRTH2
no GI
GI
F
r = - 0.77, p < 0.01
MFI CRTH2
mRSS

Figure 3

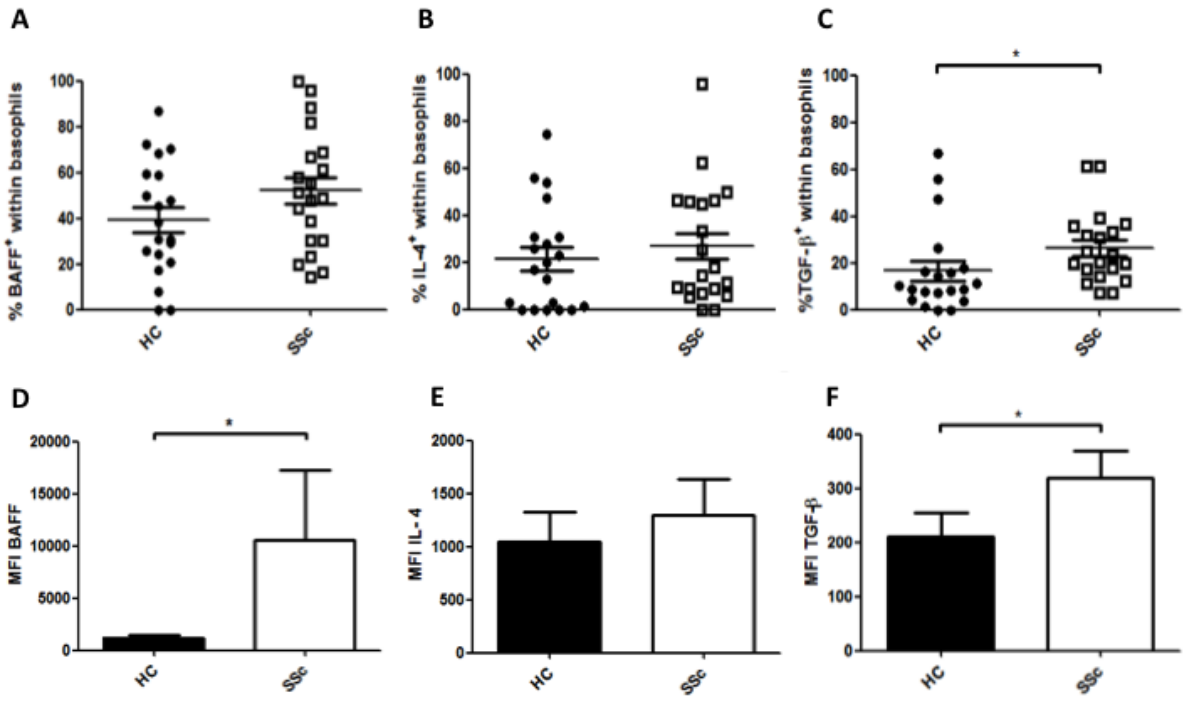

A
% BAFF+ within basophils
100
80
60
40
20
0
HC
SSc
B
% IL-4+ within basophils
100
80
60
40
20
0
HC
SSc
C
%TGF-β+ within basophils
*
100
80
60
40
20
0
HC
SSc
D
MFI BAFF
*
20000
15000
10000
5000
0
HC
SSc
E
MFI IL-4
2000
1500
1000
500
0
HC
SSc
F
MFI TGF-β
*
400
300
200
100
0
HC
SSc

Figure 4

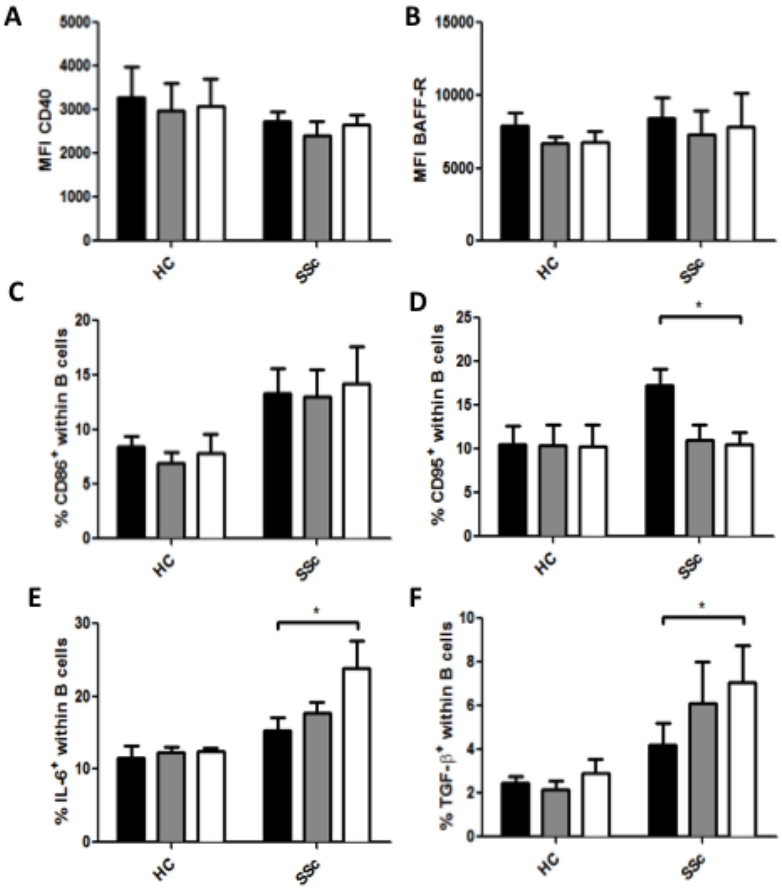

A
MFI CD40
5000
4000
3000
2000
1000
0
HC
SSc
B
MFI BAFF-R
15000
10000
5000
0
HC
SSc
C
% CD86+ within B cells
20
15
10
5
0
HC
SSc
D
% CD95+ within B cells
25
20
15
10
5
0
*
HC
SSc
E
% IL-6+ within B cells
30
20
10
0
*
HC
SSc
F
% TGF-β+ within B cells
10
8
6
4
2
0
*
HC
SSc

Figure 5

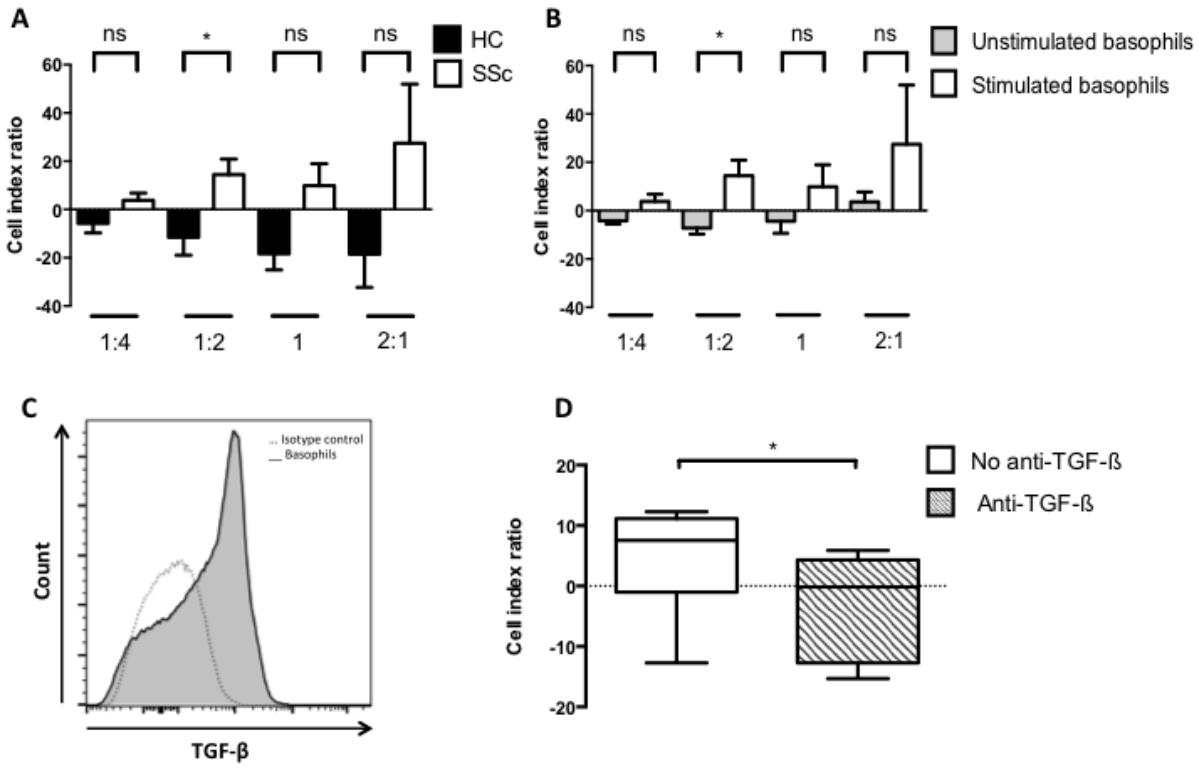
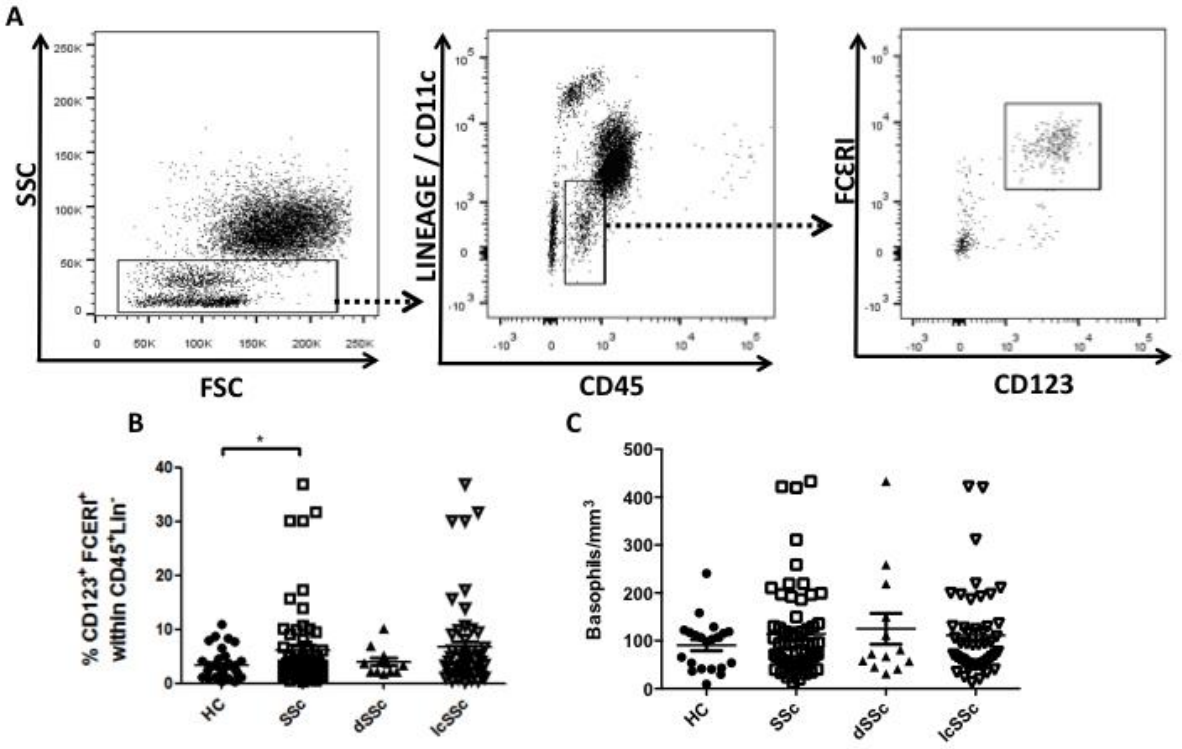
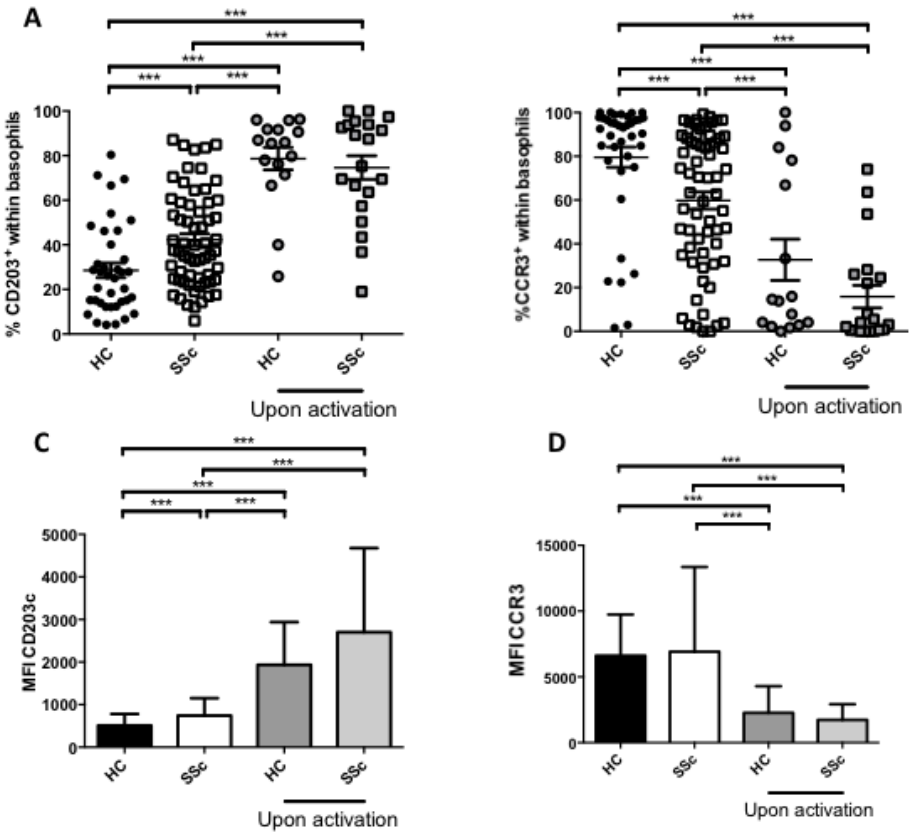

