VASCULAR INFLAMMATION IN SYSTEMIC AUTOIMMUNITY

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VASCULAR INFLAMMATION IN SYSTEMIC AUTOIMMUNITY

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Detail of a flame. Photo by Giuseppe A. Ramirez.

Plasticity and dynamism characterize the immune system as a tissue-integrating network with defensive functions. Blood and lymphatic vessel trees constitute the most evident and intuitive physical platform for the development of the net of interactions between immune cells, body tissues and foreign agents. Moreover vessel repair and immune patrolling are intimately linked physiological functions with common evolutionary roots. Not surprisingly variable degrees of vascular inflammation are often detectable in the setting of systemic inflammation and autoimmunity, whereas research in the field of cardiovascular pathology is progressively converging towards the identification of a common inflammatory background. The definition of the role of vascular inflammation in causing, sustaining and/or predicting the development of systemic autoimmunity constitute a challenging, unexplored frontier towards the development of a new generation of treatments and a better patient care.

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Editorial: Vascular Inflammation in Systemic Autoimmunity

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Keywords: vascular inflammation, autoimmunity, vasculitis, systemic inflammation, remodeling

The Editorial on the Research Topic

Vascular Inflammation in Systemic Autoimmunity

The immune system evolutionarily arises to integrate distant tissues and coordinate responses toward environmental or endogenous threats in complex living beings (1). To this purpose, innate and adaptive immune cells exploit the vast network provided by the vasculature, which enables information exchange and physical communication between threatened tissues and tissues in charge of immune cell generation and maturation as well as metabolism regulation (2). Clinical evidence of vascular inflammation can be easily found in settings of persisting inflammation and autoimmunity. Conversely, inflammation is a hallmark of disease progression in metabolic and cardiovascular diseases, which are the major causes of morbidity and mortality in the general population (3). If we translate these evidences into a prospect toward the future of research and clinical practice in immunology, two main questions arise:

- Can we identify shared pathogenic events among apparently unrelated human diseases that lead to vascular inflammation?
- Can we tackle these events to improve patient diagnosis, risk stratification, and treatment?

Although we are still far from satisfactorily answering these questions, here we highlight some of the emerging connections between vascular inflammation and systemic autoimmunity.

The first set of articles draws attention to some emerging players in the development and the maintenance of vascular inflammation (**Figure 1**). Khaib Dit Naib et al. report on the role of IL10 promoter variants in the susceptibility to Behçet's disease, adding evidence to the postulate of a common pathogenic background between Behçet's disease and other autoinflammatory conditions, such as Crohn's disease (4–6), where platelet activation and smoldering vascular inflammation may occur (7, 8). Additional hints on common genetic patterns of susceptibility among diseases characterized by vessel inflammation are emerging from multicenter studies (9). Bonatti et al. and Pattanaik et al. provide a thorough and updated appraisal on this topic by analyzing two paradigmatic conditions: anti-neutrophil cytoplasmic antibodies-associated vasculitides and systemic sclerosis, the former being characterized by necrotizing vessel inflammation, the latter by chronic vessel remodeling.

Maugeri et al. and Piotti et al. touch the pathophysiology of vascular inflammation and elegantly discuss the role of a tripartite innate immune network composed of the endothelium, platelets, and neutrophils in triggering and maintaining vessel injury as well as thromboembolic complications. In parallel, the articles by Hilhorst et al. and Lintermans et al. taken together constitute a comprehensive, cross-sectional analysis of the role of T-cells and macrophage/T-cells interactions in a wide

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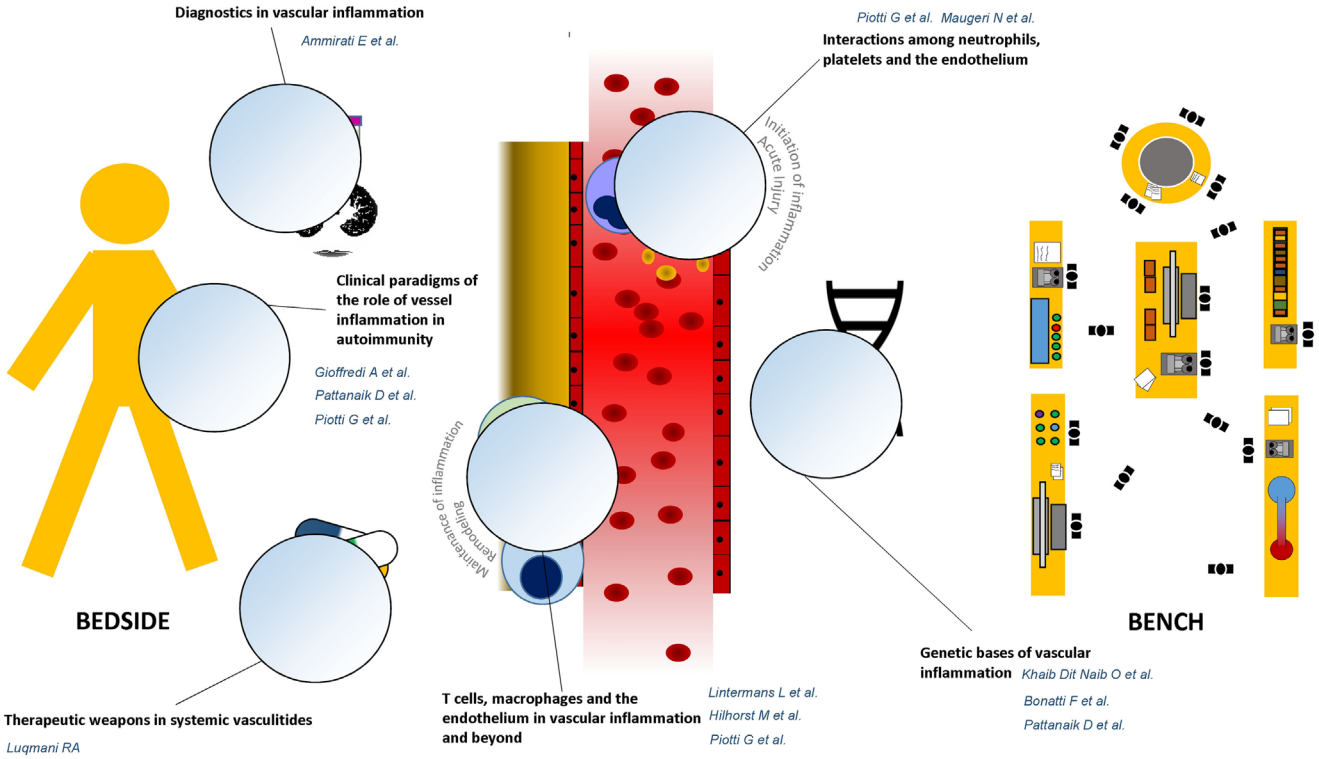

FIGURE 1 | Graphical guide to the research topic. The research topic "Vascular inflammation in systemic autoimmunity" is built on a bidirectional cross talk between clinical evidence and unsolved issues emerging from the daily rheumatology practice, at patient's bedside, and novel discoveries coming from the bench. The Reader is, thus, invited to move from dissertations about the genetic bases of vascular inflammation toward therapeutic and diagnostic applications of most significant innovations in the field of vascular pathophysiology.

range of inflammatory conditions, from autoimmune diseases to atherosclerosis.

The article by Piotti and colleagues and the ones by Gioffredi et al. and Pattanaik et al. demonstrate how these core mechanisms deploy in clinical settings, such as renal transplant rejection, eosinophilic granulomatosis with polyangiitis, and systemic sclerosis (Pattanaik et al.; Piotti et al.; Gioffredi et al.). Along with this line, Ammirati et al. discuss how increasing insight about the pathogenesis of vessel inflammation could be translated into clinical practice through the development of novel imaging techniques as well as novel biomarkers. The article by Luqmani moves further from bench toward bedside by reporting most updated therapeutic strategies to contrast vessel inflammation and by critically discussing current therapeutic aims and clinical tools to be considered in the treatment of systemic vasculitides, based on most recent pathogenic achievements.

In summary, we are glad to introduce the reader to this translational research topic on vascular inflammation in systemic autoimmunity, a bird's eye view on current research trends and, in our mind, a stimulus for discussion and for future deeper investigation on the way the blood vessel and immune cells cooperate to determine vessel injury and inflammation. We really feel that this field – which had been so rewarding for scientists in the last decades – holds promises for major breakthroughs in the next future.

AUTHOR CONTRIBUTIONS

All authors contributed in writing and revising the manuscript.

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Association analysis of *IL10*, *TNF*-α, and *IL23R-IL12RB2* SNPs with Behçet's disease risk in Western Algeria

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Objective: We have conducted the first study of the association of interleukin (IL)-10, tumor necrosis factor alpha (*TNF*-α), and *IL23R-IL12RB2* region single nucleotide polymorphisms (SNPs) with Behçet's disease (BD) in Western Algeria.

Methods: A total of 51 BD patients and 96 unrelated controls from West region of Algeria were genotyped by direct sequencing for 11 SNPs including 2 SNPs from the *IL10* promoter [c.-819T > C (rs1800871), c.-592A > C (rs1800872)], 6 SNPs from the *TNF*-α promoter [c.-1211T > C (rs1799964), c.-1043C > A (rs1800630), c.-1037C > T (rs1799724), c.-556G > A (rs1800750), c.-488G > A (rs1800629), and c.-418G > A (rs361525)], and 3 SNPs from the *IL23R-IL12RB2* region [g.67747415A > C (rs12119179), g.67740092G > A (rs11209032), and g.67760140T > C (rs924080)].

Results: The minor alleles c.-819T and c.-592A were significantly associated with BD [odds ratio (OR) = 2.18; 95% confidence interval (CI) 1.28–3.73, $p = 0.003$]; whereas, there was weaker association between *TNF*-α promoter SNPs or *IL23R-IL12RB2* region and disease risk.

Conclusion: Unlike the *TNF*-α and the *IL23R-IL12RB2* region SNPs, the two *IL10* SNPs were strongly associated with BD. The -819T, and -592A alleles and the -819TT, -819CT, and -592AA and -592CA genotypes seem to be highly involved in the risk of developing of BD in the population of Western Algeria.

Keywords: Behçet's disease, genetic association, *IL10*, *TNF*-α, *IL23R-IL12RB2*, single nucleotide polymorphism, Western Algeria

INTRODUCTION

Behçet's disease (BD) is a systemic inflammatory multifactorial disease (1). It is characterized by recurrent episodes of oral and genital ulceration, skin, and ocular lesions (2). The disease is now recognized as a systemic vasculitis, given that it can affect other tissues and organs including blood vessels, the digestive tract, and nervous system (3, 4).

The etiology of BD is not fully elucidated. There is a hypothesis that a pathogenic autoimmune process of BD is triggered by an infectious or environmental agent, in individuals genetically predisposed (5). The most strongly genetic factor associated with the disease is HLA B51 (5, 6). This association was initially described in 1973 (7) and subsequently confirmed in different ethnic groups (6, 8, 9).

However, the association between BD and HLA B51 represents only 20% in the siblings of patients with the disease (10), and 50% of cases with BD are negative for this allele (8, 9). These observations suggest the existence of other risk factors outside the HLA region. In fact, several recent genome-wide association studies have identified additional and new genomic regions that predispose to the disease (11–14).

The BD causes inflammation and chronic immune activation within small blood vessels (15–17). The site of inflammation is usually characterized by infiltration of immune cells as well as by highly elevated levels of different cytokines (18). IL-10 is one of the most important cytokines that has been observed at increased level in the serum and active lesions of BD patients (19–21). TNF-α and soluble TNF-α receptors are also elevated in the sera of patients with BD (22–24). Both IL-10 and TNF-α cytokines have been shown to play an important role in the immunopathology of autoimmune diseases (25–28), and an opposite roles in the inflammatory responses (29, 30). An autoregulatory loop appears to exist in whereby TNF-α induces IL-10 production, which ultimately reduces TNF-α synthesis (31, 32). It has been

suggested that the increase of IL-10 may down-regulate the expression of NO, prompting the protective role of elevation of IL-10 (33). Additionally, treatment with anti-TNF-α monoclonal antibodies has resulted in improvement of various manifestations of BD (34, 35).

IL-10 and TNF-α production may be regulated at the transcriptional level. Thus, several single nucleotide polymorphisms (SNPs) at the promoter of *IL10* and *TNF*-α gene have been shown to be associated with changes in the expression levels of IL-10 and TNF-α production (36, 37). On the other hand, numerous recent studies have demonstrated an association between BD and several *IL10* (13, 14, 38, 39) and *TNF*-α (39–43) SNPs in different ethnic groups. However, to date, there are no analogous or identical investigations in Algeria.

Two others cytokines, IL-23 and IL-12, may play an important role in BD pathogenesis; their levels are elevated in BD patients (44–46). IL-23 drives and promotes the development of a unique T-helper cell population that produces IL-17, Th17 cells. These IL-23-driven Th17 cells are highly pathogenic and elicit IL-17-dependent inflammation in autoimmune diseases (47). IL-12, a heterodimeric cytokine, is of crucial relevance to cell-mediated immunity and Th1 differentiation (48). This cytokine exerts its biological effects *via* binding to a heterodimeric receptor consisting of IL12RB2 and IL-12RB1 subunits.

The effect of IL-23 and IL-12 is mediated through the IL-23 and the IL-12 receptor (IL-23R, IL-12RB1). The genes that encode these receptors are adjacent on chromosome 1p31; a GWAS Studies revealed that *IL23R-IL12RB2* region is associated with BD (13, 14). Nevertheless, its association in the pathogenesis of BD remains to be confirmed in different ethnic groups. In this context, we examined genetic association for 11 SNPs in *IL10*, *TNF*-α, and *IL23R-IL12RB2* candidate genes with BD in Western Algeria.

MATERIALS AND METHODS

PATIENTS AND SUBJECTS

Fifty-one (51) unrelated BD patients and age- and sex-matched 96 healthy controls originate from the Western Algeria were recruited for a case-control study at the Oran Ophthalmic Hamou Boutlelis Hospital, the Department of Dermatology of Oran Medical Centre University, and the Oran Blood Transfusion Centre (Algeria). Among the 51 patients, 11 DNA belonging to Algerian origin, were selected from the biobank DNA for Genetics Laboratory of Autoinflammatory Diseases, Arnaud de Villeneuve Hospital, Montpellier (France).

Consent was signed by each participant or participant's parent or legal guardian if entrant is a minor, under the Rules of Ethics and Professional Conduct. Patient characteristics were recorded using a questionnaire. The diagnosis of patients was based especially on the criteria proposed in 1990 (49). The control group was composed of healthy subjects without a family history of autoinflammatory diseases, and selected from the same population. This work was approved by the Institutional Ethics Board of Tlemcen Abou-Bekr Belkaïd University.

GENOTYPING

Each DNA was genotyped for 11 SNPs, including two *IL10* promoter SNPs [c.-819T > C (rs1800871), c.-592A > C (rs1800872)], six SNPs from the *TNF*-α promoter [c.-1211T > C (rs1799964), c.-1043C > A (rs1800630), c.-1037C > T (rs1799724), c.-556G > A (rs1800750), c.-488G > A (rs1800629), and c.-418G > A (rs361525)], and three SNPs from the *IL23R-IL12RB2* region [g.67747415A > C (rs12119179), g.67740092G > A (rs11209032), and g.67760140T > C (rs924080)].

Genotyping was performed at the Laboratory of Genetics of Autoinflammatory Diseases, Arnaud de Villeneuve Hospital, Montpellier (France). Genomic DNA was isolated from peripheral blood, drawed on EDTA anti-coagulant, using QIAamp DNA Blood Kits (Qiagen, Valencia, CA, USA). The DNA samples were then dosed by spectrophotometry ND-1000 (Nano Drop Technologies, Wilmington, DE, USA) at 260 and 280 nm. The DNA concentration and ratio OD260/OD280 were estimated for each sample (50).

The DNA samples were subsequently amplified in a Applied Biosystems Thermocycler (Applied Biosystems, Foster City, CA, USA) in a 15 μL reaction volume containing 50 ng DNA, 2X Promega PCR Master Mix, and 25 μM of each primer (**Table 1**). The PCR programs were as follows: after a denaturation phase of 15 min at 95°C, the samples were subjected to 35 amplification cycles followed by a final elongation step of 7 min at 72°C. Each cycle comprises 30 s denaturation at 95°C, 30 s of primer annealing at 60°C, and 1 min extension at 72°C.

Table 1 | Primers sequence and length product.

Loci	SNPs	Forward primer	Reverse primer	Product length (bp)
IL10	rs1800871 rs1800872	TTAGACTCCAGCCACAGAAGC	GGGGGACCCAATTATTTCTC	597
TNF-α	rs1799964 rs1800630 rs1799724	GTGTGTCTGGGAGTGAGAACTTC	CTTCTTTCATTCTGACCCGG	570
	rs1800750 rs1800629 rs611525	CTCAGGACTCAACACAGCTTTTC	GAAAGAATCATTCAACCAGCG	438
IL23R-IL12RB2 region	rs11209032	GGAGTTAAACCTCTTGCTATCCTG	GATGCACAATGAGTTGATAAGG	164
	rs12119179	TACCCAGGGCATTCAGCTAC	GCTTGAGCTCCTGGATCAAG	701
	rs924080	GCACGTATGCCTTTTTGCATA	ATTTGAATGTGCCTTGGCAT	364

bp, base pair; IL, interleukin; rs, reference SNP; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

After checking the quality and size of the PCR products by agarose gel (1.5%) electrophoresis, SNPs genotyping was performed by direct sequencing using the BigDye Terminator version 3.1 (BDT v3.1) Cycle Sequencing Kit, followed by capillary electrophoresis on an ABI 3100XL Genetic Analyzer, according to the manufacturer's recommendations (Applied Biosystems, Foster City, CA, USA) (**Figure 1**).

STATISTICAL ANALYSIS

Comparisons of allele and genotype frequencies between groups (patients versus control subjects, and between the patient's groups according to different clinical features) were performed using the Chi-square or Fisher's exact tests. The association analysis was carried out by Odds ratio (OR) and corresponding 95% confidence interval (95% CI). Statistical analyses were performed using

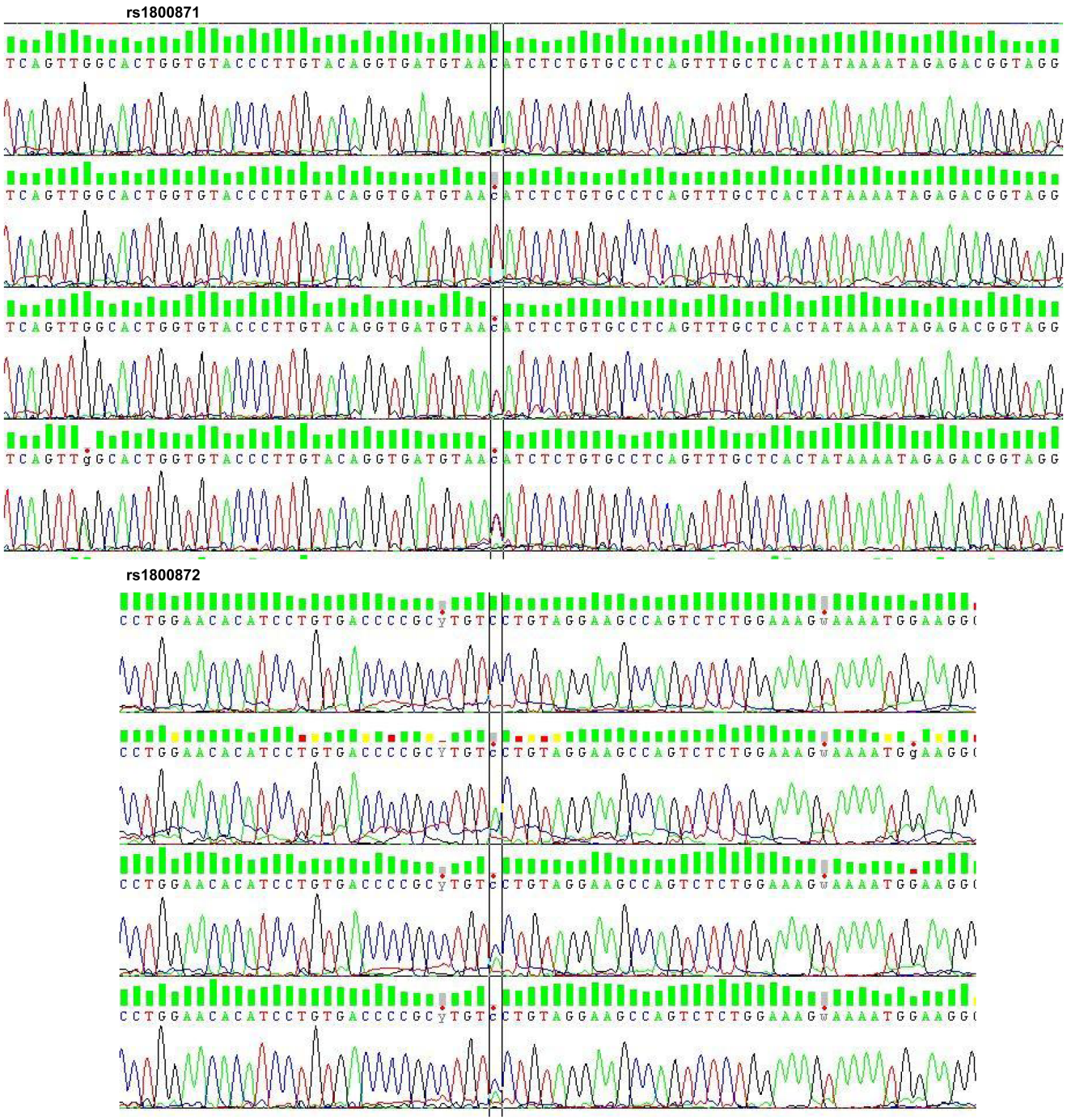

FIGURE 1 | Electropherogram of rs1800871 and rs1800872. rs, reference SNP; SNP, single nucleotide polymorphism.

GraphPad Prism Version 5.04 (GraphPad Software, Inc., La Jolla, CA, USA) and Epi Info 2000 Version 1.0 for Windows (Epi Info, Atlanta, GA, USA) software.

RESULTS

Table 2 shows the description of the clinical characteristics of the patients with BD of the current study. The mean age (±SD) of the patients at disease onset was 26 ± 11 years. Predominant lesions were oral ulcers (100%), cutaneous lesions (86.27%), genital ulcers (82.35%), eye lesions (62.74%), and arthritis (58.82%).

The distribution of alleles and genotypes frequencies of *IL10* promoter SNPs c.-819C > T (rs1800871) and c.-592C > A (rs1800872) showed that the two SNPs were in total linkage disequilibrium in our sample. For this, reason the results of one SNP c.-819C > T will be considered (**Table 3**).

The allele frequencies were significantly different in patients compared to controls. As indicated in **Table 3**, the frequencies of c.-819T (rs1800871) allele, and of the -819TT, -819CT (rs1800871) genotypes were significantly increased in patients than in controls ($p = 0.003$ and $p = 0.005$, respectively). Additionally, these SNPs was significantly associated with the disease (c.-819T; OR = 2.18, 95% CI 1.28–3.73, $p < 0.01$; -819TT and -819CT, OR = 2.17, 95% CI 1.01–4.69, $p < 0.05$) (**Figure 2**).

A subset analysis was performed to examine the difference in allele frequencies in clinical subsets of BD (**Table 4**). We observed a significant association between c.-819T and all classes; nevertheless, the association was slightly lower for the ocular lesion (OR = 1.55, 95% CI 0.81–2.96, $p > 0.05$). Additionally, the association was more significant for the Genital ulcers (OR = 2.21; 95% CI 1.29–4.04, $p = 0.002$).

We reported in **Tables 5** and **6** that all *IL23R-IL12RB2* SNPs alleles and genotypes, respectively, were not significantly associated with the disease (OR > 1, $p > 0.05$). The minor allele frequencies were different in the two groups, but this difference did not reach statistical significance ($p > 0.05$).

As indicated in **Tables 5** and **7**, alleles and genotypes of the *TNF*-α polymorphisms display similar distributions in patients and controls ($p > 0.05$). Except for c.-1037T and c.-488A all others *TNF*-α alleles were not associated with BD (OR < 1).

Table 2 | Clinical and demographic features of the Behçet patients of the current study.

Characteristics	Frequency (*n* = 51)
Mean age at disease onset ± SD (year)	26 ± 11
Sex ratio M/F (%, *n*)	56.9/43.1 (29/22)
Oral ulcers (%, *n*)	100 (51)
Genital ulcers (%, *n*)	82.4 (42)
Cutaneous lesions (%, *n*)	86.3 (44)
Eye lesions (%, *n*)	62.7 (32)
Neurological symptoms (%, *n*)	35.3 (18)
Venous thrombosis (%, *n*)	25.5 (13)
Arthritis (%, *n*)	58.8 (30)
Multiplex family (%, *n*)	35.3 (18)
pediatric case (%, *n*)	19.6 (10)
consanguinity (%, *n*)	43.1 (22)

SD, standard deviation.

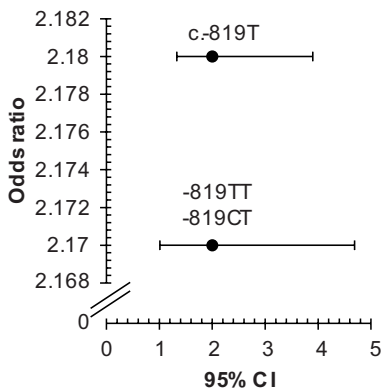

FIGURE 2 | Odds ratios for associations between *IL10* c.-819C > T, and -819TT, -819CT (rs1800871) with Behçet's disease. The two SNPs c.-819C > T and c.-592C > A are in total linkage disequilibrium in our sample; therefore, the results of only one SNP c.-819C > T is considered. CI, confidence interval; rs, reference SNP; SNP, single nucleotide polymorphism.

Table 3 | Allelic and genotypic frequencies of rs1800871 variant in BD patients and controls.

Alleles and genotypes	Frequencies (%)		*p*
	Controls (*n* = 96)	Cases (*n* = 51)	
C	141 (73.4)	57 (55.9)	0.003**
T	51 (26.6)	45 (44.1)	
CC	50 (52.1)	17 (33.3)	0.005**
CT	41 (42.7)	23 (45.1)	
TT	5 (5.2)	11 (21.6)	

*BD, Behçet's disease; SNP, single nucleotide polymorphism; rs, reference SNP. **p < 0.01.*

Table 4 | Association analysis of clinical subclasses with *IL10* c.-819T SNP in patients with Behçet's disease.

Clinical subset	OR	95% CI		*p*
		LL	UL	
Eye disease	1.55	0.81	2.96	0.152
Genital ulcers	2.21	1.29	4.04	0.002**
Skin lesions	2.07	1.17	3.68	0.007**
Arthritis-arthralgia	2.06	1.11	3.81	0.013*
Neurologic signs	2.5	1.18	5.32	0.009**

*CI, confidence interval; LL, lower limit; UP, upper limit; OR, odds ratio; SNP, single nucleotide polymorphism. *p < 0.05, **p < 0.01.*

Table 5 | *IL23R-IL12RB2* and *TNF*-α allelic frequencies in patients with Behçet's disease.