A
ns
*
ns
ns
HC
SSc
Cell index ratio
60
40
20
0
-20
-40
1:4
1:2
1
2:1
B
ns
*
ns
ns
Unstimulated basophils
Stimulated basophils
Cell index ratio
60
40
20
0
-20
-40
1:4
1:2
1
2:1
C
... Isotype control
__ Basophils
Count
TGF-β
D
*
No anti-TGF-ß
Anti-TGF-ß
Cell index ratio
20
10
0
-10
-20

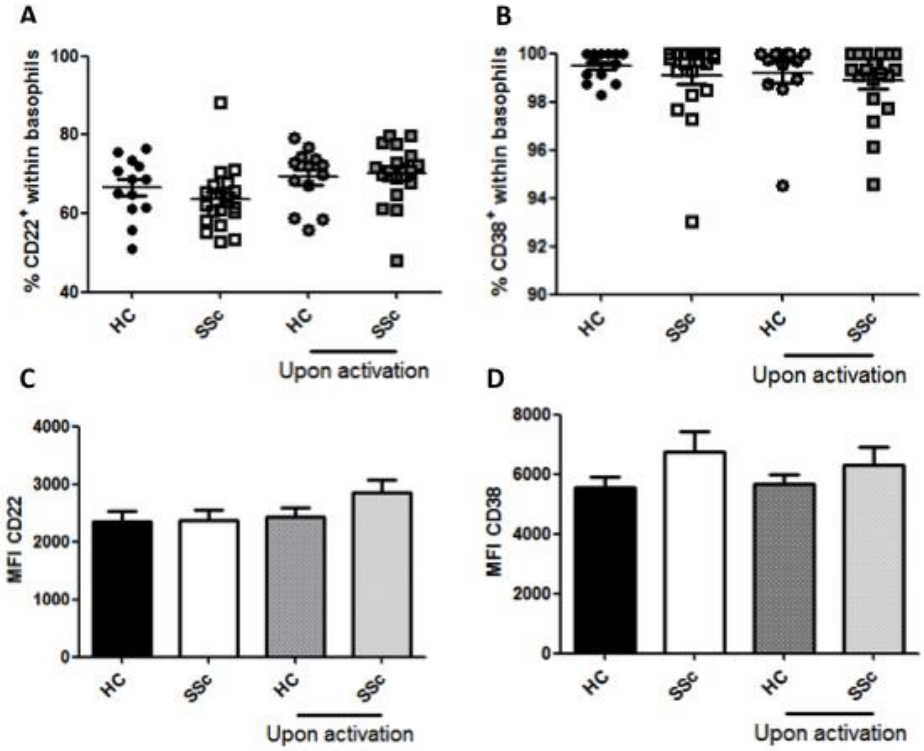
Figure 6


A
SSC
FSC
LINEAGE / CD11c
CD45
FCERI
CD123
B
% CD123+ FCERI+ within CD45+Lin-
HC
SSc
dSSc
lcSSc
C
Basophils/mm3
HC
SSc
dSSc
lcSSc

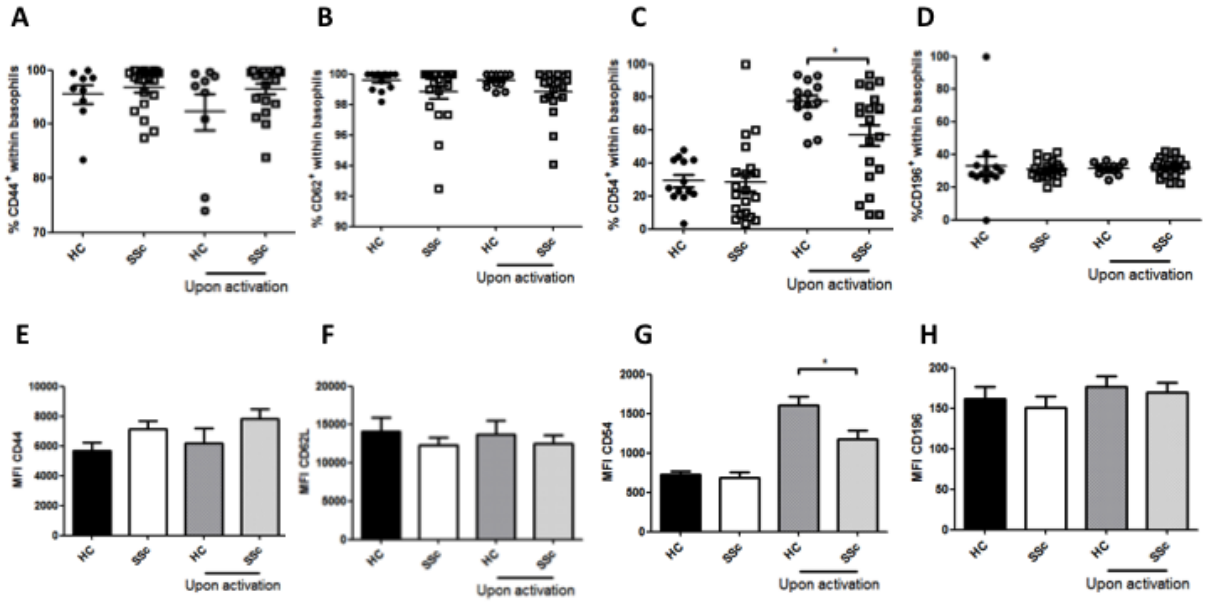
E Fig 1


A
% CD203⁺ within basophils
HC
SSc
HC
SSc
Upon activation
%CCR3⁺ within basophils
Upon activation
C
MFI CD203c
Upon activation
D
MFI CCR3
Upon activation

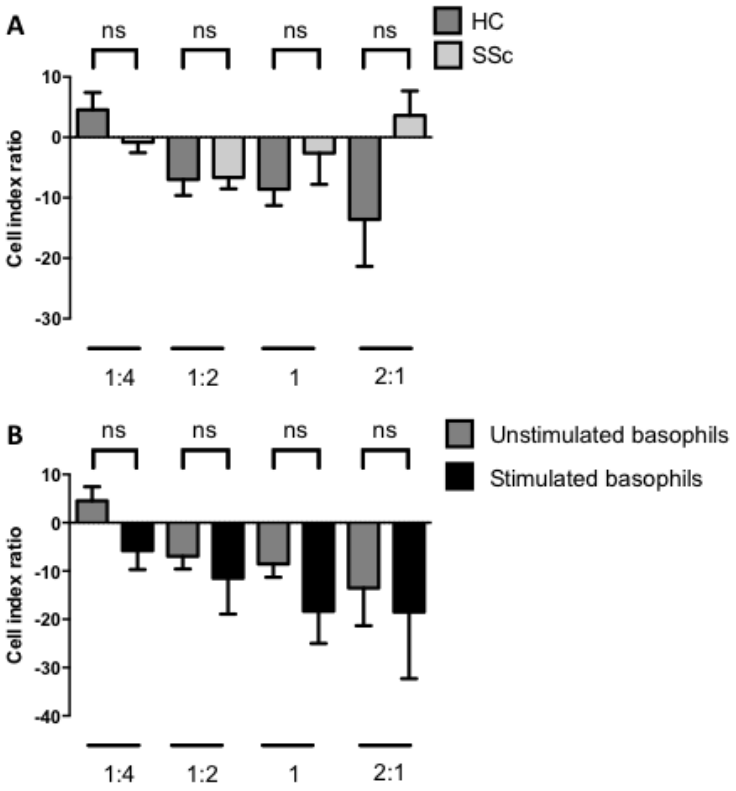
E Fig. 2


A
% CD22+ within basophils
100
80
60
40
HC
SSc
HC
SSc
Upon activation
B
% CD38+ within basophils
100
98
96
94
92
90
HC
SSc
HC
SSc
Upon activation
C
MFI CD22
4000
3000
2000
1000
0
HC
SSc
HC
SSc
Upon activation
D
MFI CD38
8000
6000
4000
2000
0
HC
SSc
HC
SSc
Upon activation

E Fig. 3


A
B
C
D
E
F
G
H
% CD44+ within basophils
% CD62+ within basophils
% CD54+ within basophils
%CD196+ within basophils
MFI CD44
MFI CD62L
MFI CD54
MFI CD196
HC
SSc
Upon activation
*

E Fig. 4


A
ns
ns
ns
ns
HC
SSc
Cell index ratio
10
0
-10
-20
-30
1:4
1:2
1
2:1
B
ns
ns
ns
ns
Unstimulated basophils
Stimulated basophils
Cell index ratio
10
0
-10
-20
-30
-40
1:4
1:2
1
2:1

E Fig. 5

DISCUSSION

&

PERSPECTIVES

Discussion and perspectives

B lymphocyte deregulations have been reported in several autoimmune diseases and particularly those with detectable autoantibodies in which B lymphocyte terminal differentiation is commonly altered. However, autoimmunity may also be defined as a rupture of tolerance independently of antibodies targeting the "self" or autoreactive plasmocytes expansion.

In AAV, we observed that B-cell compartment was unbalanced, notably in MPA patients, with increased proportion of naïve B cells and decreased circulating memory B cells, whereas no significant difference was found in GPA patients. This result is in agreement with previous studies that we recently reviewed (Dumoitier et al., 2015). Besides alteration of the B-cell maturation, we identified for the first time that MPA patients had an increased proportion of $CD21^{-/lo}$ B lymphocytes, which were previously found enriched in autoreactive B cells in Sjögren's syndrome, hepatitis C virus-related cryoglobulinemia vasculitis, systemic lupus erythematosus and rheumatoid arthritis (Isnardi et al., 2010; Saadoun et al., 2013). Moreover, this subset of autoreactive B cells was more frequent in MPO-positive vasculitis in spite of a differentiation between GPA and MPA. This result argues for a very specific deregulation of B cells associated with MPO antigens while not in PR3 positive AAV. Furthermore, if ANCA pathogenicity suggests various roles for B lymphocytes in patients with AAV, this observation also reinforces the interest of screening antigen specificity which patently influences B lymphocyte response in AAV.

In our study, we did not observe important bias in the maturation status of B lymphocytes in patients with SSc conversely to what was found in MPA patients. However, we identified the $CD69^{+}$ $CD95^{+}$ subpopulation as the prevalent activated subset in patients with early SSc. This

report is, to our knowledge, the first identifying an immune adaptive response correlated with disease progression in systemic sclerosis. We further identified increased expression of nearly all the activation markers that we tested, showing that B cells are particularly activated in SSc patients. Moreover, in SSc patients, expression of CD69, CD95 and IL-6-R allowed discrimination between the various forms of the disease, with particular emphasis in the most severe, diffuse forms, and in the associated PAH. These data constitute the first report suggesting a different activation of B lymphocytes regarding the form and the severity of the disease. It would be of high interest to evaluate the potential deleterious roles of these B cell subpopulations in subsequent studies.

In addition, we observed in both GPA and MPA a B-cell activated phenotype with increased expression of CD69 and CD40. Both of these activation markers are known to play a role in B- and T-cell cooperation and were found increased in B cells from patients with SLE or rheumatoid arthritis (Zhang et al., 2012; Medgyesi et al., 2014). These receptors also influence the development of autoreactive B cells in several experimental models like EAE and rheumatoid arthritis (Bonomo et al., 1995; Sancho et al., 2003; Monneaux et al., 2000).

Differences between GPA and MPA patients reinforced the paradigm of GPA and MPA toward autoimmunity. B cells from MPA patients were thus potentially enriched in autoreactive B cells while B cells from GPA patients displayed classical maturation subpopulations contrasting with markedly deregulated neutrophils and innate immunity (Millet et al., 2013). In the present work, we provide new evidences for a deregulation of lymphocyte subpopulations that presents similarity to what was observed in innate immune cells compartment. For instance, in GPA, we can hypothesize that neutrophil and innate immunity are the first deregulated cells whereas both T and B lymphocytes were activated through the presentation of PR3 in patients' granuloma as previously reported by Voswinkel and colleagues (Voswinkel et al., 2006). Indeed, this was not the case for MPA patients. We also found that BAFF receptor expression on B cells was

dramatically decreased in both GPA and MPA which could be related to the increased activation of the BAFF pathway in AAV (Vincent et al., 2013). This finding also revealed a potential interplay between neutrophils and B cells, as neutrophils highly express BAFF in patients with GPA, which could participate in B cell activation (Holden et al., 2011b; Scapini et al., 2008). In SSc, presence of activated basophils also participates in the activation of B cells and fibroblasts through the secretion of IL-4, TGF-β1 and BAFF, together with high expression of CD40L. Moreover, the secretion of TGF-β1 by basophils could be an interesting new field of investigation in fibrotic diseases such as SSc. Altogether these data highlight the potential interplay between innate immune cells and B lymphocytes.