Loci	SNPs	Alleles frequency (proportion, %)		OR (95% CI)	*p*
IL23R-IL12RB2	rs12119179 (g.67747415A > C)	A	C	1.24 (0.72–2.13)	0.415
	Patients	67 (65.7)	35 (34.3)		
	Controls	135 (70.3)	57 (29.7)		
	rs11209032 (g.67740092G > A)	G	A	1.18 (0.68–2.03)	0.530
	Patients	67 (65.7)	35 (34.3)		
	Controls	133 (69.3)	59 (30.7)		
	rs924080 (g.67760140T > C)	T	C	1.45 (0.87–2.41)	0.133
	Patients	54 (52.9)	48 (47.1)		
	Controls	84 (43.8)	108 (56.3)		
TNF-α	rs1799964 (c.-1211T > C)	T	C	0.92 (0.52–1.61)	0.751
	Patients	73 (71.6)	29 (28.4)		
	Controls	134 (69.8)	58 (30.2)		
	rs1800630 (c.-1043C > A)	C	A	0.83 (0.41–1.67)	0.584
	Patients	86 (84.3)	16 (15.7)		
	Controls	157 (81.8)	35 (18.2)		
	rs17999724 (c.-1037C > T)	C	T	1.01 (0.35–2.84)	0.976
	Patients	95 (93.1)	7 (6.9)		
	Controls	179 (93.3)	13 (6.7)		
	rs1800750 (c.-556G > A)	G	A	0.61 (0.14–2.09)	0.402
	Patients	98 (96.1)	4 (3.9)		
	Controls	180 (93.7)	12 (6.3)		
	rs1800629 (c.-488G > A)	G	A	1.12 (0.56–2.26)	0.726
	Patients	85 (83.3)	17 (16.7)		
	Controls	163 (84.9)	29 (15.1)		
	rs361525 (c.-418G > A)	G	A	0.93 (0.39–2.21)	0.869
	Patients	92 (90.2)	10 (9.8)		
	Controls	172 (89.6)	20 (10.4)		

CI, confidence interval; IL23R-IL12RB2, region of the interleukin-23 receptor and interleukin-12 receptor beta 2 chain-encoding gene; OR, odds ratio; rs, reference SNP; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

Table 6 | The distribution of *IL23R-IL12RB2* genotypes in patients with Behçet disease.

SNPs	Genotype distribution (frequency, %)			*p*-Value	OR (95% CI) *p*-value	MAF (%)	OR^{MAF} (95% CI) *p*-value
rs12119179 (g.67747415A > C)	AA	AC	CC	0.522	1.13 (0.54–2.35) 0.734	C	1.24 (0.58–2.67) 0.580
Patients	24 (47.1)	19 (37.2)	8 (15.7)			34.31	
Controls	48 (50)	39 (40.6)	9 (9.4)			29.69	
rs11209032 (g.67740092G > A)	GG	GA	AA	0.721	1.12 (0.57–2.21) 0.744	A	1.18 (0.71–1.97) 0.560
Patients	23 (45.1)	21 (41.2)	7 (13.7)			34.31	
Controls	46 (47.9)	41 (42.7)	9 (9.4)			30.73	
rs924080 (g.67760140T > C)	CC	CT	TT	0.327	1.46 (0.64–3.35) 0.326	C	1.58 (0.71–3.54) 0.264
Patients	13 (25.5)	22 (43.1)	16 (31.4)			47.1	
Controls	32 (33.4)	44 (45.8)	20 (20.8)			56.25	

ORs were calculated for the minor versus major alleles. CI, confidence interval; IL23R-IL12RB2, interleukin-23 receptor and interleukin-12 receptor beta 2 chain-encoding gene; MAF, minor allele frequency; OR^{MAF}, OR of the MAF; OR, odds ratio; rs, reference SNP; SNP, single nucleotide polymorphism.

Table 7 | The distribution of *TNF*-α genotypes in patients with Behçet disease.

SNPs	Genotype distribution (frequency, %)			*p*-Value	OR (95% CI) *p*-value	MAF (%)	OR^{MAF} (95% CI) *p*-value
rs1799964 (c.-1211T > C)	TT	TC	CC	0.114	0.67 (0.32–1.4) 0.249	C	0.8 (0.37–1.75) 0.580
Patients	29 (56.9)	15 (29.4)	7 (13.7)			28.43	
Controls	45 (46.9)	44 (45.8)	7 (7.29)			30.21	
rs1800630 (c.-1043C > A)	CC	CA	AA	0.826	0.79 (0.35–1.78) 0.544	A	0.78 (0.31–1.97) 0.600
Patients	37 (72.5)	12 (23.5)	2 (4)			15.69	
Controls	65 (67.7)	27 (28.1)	4 (4.2)			18.23	
rs17999724 (c.-1037C > T)	CC	CT	TT	ND	1.23 (0.4–3.74) 0.690	T	1.26 (0.34–4.69) 0.733
Patients	44 (86.3)	7 (13.7)	0 (0)			6.86	
Controls	83 (86.5)	13 (13.5)	0 (0)			6.77	
rs1800750 (c.-556G > A)	GG	GA	AA	ND	0.6 (0.13–2.12) 0.388	A	0.6 (0.12–3.07) 0.536
Patients	47 (92.2)	4 (7.8)	0 (0)			3.92	
Controls	84 (87.5)	12 (12.5)	0 (0)			6.25	
rs1800629 (c.-488G > A)	GG	GA	AA	0.909	1.17 (0.52–2.61) 0.680	A	1.18 (0.47–2.97) 0.721
Patients	35 (68.6)	15 (29.4)	1 (2)			16.67	
Controls	69 (71.9)	25 (26)	2 (2.1)			15.1	
rs361525 (c.-418G > A)	GG	GA	AA	0.299	0.75 (0.28–2.02) 0.541	A	0.9 (0.29–2.79) 0.849
Patients	43 (84.3)	6 (11.8)	2 (3.9)			9.8	
Controls	77 (80.2)	18 (18.8)	1 (1)			10.42	

ORs were calculated for the minor versus major alleles. CI, confidence interval; MAF, minor allele frequency; ND, not defined; OR, odds ratio; OR^{MAF}, OR of the MAF; rs, reference SNP; SNP, single nucleotide polymorphism; TNF, tumor necrosis factor.

DISCUSSION

To date, the etiopathogenesis of BD is not fully elucidated. Researches in recent decades have shown the complex role of genetic factors in the development of the disease. We analyze the association between BD and 11 SNPs in *IL10*, *TNF*-α, and *IL23R-IL12RB2* candidate genes in the Western Algeria population.

This is the first report demonstrating that the c.-819T and c.-592A alleles were associated with BD in Algeria.

Previous genetic studies have shown a strong association of many *IL10* variants with BD in different ethnic groups. Recent genome-wide association study conducted by Mizuki et al. (14) in a Japanese cohort, including 612 individuals with BD and 740 unaffected individuals controls from different ethnic groups, has shown a significant difference between the two groups for five *IL10* SNPs. The two SNPs analyzed in our study showed an association (OR = 2.18, 95% CI 1.28–3.73, $p < 0.01$). On the other hand, a strong association has also been highlighted for *IL10* rs1518111 (OR = 1.45, 95% CI 1.34–1.58) in a genome-wide study performed in 311,459 SNPs in 1215 individuals with BD and 1278 healthy controls from Turkey (13). Our results are consistent with those obtained by Wallace et al. (38) who studied the segregation of two *IL10* promoter SNPs, rs1800871 and rs1800896 in 178 cases and 295 controls from two populations, including Arab Middle East and United Kingdom. A strong association of the 819T allele has been observed in United Kingdom patients (OR 1.5, 95% CI 1.1–2).

Our results are, however, in disagreement with those of the study of Ates et al. (39) in which no significant association was revealed by exploring three *IL10* SNPs (-1082G > A, rs1800896, c.-819C > T, rs1800871, and c.-592C > A, rs1800872) in 102 patients with BD and 102 controls from Turkey. These conflicting results may be explained by ethnic differences.

Several studies have shown the association of the two (c.-819 C > T, c.-592C > A) studied SNPs with various inflammatory diseases, but also with cancer (51–53), periodontitis (54), and docetaxel-induced liver injury (55). These associations suggested that the two SNPs can play an important role in the expression of *IL10*. In fact, it has been previously reported that the SNP c.-819C > T and/or c.-592C > A alleles affect the transcription of *IL10* (56). Other studies performed on three SNPs at position -1082 A > G, -819C > T, and -592C > A in the promoter region of the *IL10* gene have shown that the expression levels of IL-10 was significantly different according to the some haplotypes (57, 58). Finally, it has been reported that the disease-associated rs1518111 allele seems to be associated with low IL-10 mRNA expression and protein production (13). Indeed at the SNP rs1518111 locus, the rate of transcript of the G allele is higher than that of the A allele and patients homozygous for the A allele produce less IL-10 than those who are heterozygous or homozygous for the G allele.

The result of subset analysis suggests that the risk allele might predispose to genital ulcers, skin lesions, neurologic signs, and arthritis-arthralgia, but weakly to eye complications; the association was more significant for the genital ulcers (OR = 2.21, $p = 0.002$). Our results are in agreement with those of previous study (21) that showed a variable increase in mRNA

expression within all BD lesions, including oral and genital ulcers, pseudofolliculitis lesions, and lesions at the site of pathergy testing.

Recent GWAS study from Turkey and Japan revealed *IL12R-IL23RB2* SNPs in association with BD. Three SNPs were strongly associated with the disease, including rs924080 (OR = 1.28, $p = 6.69 \times 10^{-9}$) (13), rs12119179 ($p = 2.7 \times 10^{-8}$), and rs1495965 (OR = 1.35, $p = 1.9 \times 10^{-11}$) (14), but no significant association was found in a Korean cohort.

Our results showed no significant association between BD and rs12119179, g.67740092G > A (rs11209032), and g.67760140T > C (rs924080) SNPs in the *IL23R-IL12RB2* region. In Iranian study (59), six SNPs in *IL23R-IL12RB2* were found to be associated with BD; the most significant of which were rs17375018 (OR = 1.51, $p = 1.93 \times 10^{-6}$), rs7517847 (OR = 1.48, $p = 1.23 \times 10^{-6}$), and rs924080 (OR = 1.29, $p = 1.78 \times 10^{-5}$). Others studies have also identified a strong relationship between polymorphisms of *IL23R* and BD (60–62). These associations may suggest an important role of Th17 cells that express the IL-23R on their surface. Kim et al. (62) studied the interaction of specific *IL17A*, *IL23R*, and STAT4 (signal transducers and activators of transcription 4) SNPs in intestinal BD Korean patients; they suggest that the IL-23/IL-17 axis plays a significant role in disease pathogenesis.

IL-12 has been implicated in the pathogenesis of a multitude of diverse autoimmune diseases (63, 64). *IL12RB2* constitute a risk factor for primary biliary cirrhosis, with the reported top associated SNPs mainly located in intronic sequences (65–67).

The genetic architecture and modularity of human autoimmune diseases is very complex. The functional implications of most of these associations are not yet clarified. Identify candidate causal SNPs and pathways (ICSN Pathway) analysis may act as a powerful guide to further research into the functional and immunological ramifications of these associations.

No significant associations were found between BD and studied *TNF*-α polymorphisms. These SNPs have been studied in various ethnic groups for possible association with BD. However, the allelic and genotypic associations of these studies have been contradictory. In Korean patients, *TNF*-α c.-1043A (rs1800630) allele was associated with an increased risk of BD (OR = 1.4, $p = 0.030$) (68). However, no significant association was found in meta-analysis studies for this SNP (42). Additionally, it has been reported a significant associations between c.-1037T allele (rs1799724) (OR = 0.76, 95% CI 0.58–0.98), c.-488G allele (rs1800629) (OR = 1.8, $p = 0.010$) (68), and c.-418A allele (rs361525) (OR = 1.51, 95% CI 1.12–2.04) (42), and BD. Moreover, no significant associations were identified with other *TNF*-α promoter polymorphisms, such as c.-1037T (rs1799724), c.-488A (rs1800629), and c.-556G > A (rs1800750) alleles with BD in Moroccan patients (43). The *TNF*-α c.-1211C allele (rs1799964) presented a significant association with BD in several populations, including Turkish ($p = 0.023$) (69), Korean ($p = 0.030$, OR = 1.4) (68), and UK white Caucasoid population (RR = 2.3, $p = 0.00004$) (40). The frequency of the *TNF*-α c.-1211C allele was significantly higher in Behcet's patients than in healthy controls in Moroccan and Tunisian populations (OR = 1.65, $p = 0.015$; OR = 1.68, $p = 0.02$, respectively) (41, 43) and in meta-analysis (OR = 1.35, 95% CI 1.09–1.68) (42). This polymorphism has been associated with several extra-intestinal manifestations of Crohn's disease, including uveitis, erythema nodosum, and large joint arthropathy (70), all of which are known to be associated with BD. Further investigation is necessary to determine the functional significance of *TNF*-α c.-1691042C and how it participates in the inflammatory dysregulation associated with BD.

Thus, polymorphisms at positions c.-1211T > C, c.-1043C > A, c.-1037C > T, and c.-488G > A have been associated with increased transcriptional activity and production of TNF-α in some studies (37, 71), in contrast to others (72–75). The over production of TNF-α during the course of BD may result in other *TNF*-α polymorphisms or post-transcriptional mechanisms. Furthermore, TNF-α production is not only under the control of the promoter region of *TNF*-α, and it may also result from complex cis and trans interactions among other cytokines.

TNF-α is encoded in the HLA complex on chromosome 6, a region that has long been known to be associated with BD. This gene-dense region, presentes a strong linkage disequilibrium (76). The association between BD and *TNF*-α could therefore be a result of linkage disequilibrium with alleles within this group. So it will be interesting to investigate other genes polymorphism among this region in our population.

In conclusion, we replicate the associations between BD and the SNPs from the *IL23R-IL12RB2* region and c.-1037C > T and c.-488G > A *TNF*-α promoter SNPs. *IL10* promoter SNPs (rs1800871 and rs1800872) is strongly associated with BD in the population of the Western Algeria. It would be interesting to study other SNPs to identify additional associations with BD in the studied population.

AUTHORS CONTRIBUTION

Mourad Aribi, Gérard Lefranc are Principal Investigators of the study, participated in its design and execution and helped draft the manuscript and critically reviewed it for intellectual content; Mouna Barat-Houari participated in the design of the study, carried out genetic analyses, and helped draft the manuscript; Ouahiba Khaib Dit Naib wrote the manuscript and carried out genetic analyses; Aicha Idder, Amel Chiali, and Hakim Sairi are responsible for the recruitment of eligible patients and their families; Isabelle Touitou conceived of the study, participated in its design, and coordination. All the authors read and approved the final manuscript.

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Genetic susceptibility to ANCA-associated vasculitis: state of the art

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ANCA-associated vasculitis (AAV) is a group of disorders that is caused by inflammation affecting small blood vessels. Both arteries and veins are affected. AAV includes microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) renamed from Wegener's granulomatosis, and eosinophilic granulomatosis with polyangiitis (EGPA), renamed from Churg–Strauss syndrome. AAV is primarily due to leukocyte migration and resultant damage. Despite decades of research, the mechanisms behind AAV disease etiology are still not fully understood, although it is clear that genetic and environmental factors are involved. To improve the understanding of the disease, the genetic component has been extensively studied by candidate association studies and two genome-wide association studies. The majority of the identified genetic AAV risk factors are common variants. These have uncovered information that still needs further investigation to clarify its importance. In this review, we summarize and discuss the results of the genetic studies in AAV. We also present the novel approaches to identifying the causal variants in complex susceptibility loci and disease mechanisms. Finally, we discuss the limitations of current methods and the challenges that we still have to face in order to incorporate genomic and epigenomic data into clinical practice.

Keywords: ANCA-associated vasculitis, granulomatosis with poliangiitis, microscopic poliangiitis, eosinophilic granulomatosis with poliangiitis, pharmacogenetics, rituximab, genome-wide association studies

INTRODUCTION

Vasculitis is a group of disorders that is caused by inflammation affecting blood vessels; it is primarily due to leukocyte migration and resultant damage (1). It is a multi-system inflammatory-autoimmune disease that affects both arteries and veins with consequent tissue damage, especially to the respiratory tract and kidneys; it causes early mortality, organ failure including end stage renal disease and chronic morbidity. Children and adults, males and females, and individuals of any ethnic background can be affected.

According to the size of the vessel affected, vasculitis can be classified into large, medium, and small vessels.

Small-vessel vasculitis predominantly involves microscopic blood vessels with absent or insufficient immune deposits; its association with circulating autoantibodies to neutrophils (ANCA) has led to these conditions being grouped together as ANCA-associated vasculitis (AAV) (2, 3).

ANCA-associated vasculitis includes microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA) renamed from Wegener's granulomatosis, and eosinophilic granulomatosis with polyangiitis (EGPA), renamed from Churg–Strauss syndrome (4).

Microscopic polyangiitis is associated with proteinase 3 (PR3)-ANCA in 26% of cases and with myeloperoxidase (MPO)-ANCA in 58% of cases (5), while GPA is characterized by PR3-ANCA in 66% of patients and MPO-ANCA in 24% of patients (6).

Interestingly, some patients do not present ANCA; in particular, in EGPA more than an half are ANCA-negative (7).

ANCA-associated vasculitis has an approximate prevalence of 200 per million and an annual incidence of 20 per million.

The causes of AAV are unknown; it can be considered a complex disease, because multiple genetic factors, combined with yet unknown environmental factors, are able to influence its susceptibility (8, 9).

In addition, different phenotypes are influenced by several triggers and environmental factors (10).

Ethnicity affects the type of AAV, with Chinese and Japanese populations having more MPA and less GPA and EGPA than Caucasian, and the latter more than the African; geographical disparities in AAV have been found, with GPA and EGPA more common in colder climates. In Europe, GPA is more frequent in the north, and the reverse tendency has been found for MPA (11).

Similar treatment strategies used in trials involving patients with AAV, considered GPA and MPA as a single disease spectrum (6), hence the hypothesis that future genetic studies should group GPA and MPA together (12).

FAMILIAL AAV CASES

The etiology of AAV theoretically involves interaction between genetics and environmental factors; however, knowledge about whether AAV clusters in families is poor.

Family recurrence is a strong indicator of a possible heritability in multifactorial disorders (13). Furthermore, information on the possible heritability of AAV is of clinical importance, because family members often want to know whether AAV increases their closest relatives at increased risk.

Familial AAV cases have been described but a putative hereditary factor has never been demonstrated.

The presence of EGPA in a family was described in two sisters with atopic-type bronchial asthma and negative perinuclear-ANCA results. The human leukocyte antigen (HLA) complex and six siblings were investigated, but the authors did not found a clear pattern of heritability (14).

An interesting study investigated the risk of GPA in relatives of patients with GPA, because several GPA families have been described (15). Using Swedish nationwide registers, the authors compared the occurrence of GPA among 6,670 first-degree relatives and 428 spouses of 1,944 Swedish patients with GPA, with the occurrence among 68,994 first-degree relatives and 4,812 spouses of 19,655 control subjects.

Granulomatosis with polyangiitis was present in 2/6,670 first-degree relatives of patients and in 13/68,994 first-degree relatives of the controls, with a relative risk of 1.56 (95% confidence interval 0.35–6.90).

The authors concluded that there is no evidence of an increase in familial risk of GPA, while it was demonstrated in other studies for other autoimmune disorders, such as inflammatory bowel disease (IBD), systemic lupus erythematosus (SLE), and multiple sclerosis (MS).

In several other cases, the familial members were affected by AAV and another type of vasculitis, underlying the possibility of common genetic triggers at the basis of vasculitis. In a first case, polyarteritis nodosa and GPA were observed in several members of two different families. The authors concluded that genetic factors are important but not sufficient to express clinical features of these diseases (16).

In another reported case, a man was affected by EGPA and his son by GPA (occurred 5 years later). These two patients lived together in Northern Italy and shared the HLA haplotype A*03-B*07-C*w07-DRB1*0404-DQB1*0302, a putative marker of autoimmunity (17). Recently, in an Indoasian family, three members of the same family with GPA who share the allele HLA-DPB1*04:01 were described, furthermore demonstrating the HLA locus is involved in AAV genesis (18).

Pre-GWAS ASSOCIATION STUDIES IN AAV

NON-HLA ASSOCIATIONS

The classical approach for genetic studies applied to AAV is the case–control strategy, performed investigating candidate-gene polymorphisms. The dimension of the population, the ethnicity, the number of the investigated polymorphisms and the allele frequencies mainly determine the power of a study. The non-HLA single-nucleotide polymorphism (SNP) markers associated with AAV may contribute to the total variance in susceptibility, in addition to the HLA genes. The pre-GWAS non-HLA association studies are listed in **Table 1**.

Protein tyrosine phosphatase, non-receptor type 22 (lymphoid) (PTPN22)

The rs2476601 PTPN22 SNP is linked with several autoimmune diseases. The first associations between rs2476601 SNP of PTPN22 gene and autoimmune diseases were demonstrated with type 1 diabetes (T1D) (19) and rheumatoid arthritis (RA) (20); after that, the finding expanded to other autoimmune diseases, enhancing the importance of signaling thresholds in disease susceptibility; even if it is difficult to determine how threshold effects change disease risk. PTPN22 gene encodes for an intracellular tyrosine phosphatase that dephosphorylates activating tyrosines, as well as other molecules involved in TCR signaling. The transgenic mouse homolog of PTPN22 shows hyperactive T cells (21). In human beings, the PTPN22 gene can present a SNP at the codon 620 (Arginine to Tryptophan), a change able to destroy the binding of PTPN22 with the binding partner, the intracellular tyrosine kinase, Csk. However, the consequence of this for T cell activation thresholds remains difficult to interpret. The first studies of PTPN22 risk allele carriers an increased phosphatase activity with reductions in TCR and BCR signaling (22). Recent studies have supported the hypothesis that the 620W allele acts as the knock-out mouse, with loss of function leading to hyper-responsiveness. The lack of PTPN22 results in changes in T cell populations in mice, particularly an expansion of T reg cells (23). In addition to T cell influence, also B cell receptor signaling appears to be affected by PTPN22 SNP (24). The protein Csk has recently been demonstrated as a susceptibility gene for several autoimmune diseases such as Celiac disease (CD) (25), Scleroderma (26) and SLE (27). Thus, both PTPN22 and Csk may influence disease risk affecting the B cell receptor repertoire development. Taken together, these data suggest that groups of genes are able to influence receptor signaling thresholds; PTPN22 and Csk, in fact, may influence the risk of autoimmunity influencing the autoimmune repertoire selection and the abundance of regulatory cell subsets. (28). It is highly probable that different mechanisms explain the genetic associations for every autoimmune disease.

The first study of the rs2476601 SNP in AAV has been performed in a German GPA cohort, demonstrating a statistical association; interestingly, stratifying the GPA patients in ANCA-positive and ANCA-negative, the association was stronger in the ANCA-positive subgroup (29). Later, the result was confirmed in British GPA and MPA patients (30) and Italian AAV cases (31). The Italian study confirmed the strong association with the ANCA-positive subset, while suggested that EGPA is not associated with the PTPN22 SNP.

Cytotoxic T lymphocyte associated antigen-4

Cytotoxic T lymphocyte associated antigen-4 (CTLA-4) is a regulatory molecule expressed on T cells that plays a major role in peripheral tolerance and inhibiting T cell activation (32).

CTLA-$4^{-/-}$ knock-out mice show a phenotype rapidly developing in lymphoproliferative disease with multiorgan lymphocytic infiltrations, tissue destruction, splenomegaly, lymphadenopathy, and elevated serum immunoglobulin with early lethality, demonstrating a predominant role in suppressing T cell function (33). It is likely that defective CTLA-4 expression and function are associated with autoimmune diseases. Furthermore, CTLA-4 activity influences a negative regulator of both cellular and humoral responses and mediates antigen-specific apoptosis.

Negative signaling of CTLA-4 has an active role in the regulation of autoreactive T cells, and disturbance of the normal control of the CTLA-4 may cause the pathogenesis of autoimmune diseases.

The CTLA-4 genetic polymorphisms alter activity influencing the CTLA-4 gene expression.

Table 1 | Positive genetic association studies in anti-neutrophil cytoplasmic antibody-associated systemic vasculitis.

Gene and variation	Population	Cases	Controls	OR	*P*-value	Other associated autoimmune diseases	Reference
AAT (Z allele)	German	79 GPA	752	3.8[a]	<0.0001	CD	(41)
AAT (Z allele)	Swedish	88 (66 GPA)	No controls	6.0	<0.0001	CD	(40)
CD18 (AvaII)	German	31 MPO^+	120	2.56	<0.005	/	(44)
CD40 (rs4810485)	European Descent	895 GPA	1976	0.81	0.002	AT, T1DM, IBD, PS, MS, RA, and SLE	(90)
CTLA4 (rs231735)	European Descent	895 GPA	1976	0.82	0.001	RA	(90)
CTLA (AT)86 repeat	European Descent	117 GPA	123	Not available	0.0005	/	(40)
CTLA4 (rs3087243)	European Descent	895 GPA	1976	0.79	0.000098	GD, RA, T1DM, AITDs, and HA	(90)
CTLA4 (rs3087243)	British	641 (GPA, MPA, and EGPA)	9,115	1.19	6.4×10^{-3}	GD, RA, T1DM, AITDs, and HT	(30)
*HLA-DPB1*0401*	German	282 GPA	380	2.47	6.4×10^{-8}	/	(45)
*HLA-DQB1*0303*	Japanese	50 MPA	77	2:35	0.017	ACLT, SSc	(50)
HLA-DQw7	British	34 GPA, 25 MPA	1,103	2.9	<0.0025	/	(47)
HLA-DR3	British	34 GPA, 25 MPA	1,103	0.31	<0.01	/	(47)
HLA-DR4	Dutch	241 GPA, 30 MPA, 12 EGPA	9,872	1.7	<0.0001	/	(48)
HLA-DR6	Dutch	241 GPA, 30 MPA, 12 EGPA	9,872	0.3	<0.0001	/	(48)
*HLA-DRB1*04*	German	102 EGPA	341	1.86	0.0028	RA, GCA, AD	(52)
*HLA-DRB1*07*	Italian	48 EGPA	350	2:42	0.0042	SLE, AD	(51)
*HLA-DRB1*07*	German	102 EGPA	341	1.57	0.046	SLE, AD	(52)
*HLA-DRB1*09*	European Descent	76 GPA	4,039	4.00a	0.005	/	(49)
*HLA-DRB1*0901-DQB1*0303*	Japanese	50 MPA	77	2:44	0.0037	GD, MG, T1DM, RA, and SLE	(50)
*HLA-DRB1*13*	German	102 EGPA	341	0.50	0.019	Early childhood, MG	(52)
HLA-DRB3	Italian	48 EGPA	350	0:54	0.028	childhood ALL	(51)
HLA-DRB3	German	102 EGPA	341	0.61	0.004	childhood ALL	(52)
HLA-DRB4	Italian	48 EGPA	350	2:49	0.00023	HT	(51)
HLA-DRB4	German	102 EGPA	341	1.87	0.0002	HT	(52)
IL-10	Swedish	32 GPA	109	/	<0.05	SLE, SSc, KD, PM, DM, PV, UC, SS, GD, MG, PS, and ALPS.	(39)
IL-10 haplotype	German	103 EGPA	507	2:16	0.0003	SLE, SSc, KD, PM, DM, PV, UC, SS, GD, MG, PS, and ALPS.	(38)

(Continued)