In line with these observations, patients affected by GPA presented with important activation of innate immune system altogether with an increased production of IL-6 by B lymphocytes correlated with T lymphocytes activation, and particularly for Th2 and Th9 subsets. These observations suggest that B and T cells deregulations could act as counterparts in patients with GPA which could enhance and maintain the inflamed context.

In the present work, we observed skewed T-cell polarization toward Th2, Th9 and Th17 responses in GPA, whereas patients with MPA presented with decreased percentages of Th1 within T cells. Moreover, in both AAV, we reported deregulation of Tfh compartment, with significant differences between MPA and GPA patients, which could explain both different B lymphocyte response and maturation status in AAV.

Patients with systemic sclerosis showed several antibodies targeting mostly fibroblasts and endothelial cells. However, B lymphocytes implication in SSc is poorly documented while evidences for a deleterious role of B cells on fibroblasts have been recently reported (François et al., 2013). We reported for the first time, increased production of IL-6 and TGF-β1 in patients with SSc. Moreover, we highlighted the potential role of the IL-6/IL-6R pathway in the SSc

context, as IL-6R seems particularly expressed at the membrane of B cells from patients with PAH associated with SSc. This observation is of peculiar interest since IL-6 is strongly implicated in endothelial cells remodeling.

Unexpectedly, B cells from patients with iPAH did not present similar phenotype as patients with PAH associated with SSc. However, deregulation of the IL-6/IL-6R pathway was observed in our small cohort of patients. These data highlight the critical role of IL-6 in both iPAH and SSc-PAH (Ricard et al., 2014).

In SSc, a basal activation of the B cells was observed, allowing an important secretion of TGF-ß1. The latter contributed to the proliferation of fibroblasts and to the secretion of collagen, responsible for the induction of fibrosis as observed in the pathology. This report reinforces the interest of B cell/fibroblast interplay which was previously highlighted in rheumatoid arthritis (Störch et al., 2016). These data also suggest that drugs targeting this cytokine pathway could be an interesting opportunity in systemic sclerosis while B cells could be preferentially targeted in diffuse SSc.

Vascular autoimmune pathologies regroup a vast amount of diseases related or not to B lymphocytes deregulation. However, in most of the cases, targeting B lymphocytes using anti-CD20 monoclonal antibody or B cell physiology (BAFF, IL-6, IL-4…) through immunomodulation drugs, induce promising results in animal models and clinical studies. Our results place B lymphocytes, besides their role in antibody production, as important pathophysiological players through the secretion of pro-inflammatory and pro-fibrotic cytokines such as IL-6 and TGF-ß1 which are both implicated in endothelial cells activation in autoimmune vascular diseases. Based on these findings, we might now propose a new classification of autoimmune vascular diseases: those with deregulation in B cells maturation and differentiation (MPA), those with strong pro-inflammatory response related to innate

immune cells (SSc and GPA), those with important activation of IL-6 pathway and endothelial cell remodeling (SSc-PAH and iPAH) and finally those with important pro-fibrotic secretions (SSc).

To conclude, in these settings, evaluation of anti-cytokine antibodies efficiency should be an additional therapeutic strategy in addition to the direct targeting using anti-B cell monoclonal antibodies. This is particularly the cases for severe manifestation of SSc, like SSc-PAH or early diffuse SSc, in which both B cells, IL-6 and TGF-β1, seem particularly implicated. These strategies could also interrupt potential interplays between innate immune cells, T cells and pathological B cells like in GPA. This could allow the management of the various roles of B lymphocytes and not only those of auto-antibody production.

ANNEXES

Annexe 1

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Quarterly Medical Review

Pathophysiology of systemic sclerosis: State of the art in 2014

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In this issue

Systemic sclerosis: views and thoughts for the future
Luc Mouthon, Paris, France

Pathophysiology of systemic sclerosis: state of the art in 2014
Nicolas Dumoitier et al., Paris, France

Gastrointestinal involvement in systemic sclerosis
Edoardo Savarino et al., Padua, Italy

Update in systemic sclerosis-associated pulmonary arterial hypertension
Mohamed A. Gashouta et al., Baltimore, United States

Kidney involvement in systemic sclerosis
Virginia D. Steen et al., Georgetown, United States

Musculoskeletal involvement in systemic sclerosis
Veronika Lóránd et al., Pécs, Hungary

Interstitial lung disease in systemic sclerosis
Athol U. Wells, London, United Kingdom

Lung and heart-lung transplantation for systemic sclerosis patients
David Launay et al., Lille, France

Summary

Major work has been done in order to improve the understanding of systemic sclerosis (SSc) pathogenesis. A number of new experimental models have been set up, that should help to understand the disease pathogenesis and test new therapeutic targets. Reactive oxygen species represent a hallmark of the pathogenesis of SSc, both at the fibroblast and at the endothelial cell levels. Although a large number of genetic studies have been conducted, it is still difficult to identify a genetic background specific to SSc, and the major progress in this setting is probably the identification of an interferon signature. Besides endothelial cells and fibroblasts, major development has been made in the understanding of the role of B cells and autoantibodies in the pathogenesis of SSc. Plasmacytoid dendritic cells seem to play a major role in the pathogenesis of SSc through the secretion of CXCL4, although these data will need to be confirmed in the near future.

Systemic sclerosis (SSc) is a rare connective tissue disease characterized by vascular involvement responsible for vascular hyperactivity and remodeling, together with fibroblasts activation and extra-cellular matrix synthesis [1].
SSc mostly occurs in females (3 to 8 females for 1 male). Its prevalence varies between 30 and 240 per million inhabitants, being higher in North America and Australia than in Japan [2]. In Europe, the prevalence varies between 50 and more than 150, with 158 per million in France [3]. Reports of sporadic clusters of higher prevalence suggest the existence of environmental risk factors, but only silica and solvents exposure have been consistently associated with SSc [2].
Patients with SSc are usually classified into two main groups, according to the extent of skin involvement: limited SSc (lSSc), with skin involvement essentially limited to the hands and face; and diffuse SSc (dSSc), with skin involvement proximal to the elbows and knees. In patients with

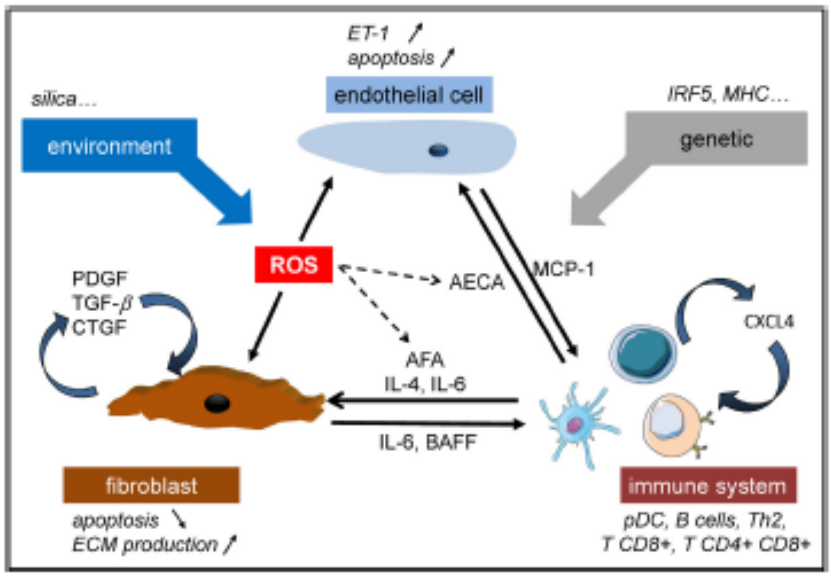

FIGURE 1

Key elements in the pathophysiology of systemic sclerosis

Dotted arrow: possible modulation; Solid arrow: activation.

AECA: anti-endothelial cell antibodies; AFA: anti-fibroblast antibodies; BAFF: beta cell activated factor; CTGF: connective tissue growth factor; ECM: extracellular matrix; ET-1: endothelin-1; IL: interleukin; IRF-5: interferon response factor 5; MCP-1: monocyte chemotactic protein 1; MHC: major histocompatibility complex; pDC: plasmacytoid dendritic cells; PDGF: platelet derived growth factor; ROS: reactive oxygen species; TGF-β: transforming growth factor beta.

lSSc, visceral involvement is rare, whereas patients with dSSc more frequently experience visceral involvement [4]. It is essential to distinguish between limited and diffuse SSc in order to elucidate the pathophysiology of the disease, since distinct mechanisms probably contribute to the occurrence of the two forms of the disease.

In recent years, significant progress has been made in the understanding of mechanisms contributing to the occurrence of vasculopathy and fibrosis, both in human samples and animal models. However, experimental models, although very useful, do not allow to characterize all the mechanisms at play in SSc, contributing to explain why we are still lacking a universal treatment of SSc. In this review article, we will provide the reader with an overview of the pathogenesis of SSc, with emphasis of recent acquisitions in the field *(figure 1)*.

Environmental factors

A number of environmental factors that may contribute to the occurrence of SSc have been identified. Various activities, including manufacturing and rural activities have been associated with the occurrence of SSc, notably the exposure to silica, dust and hydrocarbons [2]. The first reports of the association between silica and SSc were made in limited numbers of patients [5] followed by retrospective comparative studies, which confirmed that exposure to silica conferred a high risk to develop SSc [6]. Odds Ratio of 3.93 (1.84–8.54) [7] and 5.57 (1.69–18.37) [8] were calculated in studies conducted in Australia and France, respectively, leading a number of countries including France, Germany, Canada and South Africa to consider SSc as an occupational disease. In addition to silica, case control studies pointed out that past exposure to solvents was associated with SSc [5], with discrepancies among studies regarding the types of solvents involved and/or patient gender. Thus, paint thinner or removers, mineral spirits, trichloroethylene, trichloroethane, perchloroethane, gasoline, aliphatic hydrocarbons, halogenated hydrocarbons, benzene, toluene or xylene-solvents have been proposed as the most at risk solvents, although discrepancies were identified among studies. Nietert et al. found that SSc males were more frequently exposed to organic solvents (in particular trichloroethylene) than controls (OR 2.9 [1.1–7.6]), which was not the case for females [9]. In a recent meta-analysis of the literature, the occurrence of SSc was associated with increased ORs for silica, chlorinated solvents, trichloroethylene and welding fumes for male patients, aromatic solvents and ketones for female patients and white spirit for both [10]. Several others toxic

products including epoxy resins and pesticides, as well as paints, adhesive, hair dye, contact lenses and fabric dyes have been investigated in SSc case controls studies but were never found significantly at risk for SSc. Finally, although silicone breast implants were suspected as potentially responsible for SSc induction [11], two meta-analyses [6,10] concluded to the absence of association between SSc and breast implants.

In addition, outbreaks of SSc-like disorders have clearly been linked to chemical exposure, such as polyvinyl chloride intoxication [12] or toxic oil syndrome [5].

Some viruses or certain chemicals, such as solvents, may also play a triggering role in the initiation and maintenance of the disease. Notably, Lunardi et al. have reported the presence of a "SSc peptide" recognized by serum immunoglobulin from 93% of patients with SSc. Interestingly, this peptide shows an important homology with UL94, a cytomegalovirus (CMV) late protein that can be recognized by purified IgG from patients [13]. *In vitro*, patient antibodies targeting UL94 also seem to induce apoptosis of endothelial cells (EC) similarly to SSc specific antibodies, suggesting a possible role of CMV in the triggering and the maintenance of SSc. Thus, it turns out that a cascade of extrinsic and intrinsic events seems necessary for the induction of the disease [14].

Genetic factors

General approaches

Many studies have been conducted in recent years in an attempt to identify genetic risk factors in SSc. From three U.S. cohorts, involving 703 families, including 11 multiplex SSc, first degree relatives were identified to get increased susceptibility to develop SSc, with a relative risk close to 13 (10–16 across cohorts), with a recurrence rate of 1.6% versus 0.026% in the general population [15]. For siblings, the relative risk in siblings (λs) is increased to 15 (10–27 across cohorts). For comparison, the risk (λs) varies between 3 and 15 in rheumatoid arthritis (RA) and between 20 and 29 in systemic lupus erythematosus (SLE). Only one study has been performed in twins. Based on the analysis of 42 twin pairs (24 monozygotic), this study demonstrates a poor agreement with the clinical expression of the disease (4.7%) [16]. However, there is a higher concordance for the presence of anti-nuclear antibodies (ANA): 40% for dizygotic and 90% for monozygotic. These results suggest that genetic predisposition alone is not sufficient to develop SSc but might influence the autoantibody profile.

Following the development of molecular biology, many data have been published on association studies. Unfortunately, a number of them were not repeated in large cohorts and/or could not be independently replicated by another group, which is essential for the validation of a genetic association signal. This was particularly the case for genes suspected to be involved in the regulation of fibrosis such as *SPARC* (encoding the secreted protein acid rich in cysteine), *CTGF* (encoding connective tissue growth factor), *TGFB* (encoding the tissue growth factor β) or *FBN1* (encoding fibrillin 1) [17] or vascular factors including *KCNA5* (encoding the potassium voltage-gated channel 5) or *uPAR* (encoding the urokinase-type plasmogen activator receptor) [18,19]. Interestingly, *uPAR* extinction in a mouse model led to dermal and pulmonary fibrosis together with microvasculopathy [20]. In parallel, a decrease of full-length *uPAR* protein expression in dermal biopsies of patients with SSc as compared to normal skin has been reported, suggesting a protective role of this protein in SSc. In addition, the potential influence of *KCNA5* SNP on SSc prevalence remains controversial [21].