Table 1 | Continued

Gene and variation	Population	Cases	Controls	OR	*P*-value	Other associated autoimmune diseases	Reference
IL2RA rs41295061	British	675 (GPA, MPA and EGPA)	8,936	0.77	0.012	T1DM, SLE	(30)
PRTN3 -564G	German	66 GPA	106	0.5	<0.01	/	(44)
PTPN22 (rs2476601)	European Descent	895 GPA	1976	1.35	0.002	CD, PS, RA, SLE, T1DM, GD, HT, and some forms of JA	(90)
PTPN22 (rs2476601)	German	199 GPA	399	1.8	0.002	CD, PS, RA, SLE, T1DM, GD, HT, and some forms of JA	(46)
PTPN22 (rs2476601)	Italian	143 GPA, 102 MPA, and 99 EGPA	945	1.91 GPA; 2.31 GPA ANCA$^+$	0.005 GPA; 0.00012 GPA ANCA$^+$	CD, PS, RA, SLE, T1DM, GD, HT, and some forms of JA	(31)
PTPN22 (rs2476601)	British	641 (GPA, MPA and EGPA)	9,115	1.4	1.4×10^{-4}	CD, PS, RA, SLE, T1DM, GD, HT, and some forms of JA	(30)
TLR9 3-SNP haplotype	German, Dutch, British	646 GPA, 164 EGPA, and 53 MPA German AAV; 273 GPA, 53 EGPA and 100 MPA Dutch and British AAV cases	1898	1.64	0.000044	IBD	(37)
FCGR3B CNVs number high	British	556 (GPA, MPA and EGPA)	286	/	1×10^{-8}	RA	(57)
FCGR3B CNVs number low	British	80 GPA	190	/	0.003	SLE	(69)
FCGR3B CNVs number low	British	76 MPA	190	/	0.0003	SLE	(69)
FCGR3B CNVs number low	French	84 GPA	181	/	0.0001	SLE	(69)
DEFB4 CNVs number high	Chinese	112 AAV (ANCA$^+$ and biopsy proven necrotizing glomerulonephritis)	523	/	0.009	SLE, PS, COPD, IBD, HIV infection, and SAP	(65)

ANCA, autoantibodies to neutrophils; OR, odds-ratio; CNVs, copy number variations; GPA, granulomatosis with poliangiitis; MPA, microscopic poliangiitis; EGPA, eosinophilic granulomatosis with poliangiitis; CD, Crohn's disease; T1DM, diabetes mellitus type1; CD, celiac disease; RA, rheumatoid arthritis; MS, multiple sclerosis; pSS, primary sicca syndrome; AT, autoimmune thyroiditis; IBD, inflammatory bowel disease; AITD, autoimmune thyroid disease; GB, Guillain–Barrè syndrome; MG, myasthenia gravis; ACLT, autoimmune chronic lymphocytic thyroiditis; SSc, systemic sclerosis; GCA, giant cell arteritis; ACPAs, anti-citrullinated protein antibodies; KD, Kawasaki disease; DM, dermatomyositis; PV, pemphigus vulgaris; UC, ulcerative colitis; SS, Sjogren's syndrome; ALPS, autoimmune lymphoproliferative syndrome; SAP, severe acute pancreatitis; GD, Grave's disease; COPD, chronic obstructive pulmonary disease; PS, psoriasis; HT, Hashimoto thyroiditis; JA, juvenile arthritis; AD, atopy.

An AAV associated polymorphism is the allele (named allele 86) of the CTLA-4 microsatellite polymorphism (AT)n, which maps in the 3′-untranslated region (3′-UTR) of exon 3. Individuals with long CTLA-4 (AT)n repeat alleles show hyper-reactive T cells.

The CTLA-4 (AT)n 86 allele has been demonstrated to be important for maintenance of normal levels of CTLA-4 expression and seems to balance T cell activation/inhibition. A study in European descent GPA patients (34) confirmed findings from a Scandinavian cohort in which a positive association with the long alleles of (AT)n in the CTLA-4 3′-UTR was demonstrated. Evidences of a blockade of T cell costimulation using CTLA-4Ig seems to be a potential therapeutic intervention, alternative to standard treatment with immunosuppressive agents. In a recent open-label trial of 20 patients with non-severe relapsing GPA, the safety and efficacy of abatacept were investigated. The authors concluded abatacept was well tolerated and an high frequency of disease remission and prednisone discontinuation were observed. This study demonstrated anti-CTLA4 may be an option in the treatment of some subgroups of AAV (35).

A second polymorphism in CTLA-4 gene investigated in AAV is the SNP rs3087243. A large British study found the minor allele (A) of this SNP is protective for AAV (30).

Toll-like receptors

Evidences are emerging about the role of Toll-like receptors (TLRs) in the development of the autoimmune diseases (36).

Toll-like receptors are a family of innate receptors whose specificities are predetermined in the germline. Therefore, TLRs have evolved to recognize conserved features of microbes. Viruses typically lack the conserved features common to other pathogen classes, so the innate immune system has evolved to recognize viral nucleic acid as a hallmark of viral infection.

TLR9 signaling may be involved in disease pathology, favoring models of infectious agents triggering AAV development. Unmethylated CpG motifs within bacterial nucleic acid were the first ligands identified to activate TLR9. Since then, many DNA viruses have also been shown to activate this TLR, including those from the herpesvirus and adenovirus. Both α- and β-herpes viruses have genomes that are rich in CpG motifs. The contribution of TLR9 recognition of these viruses has clearly been reported *in vitro* (37).

In order to investigate the genetic contribution of TLR9 on the susceptibility and clinical manifestation of AAV, four SNPs in TLR9 have been genotyped in 863 German AAV cases and 1344 healthy controls (38). In the replication step, significant results were investigated in a cohort of 426 Dutch and British AAV cases. Interestingly, in GPA patients the association with genotypes and haplotypes was predisposing (OR >1), while in MPA was protective (OR <1). When cases were stratified according to ANCA status rather than to clinical entity, the association was confirmed; in fact, the results showed a strong overall difference in TLR9 allele/haplotype frequencies between PR3-ANCA and MPO-ANCA cases. These results confirmed the findings of the EVGC GWAS about the genetically differences between PR3-ANCA and MPO-ANCA AAV.

Interleukin-10

Interleukin-10 (IL-10), which was originally named cytokine synthesis inhibitory factor, is a cytokine that is produced by type T-helper cells. IL-10 presents anti-inflammatory properties, with the inhibition of immune mediator secretion, antigen presentation, and phagocytosis.

A study investigated the presence of IL10 gene polymorphisms in EGPA and GPA patients of European ancestry (39). Three SNPs in the IL10 gene promoter (IL10-3575, IL10-1082, and IL10-592) were analyzed in 403 GPA patients and 103 EGPA patients, compared with 507 German controls. The IL10-3575/-1082/-592 TAC haplotype, part of the extended ancient haplotype IL10.2, was highly significantly associated with ANCA-negative EGPA, providing further evidences that AAVs have distinct genetic backgrounds. Other studies of the IL-10 SNPs were performed both in German and Swedish population, suggesting the IL-10 may influence the production of autoantibodies (40).

SERPINA1

The SERPINA1 gene encodes for α1-antitrypsin, a neutral serine protease inhibitor, which includes proteinase 3; this is the major inhibitor of PR3 activity, and is supposed to limit the damage to the local tissues. This mechanism may be crucial in AAV, because the decreased function of α1-antitrypsin potentially results in persistence of PR3 in inflammatory tissue, with the final consequence of ANCA generation.

The SERPINA1 gene is highly polymorphic; the Z allele (Glu342Lys) in α1-antitrypsin (AAT) deficiency is a combined deficiency and dysfunctional allele.

Several studies investigated the role of the Z allele in AAV, showing that heterozygous patients for the Z variant of the SERPINA1 gene have an increased risk than the general population of developing GPA (41, 42).

PRTN3

PRTN3 gene encodes for proteinase 3 (PR3) protein, a neutrophil intracellular protease that is the main antigen of ANCA autoantibodies; it is located on the plasma membrane of a subset of neutrophils. The rate of this membrane PR3-positive neutrophils subset is characteristic of an individual, and this semms to be genetically determined (43).

In GPA patients, there is an increased number of PR3-positive neutrophils and an elevated level of PRTN3 expression compared with controls. Increased levels of PRTN3 expression are associated with an elevated risk of relapse and with an increased relapse rate; this supports the idea that PR3 expression on the membrane of neutrophils plays a role in the pathophysiology of PR3-AAV (44).

The promoter and coding regions of the PRTN3 gene were sequenced searching for genetic variants in 79 GPA patients and 129 healthy controls. Seven SNPs, one amino acid change (Val119Ile), one 84 bp insertion/deletion, and a microsatellite were found. An association with GPA was demonstrated for the A-564G polymorphism in the PRTN3 promoter; this variation affects a candidate transcription factor-binding site. The authors suggested the overexpression of PRTN3, might predispose the patient to the development of AAV (45).

HLA ASSOCIATIONS

The HLA region, located on chromosome 6p21 and extended for 7.6 Mb, is the most dense gene region within the human genome encoding more than 250 expressed loci including several key immune response genes. The region can be subdivided in class I, II, and III regions and contains the highest number of polymorphisms and the most dense linkage disequilibrium in the human genome. In the last 50 years, numerous associations between the HLA region and autoimmune disease have been demonstrated. The association between HLA and AAV has been largely investigated, for different populations and with several methods. The pre-GWAS HLA association studies are listed in **Table 1**.

The most studied AAV has been GPA, with demonstrated association for DPB1*0401 (46, 47), DQw7 (48), and different DRB1 genes, some with protective effects [DRB1*03 (47), DRB1*06 (48)], other with causative effects [DRB1*04 (49), DRB1*09 (50)]; in some cases the populations were small and the results have not been replicated.

Microscopic polyangiitis has been investigated in few studies, mainly because the incidence of the disease is smaller than GPA. Associations were demonstrated with DRB1*03 (48) and DRB1*06 (49) (protective effects), while DRB1*04 (49) and the haplotype DRB1*0901-DQB1*0303 (51) have causative effects.

Finally, EGPA has been mainly investigated in Italian (52) and German (53) populations, with the same results. The HLA genes DRB4 and DRB1*07 have causative effects while DRB3 and DRB1*13 with protective effects. Furthermore, the first study stratified EGPA in the two major clinical subsets (54), ANCA-negative, with organ damage mainly mediated by tissue eosinophilic infiltration and ANCA-positive, with features of small-vessel vasculitis; analyzing the HLA-DRB4 in patients categorized by different numbers of vasculitic manifestations (purpura, alveolar hemorrhage, mononeuritis multiplex, rapidly progressive glomerulonephritis, and constitutional symptoms) suggested that the gene frequency correlates with the different number of vasculitis symptoms.

All the studies examined relative small population, and need to be confirmed in large studies with focused GWAS. Furthermore, the different associations between GPA, MPA, and EGPA demonstrate the three AAV have a different genetic background.

GWAS AAV GENETICS

The main aim of a GWAS is to identify common genetic variation associated with well phenotyped disease in a non-biased method. Inherent to the properties of association-based statistics, the premise for conducting GWAS is based on the assumption that disease risk is associated with relatively common variants with a minor allele frequency greater than 2–5%. The goal is to identify frequency differences in polymorphisms between cases and controls. As a result, the study analyses a large sample size with a strong statistical correction in order to avoid spurious associations due to false-positive findings. The occurrence of linkage disequilibrium is used because not all the polymorphisms can be genotyped to detect an association. The larger the population studied for a disease, the more associated variants can be discovered (55).

To date, two different GWASs have been performed in AAV. The first one was performed by the European Vasculitis Genetic Consortium (EVGC) (56) and the second one by the VCRC (Vasculitis Clinical Research Center) (57). The two GWASs differed for the study design.

In 2012, the EVGC performed the first GWAS of AAV (ANCA-positive GPA and MPA) in order to identify common or specific genetic-risk factors.

The clinical diagnosis of AAV was evaluated according to the European Medicines Agency algorithm, supported by either a positive ANCA assay or a diagnostic biopsy.

The EVGC GWAS was performed in 1233 UK patients with AAV and 5884 controls (from the Wellcome Trust Case–Control Consortium) and was replicated in 1454 Northern European case patients and 1666 controls. A total of 2687 AAV patients of European ancestry and 7650 controls were analyzed. The main results of the EVGC GWAS are listed in **Table 2**.

In order to confirm the GWAS data, 156 SNPs were genotyped in a replication cohort of 1454 AAV patients and 1666 controls.

In addition to the SNPs genotyped in the chip, three additional SNPs were included: the proteinase 3 gene (PRTN3), which is a major ANCA autoantigen, the interleukin-2-receptor alpha gene (IL2RA) and the protein tyrosine phosphatase, non-receptor type 22 gene (PTPN22) because were found to be associated with AAV in previous studies.

Combined analysis of the discovery and replication cohorts showed that these SNPs were statistically significant. Three of them mapped in the HLA locus, with the most significant, which maps in the HLA-DPB1 gene, while a fourth associated SNP maps in the SERPINA1 locus at 14q32; this demonstrates that the susceptibility loci are located bot on HLA and in non-HLA regions. The SERPINA1 gene encodes for the protein α1-antitrypsin, a neutral serine protease inhibitor, which includes proteinase 3. Associations with the rare Z (null) allele of SERPINA1 have been described in previous studies, but the significance were low (58).

The SNP in the SERPINA1 locus is in linkage disequilibrium with the Z allele, suggesting that the causal variant at the SERPINA locus is either the Z allele of SERPINA1 or is in close linkage disequilibrium with it.

In order to investigate whether GPA and MPA are genetically distinct clinical diseases, a further statistical analysis of the discovery cohort was subdivided in the two diseases according with clinical data comparing the seven most significant SNP associated with AAV in the two diseases. Interestingly, the three HLA and SERPINA1 SNPs differed significantly between GPA and MPA, with almost all the association related to GPA.

Finally, a comparison between the subgroups of patients with PR3-ANCA and with MPO-ANCA, evidenced significant differences at the HLA, SERPINA1, and PRTN3 loci, demonstrating a genetic association with PR3-ANCA but not with MPO-ANCA.

In the GPA subgroup, the associations were found only in PR3-ANCA patients, not in MPO-ANCA patients. Within the MPA group, the findings were the same: an association of PR3-ANCA and the HLA-DP and SERPINA1 SNPs. The most significant SNP associated with AAV in non-MHC regions was the rs7151526, both in GPA and PR3-ANCA subgroups; the Z allele of this SNP maps in the SERPINA1–SERPINA11 locus at 14q32.

The major finding of the EVGC GWAS was that the strongest association of all these genetic polymorphisms appeared to be related with ANCA specificity rather than the clinically defined

Table 2 | Associations of single-nucleotide polymorphisms (SNPs) and antineutrophil cytoplasmic antibody (ANCA)-associated vasculitis (GPA$^+$ MPA), According to Cohort.

				Combined cohort ($N = 2267$ case patients, 6858 controls)	
Gene	**Chromosome**	**SNP (Minor Allele)**	**Nucleotide change**	**OR**	***P*-value**
SNPs WITH GENOME-WIDE SIGNIFICANCE IN COMBINED COHORT					
HLA-DP	6	rs3117242	A > G	3.67	1.5×10^{-71}
HLA-DQ	6	rs5000634	A > G	0.80	2.9×10^{-9}
COL11A2	6	rs3130233	A > G	1.51	7.8×10^{-15}
COL11A2	6	rs3117016	A > G	1.83	6.4×10^{-24}
SERPINA1	14	rs7151526	A > C	0.59	2.4×10^{-9}

				Clinical syndrome			
				GPA vs. control ($N = 1683$ vs. 6858)		MPA vs. control ($N = 489$ vs. 6858)	
Gene	**Chromosome**	**SNP**	**Nucleotide change**	**OR**	***P*-value**		
ASSOCIATIONS OF SNPs AND ANCA – ASSOCIATED VASCULITIS, ACCORDING TO CLINICAL AND ANCA SUBGROUPS							
HLA-DP	6	rs3117242	A > G	5.39	3.1×10^{-85}		
HLA-DQ	6	rs5000634	A > G	0.83	2.2×10^{-6}		
ARHGAP18	6	rs1705767	A > C	0.78	3.3×10^{-7}		
SERPINA1	14	rs7151526	A > C	0.54	4.4×10^{-10}		
PRTN3	19	rs62132295	A > G	0.78	2.6×10^{-5}		
MOSPD2	X	rs6628825	A > G	0.80	2.6×10^{-6}		

				ANCA specificity			
				Proteinase 3 vs. control ($N = 1521$ vs. 6858)		Myeloperoxidase vs. control ($N = 556$ vs. 6858)	
Gene	**Chromosome**	**SNP**	**Nucleotide change**	**OR**	***P*-value**	**OR**	***P*-value**
HLA-DP	6	rs3117242	A > G	7.03	6.2×10^{-89}	1.55	3.2×10^{-2}
HLA-DQ	6	rs5000634	A > G	0.86	3.3×10^{-5}	0.65	2.1×10^{-8}
ARHGAP18	6	rs1705767	A > C	0.73	5.2×10^{-8}	0.87	1.0×10^{-2}
SERPINA1	14	rs7151526	A > C	0.53	5.6×10^{-12}	0.84	2.8×10^{-1}
PRTN3	19	rs62132295	A > G	0.73	2.6×10^{-7}	1.10	2.2×10^{-1}
MOSPD2	X	rs6628825	A > G	0.77	6.1×10^{-7}	0.86	6.3×10^{-1}

GPA, granulomatosis with poliangiitis, MPA, microscopic poliangiitis, EGPA, eosinophilic granulomatosis with poliangiitis.

syndromes. The genetic background with autoantibody specificity evidenced in this study could impact the clinical classifications of GPA and MPA and therapeutic options (59).

Further GWAS with large ANCA-negative AAV are needed in order to find the genetic basis of ANCA-negative GPA and MPA.

A second GWAS was performed by the VCRC in 492 GPA patients (white subject of European descent) and 1,506 healthy controls; the data were replicated in a second cohort of 528 GPA patients and 1,228 controls. The main results of the VCRC GWAS are listed in **Table 3**. Association analysis of the two combined cohort (750 patients and 1,820 controls) showed that the strongest association mapped in the HLA region, with the SNP rs9277554 (HLA-DPB1 gene, $P = 1.92 \times 10^{-50}$), and rs9277341 (HLA-DPA1 gene, $P = 2.18 \times 10^{-39}$). An independent single SNP, rs26595, showed also association with GPA in the combined cohort ($P = 2.09 \times 10^{-8}$). This SNP maps on chromosome 5 in proximity of the SEMA6A gene, which encodes for a protein called semaphorin 6A. Semaphorins are involved in many physiologic processes, such as vasculogenesis, cardiogenesis, osteoclastogenesis, tumor metastasis, and immune regulation (60, 61). The role of SEMA6A in GPA is still unclear; a possible theory explains that semaphorins have an important role in the immune response in autoimmune and allergic disorders (62). The association between the rs26595 SNP of the SEM6A6 gene and GPA was recently investigated in 879 GPA patients, 150 MPA patients and 191 EGPA patients, compared with 1,376 healthy control subjects (63). All

Table 3 | Results of genome-wide association (stage 1) and replication (stage 2a) analyses of GPA association.

				Combined analysis ($n = 750$ case patients, $n = 1820$ controls)	
Gene	**Chromosome**	**SNP**	**Nucleotide change**	**OR**	***P*-value**
HLA-DPB1	6	rs9277554	T > C	0.24	1.92×10^{-50}
HLA-DPA1	6	rs9277341	C > T	0.33	2.18×10^{-39}
Non-HLA region					
WSCD1	17	rs7503953	A > C	1.50	1.93×10^{-7}
COBL	7	rs1949829	T > C	1.78	4.19×10^{-7}
CCDC86	11	rs595018	A > G	1.46	1.60×10^{-7}
DCTD	4	rs4862110	C > T	1.44	2.14×10^{-6}
				Combined analysis ($n = 987$ case patients, $n = 2731$ controls)	
SEMA6A	5	rs26595	C > T	0.74	2.09×10^{-8}
				c-ANCA-positive patients vs. controls (patients, $n = 578$; controls, $n = 1,820$)	
Gene	**Chromosome**	**SNP**	**Nucleotide change**	**OR**	***P*-value**
VCRC GWAS: associations of MHC Loci with c-ANCA-positive GPA					
HLA-DPB1	6	rs9277554	T > C	0.16	4.7×10^{-57}
HLA-DPA1	6	rs9277341	C > T	0.27	2.30×10^{-42}

GPA, granulomatosis with poliangiitis; SNP, single-nucleotide polymorphism; OR, odds-ratio; VCRC, Vasculitis Clinical Research Consortium; ANCA, autoantibodies to neutrophils.

of the subjects were white Europeans. ANCA status was available for 90% of the patients. The authors did not observe any statistically significantly different allele frequencies between AAV patients and controls, not confirming the findings from the initial VCRC GWAS. Between the two studies the approaches of analysis is different, raising the need for further genetic association studies in GPA.

SERPINA1 has been the only non-MHC locus associated with AAV. However, the SNP strongly associated in that study (rs7151526) is located 557 bp from rs1956707 and was not independently associated in that population; the reason may be the two SNPs are not in linkage disequilibrium.

The potential HLA associations to specific GPA subsets were also investigated. Comparisons of the HLA risk allele frequencies between the PR3-cANCA positive subgroup (88% of cases) and the ANCA-negative subgroup showed that the associations with HLA–DPB1 (rs9277554, $P = 4.7 \times 10^{-57}$) and HLA–DPA1 (rs9277341, $P = 2.30 \times 10^{-42}$) were caused by the PR3-cANCA positive group, a finding, which confirms previous data suggesting genetic differences between ANCA-positive and ANCA-negative GPA.

The differences between the two GWAS results may be explained to different factors, such as phenotypes in the patient cohorts (i.e., ANCA-positive GPA and MPA in the EVGC versus ANCA-positive and -negative GPA in VCRC) and in the genetic and statistical approaches.

Further genetic studies of additional cohorts are needed to clarify the AAV genetic background. The two different GWAS have identified SERPINA1, SEMA6A, and HLA loci as significant factors related to AAV risk and demonstrate that the strongest association is linked to HLA–DPB1*04. Although the results must be confirmed in further studies, they highlight that MPA and GPA might be considered two distinct clinical entities, and that ANCA specificity might be segregated in different subgroups, probably better than the distinction actually made between GPA and MPA; as well, the autoantigen PR3 might be a direct player/trigger in the pathophysiology of GPA.

COPY NUMBER VARIATIONS

Copy number variations (CNVs) have been defined by the presence of variable copies (deleted or duplicated) of genomic regions of 1 kb or more in different individuals; CNVs represent an important source of genetic variation in the human genome (64).

Approximately 360 Mb pairs (12% of the human genome is represented by CNVs), with a preponderance of smaller size rearrangements (<20 kb). The genomic regions with these CNVs contain hundreds of genes and other functional elements, and many CNVs have a population frequency higher than 1% [copy number polymorphisms (65)].

CNVs can change the gene dosage of particular gene, influencing the susceptibility to a specific disease.

Immunological genes are frequently involved in CNVs, perhaps because this represents a mechanism to expand the recognition repertoire. To date, a low number of studies have been performed

in autoimmune diseases, even if immunological genes have the potential to be affected by CNVs.

A first demonstration that CNVs are linked with AAV has been found in a Chinese population; in a study of 112 AAV (ANCA positive and biopsy proven necrotizing glomerulonephritis) patients and 523 controls, an association of CNVs in the DEFB4 gene has been statistically demonstrated (65). This gene belongs to the β-defensin family genes, underlying a possible mechanism of susceptibility in some inflammatory disorders, such as psoriasis (Ps), chronic obstructive pulmonary disease, IBD, HIV infection, and severe acute pancreatitis. Furthermore, at mRNA level, there is a correlation between CNVs and β-defensin gene expression (66).

Defensins show a vast spectrum of anti-microbial activity, representing an important factor in the first defense against microorganisms and connect the innate and adaptive immune system. These functions are performed with the production of chemotactic factors inducing pro-inflammatory cytokines.

A further CNV analysis in AAV has been performed studying the FCGR3B gene.

Low-affinity Fc-receptors (FcgR) play a crucial role in the initiation and regulation of the antibody-mediated immune response, linking humoral, and cellular immunity. These receptors are able to recognize the constant domain of IgG and are involved in the mobilization of macrophages, natural killer T-cells, and neutrophils to regions of immune complex deposition. FCGRs also play an important role in the recognition and clearance of immune complexes, and as modulators of B-cell activity. In particular, FCGR3B CNV has been investigated in risk of autoimmunity (67).

Association between FCGR3B CNV and a number of autoimmune disorders has been reported in European populations, including SLE (68), RA (69), and in Japanese population for ulcerative colitis (64).

In the investigation of the association between FCGR3B CNV and AAV, it is noteworthy that the approach they used for statistical analysis; in a previous paper, an association with AAV and low FCGR3B CNV by Quantitative Polymerase Chain Reaction was utilized (70), but analyzing a UK Caucasian vasculitis cohort with a triple paralog ratio test the association was not confirmed, suggesting that the initial finding may have been a false-positive resulting deriving from the lack of robustness in the qPCR assay (71).

EPIGENETICS

Epigenetics can be defined as those mechanisms that cause stable changes of gene expression without influencing the primary nucleotide sequence; it might explain why human body cells have highly specific phenotypes, even though they all carry an identical genome (72).

Epigenetic mechanisms are particularly important for autoimmunity, since the expression of pro-inflammatory genes (like TNF-α) is regulated at the chromatin level.

Little is known about epigenetics in AAV; in 2010 a study investigated the role of epigenetics in AAV (73), this was based on the concept that the development of AAV basically needs two things: a dysregulation in immune response able to produce autoantibodies directed to PR3 and MPO and high expression of these major ANCA autoantigens in mature neutrophils. When the autoantibodies find the aberrantly autoantigens presented on neutrophils they can stimulate activation of neutrophils causing vascular inflammation. The data suggested that epigenetic modifications (mainly DNA methylation and histone modification) associated with gene silencing are able to influence ANCA autoantigen encoding genes, potentially influencing the expression of PR3 and MPO in AAV patients, and suggesting that epigenetic events may be important in the development of autoimmunity.

This study and other investigations in autoimmune diseases suggest epigenetics globally affects these disorders and that the persistence of the epigenomic changes could lead to an aberrant gene expression, contributing to the perpetuation of the disease mechanisms.

PHARMACOGENETICS

The pharmacogenetics is the study of the role of inherited and acquired genetic variation in drug response (74). These type of studies scans for candidate genetic variants of subjects based on the different type of response for a specific drug, in a case–controls design. Pharmacogenetics has the potential to predict response and/or toxicity based on genetic background and has the potential to personalize therapy. The aim of the pharmacogenetics is to personalize the specific theraphy, enhancing the response and minimizing the side effects of the chemotherapeutic drugs. In recent years, the development of monoclonal antibodies directed against a particular immunological protein has permitted the treatment of several autoimmune disorders [i.e., RA and AAV, autoimmune hemolytic anemia, T1D, Sjogren's syndrome (Sj)]. One of these most used drugs is rituximab (RTX), a chimeric monoclonal antibody directed against the protein CD20. RTX is able to destroy normal and malignant B cells that present CD20 (primarily found on the surface of the B cells) on their surfaces; this is useful for the treatment of those diseases characterized by a high number of iperactive or dysfunctional B cells.

Unfortunately, RTX has several side effects, such as disability, cardiac arrest, syndrome of tumor lysis (which can cause acute renal failure), infections (Hepatitis B reactivation), progressive multifocal leukoencephalopathy, immune toxicity (with depletion of B cells), and pulmonary toxicity. Sometimes RTX can cause death. RTX acts with a mechanism mediated by the Fc portion, including apoptosis of CD20-positive cells, complement-dependent cytotoxicity (CDC), Fcg receptor (FcgR)-mediated mechanisms, antibody-dependent cellular cytotoxicity (ADCC), and phagocytosis. These combined effects can stimulate elimination of B cells with the generation of normal B cells developed from lymphoid stem cells (75).

Several polymorphisms able to influence the RTX activity have been identified. FCGR3A gene, which encodes the FcgRIIIa protein, presents the c.559 SNP, which can change the aminoacid in position p.158 of the FcgRIIIa, affecting the affinity for human IgG1 (76, 77); as a consequence, human IgG1 binds more strongly the NK cells homozygous for FCGR3A-158V than to NK cells homozygous for FCGR3A-158F.

Because FcgRIIIa is expressed only by monocytes/macrophages and NK cells (the main actors in ADCC), it has been postulated that patients homozygous for FCGR3A-158V show significantly

better responses to RTX because they have enhanced ADCC activity compared with FCGR3A-158F carriers. This was confirmed in other studies using RTX (78, 79).