Immune system and molecules of intracellular signaling

The tumor necrosis factor ligand superfamily 4 gene (*TNFSF4*) encodes OX40L (CD252), the ligand of OX40 (CD134). OX40L, involved in antigen presentation in addition of T and B lymphocyte activation, is known as a susceptibility factor for SLE [22]. Interestingly, a study performed in 1,059 patients with SSc and 698 controls showed an association between OX40L polymorphism and SSc and the major disease subgroups [23]. This result has been confirmed in replication and meta-analysis studies [24,25].

Type I interferons (IFN) are key mediators of innate immunity and antimicrobial defense. IRF5 (interferon-regulatory factor 5 encoded by *IRF5*) is a transcription factor involved in the signaling of toll-like receptors (TLRs) and activation of target genes of interferon. Candidate genes studies have shown its association with the disease and its contribution to pulmonary fibrosis associated with SSc, independently of other risk factors identified [26,27]. These studies permanently identified IRF5 as a susceptibility factor to SSc. Moreover, recent updates highlighted the major role of IRF5 in the progression and in the occurrence of interstitial lung disease (ILD) in SSc [28,29].

Signal transducer and activator of transcription 4 (STAT4) is a transcription factor of the STAT family that induces the expression of type I IFN, interleukin (IL)-12 and IL-23, and stimulates the synthesis of IFN-γ and IL-17. Thus, a variant of the gene located in the third intron (rs7574865), without specific biological function, has independently been found to be associated with several autoimmune diseases in two European, one U.S. and a Japanese cohorts [30]. However, *STAT4* is probably not directly responsible for the autoimmune state, since the combination of *IRF5* and *STAT4* [27] variants was associated with an increased risk of developing pulmonary fibrosis in patients with SSc (OR 2.72 [1.86–3.99], $P = 3.07 \times 10^{-7}$ in presence of 3 or 4 at-risk alleles). In that study, *STAT4* was found to be the second outside association major histocompatibility complex (MHC)

signal (rs3821236 $P = 3.37 \times 10^{-9}$, OR 1.30 [1.18–1.44]). Interestingly, *Stat4*$^{-/-}$ mice exposed to bleomycin developed reduced dermal fibrosis as compared to controls, as assessed by a 65% decrease of dermis thickness, 68 ± 5% decrease of hydroxyproline content and 71 ± 6% decrease of myofibroblast numbers [31]. In addition, leukocyte infiltration was also reduced (63 ± 5%) in skin lesions, as well as the pro-inflammatory cytokines production including TNF-α, IL-2 and IL-6. Thus, STAT4, which plays a major role in an inflammatory model of fibrosis, might represent a potential therapeutic target in SSc.

Two genes, *PTPN22*, encoding the protein tyrosine phosphatase called Lyp, and *CSK* encoding the c-src tyrosine kinase also called CSK, which modulates signaling through the T cell receptor (TCR), have been found to increase susceptibility to SSc. *PTPN22* was one of the first susceptibility genes associated with several autoimmune diseases. Its main variant (rs2476601, 1858C > T, R620 W) has been reported to be modestly associated with SSc [32,33], particularly in patients with anti-centromere autoantibodies; however this association is attenuated by the fact that a significant proportion of patients with SSc also develop autoimmune thyroiditis, which is associated with polymorphisms of *PTPN22*. Furthermore, the genome wide association studies (GWAS) identified an association of a *CSK* variant (rs1378942) with SSc frequency which was unrelated to auto-antibody specificities ($P = 5.04 \times 10^{-12}$; OR = 1.20) [34]. Interestingly, in the 2 GWAS performed, *CD247* (rs2056626; OR 0.86 [0.81 to 0.90] $P = 2.09 \times 10^{-7}$), which encodes a subunit of the TCRζ (component of TCR/CD3 complex), was significantly associated with a risk of developing SSc [35,36].

Several studies confirmed the major role of B cells in SSc. Thus, two variants (rs10516487 and rs3733197) of B cell-specific scaffold protein with ankyrin repeats (*BANK1*) gene, a promoter of the tyrosine kinase LYN phosphorylation, were found to be associated with dSSc [37,38], whereas another B lymphocytes specific gene, *BLK* (B lymphocyte kinase), which transduces the signal downstream of the BCR and is also involved in SLE (*C8orf13-BLK* region, rs13277113 and rs2736340) was associated with subgroups of lSSc and anti-centromere positive patients [39,40].

The variant of *TNFAIP3* (6q23) was also identified through the GWAS as a susceptibility factor for autoimmune diseases [41]. *TNFAIP3* is an intracellular protein that regulates negatively the NF-κB signaling pathway (by deubiquitination) downstream to the TNF-R super-family, TLRs, IL-1R and nucleotide-binding oligomerization domain protein 2 (NOD2) receptor. The rare G allele of rs5029939 was associated with SSc with an OR of 2.08 [1.59 to 2.72], $P = 1.16 \times 10^{-7}$, with dSSc with an OR of 2.71 [1.94 to 3.79], $P = 5.2 \times 10^{-9}$ and with pulmonary arterial hypertension (PAH) with an OR of 3.11 [1.86 to 5.17], $P = 1.3 \times 10^{-5}$.

Taken together, these data reinforce the autoimmune component in SSc, with evidence for involvement of different pathways contributing to autoimmunity in other diseases, although fine mechanisms contributing to the occurrence of SSc remain to be identified.

Genetics might help to identify new biomarkers that could allow better stratification of patients at risk of developing visceral manifestations and offering them targeted therapies before the occurrence of severe organ involvement. However, identified OR are often weak even if they combine several gene polymorphisms. Thus, new genetic approaches might probably be helpful in the near future to go further in this direction.

Major histocompatibility complex

As for the vast majority of autoimmune diseases, the association of human leukocyte antigen (*HLA*) locus with SSc has been identified many years ago. It has been clarified by working specifically on HLA class II from 1300 SSc patients from USA and 1000 controls [42]. The strongest associations with SSc in Caucasians and Hispanics were found for *DRB1*1104*, *DQA1*0501*, *DQB1*0301*, *DQB1* alleles encoding a protein without leucine at position 26 (*DQB126*epi*). On the other hand, *DRB1*0701*, *DQA1*0201*, *DQB1*0202* and *DRB1*1501* alleles were protective. In a recent GWAS, several SNPs located in 6p.21 in the MHC region were strongly associated with SSc, whereas the rs6457617 variant SNP located in the *HLA DQB1* gene was strongly associated with SSc ($P = 2.31 \times 10^{-18}$) [43]. These results are associated with a strong weight of ethnicity and heterogeneity of the disease.

In addition, many other alleles or haplotypes appear very specific to certain subgroups, particularly characterized by autoantibody status [44]. Notably, *DRB1*1101* and *DPB1*1301* alleles have been associated with anti-topoisomerase I positivity when *DRB1*0401-22* and *DRB1*0801-11* alleles are associated with anti-centromere positivity [45].

Experimental models

A relatively large number of animal models of SSc have been set up over the years, including at least 5 genetic and 3 inducible models that reproduce, at least partly, human SSc [46–65]. Most of them are listed in *(figure 2)*. For a purpose of clarity we will not detail them all.

Tsk-1 mouse model is very well described and results from a partial duplication of the fibrillin 1 gene (*Fbn1*) leading to a characteristic phenotype of dermal fibrosis associated with increased extracellular matrix (ECM) production by fibroblasts and detection of SSc specific and other autoantibodies (anti-topoisomerase, anti-deoxyribonucleic acid [DNA]...) [46–48]. Fibroblasts cultured from the skin of Tsk-1 mice also display a pro-fibrotic phenotype and can thus be used for the assessment of drugs *in vitro* [49]. Interestingly, in this mouse model, B cell deficiency can attenuate fibrosis progression by modulating IL-6 production [50]. Finally, Tsk-1 mouse is associated with pulmonary emphysema and not with pulmonary fibrosis, which has to be considered with the recent description of

		Experimental model	Vasculopathy	Fibrosis	Inflammation	Autoantibodies	Limits/specificities
Genetic models	Spontaneous	Tsk-1 [46]					Unclear various organs defects
		Tsk-2 [52]					Unclear various organs defects
		UCD-200 UCD-206 [54]					Chicken model with poor background
		TβRIIΔk and TBRI^{CA} [56, 57]					Absence of autoimmunity
	Genetic modification	Caveolin 1$^{-/-}$ [61]					Not well characterized
		Fra-2$^{+/-}$ [61]					Not well characterized
		Fli1$^{\Delta CAT/\Delta CTA}$ [58]					Not well characterized/ absence of autoimmunity
		Fli1 endothelial cell KO [58]					Not well characterized/ absence of autoimmunity
Inducible models		Bleomycin induced model [65]					Overestimation of drug effects
		ROS-induced model [62]					TGF-β independent model
		Topo I/CFA's adjuvant induced SSc [61]					Unclear function of anti-topoisomerase I antibody
		Angiotensin II induced SSc [61]					Poor links with human SSc
		Scleroderma tous GVHD [61]					Not well characterized

FIGURE 2

Genetic and inducible systemic sclerosis (SSc) experimental models [46,52,54,56-58,61,62,65]

Dark grey: symptom similar to human SSc; Light grey: symptom partially mimicking human SSc; White: no evidences of symptom similar to SSc; CFA: complete Freund's adjuvant; GVHD: graft versus host disease; Fra: fos related antigen, Fli1: friendleukemia integration 1, KO: knock out; ROS: reactive oxygen species; SSc: systemic sclerosis; TGF-β: transforming growth factor-β; Topo-1: topoisomerase 1; Tsk: tight skin, UCD: University of California at Davis.

combined pulmonary fibrosis and emphysema (CPFE) in SSc [51]. Another animal model, the Tsk-2 mouse model occurs after administration of ethyl-nitrosourea, a mutagenic agent [52]. This model is associated with systemic inflammation. However, these two mouse models lack typical vascular phenotype and combine diverse organ involvements unclearly related to SSc [53].

UCD-200 and UCD-206 chicken models closely reproduce the human disease with vasculopathy characterized by Raynaud's phenomenon or ischemic lesions on toes, skin fibrosis with activated fibroblasts, inflammation and autoantibodies. Nevertheless, the absence of molecular background in non-mammalian, particularly in chicken, drastically limits the development of researches on this model [54,55].

As TGF-β has been identified as a profibrotic mediator in human SSc and an activator of fibroblasts, two mouse models are focused on TGF-β pathways: TBRI^{CA} (Cre-ER) mice and TβRIIΔk mice. In the first case, under the control of tamoxifen, the active

form of TFG-β is overexpressed after birth [56]. Upon this increased secretion of active TGF-β, mice develop progressive and generalized skin fibrosis with activation of fibroblasts and vasculopathy in small arteries in lungs, kidneys and several organs also affected in human SSc. Unexpectedly, histological analysis of TBRI^{CA} mice differ from human SSc lesion, which limits the understanding of TGF-β pathways dysregulation in SSc patients. TβRIIΔk construct encodes for the cellular and transmembrane domains of human TGF-βRII, specifically in fibroblasts. Increased expression of this receptor leads to fibroblasts activation and stimulates ECM protein secretion. Interestingly, this construct affects mice only 6 weeks after birth and reproduces human dermal and pulmonary fibrosis [57].

Fli1$^{\Delta CTA/\Delta CAT}$ and Fli1 endothelial cell knock out (ECKO) are 2 recently described genetic models implicating the same transcription factor identified in Friend virus-induced erythroleukemia model in mice [58]. This protein is known to be epigenetically down-modulated in skin lesion of patients with SSc [59,60]. In C57BL/6 mice, an ECKO or a specific deletion of a potent transcriptional repressor through its carboxy-terminal activation (CTA) domain leads to significant up regulation of collagen synthesis in mouse skin. However, if mouse skin presents ultrastructure with collagen fibrils similar to human dermal lesion in SSc and vasculopathy, skin thickness is comparable with that of *wt* mice. These results suggest a potential role of Fli1 in the genetic predisposition to SSc. However, the absence of autoimmunity and systemic inflammation represent true limits for these models [61].

An interesting new model of SSc has also been developed using reactive oxygen species (ROS) in BALB/c strain [62]. Subcutaneous injections of a ROS solution, containing HOCl, O^-_2, $ONOO^-$ and/or OH^-, everyday for 6 weeks leads to the development of skin fibrosis but also systemic reaction with lung fibrosis and renal injury. Interestingly, exposure to these oxidative molecules triggers a systemic autoimmune response characterized by the detection of anti-topoisomerase I and anti-centromere antibodies in ROS-treated mice. This autoimmune compound is not obtained in severe combined immunodeficiency (SCID) mice, which present only skin fibrosis after ROS treatment. Moreover, sera of *wt* mice treated with ROS solution could induce H_2O_2 production by mouse EC, similarly to what we observe when human EC exposed to SSc patient's serum [63]. Recent study in ROS induced SSc have also shown an increase of splenic T cells with higher production of IL-4 and IL-13, two cytokines implicated in human SSc [64]. Thus, TGF-β independent model suggests a possible new role of ROS in the oxidation of self-antigens that could lead to auto-immune response against modified self-antigens and particularly, topoisomerase 1 and centromere antigens.