Weng and Levy (78) also showed that the FCGR2A-131H/R polymorphism significantly affects response and time to progression after RTX treatment, with better results for FCGR2A-131H homozygous patients. These results were not confirmed in other studies, that found no impact on response (80), suggesting the association may arise from linkage disequilibrium between FCGR3A/FCGR2A polymorphisms (81, 82).

In addition to the influence of FCGR2A and FCGR3A in RTX action, it is important to consider that the drug binds to aminoacids 170–173 and 182–185 on CD20 protein; these residues are physically close to each other for the creation of a disulfide bond between amino acids 167/183, creating a potential mechanism of different response to RTX based on CD20 mutations.

To date, there is no evidence that inherited mutations or polymorphisms of the CD20 gene (called MS4A1) influence the response to RTX (83).

Recently, an Italian group selected 132 patients with systemic autoimmune diseases treated with RTX.

Of these patients, 81 (61.4%) were SLE patients, 16 (12.1%) presented different inflammatory myopathies such as polymyositis and dermatomyositis, 13 (9.8%) were AAV patients (GPA, EGPA and MPA); the remaining 22 patients suffered of other systemic autoimmune diseases such as Sj, systemic sclerosis (SSc), or autoimmune hemolytic anemia.

Genotyping for FCGR3A-158F/V (rs396991) gene polymorphism was performed, in order to determine whether the SNP 158F/V in the FCGR3A gene, influences the RTX response in systemic autoimmune disease patients.

The type of drug response after RTX infusion was evaluated: 61% of the patients had a complete response, partial response 27 and 12% did not respond to the treatment. A statistically significant difference was observed in 158 V allele frequency between responder (38%) and non-responder (16%) patients ($P = 0.01$; OR = 3.24). RTX was also more effective in the 158 V allele carriers (94%) than in homozygous 158FF patients (81%): $P = 0.02$; OR = 3.96. This study shows that FCGR3A-158F/V SNP influences the response to RTX in autoimmune diseases, including AAV (results need to be replicated in a larger population, because the AAV population was small) (84).

Another drug used in the treatment of AAV is the cyclophosphamide (CP), a bifunctional DNA alkylating agent; this drug is used for the treatment of pediatric and adult malignancies, because it is characterized by anti-tumor activity in human beings (85). At low dosage, CP is a potent immunomodulatory used as second-line therapy (86). Like other chemotherapy, CP shows difference in efficacy and toxicity in patients. CP side effects include cardiotoxicity, nephrotoxicity, neurotoxicity, infertility, bladder toxicity, myelosuppression, and leukemogenesis. CP bioactivation and/or detoxification has demonstrated to be influenced by different SNPs; with the era of –omics, however, new methods can increase knowledge about the genetic background able to influence CP activity (87). Finally, genes found having pharmacokinetic and pharmacodynamic effects, require replication in larger and more diverse patient populations.

OVERLAP WITH OTHER AUTOIMMUNE DISEASES

Several HLA and non-HLA susceptibility variants appear to increase risk in autoimmune diseases, which share the same pathway even if the diseases are different. In recent years, candidate-gene studies in AAV have often investigated variants previously associated to other autoimmunity diseases.

The two most investigated genes, which are potentially associated with autoimmune diseases are CTLA-4 and PTPN22.

The CTLA-4 SNPs rs3087243 and rs231735 have been previously found to be associated with RA and T1D, and replicated in GPA (34, 88–90).

In 2012, a study examined the association between previously identified autoimmune disease susceptibility loci and GPA, trying to demonstrate shared genetic susceptibility variants (91). The study examined 431 GPA patients and 473 controls, all of European ancestry. Multiple SNPs were assessed to be associated with GPA, shared with other autoimmune diseases such as Crohn's disease, T1D, SLE, RA, CD, and ulcerative colitis. SNPs in CTLA-4 gene were significantly associated with GPA in the single-marker meta-analysis [odds ratio (OR) = 0.79, $P = 9.8 \times 10^{-5}$]. The genetic-risk score for RA susceptibility markers was significantly associated with GPA ($P = 5.1 \times 10^{-5}$). The results may demonstrate that RA and GPA share a similar genetic background.

This study supports the findings of a previous epidemiologic study, which showed an increased frequency of RA among offspring of GPA patients (92). GPA and RA present several differences, such as the pulmonary and renal manifestations (not commonly observed in RA, but frequent in GPA); furthermore, inflammatory arthritis (characteristic of RA), is not present in every patients with GPA.

Interestingly, this meta-analysis showed that other loci different from CTLA-4 previously found to be associated with common autoimmune diseases were not statistically significantly associated with GPA in this study.

The PTPN22 rs2476601 (R620W) polymorphism has been found to be associated with GPA (29) and with multiple other autoimmune diseases (23).

Recently, a comprehensive meta-analysis (93) showed that PTPN22 C1858T SNP is strongly associated with nine autoimmune diseases: T1D, RA, SLE, Graves' disease, Addison's disease, AAV, juvenile idiopathic arthritis, myasthenia gravis and Graves' disease with other concomitant autoimmune diseases, with the T allele increasing the risk of disease. The association suggests that is possible the presence of a common mechanism shared by these autoimmune diseases. Further investigation of these mechanisms may in the future shed light in the pathogenesis of the autoimmune diseases. The studies related to the PTPN22 R620W variant suggest that there is a correlation between the presence of autoantibodies and the development of self-reacting antibodies, with the PTPN22 620W, which may affect autoimmune response on several levels, including generation and activation of autoimmune cells. Based on this theory, the PTPN22 function in autoimmune disease manifestations seems to affect generation and activation of immune cells (93).

In conclusion, the sharing of a similar genetic background suggests that different autoimmune diseases may have similar pathogenic mechanisms, and the shared association with CTLA-4

and PTPN22 variants affects the threshold for activation or deactivation of autoreactive T cells.

Further GWAS results and other genomic approaches can increase the knowledge of the genetic background of AAV, searching for rare variants that would be not investigated in previous studies.

FUTURE PERSPECTIVES

In complex diseases, it is necessary to search new associated loci, but also replicate already known susceptibility variants. Novel demonstrated loci confer low increases in susceptibility to the disease, but they may evidence new pathways important in etiology or that may be crucial for pharmacogenetic applications. To date, the HLA complex still show the strongest association for several autoimmunity diseases (like AAV), but different non-HLA genes associated with autoimmunity are now under investigation.

There are now several methods available for investigating more deeply the genomic regions associated with autoimmunity.

IMMUNOCHIP

In recents years, Illumina produced ImmunoChip genotyping SNPs microarray, which allows immunogenetics studies of the major inflammatory and autoimmune diseases, mapping with high-density previous established GWAS significant loci. The Immunochip contains 196,524 polymorphisms, with 195,806 SNPs and 718 small insertion/deletions, enabling cheap fine mapping of loci for common and rare variants. Being immunogenetics highly involved in inflammatory and autoimmune disorders, Immunochip includes a dense coverage of the HLA Complex SNPs; this enables deep replication and imputation of the major classical HLA loci (94).

Several autoimmune diseases have already been investigated with Immunochip, such as atopic dermatitis (95), ankylosing spondylitis (96), CD (97), selective IgA Deficiency (98), juvenile idiopathic arthritis (99), MS (100), RA (101), Sj (102), Ps (103), SSc (104); the unique vasculitis analyzed with Immunochip has been Takayasu Arteritis (105). In all these cases, statistically significant immunogenetic variants have been demonstrated; at this time, no Immunochip study has been performed in AAV. Such an analysis may confirm the results obtained from the two GWAS and possibly identifying more associated loci with AAV.

TARGETED SEQUENCING

Targeted sequencing is focused on regions statistically associated by previous studies, such as GWAS. In the case of rare mutations with high penetrance, the addition of more variants and increased sample size may be required, involving deep sequencing in a large number of affected individuals. This approach may add markers to those already known.

Recently has been demonstrated that targeted sequencing of pooled samples increases the chance to efficiently and cost-effectively capture all variation in a more limited target region selectively amplified in multiple DNA samples (106, 107). Such an approach allows efficient use of Next Generation Sequencing technologies, which generate billions of base pairs per experiment, yet introducing challenges in data processing and analysis to discover novel variants and assessing their potential association to disease. In AAV, resequencing will be feasible when several variants will be discovered from different GWAS or Immunochip analysis.

WHOLE EXOME SEQUENCING

In the human genome, there are about 18,000 genes, which contain exons and introns. Exons are short, functionally important sequences of DNA which, together, represent only the portion of the genome that is translated in protein. Whole Exome sequencing (a targeted exome capture) is a recent strategy designed to sequence only the coding regions of the genome (which represents 1% of the human genome or about 30 megabases); this is an effective method alternative to whole genome sequencing, cheaper and less complicated in the bioinformatical/statistical analysis.

The whole exome sequencing potentially may identify the coding variants responsible for both mendelian and common diseases (108).

Whole exome sequencing study may be applied to AAV cases for several topics, such as for familial cases, the investigation of extreme phenotypes, for the finding of mutations or rare variants of already known or novel susceptibility genes and for pharmacogenomic study. Furthermore, the increasing number of susceptibility loci will improve bioinformatic pathway analyses. Investigation of genetically modified animals genetically engineered with identified susceptibility genes may also provide more knowledge to the pathophysiology and potentially validate novel treatment targets.

The elucidation of these novel components may have clinical relevance, as they can be included in genetic-risk modeling approaches. Moreover, they might represent novel biomarkers for AAV, enabling physicians to diagnose patients at risk, preferably before the onset of symptoms, which would greatly reduce the overall cost to society and the burden on patients.

Most importantly, the causal variants, or other molecules that have been identified as playing a role in the same pathway, represent new potential therapeutic targets, not only for AAV but also other autoimmune diseases.

META-ANALYSIS AND THE -OMICS

Meta-analysis is an important tool to increase the statistical power and analyze the effect of gene variations across groups of different ancestries. A huge amount of GWAS data is becoming available, opening the possibility that future meta-analyses will estimate effect sizes, determining which genes may predispose to different subsets of AAV. Predictive mathematical models integrating the contributing loci may also be helpful. In addition, it is necessary to understand how specific genetic variants are responsible for the association and the biological effect. Fine mapping, targeted sequencing, transcriptomics, proteomics, and metabolomics may be the future goal for being able to stratify risk patients to truly develop a personalized approach to care.

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REVIEW
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Disruption of a Regulatory Network Consisting of Neutrophils and Platelets Fosters Persisting Inflammation in Rheumatic Diseases

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A network of cellular interactions that involve blood leukocytes and platelets maintains vessel homeostasis. It plays a critical role in the response to invading microbes by recruiting intravascular immunity and through the generation of neutrophil extracellular traps (NETs) and immunothrombosis. Moreover, it enables immune cells to respond to remote chemoattractants by crossing the endothelial barrier and reaching sites of infection. Once the network operating under physiological conditions is disrupted, the reciprocal activation of cells in the blood and the vessel walls determines the vascular remodeling *via* inflammatory signals delivered to stem/progenitor cells. A deregulated leukocyte/mural cell interaction is an early critical event in the natural history of systemic inflammation. Despite intense efforts, the signals that initiate and sustain the immune-mediated vessel injury, or those that enforce the often-prolonged phases of clinical quiescence in patients with vasculitis, have only been partially elucidated. Here, we discuss recent evidence that implicates the prototypic damage-associated molecular pattern/alarmin, the high mobility group box 1 (HMGB1) protein in systemic vasculitis and in the vascular inflammation associated with systemic sclerosis. HMGB1 could represent a player in the pathogenesis of rheumatic diseases and an attractive target for molecular interventions.

Keywords: platelets, neutrophils, HMGB1, inflammation, rheumatic diseases

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NEUTROPHILS, PLATELETS, AND VASCULAR INFLAMMATION

Neutrophils are terminal cells with a relatively short half-life in the circulation. They are effective as a first barrier toward various invading noxae. To carry out this function, neutrophils cross the vessel wall and migrate to the inflamed/injured tissues. This step requires the recognition of P-selectin on the activated endothelium and the asymmetric polarization of the leukocyte β2 integrins that generates the unidirectional movement associated with the infiltration of the surrounding perivascular tissues. These events influence the immune function of transmigrating leukocytes, thus contributing to the overall outcome of the inflammatory response: effective resolution versus persistence of vascular inflammation, healing of the injured vessel wall versus active remodeling, intimal hyperplasia, or aneurism formation. Conversely, transmigrating neutrophils both damage and activate endothelial cells, enforcing a self-sustaining positive feedback loop that contributes for example in patients with systemic small-vessel vasculitis or with systemic sclerosis (SSc) to vascular remodeling and inflammation (1–4).

At sites of infection, neutrophils dispose of invading microorganisms. This action depends partially on the engulfment into a phagosome upon reorganization of the actin-based cytoskeleton and on the activation of the NADPH oxidase system with generation of reactive oxygen species (ROS) (5, 6). The oxygen species combined with the granules microbicidal moieties released into the phagolysosome limits the pathogen viability (6–8). Non-phagocytic neutrophils' microbicide mechanisms have also been described, which involve the release of decondensed chromatin threads in the extracellular space. This phenomenon is referred to as neutrophil extracellular traps (NETs) generation (8–11). Neutrophils preferentially generate NETs when they fail to engulf the pathogen because they are immobilized, tightly adherent to a substrate, or near to apoptosis (8, 12–15). Primary granules fuse with the nuclear membrane, causing the formation of myeloperoxidase–DNA and elastase–DNA complexes (14), while the physicochemical properties of the chromatin change dramatically upon the citrullination of histones by the peptidylarginine deiminase 4 (PAD4) enzyme (16, 17). Both granule redistribution and PAD4-mediated histone citrullination are required for NET generation. During experimental sepsis, NETs play a role in bacterial trapping ensnaring circulating bacteria and restricting their dissemination to distant organs (18).

Neutrophils and platelets colocalize at sites of vascular injury, hemorrhage, and thrombosis. In these conditions, various inflammatory and thrombogenic signals are integrated, resulting in the productive interaction between platelets and leukocytes, yielding the formation of aggregates (15, 19, 20). Neutrophils/platelets heterotypic aggregates depend on platelet P-selectin, are endowed with inflammatory and thrombogenic actions, and represent a shared feature of acute cardiovascular diseases and of systemic inflammatory, neoplastic, and autoimmune diseases (21).