Finally, bleomycin-induced dermal fibrosis model is the widest used model in of SSc. Pro-fibrotic effects of bleomycin have been known for a long time since high doses of bleomycin instillation during cancer treatments frequently promote lung injury and pulmonary fibrosis [65]. Subcutaneous high doses of bleomycin typically induced localized dermal fibrosis and also induced systemic manifestation like lung injury and pulmonary fibrosis and inflammatory changes in skin. Few studies report activation of fibroblasts and SSc-like phenotypes characterized by the presence of autoantibodies, increased ECM secretion and systemic inflammation [66,67]. Easy handling model, bleomycin-induced dermal fibrosis remains nevertheless an artificial model in which anti-inflammatory drug effects are frequently overestimated as compared to results obtained in SSc patients [68].

Fra-2, caveolin 1$^{-/-}$, sclerodermatous GVHD mice models are also available and are characterized by SSc-like symptoms. However, in these models skin fibrosis is dominant, whereas the autoimmune reaction is limited [61].

Oxidative stress

As suggested in the experimental model chapter, oxidative stress plays a major role in the pathogenesis of SSc, and data have accumulated over the past 15 years to document it. Thus, ischemic phenomena leading to superoxide anions production do occur in SSc patients [69]. Interestingly, silica, an environmental agent responsible for the occurrence of SSc, is responsible for the induction of oxidative stress and NF-κB pathways in lungs of a luciferase reporter mouse model of respiratory insufficiency [70]. Indirect markers of ROS involvement have also been reported in sera from patients with SSc, such as oxidative proteins and lipid peroxydation [71,72]. Finally, monocytes and fibroblasts isolated from patients with SSc show an increased synthesis of superoxide anions [73] that could directly increase fibroblasts proliferation and ECM production [63].

More recently, exposure of EC and fibroblasts to sera from patients with SSc has been shown to induce a higher production of H_2O_2 by both HUVEC, an EC line, and the two fibroblast cell lines HEP-2 and NIH 3T3, than the exposure to sera from healthy controls [74]. Interestingly, clinical status of patients correlated with impact of the serum on ROS production by EC and fibroblast. This production of ROS is combined with an increased proliferation of fibroblasts. The deleterious effects of SSc patients' sera could be reversed by N-acetyl cysteine and reduced glutathion for H_2O_2 production by EC, by bosentan for NO production by EC and D-penicillamine for fibroblasts proliferation.

This amplification of ROS production has also been linked to Ras protein and ERK1/2 activation pathways in patients with SSc, in synergy with platelet derived growth factor (PDGF) excess in the serum of patients [75]. A direct action of ROS on DNA has also been suggested since urine levels of 8-hydroxy-2′-deoxyguanosine, a marker of endogenous oxidative damage of DNA,

have been found higher in patients with SSc than in controls [76].

Thus, modifications of ROS targets, and particularly topisomerase 1 and centromere proteins, two targets of SSc antibodies, have been proposed to contribute to the dysregulations observed in SSc [62]. Moreover, autoantibody binding seems to be dependent on specific metal deposits and ROS production in patients with SSc [77].

Endothelial cell activation

In patients with SSc, arterial and arteriolar hyperreactivity and myointimal proliferation can lead to vascular occlusion occurring in digital arteries, as well as in heart, lung and kidney arteries. The equilibrium of vascular endothelium in physiological conditions results from the balance between vasodilator and vasoconstrictor molecules, as well as the expression of adhesion molecules at the surface of EC. In patients with SSc, the vascular tone is dysregulated, leading to perturbed interactions between EC, vascular smooth muscle cells (VSMC) and ECM components participating in vascular remodeling and occlusion [78].

In patients with SSc, EC lesions are associated with increased plasma levels of factor VIII, decreased angiotensin converting enzyme production, increased activity of von Willebrand factor (vWf) and detection of circulating platelet aggregates [79]. At an early stage of the disease, EC involvement in SSc is characterized by increased apoptosis, loss of physiological barrier with permeabilisation of blood vessels and abnormal vascular tone regulation [80].

It has been proposed that EC activation and proliferation might occur as a consequence of the binding of anti-endothelial cell antibodies (AECA). Thus, in patients with SSc, AECA have been reported to induce EC apoptosis through antibody dependent cell cytotoxicity, involving NK cells and Fas/FasL pathway [81]. However, although of interest, these *in vitro* data do not preclude the pathogenic role of these antibodies *in vivo*. In addition, AECA are not specific for SSc and can be found in various auto-immune diseases, binding to a relatively large number of EC antigens. Finally, AECA failed to be identified in a number of experimental models of SSc including the Tsk-1 mouse model. In the same line, the prevalence of antiphospholipid syndrome is very low in SSc, although anti-cardiolopin antibodies and increased vWf activity have been associated with the detection of PAH, suggesting that anti-cardiolipin antibodies could represent a marker of EC damage in PAH associated with SSc [83].

Other mechanisms are involved in the perturbation of the vascular tone in patients with SSc. Thus, increased endothelin-1 (ET-1) levels, which are known to enhance vasoconstriction, inflammation, and mediate fibrosis and vascular remodeling, have been detected in the serum of SSc patients [82]. ET-1 is known to also activate fibroblasts and induce *in vitro* ECM production [83]. In patients with SSc, ET-1 levels have been shown to correlate with the severity of PAH. Moreover, ET-1 has been found to be increased in the lung at the mRNA and protein levels in patients with idiopathic PAH as compared to healthy controls. In kidney biopsies from SSc patients who experienced scleroderma renal crisis, immunohistochemistry staining demonstrated the presence of ET-1 in glomeruli, arterioles and arcuate arteries, which was not the case in other vascular diseases involving the kidney [84]. Interestingly, ET-1 receptors (ETR) inhibitors, including bosentan, ambrisentan and more recently macitentan, have demonstrated efficacy in the treatment of idiopathic PAH and PAH associated with SSc [85]. In addition, defective prostacyclin synthesis and perturbed NO synthesis have also been reported to a possible key elements to explain EC dysfunction in patients with SSc [86].

Finally, vascular epidermal growth factor (VEGF) levels have been reported to be decreased in patients with SSc as compared to healthy control [87]. Increased synthesis of MCP-1 and VCAM-1 by EC could also increase the recruitment of lymphocytes and promote the local and systemic inflammation in patients with SSc [88].

Fibroblasts involvement and extra-cellular matrix synthesis

Collagen accumulation in the dermis is one of the hallmarks of SSc. Extra-cellular matrix (ECM) accumulation is mostly the consequence of fibroblasts activation. *In vitro*, fibroblasts isolated from involved skin of patient with SSc produce more collagen IV, proteoglycan and fibronectin than those obtained from the skin of healthy controls. Phenotypical changes of these fibroblasts have also been described, with the expression of alpha-smooth muscle actin (α-SMA), a marker of differentiation into myofibroblast [89]. These myofibroblasts are known to secrete more collagen and display VSMC properties including migration phenotype. Distinctly from EC, which experience increased apoptosis, fibroblasts isolated from SSc patients have a defect in Fas/FasL induced apoptosis pathway which probably participates to the maintenance of the disease process [90]. Interestingly, a defect in metalloproteinase synthesis, which regulates formation of ECM, has also been reported [91].

In addition to intrinsic phenotype modifications of fibroblasts, extrinsic factors also contribute to explain excessive fibroblast activation in patients with SSc.

Transforming growth factor β (TGF-β) has been proposed as an utmost important factor in fibroblast activation. Thus, fibroblasts phenotype modification in patients with SSc could be easily reproduced with incubation of normal fibroblasts with TGF-β [92]. As in TGF-β-dependent experimental models, in patients with SSc, the expression of TGF-β receptors at the fibroblast surface has been found increased as compared to healthy control fibroblasts [93]. This result, together with the

observation of increased levels of endogenous TGF-β in lung and dermal SSc lesions suggests an over stimulation of this pathway, leading to fibroblast phenotypical modifications including over proliferation, ECM production and differentiation into myofibroblasts. In addition, the expression of endogenous inhibitors of TGF-β pathway, including Fli-1 and SMAD7, is also defective in SSc patient's fibroblasts [94,95]. However, the persistence of phenotypical modifications of fibroblasts from patients with SSc despite multiple passages, *in vitro*, and recent updates on epigenetic and DNA methylation mechanisms, suggest the existence of other mechanisms independent of TGF-β stimulation [96].

In addition to TGF-β, IL-4, a cytokine secreted by T helper 2 (Th2) cells has been identified as a key cytokine in SSc. IL-4 express pro-fibrotic properties on fibroblasts and increased IL-4 production has been documented in the spleen of ROS-induced SSc mouse model as well as in the serum of patients with SSc [97]. Interestingly, in bronchoalveolar fluid of SSc patients with interstitial lung disease, Luzina et al. have observed activated $CD8^+$ T lymphocytes that present an activated phenotype and expression of mRNA encoding IL-4 and latent TGF-β mRNA [98]. Another important local source of IL-4 could also be a subset of double positive CD4/CD8 T lymphocytes, present in dermal lesions of patients with SSc [99]. In addition to IL-4, other growth factors have been identified in patients with SSc, including connective tissue growth factor (CTGF) or PDGF [100]. Alternatively to these growth factors, autoantibodies may activate fibroblasts. Anti-fibroblast antibodies (AFA) are not specific to patients with SSc and could lead to fibroblasts activation by direct targeting surface adhesion molecules. Two studies notably reported that exposure of normal fibroblasts to AFA leads to the internalization of the antibody that could interact with caveolin pathway and induce the secretion of pro-fibrotic chemokines including CCL2, CXCL1, CXCL8, CKLF and ECGF1 [101,102].

AFA directed at the PDGF receptor have been detected in patients with SSc. These autoantibodies have been reported to enhance *in vitro* the activation and pro-fibrotic phenotype of isolated fibroblasts [75,101]. Unfortunately, these results remain controversial [103].

PDGF can also be targeted by an inhibitor of the PDGF receptor, imatinib, a serine-protein kinase receptor inhibitor, that has been developed in the treatment of chronic myeloid leukemia [104,105]. Imatinib binds to c-Abl, a signaling protein downstream to TGF-β receptor, and blocks both TGF-β receptor and PDGF receptor. Because of promising results in the bleomycin induced model of SSc [106], clinical trials investigated the efficacy of imatinib in patient with SSc [107,108]. However, the results were disappointing in terms of tolerance, with a large proportion of dropouts and poor efficacy [109].

Novel therapeutic strategies have been proposed in experimental models of SSc and fibroblasts from patients with SSc in culture including inhibitors of SRC kinase family, Rho associated kinases, Fos related antigen-2 (Fra2) or histone acetylases and DNA methyl transferases [110,111]. Several of these strategies will be investigated in the treatment of patients with SSc in the near future.

Immune system activation

In addition to EC and fibroblasts, the immune system plays an important role in the pathogenesis of SSC. Thus, autoantibodies, imbalance in B and T lymphocytes subpopulations and perturbations of dendritic cells have been reported in SSc. Although the disease cannot be qualified as autoimmune, since autoantibodies have not been shown to induce/transfer the disease, the identification of specific autoantibodies is of major help in the diagnosis and/or the evaluation of the prognosis of SSc [112].

The distribution of autoantibodies in SSc patients correlates with phenotypes. Thus, anti-topoisomerase 1, anti-RNA polymerase III and anti-U3 RNP correlate with dSSc, while anti-centromere, anti-Pm/Scl, anti-Th/To and anti-U1 RNP are associated with lSSc. Various other antibodies have also been found in SSc patients, directed against several targets such as fibrillin-1, metalloproteinases or PDGF receptor. These autoantibodies argue for a pathogenic role of B cells [113]. Thus, circulating B cells from SSc patients differ in their phenotype as compared to healthy controls, with increased proportions of naïve B cells and decreased numbers of memory B cells and plasma cells [114]. CD19 and CD21, two activation co-receptors of the B-cell receptor (BCR), are overexpressed in these naïve and memory B cells. The activation receptors CD80, CD86 and CD95 are upregulated on memory B cells, suggesting their participation to the pathogenetic process.

High levels of B cell activating factor (BAFF) have been measured in the serum of patients with SSc, together with an overexpression of BAFF-R at the surface of peripheral B cells from SSc patients [115]. BAFF activates the NF-κB pathway, promotes B cells survival and participates to the differentiation of autoreactive B cells. In addition, it has been documented that B cells infiltrate the dermis of SSc patients [116,117], that circulating levels of IL-6 are increased in SSc patients as compared to healthy controls and correlated with the extent of skin fibrosis [118]. In Tsk-1 mouse, the depletion of B cells leads to a decrease of total IL-6 mRNA and improvement of fibrotic lesions [119]. Finally, IL-6 is known to stimulate collagen secretion by fibroblasts and represent a potential ''B cell link'' to fibroblast activation.

T cells are also involved in the pathogenesis of SSc. Thus, a Th-2 bias has been documented in SSc patients [120]. Increased levels of IL-4 and IL-13 have been measured in the serum of SSc patient. These two cytokines have been classically described as synergic Th-2 polarization cytokines via STAT6. Although the trigger contributing to the initiation of secretion of these

cytokines is not identified, it has been documented that mature and activated Th-2 secrete IL-4, IL-6 and IL-13, enhancing a positive loop of Th-2 polarization and B cells stimulation [121]. Interestingly enough, the treatment of Tsk-1 mice with an anti-IL-4 monoclonal antibody prevented the induction of skin lesions [122], underlining the major role this cytokine in SSc physiopathology.

Taken together, targeting B cells (anti-CD20), IL-6, IL-4 and also T cells and mature lymphocytes (anti-CD52) may lead to substantial clinical improvement in mouse models and in open series of patient with SSc [119,122–124]. These findings argue for the important role of B and T cells in the pathogenesis of SSc.

In addition to B and T lymphocytes, dendritic cells appear of utmost importance in SSc. Thus, a recent proteome-wide analysis of the culture supernatant of plasmacytoid dendritic cells (pDC) isolated from patients with SSc allowed identification of increased levels of CXCL4, with higher levels in diffuse than in limited forms, and increased levels in early diffuse vs. late diffuse SSc patients [125]. Interestingly, CXCL4 was also detected in the skin of SSc patients and not in healthy control skin. Serum levels of CXCL4 correlated with clinical status of SSc patients. Notably, in patients with lung fibrosis and/or PAH, high levels of CXCL4 correlated with a poor prognosis. Finally, infusion of CXCL4 leads to increased leukocytes infiltration, skin thickening and CCL2 mRNA expression in the bleomycin-induced model of SSc. Taken together, these observations argue for a possible role of CXCL4 in the pathogenesis of SSc. Further studies will aim at confirm the interest of targeting CXCL4 in SSc patients.

Conclusion

Major work has been done in order to improve the understanding of SSc pathogenesis. A number of new experimental models have been set up, that should help to test new therapeutic targets. Although a large number of genetic studies have been conducted, it is still difficult to identify a scleroderma specific genetic background, and the major progress in this setting is probably the identification of an interferon signature, as reported in SLE and Sjogren's syndrome. Besides EC and fibroblasts, B cells and autoantibodies actually probably represent major actors in the pathogenesis of SSc. Finally, the role of dendritic cells and CXCL4 seem to be promising.

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Annexe 2

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Review

Implication of B lymphocytes in the pathogenesis of ANCA-associated vasculitides

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ABSTRACT

ANCA-associated vasculitis (AAV) is a subgroup of vasculitides characterized by the detection of anti-neutrophil cytoplasm antibodies (ANCA). Until recently, the pathogenesis of AAV mainly involved neutrophils, T cells, and ANCA. Importantly, data were recently published supporting B-cell implication in this setting. Thus, the identification of activated B lymphocytes in granulomatous lesions and the efficacy of B-cell depletion using rituximab in the treatment of patients with AAV changed our mind. However, the impact of B lymphocytes on disease activity and its specific role in the pathogenesis of AAV remains unclear, at least in part as the consequence of the limited number of patients investigated and the restricted number of studies investigating B-cell subsets. Perturbations of B-cell homeostasis have been identified in AAV with increased expression of CD38 and decreased expression of CD5 in active phase, contrasting with increased expression of CD25 and CD86 in remission state. Although decreased secretion of interleukin (IL)-10 has also been reported during disease flares, these data remain controversial and the cytokines secretion profile of B-cells needs to be further investigated. Herein, we summarize recent advances in the understanding of the implications of B-cells in the field of AAV and propose new fields of investigation for a better understanding of B lymphocytes in the pathogenesis of AAV.

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Contents

1. Introduction . . . 997
2. Methods . . . 997
3. General aspects on B-cells . . . 997
4. ANCA production by B-cells . . . 997
5. *In situ* properties of B-cells in AAV . . . 998
6. Circulating B-cell subsets in AAV . . . 999
   6.1. Classical B-cell subsets . . . 999
   6.2. Autoreactive B-cells . . . 999
   6.3. Regulatory B-cells . . . 1000
   6.4. Proinflammatory B-cells . . . 1000
7. Present and future treatments targeting B-cells . . . 1000
   7.1. Cyclophosphamide . . . 1000
   7.2. Rituximab . . . 1000

Abbreviations: Aa, amino acid; AAV, ANCA-associated vasculitides; ANCA, anti-neutrophil cytoplasm antibodies; BAFF, B-cell activated factor; BAFF-R, BAFF receptor; BCR, B-cell receptor; c-ANCA, cytoplasmic ANCA; CLL, chronic lymphocytic leukemia; CTLA-4, cytotoxic T lymphocyte associated protein 4; EGPA, eosinophilic granulomatosis with polyangiitis; ENT, ear nose and throat; GPA, granulomatosis with polyangiitis; IL, interleukin; INF, interferon; IVIG, intravenous immunoglobulin; MPA, microscopic polyangiitis; MPO, myeloperoxidase; p-ANCA, perinuclear ANCA; PR3, proteinase 3; SLE, systemic lupus erythematosus; TACI, transmembrane activator and CAML interactor; TLR, toll-like receptor; TLS, tertiary lymphoid structures; TNF-α, tumor necrosis factor alpha.

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7.3. Other B-cell targeting therapy . . . 1001
7.3.1. TNF-α blocking agents . . . 1001
7.3.2. Belimumab . . . 1001
7.3.3. Tocilizumab . . . 1001
7.3.4. Intravenous immunoglobulin (IVIg) . . . 1001
8. Conclusions . . . 1001
Take-home messages . . . 1001
Conflicts of interest . . . 1001
Acknowledgements . . . 1001
References . . . 1002

1. Introduction

Primary vasculitides constitute a heterogeneous group of diseases characterized by inflammation of blood vessels [1]. These rare diseases are characterized by distinct pathophysiological mechanisms and involve blood vessels of different sizes, resulting in a wide spectrum of clinical symptoms [1–4]. Anti-neutrophil cytoplasm antibodies (ANCA)-associated vasculitides (AAV) are pauci-immune necrotizing vasculitides involving small-sized vessels which include granulomatosis with polyangiitis (GPA) (formerly named Wegener's granulomatosis), eosinophilic granulomatosis with polyangiitis (EGPA) (formerly named Churg–Strauss syndrome), and microscopic polyangiitis (MPA) [5]. The immunofluorescence pattern of ANCA distinguishes cytoplasmic ANCA (c-ANCA), targeting proteinase 3 (PR3) in most cases, and perinuclear ANCA (p-ANCA), usually targeting myeloperoxidase (MPO). Inflammation of small-sized vessels is responsible for organ damage with granulomatous involvement of upper and lower respiratory tract in GPA and EGPA [6], and glomerulonephritis, intra-alveolar hemorrhage, and/or peripheral neuropathy in GPA, MPA, and EGPA [7].

Most of the research that has been conducted on AAV has focused on the production, secretion, and pathogenicity of ANCA and its impact on relapses [8]. Besides the pathogenic role of ANCA, previous studies have investigated the role of genetic predisposition [9], silica exposure [10] or autoimmune overlaps [11] and have demonstrated the implication of T cells in AAV, showing a predominant Th1 polarization in GPA contrasting with a predominant Th2 polarization in EGPA [12,13]. Only few studies were dedicated to analyze the potential role of B-cells in AAV. One of the first reports on the implication of B-cells in AAV focused on the analysis of B-cell infiltrates proximal to PR3-positive cells and plasma cells within endonasal inflammatory lesions of patients with GPA [14]. Within these lesions, Voswinkel et al. reported on the activation and maturation of aggregated B-cells [14,15], arguing for a key role of B lymphocytes in the pathogenesis of AAV. This concept was reinforced by the results of two prospective randomized trials demonstrating the efficacy of rituximab, an anti-CD20 monoclonal antibody, in the induction of remission in patients with GPA or MPA [16–18], and more recently for the maintenance of remission in the same diseases, with the superiority of rituximab compared to azathioprine [19].

The purposes of this systematic review are to summarize data supporting the role of B-cells in the pathogenesis of AAV and to explore new fields of investigation for the targeting of B-cells in the treatment of AAV.

2. Methods

Original and review articles in English or French with an abstract in the English language published from 1950 to January 15, 2015, were identified by completion of electronic database searching (Medline, ClinicalTrials.gov, Web of Science). Keyword combinations used included one of the following: anti-proteinase 3, anti-myeloperoxidase, ANCA-associated vasculitides, granulomatosis with polyangiitis, microscopic polyangiitis, eosinophilic granulomatosis with polyangiitis, together with one of the following: B lymphocytes, B-cells, regulatory B-cells, autoreactive B-cells, inflammatory B-cells, memory B-cells, naive B-cells, B-cells subsets, pathogenesis, plasmocytes, rituximab, anti-CD20, resulting in 408 unique references, including 357 original and 51 review articles.

3. General aspects on B-cells

B-cells derive from lymphoid precursors and are characterized by the membrane expression of the B-cell receptor (BCR) [20]. The BCR is composed of two identical heavy chains of immunoglobulins (Ig), which can be distinguished into five different isotypes (α, β, δ, γ, or μ), and two identical light chains (κ or λ), associated with co-receptors including CD19, CD21, CD79a, and CD79b. The heavy and light chains of Ig of the BCR form a tetramer connected by intra- and inter-disulfide bonds [21]. Specific Ig isotypes reflect the stage of differentiation of B-cells. Naive B-cells are characterized by high expression of IgM and IgD, whereas marginal zone B-cells (MZ B-cells) are characterized by high expression of IgM and low expression of IgD on the surface of $CD19^+$ B-cells [22]. BCR complex, in association with its co-receptors, recognizes the antigen by its tridimensional structure and promotes B-cell activation and differentiation into plasmablasts and plasma cells producing high-affinity antibodies (Fig. 1).

In mice, B-cells can be divided into two different subsets, including B1 and B2 cells [23]. B1 cells produce low affinity and T-independent polyreactive antibodies mainly directed against viruses and bacteria. These cells are restricted to pleural and abdominal cavities and excluded from secondary lymphoid organs (spleen and lymph nodes). These cells represent the first line of defense in mice by secreting high levels of IgM and low levels of IgG [24]. Recently, evidences of B1 cells exerting a protective effect in various inflammatory and/or autoimmune diseases, including atherosclerosis and multiple sclerosis, have been identified in humans [25,26]. In contrast, B2 cells require the presence of T cell signals to promote their activation and differentiation into antibody-producing cells, by inducing immunoglobulin class switching, acquisition of memory markers such as CD27, and antibody secretion [27,28]. These cells are predominantly found within secondary lymphoid organs and secrete high-affinity antibodies and are identified as MZ B-cells when they are found within the marginal zone of secondary lymphoid organs. However, the classification into B1 and B2 cells based on the expression of CD20, CD27, and CD43 is still controversial in humans [29,30].

4. ANCA production by B-cells

Detection and characterization of ANCA strongly support the implication of B-cells in the pathophysiology of AAV [31]. The prevalence of ANCA among patients with AAV differs according to the type of vasculitis. In GPA, PR3-ANCA are detected in 85% of patients while MPO-ANCA are identified in less than 10% of cases [12,32]. MPO-ANCA are detected in 60% of patients with MPA and in 31–38% of patients with EGPA [12,33,34].

In vitro studies demonstrated the induction of a respiratory burst in TNF-α primed neutrophils and monocytes after incubation with both

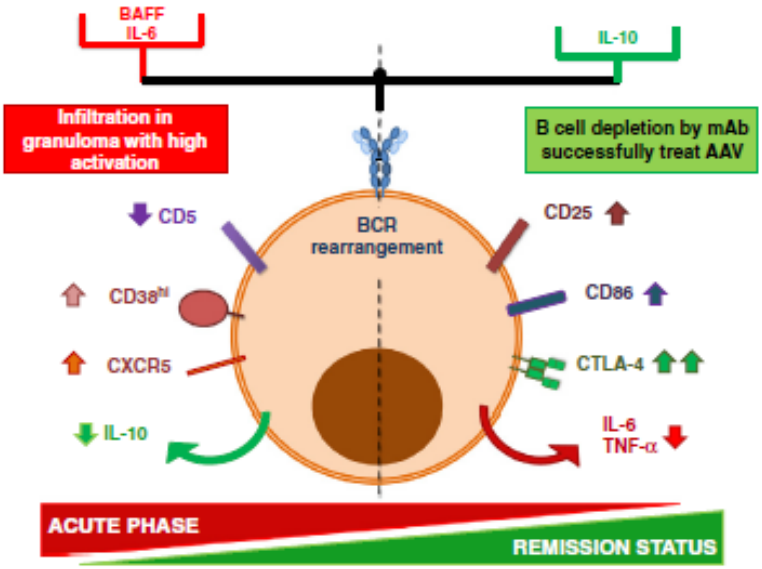

Fig. 1. General aspect of B lymphocytes. Schematic representation of main maturation steps of B-cell maturation. CD: cluster of differentiation; Ig: immunoglobulin; IL: interleukin; MHC: major histocompatibility complex; MZ B-cells: marginal zone B-cells; NFAT: nuclear factor of activated T cells; NF-κB: nuclear factor kappaB; TGF-β: transforming growth factor beta.

anti-PR3 and anti-MPO antibodies [35,36]. Sophisticated "under flow" model also demonstrated the importance of ANCA in adhesion stabilization and transmigration of neutrophils [37]. The pathogenic role of MPO-ANCA *in vivo* has been demonstrated in 2002 by Xiao and colleagues who have set up an experimental model of MPO-ANCA-associated vasculitis [38]. They used MPO knock-out mice that they immunized with murine MPO to induce the production of anti-MPO antibodies and then transferred purified splenocytes or purified IgG from these mice to Rag2$^{-/-}$ or wild-type mice. Transfer of splenocytes or purified IgG triggered the development of glomerulonephritis and pulmonary capillaritis after 6 days in transferred mice [38]. These experiments allowed to demonstrate that MPO-ANCA were pathogenic *in vivo* in mice. In humans, the pathogenic role of MPO-ANCA was also demonstrated since the materno-fetal transfer of MPO-ANCA was reported to induce pneumo-renal syndrome related to vasculitis in the newborn in individual cases [39].

If the pathogenesis of MPO-ANCA has been clearly demonstrated *in vitro* and *in vivo*, this is not the case for PR3-ANCA. Thus, MPO-ANCA were demonstrated to activate neutrophils *in vitro*, similarly as MPO-ANCA [40], but their direct pathogenic effect *in vivo* has not been definitively established to date. A recent model of humanized severe combined immunodeficiency (SCID) mice suggested that transfer of human PR3-ANCA could induce vasculitis [41]. In this model, chimeric mice were generated by injecting hematopoietic stem cells into irradiated NOD-SCID-interleukin (IL)-2R$^{-/-}$ mice. Matched chimera mice were then treated with human IgG from patients with PR3-ANCA and vasculitis, from patients with non-vasculitic renal disease or from healthy controls. PR3-ANCA-treated mice showed mild pauci-immune proliferative glomerulonephritis with human and mouse leukocytes infiltration, contrasting with the absence of glomerular changes in healthy controls. No granulomatous formation was observed in this model. Even if these results represent one of the first evidence that PR3-ANCA could exert pathogenic effect, it needs to be confirmed in other studies [42].

IgG subclasses are all represented in PR3-ANCA and MPO-ANCA in AAV patients [7], without any disease specificity or any correlation with disease activity. Interestingly, ANCA epitope specificity might be associated with distinct pathogenic effect. Thus, ANCA specific epitopes were recently identified for MPO-ANCA in patients with AAV [43]. Roth et al. identified a specific epitope (amino acid 447–459) targeted by MPO-ANCA that was detected in about 50% of patients and was exclusively associated with active AAV [44]. These antibodies, when specific for MPO$^{447-459}$, were able to activate neutrophils and triggered the release of reactive oxygen species *in vitro* and to induce nephritis when injected to mice *in vivo* [44].

5. *In situ* properties of B-cells in AAV

In other systemic autoimmune diseases, such as rheumatoid arthritis and Sjögren's syndrome, target tissue infiltration by B-cells correlates with disease activity and progression [45,46]. In GPA patients, B-cells infiltrating granulomatous lesions might be activated. Voswinkel and colleagues performed the first BCR sequencing in biopsies of endonasal lesions of active GPA patients [15]. They and others suggested an *in situ* activation and selection of B-cells in contact with PR3 at the neutrophils surface. In another study, a correlation was found between the importance of B-cell infiltration within granulomatous lesions in GPA and the occurrence of relapses, despite peripheral B-cell depletion in few patients treated with rituximab [47]. Interestingly, in a cohort of patients with AAV, including GPA and MPA patients, tertiary lymphoid structures (TLS) have been identified in involved tissues [48]. TLS were identified in glomerulonephritis lesions, composed of a germinal center containing follicular dendritic cells and memory and non-memory B-cells at the periphery of germinal center, in contact with T cells. Interestingly, highly proliferative B-cells have been found in the center of TLS, providing an important source of antigen-presenting cells favoring T cells maturation at the periphery. Nevertheless, no evidence has been obtained for antibody secretion or maturation of antibody-producing cells in renal lesions of patients with AAV. These results suggest an antibody-independent pathological role of B-cells *in situ*.

CXCL13, a B-cell specific chemokine, was detected in TLS, and CXCR5, the CXCL5 receptor, was expressed by infiltrating B lymphocytes [49]. Similar results were found regarding CCL21, expressed by perivascular stromal cells, and CCR7, expressed by B and T lymphocytes infiltrating ectopic lymphoid structure [49]. Zhao et al. [50] also investigated the expression of CCR10 at the surface of circulating and tissue infiltrating B-cells. However, they found no difference of expression,

suggesting a chemokine specificity mechanism for the migration of B-cells within nasal granulomatous lesions in GPA.

The same group reported on the detection of B-cell activating factor of the tumor necrosis factor (TNF) family (BAFF) in endonasal lesion of GPA patients [51]. These data are supported by the previous detection of increased levels of BAFF in the serum of active GPA patients as compared to healthy donors [52]. These results support the potential activation of B-cells both in peripheral blood and tissue lesions, possibly through BAFF-BAFF-R interaction resulting in increased survival and activation of B-cells and emergence of autoreactive B-cells [53,54]. Morevover, ANCA-activated neutrophils secrete increased amounts of BAFF *in vitro*, suggesting a potential crosstalk between neutrophils and B lymphocytes in patients [55]. Taken together, these data provide evidences for a key role of *in situ* infiltrating B-cells in the pathogenesis of AAV, in particular activation and maturation of potential autoreactive B-cell within tissue lesions.

6. Circulating B-cell subsets in AAV

Peripheral B-cell subpopulations have been analyzed in patients with GPA (summarized in Table 1). Besides classical B-cell subsets, data regarding the role new B-cell subsets are emerging.

6.1. Classical B-cell subsets

Moosig and colleagues failed to identify differences in absolute number and percentage of circulating B-cells between untreated GPA patients and healthy donors [56]. They also reported that B-cells from patients and healthy donors similarly express two co-stimulation receptors, i.e. CD80 and CD86 (B7-1 and B7-2). More recently, several groups reported changes in the distribution of circulating B-cell subsets in AAV patients when compared to healthy donors, together with different patterns of receptor expression and cytokine expression (Fig. 2) [56–62].

Eriksson and colleagues [57] reported on up-regulations of CD86 and CD25 at the B-cell surface in GPA patients with inactive disease under glucocorticoid treatment, these two markers having been proposed to characterize exhausted autoreactive B-cells [63,64]. In patients with active GPA, a significant increase of the expression of CD38 by B-cells has also been described in comparison to patients in remission and healthy controls [58]. In systemic lupus erythematous (SLE) patients, these cells were reported to express functional defects that probably promote disease progression [65], whereas in patients with B-cell chronic lymphocytic leukemia (CLL), CD38^{hi} B-cells express cell cycle modifications that are associated with poor prognosis [66]. In AAV patients, the role of these CD38^{hi} B lymphocytes is still unknown, and the mechanisms triggering the differentiation of mature B lymphocytes in autoreactive B lymphocytes are still unclear.

Steiner and colleagues [59] also reported on the cytotoxic T lymphocyte associated protein (CTLA)-4 (CD154) expression on T and B lymphocytes in GPA. This inhibitory receptor, binding to CD80 and CD86, was found to be expressed on peripheral B lymphocytes of 50% of patients with active disease, of all patients in remission state and not on B lymphocytes from healthy controls. Unfortunately, this report did not include functional studies or further investigations in AAV patients. However, it was reported in a recent study that CTLA-4 was expressed by B-cells in mouse thymus and that there was a difference in IgG/IgM balance in mice deficient for B-cells CTLA-4 [67].

6.2. Autoreactive B-cells

Traditionally, autoimmune disorders were divided in T lymphocytes and autoantibody mediated diseases. Autoantibody production has been reported for years in many diseases but also in healthy donors leading the scientific community to distinguish between natural and pathogenic autoantibodies [68]. As previously described, the pathogenic role of MPO-ANCA has been clearly identified in mouse models unlike PR3-ANCA. Autoreactive B-cells have been characterized as CD19^{hi} CD21^{low} CD38^{-} B-cells in patients with SLE, common variable immune deficiency with autoimmune cytopenia, and hepatitis C virus-related cryoglobulinemia vasculitis [69–71].

Culton et al. reported on a peripheral B-cell subset characterized by higher expression of CD19 and higher production of autoantibodies, suggesting that these cells could be autoreactive B-cells [72]. Interestingly, Toll-like receptor (TLR) 9 stimulation of circulating B-cells isolated from patients with AAV leads to a significant increase of ANCA secretion by B-cells from patients, particularly for PR3-ANCA-positive patients [73]. This study also suggests that infectious context could trigger the autoimmune process in AAV. These findings could support the presence of autoreactive B-cells in AAV that could be activated by TLR stimulation on the context of microbial infections.

Table 1
Overview of peripheral B lymphocyte phenotypes in granulomatosis with polyangiitis (GPA).

Total B-cells (% of PBMC)	Active (n)	Remission (n)	Healthy donor (n)	P-value
CD19^{+} [49]	10.66 (24)		12.9 (17)	ns
Activation markers (% within CD19^{+})				
CD6^{+} [103]	17^{a} (24)	26 (19)	28 (68)	***
CD25^{+} [50]	23 (18)	48^{a} (16)	29 (20)	**
CD38^{-} [53]	16.88 (21)	20.58 (16)	13.05 (12)	ns
CD38^{+} [53]	82.37 (21)	78.84 (46)	86.35 (12)	ns
CD38^{bright} [53]	0.82^{a} (21)	0.31 (16)	0.40 (12)	*
CD80^{+} (B7-1) [49]	7 (24)		2 (17)	ns
CD86^{+} (B7-2) [49]	8 (18)	31^{a} (16)	8 (20)	***
Inhibition marker (% within CD19^{+})				
CTLA-4^{+} (CD152) [56]	50% positive (9)	100% positive (8)	negative (19)	N.A.
Cytokine expression (% within CD19)				
IL-10^{+} [70]	4.7^{a} (11)	8.1 (30)	11.3 (17)	**
IL-10^{+} [71]	10.4 (6)	8.1 (15)	9.2 (18)	ns

P-values calculated using Mann-Whitney or ANOVA tests according to publications.
IL: interleukin; ns: not significant; N.A.: no application; PBMC: peripheral blood mononuclear cell;
^{a} Compared to healthy donor, patient population express significant differences.
* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

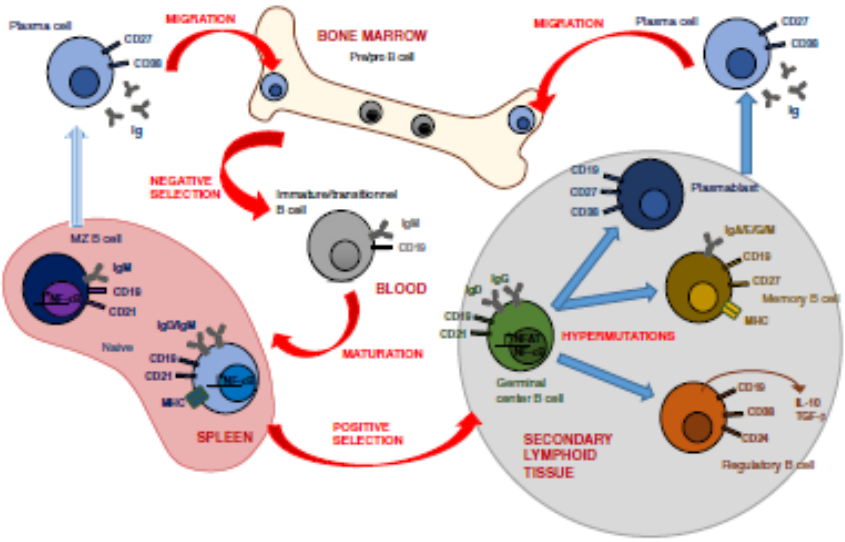

Fig. 2. B lymphocytes in ANCA-associated vasculitis: a double-edge sword. Schematic representation of phenotype present by B-cells in patients with ANCA-associated vasculitis during a disease flare (left) or in remission (right). BAFF: B-cell activating factor; BCR: B-cell receptor; CD: cluster of differentiation; CTLA-4: cytotoxic T lymphocyte associated protein 4; CXCR5: C-X-C chemokine receptor 5; IL: interleukin; mAb: monoclonal antibody; TNF-α: tumor necrosis factor alpha.

6.3. Regulatory B-cells

Regulatory B-cells (Bregs) have been identified for the first time in 1974 [74]. Regulatory B-cells are characterized by the production of IL-10 and the induction of energy or apoptosis of activated T cells [75]. Regulatory B-cells can also enhance the differentiation of regulatory T cells by cell-to-cell contact and TGF-β secretion [76]. Unfortunately, phenotypes of mouse and human Bregs are not fully defined. Blair et al. have reported the identification of $CD24^{hi}CD38^{hi}$ B-cells with regulatory functions and IL-10 production in SLE patients [77], and such $CD24^{hi}$ $CD38^{hi}$ B-cells have been also found in other inflammatory diseases. Interestingly, other markers have been also associated with Bregs phenotype, such as up-regulation of CD1d, CD5, or CD22 [78].

In AAV, increased serum levels of IL-10 were found, suggesting a potential role of Bregs in these patients [79]. However, Wilde et al. have described a decrease of IL-10-secreting B-cells in the peripheral blood of active GPA patients when compared to healthy controls [60]. This study has also shown an increase of IL-10-secreting B-cells in patients in remission phase, after glucocorticoid treatment. However, these results were not confirmed in another study in which the proportion of IL-10-producing B-cells was found to be similar in patients in remission compared to patient in active phase [61]. However, both studies demonstrated a decrease in the proportion of $CD24^{hi}CD38^{hi}$ B-cells in patients with active AAV compared to healthy controls, suggesting a decrease of classical Bregs in AAV patients. In contrast, no data are available on the production of TGF-β by B-cells in AAV patients.

6.4. Proinflammatory B-cells

In autoimmune conditions, B-cells have been found to produce IL-6, IL-21, and TNF-α, notably under the influence of type I interferon (IFN-α and IFN-β) or TLR ligands [80,81]. These cytokines exert deleterious effects within inflammatory lesions, especially in rheumatoid arthritis in which patients are dramatically improved with anti-TNF-α blocking agents. Interestingly, IL-6 and TNF-α have been reported to be increased in the serum of patients with GPA and MPA [82,83].

In AAV, TNF-α is known to increase the expression of PR3 at the surface of neutrophils [84]. Thus, Reumaux et al. reported on the increased binding of ANCA to PR3 expressed on neutrophil surface after priming with TNF-α [85]. Interestingly, activation by TNF-α also leads to activation of NADPH oxydase in neutrophils, suggesting a possible amplification of immune response against neutrophil and maintenance of inflammation in AAV patients [86]. Two open studies suggested a beneficial effect of infliximab, a chimeric monoclonal anti-TNF-α antibody in GPA patients refractory to cyclophosphamide [87,88]. However, this finding was not confirmed in a prospective randomized study using etanercept, a soluble dimerized TNF-α receptor, in the maintenance of remission [89].

B-cell depletion therapy using rituximab demonstrated a decrease of IL-6 and TNF-α serum levels in rituximab-treated patients compared to glucocorticoid-treated patients in AAV [90], suggesting that B-cells could be an important source of proinflammatory cytokines in AAV. However, evidence is lacking actually that these inflammatory cytokines are directly produced by B-cells.

7. Present and future treatments targeting B-cells

7.1. Cyclophosphamide

Cyclophosphamide, an alkylating agent, is one of the major anticancer treatments [91]. It is used as an immunosuppressive drug in patients with severe systemic autoimmune diseases [92–94]. Thus, cyclophosphamide represents the reference treatment for the induction treatment in patients with GPA [95] and has become the standard of care in association with glucocorticoids in AAV patients with severe disease. Although cyclophosphamide is known to deplete both B-cells and T cells in peripheral blood, depletion is more pronounced in B-cells than in T cells [96]. *In vitro* and *in vivo* experiments document the effect of cyclophosphamide on antibody production and secretion, in AAV and SLE, suggesting a possible impact on autoreactive B-cells and autoantibodies [97,98]. However, cyclophosphamide is responsible for a number of adverse events and alternative treatments are needed to induce remission in patients with AAV.

7.2. Rituximab

Anti-CD20 antibodies, particularly rituximab (Rituxan®, Mabthera®), were designed to target B-cells and treat B-cell lymphoproliferative disorders, particularly B-cell lymphomas and obtained an FDA agreement in

1997 in this indication [99]. Then, rituximab was tested in a number of autoimmune conditions such as immune thrombocytopenic purpura [100], cold agglutin disease [101], or thrombotic thrombocytopenic purpura [102]. Specks et al. reported in 2001 the first patient with GPA treated with rituximab in whom complete remission was documented at 1 year [103]. Limited numbers of patients with refractory MPA and EGPA were also successfully treated with rituximab in the middle of the 2000s decade [104,105]. Interestingly, the similar efficacy of rituximab and cyclophosphamide was documented in two prospective randomized trials, in the induction treatment of GPA and MPA [16–18], allowing to obtain an FDA approval in the treatment of GPA and MPA in 2011. More recently, the French Vasculitis Study Group demonstrated the superiority of rituximab over azathioprine in the maintenance treatment of patients with GPA and MPA [19].

In autoimmune diseases, rituximab treatment leads to rapid and complete depletion of naive B-cells and nearly all peripheral B-cell subsets [106]. Interestingly, an *in vitro* non-depleting study also demonstrated a better efficacy of rituximab on $CD27^-$ naive B-cells compared to $CD27^+$ memory B regarding B-cell activation, proliferation, and viability [107]. Few months after the first injection of rituximab, transitional B-cells repopulation is observed and plasma cells recirculate in the peripheral blood of treated patients [108,109]. Moreover, notably in rheumatoid arthritis patients, a high proportion of circulating memory B-cells is associated with increased risk of relapse in patients receiving rituximab [108,110]. In AAV, Bunch et al. suggested that after rituximab treatment, the number of $CD5^+$ B lymphocytes, a B-cell subset that seems to be poorly affected by anti-CD20 monoclonal antibody, could correlate with the risk of relapse and/or disease severity [62].

7.3. Other B-cell targeting therapy

7.3.1. TNF-α blocking agents

Ten years ago, infliximab, a humanized anti-TNF-α monoclonal antibody, was proposed in the treatment of patients with refractory AAV [88,111–113]. However, etanercept, a soluble TNF-α receptor combined with an IgG1 Fc portion, was evaluated in addition to classical treatment with cyclophosphamide for GPA patients and failed to prevent the occurrence of relapses at 12 months [114]. In addition, treatments with TNF-α blockers seem to be associated with increased risk of solid malignancy in patients with AAV [89].

7.3.2. Belimumab

Increased levels of BAFF, transmembrane activator and CAML interactor (TACI), and IL-21, three major cytokines which participate to B lymphocytes activation, have been found in the serum of patient with active GPA and MPA [52]. As previously indicated, BAFF seems to increase autoreactive B-cells selection and promote the activation and survival of pathogenic B-cells [53,54]. This pathogenic role of BAFF in AAV has been also suggested because of an increase secretion of BAFF by *in vitro* ANCA-stimulated neutrophils [55]. Belimumab, a humanized monoclonal antibody targeting BAFF, has been shown to improve patients with SLE with a good tolerance profile [115,116] and FDA approved belimumab in the treatment of active form of SLE in 2011. The BREVAS study was launched in 2012 to evaluate belimumab for the maintenance of remission in patients with AAV. However, the study will not be completed because of an inappropriate design.

7.3.3. Tocilizumab

Based on IL-6 overexpression in patients with AAV [117], the role of this cytokine was also suggested in the pathophysiology of AAV. Stimulation of monocytes by anti-PR3 leads to increased secretion of IL-6 and other proinflammatory cytokines [36]. Similar results were obtained with endothelial cells stimulated with antibodies from patients with GPA and MPA, leading to an increased secretion of IL-6 and E-selectin [82]. IL-6 is also involved in enhancing the maturation of B-cells [118], particularly, in the induction of antibody secretion by B-cells [119,120]. Tocilizumab, a humanized monoclonal antibody targeting the IL-6 receptor, recently obtained FDA approval for the treatment of rheumatoid arthritis [121]. Tocilizumab is also evaluated mainly in systemic vasculitis involving large vessels, including giant cell arteritis, Behçet's disease, or Takayasu arteritis. To our knowledge, Tocilizumab has not been tested in AAV.

7.3.4. Intravenous immunoglobulin (IVIg)

IVIg are preparations of normal human IgG obtained from pools of plasma from more than 10,000 healthy blood donors that are used in the treatment of a wide range of autoimmune and inflammatory diseases [122]. The evidence for a benefit of IVIg in patients with AAV initially came for small studies [123,124]. Then 34 patients with persistent disease activity were randomized to receive either a single course of IVIg or placebo in a double-blind trial. Fourteen of 17 patients in the IVIg group as compared with 6/17 patients who received placebo had a reduction in disease activity, but this effect was not sustained beyond 3 months [125]. IVIg have been proposed for the treatment of a first relapse of AAV in 22 patients. All patients initially responded to IVIg therapy and IVIg induced complete remissions of relapsed AAV in 13 of 22 patients at month 9 [126]. Recently, the potential benefits of IVIg in the treatment of patients with AAV and the maintenance of remission in these patients were highlighted [127]. Interestingly, IVIg is the only treatment available that allows controlling relapse and does not increase immunosuppression. Mechanisms of action of IVIg in patients with AAV include among others neutralization of ANCA through idiotypic mechanisms, modulation of Fcγ receptors expression and of neutrophil activation [128]. Importantly, IVIg is also able to modulate the activity, function, and differentiation of B-cells [129].

8. Conclusions

In the last years, increasing knowledge has been obtained on the role of B lymphocytes in the pathophysiology of AAV, boosted by the efficacy of B-cell-targeting therapies in the therapeutic management of AAV patients, both in induction and maintenance therapy. In addition to their role of "pathogenic ANCA-producing cells," B-cells may also produce immunomodulatory or proinflammatory cytokines that regulate immune responses, becoming key actors and a major target in AAV.

Take-home messages

- MPO-ANCA are pathogenic *in vitro* and *in vivo*, whereas the pathogenic role of PR3-ANCA *in vivo* remains controversial.
- Activated B-cells are present in granulomatous lesions form patients with AAV and are in close contact with neutrophils expressing PR3.
- Peripheral B-cells from AAV patients display higher expression of CD38, CTLA-4 and lower expression CD5 in active phase contrasting with increased CD25 and CD86 expression in remission.
- Clinical trials demonstrated the efficacy of rituximab, a B-cell depletion therapy, both in the induction and maintenance of remission in patients with GPA and MPA.
- Other B-cell targeting therapies are evaluated in animal models and humans.

Conflicts of interest

N.D, S.L, and J.L declare that they have no conflicts of interest concerning this article. B.T received honoria and consulting fees from Roche. L.M. received traveling grants and consulting fees from Roche.

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Résumé

Différents mécanismes de tolérance centraux et périphériques permettent la sélection négative des lymphocytes B auto-réactifs tout en préservant la sélection positive et la différenciation en plasmocytes producteurs d'anticorps de haute affinité. Ces mécanismes de tolérance sont altérés dans les pathologies auto-immunes et ces altérations conduisent à la production d'auto-anticorps. Ainsi, un ciblage thérapeutique des lymphocytes B autoréactifs, notamment avec les anticorps monoclonaux anti-CD20, donne des résultats prometteurs dans différentes pathologies auto-immunes. Si ces traitements ont démontré leur efficacité dans les vascularites associées aux anticorps anti-protéines cytoplasmiques des polynucléaires neutrophiles (ANCA) (VAA), d'autres maladies auto-immunes à composante vasculaire, dont la sclérodermie systémique (ScS) ou l'hypertension artérielle pulmonaire idiopathique (iHTAP), restent insensibles à ce ciblage. D'autre part, la caractérisation des sous-populations lymphocytaires B pathologiques porte essentiellement sur la détection des cellules exprimant ces auto-anticorps. Ce projet a ainsi eu pour objectif une caractérisation phénotypique et fonctionnelle comparative des sous-populations lymphocytaires B impliquées dans ces différentes pathologies auto-immunes vasculaires.

Les patients atteints de granulomatose avec polyangéite présentent une activation importante de l'immunité innée mais aussi une production augmentée d'IL6 par les lymphocytes B en lien avec une activation importante des lymphocytes T. Des modifications phénotypiques des lymphocytes B sont observées chez les patients atteints de VAA, notamment la polyangéite microscopique suggérant pour cette vascularite une composante auto-immune. Ainsi, le CD69, le CD95 et le récepteur à l'IL-6 permettent de discriminer les différentes formes de la maladie. Dans la ScS et plus particulièrement dans les formes les plus sévères, diffuses, et dans l'HTAP associée, une activation basale des lymphocytes B est observée avec une sécrétion importante d'IL-6 et de TGF-ß. Ce dernier contribue, *in vitro*, à la prolifération des fibroblastes et à la sécrétion de collagène, responsables des mécanismes fibrosants observés dans la pathologie. Enfin, les basophiles présents dans la ScS semblent également activés et participer à l'activation des lymphocytes B et des fibroblastes malades. Ces résultats montrent que le lymphocyte B, en plus de son rôle dans la production d'anticorps, peut intervenir en physiopathologie par la sécrétion de cytokines pro-inflammatoires ou pro-fibrosantes comme l'IL-6 et le TGF-ß, toutes deux impliquées dans les processus d'activation vasculaire.

Summary

Tolerance mechanisms allow the negative selection of auto-reactive B lymphocytes while protecting the positive selection and differentiation of plasmocytes that produce high affinity antibodies. Tolerance mechanisms are altered in various auto-immune diseases and allow the production of auto-antibodies. Indeed, therapeutic targeting of autoreactive B lymphocytes, notably using anti-CD20 monoclonal antibodies, gives promising results in several auto-immune pathologies. While these treatments show a relative efficacy in anti-neutrophil cytoplasmic antibodies associated vasculitis (ANCA) (AAV), other autoimmune diseases with vascular components, among which systemic sclerosis (SSc) or idiopathic high blood pressure (iPAH), remain resistant to the targeting. Previous studies have essentially addressed the characterization of auto-antibodies producing B cells sub-populations. Therefore, this thesis project aimed at delineating phenotypic and functional characterization of the lymphocytic B cells sub-populations involved in these various vascular autoimmune diseases.

Patients affected by Granulomatosis with polyangiitis presented with important activation of innate immune system altogether with an increased production of IL6 by B lymphocytes correlated with T lymphocytes activation. Phenotypic alterations of B lymphocytes were observed for AAV patients, notably with MPA, suggesting an autoimmune component. Expression of CD69, CD95 and IL-6- receptor allowed discrimination between the various forms of the disease. In SSc, with particular emphasis in the most severe, diffuse forms, and in the associated PAH, a basal activation of the B cells was observed, allowing an important secretion of IL-6 and TGF-ß1. The latter contributed to the proliferation of fibroblasts and to the secretion of collagen, responsible for fibrosis induction as observed in the pathology. Finally, presence of activated basophils in SSc also participates in the activation of B cells and fibroblasts. These results place B lymphocytes, besides their role in antibody production, as important pathophysiological players through the secretion of pro-inflammatory and pro-fibrotic cytokines such as IL-6 and TGF-ß which are both implicated in endothelial cells activation in autoimmune vascular diseases.