Upon platelet adhesion to damaged vessel walls, P-selectin expressed on their surface facilitates the leukocyte recruitment at the site of vascular injury. Signals activated downstream the recognition of platelet P-selectin promote the generation of ROS (5, 22), the activation of β2 integrins (2, 7, 15, 23, 24), the release of pentraxin 3 from the neutrophil specific (secondary) granules (25), the release of myeloperoxidase from the azurophilic (primary) granules (6, 25, 26), and the *de novo* synthesis and the surface expression of leukocyte tissue factor (1, 2, 21) (**Figure 1**).

Additionally, activated platelets release soluble inflammatory signals, including IL-1β, PDGF, and the prototypic endogenous immune adjuvant, the high mobility group box 1 (HMGB1) protein (5, 27, 28). Finally, the inflammatory action of platelets is amplified and sustained by the release of bioactive microparticles (5, 20, 27, 29, 30). Microparticles comprise small vesicles (usually ranging from 0.05 to 1 μm) shed from activated or dying cells as a consequence of the disruption of the pathway actively maintaining the asymmetry between the phospholipid layers of the plasma membrane. Most microparticles in the blood derive from platelets (30). Platelet-derived microparticles participate in blood coagulation and actively contribute to the inflammatory action of platelets (30). The array of signals expressed, generated, or released by platelets upon activation acts mostly locally influencing the microenvironment. However, these signals possibly influence the leukocyte function in the circulation, in particular in patients with systemic vasculopathy (2, 31).

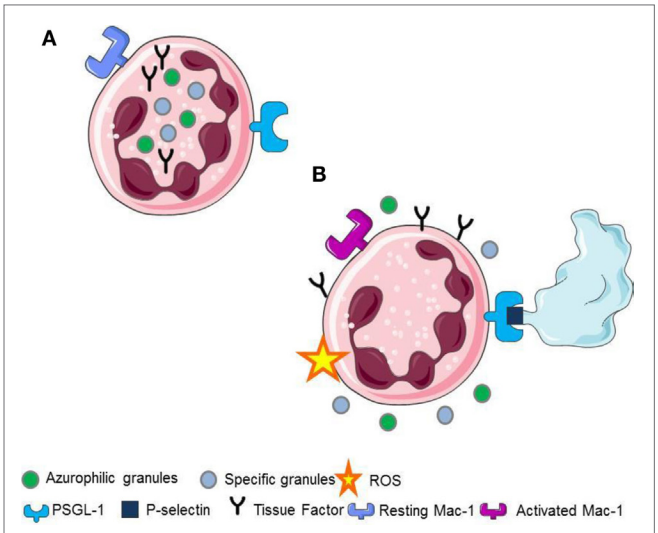

FIGURE 1 | **(A)** Resting neutrophils. **(B)** P-selectin recognizes PSGL1 promoting the generation of ROS, the activation of Mac-1 on neutrophils, the release of pentraxin 3 from the neutrophil-specific (secondary) granules, the release of myeloperoxidase from the neutrophil azurophilc (primary) granules and the surface expression, and the novo synthesis of tissue factor in neutrophils.

Of importance, the dangerous connection between neutrophils and platelets can have different outcomes depending on the context. These outcomes include the phagocytic removal of platelets in physiological conditions (6). Upon inflammatory conditions, adherent neutrophils recognizing activated platelets are committed to NET generation (12, 20, 32).

NETs CONTRIBUTE TO PROPAGATE VASCULAR INJURY AND AUTOIMMUNITY

Neutrophil extracellular traps generation comprises a physiological response of living neutrophils to various stimuli present in a specific environmental context (11, 12, 20, 33) or a form of cell death that is morphologically distinct from apoptosis (34, 35). The mechanisms regulating the type of NET formation seem to depend on the triggering stimuli and on the context of stimulation. They comprise (i) the production of ROS and the induction of autophagy, (ii) the fusion of primary granules with nuclear membrane, (iii) the interaction of elastase and MPO with the DNA, (iv) the citrullination of histones, the chromatin decondensation, and, finally, (v) the nuclear envelope and, eventually, the cell membrane integrity disruption (10, 34, 36) (**Figures 2** and **3**).

The ensuing prolonged exposure of neutrophil microbicidal proteins as well as of citrullinated histones in the extracellular environment could initiate autoimmunity (4, 10, 37, 38). NETs are cleared *via* a mechanism involving DNAses and the first component of the classical pathway of complement activation, C1q. As a consequence, the endocytotic/phagocytotic function of scavenger macrophages limits NETs inflammatory and

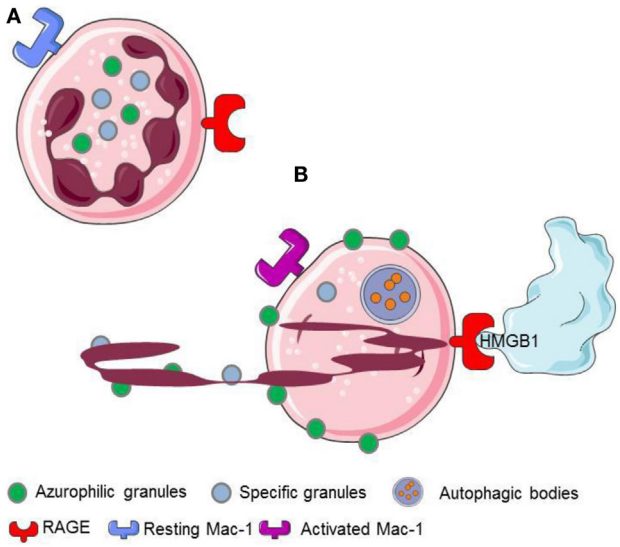

FIGURE 2 | HMGB1 released or expressed by activated platelets recognizes RAGE expressed on neutrophils. **(A)** Resting neutrophils express non activated Mac-1 and RAGE on their surface. **(B)** HMGB1 expreesed by activated platelets induces neutrophils to initiale autophagy, promotes the redistribution of neutrophil granules, induces the transactivation of Mac-1 and elicits the NETs formation.

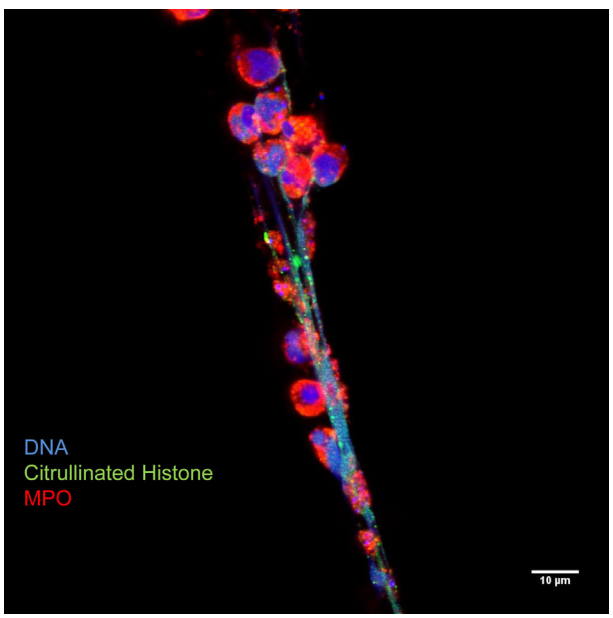

FIGURE 3 | Neutrophil extracellular traps are characterized by decondensed latices of DNA with citrullinated histones decorated with neutrophil granules proteins, such as myeloperoxidase. Originally published by Maugeri et al. (20).

potentially vessel-injuring properties (39, 40). Moreover, persistence of NETs in the tissue might prompt fibrosis *via* induction of myofibroblasts (41).

In contrast, defective clearance of NETs and activation of the alternative pathway of complement activation might be associated with persisting tissue damage and to autoimmunity (10, 42–46). Accumulation in the plasma of byproducts of NET generation/catabolism (such as complexes of DNA-MPO or soluble DNA with citrullinated histones), higher capacity of *in vitro* NETs generation, and an impaired capacity of NETs degradation were observed in patients with systemic lupus erythematosus (SLE), small vessel vasculitis, rheumatoid arthritis, and psoriasis (47), while the association of biologically active moieties, such as tissue factor, TNFα and IL-1β, with NETs might influence their action in the microenvironment (48, 49).

The formation of NETs *in vivo* seems to be directly associated with SLE. Accordingly with this hypothesis, in an animal model of lupus, the effective inhibition of PAD4 results both in a reduction of NET formation and in altered circulating autoantibody profiles, restored complement levels, and reduced glomerular IgG deposition (37, 38, 50).

ENDOGENOUS MECHANISMS MAINTAINING VASCULAR INFLAMMATION: A ROLE FOR HMGB1

High mobility group box 1 has been named after its ability to quickly migrate in polyacrylamide and triton–urea gels, a feature that depends on a high-content of charged amino acid residues. HMGB1 is located on the 13q12 human chromosome. The gene comprises six exons that encode for a 215-amino acid polypeptide, with an apparent molecular mass of 25 kDa. HMGB1 proteins from mammals are nearly identical, indicating that each residue is under selective pressure. In general, the cell type and state of activation influence levels and localization, and more differentiated cells display a lower protein content. HMGB1 consists of a long acidic carboxyterminal region (the "acidic tail") and of two positively charged domains, referred to as "box A" and "box B," that bind to DNA and contain nucleus localization signals. HMGB1 is mostly located in the nucleus of most living cells where it bends DNA, thus facilitating the assembly of proteins, including transcription factors, on their targets. HMGB1 moves constantly from the nucleus to the cytoplasm (51, 52). In response to stress, senescence, or inflammatory signals, HMGB1 is hyperacetylated at two sites in nuclear localization, and this isoform accumulates in the cytoplasm (53). HMGB1 in the cytoplasm promotes autophagy, by which cells recycle internal constituents so as to generate ATP and promote survival under conditions of environmental stress (51).

The interaction with the chromatin in living cells is transient, and HMGB1 plays relevant biological functions in the cytosol, where it behaves as a potent inductor of autophagy (54). Cell death *via* an unscheduled accidental pathway, which associates with the disruption of membrane compartmentalization, results in the redistribution of the molecule at extracellular sites, where HMGB1 behaves as a potent inflammatory signal (51, 55). Most activated cells also mobilize HMGB1. Monocytes, macrophages, and immature myeloid and plasmacytoid dendritic cells (DCs) secrete HMGB1 in response to primary inflammatory signals. Anucleated platelets also contain and upon activation release substantial amount of HMGB1, either as a soluble moiety or associated with microparticles (5, 20, 27, 28). HMGB1 released

or expressed by activated platelets commits neutrophils to autophagy (20), promotes the redistribution of neutrophil granules (5), induces the Mac-1 transactivation (5), and elicits the NETs formation (20, 56, 57) (**Figure 2**).

Posttranslational modifications, such acetylation, phosphorylation, methylation, and oxidation/reduction (58–61), and the interaction with other bioactive molecules, including LPS or chemokines (62, 63), CXCL12 in particular (64, 65), all influence the function of extracellular HMGB1 (5, 58, 66). The redox status of the three cysteine residues of the molecule (C23, C45, and C106) apparently dictates the sequential events of leukocyte recruitment, activation, and resolution of inflammation (59). The characteristics of HMGB1 biology, including its association with various events important in the natural history of vasculitis, such as necrosis, granuloma formation, and leukocytes survival and activation, as well as its ability to regulate inflammation and tissue repair and remodeling, make the protein a candidate player in this family of diseases.

Acute vascular inflammation has a well-characterized homeostatic role. Defects in the program result in self-sustaining vascular inflammatory diseases, referred to as vasculitis. Indeed, an unrelenting inflammatory process mostly restricted to the vessel wall characterizes large vessel vasculitis [Takayasu arteritis, giant-cell arteritis (GCA)] (3, 67). The productive interaction between activated adventitial DCs and T cells is an early and crucial event. The local production of T cells cytokines eventually results in IFNγ-mediated activation of macrophages and in the formation of giant cells at the intima-media junction. Giant cells and activated macrophages produce growth factors (such as vascular endothelial growth factor and platelet-derived growth factors), which sustain intimal hyperplasia and contribute to subsequent end organ ischemia (68). Circulating blood cells are also activated and might contribute to the clinical picture. For example, thrombocytosis is frequent in GCA patients (69, 70), and aspirin protects patients from cranial ischemic complications (1, 71–74). Aspirin-resistant events are, however, quite frequent, and platelet count does not identify patients at higher risk of severe ischemic events (74–76).

Blood cells of GCA patients express tissue factor, a key molecule in thrombin formation downstream activation of Factor VII, and display a greater fraction of platelets expressing P-selectin, which is associated with a procoagulant state (1, 77). Specific clinical features or the extent of biomarkers of systemic inflammation, which, however, may fail to reveal the extent of ongoing smoldering vascular inflammation (78), do not apparently influence these features (3). Despite extensive investigations, markers reflecting not exclusively inflammation but the extent of the process taking place in the affected vessels have proved elusive, with the possible exception of the soluble pattern recognition receptor PTX3, which is produced in peripheral tissues in response to signals of injury by innate immune cells, such as DCs and macrophages (79, 80).

Dendritic cells and macrophages are a critical source of HMGB1, which shapes their functional polarization and migratory properties (81–85). While PTX3 plasmatic levels seem to be associated with the entity of the disease (79, 86, 87), concentrations of HMGB1 in the blood are not an effective biomarker of large vessel vasculitis (88). Indeed, patients with Takayasu's arteritis and GCA present similar serum HMGB1 levels compared with healthy controls and seem unrelated to disease activity (88).

However, it should be considered that several posttranslation modifications influence the bioactivity of the molecule. Specifically environmental conditions, such as the redox status, influence HMGB1 inflammatory action, causing the shift from a moiety that mostly causes leukocyte recruitment or to a signal that elicits the secretion of inflammatory cytokines (53), see above. As such, the total concentrations of the molecule in the blood might not reflect the actual fraction of the bioactive molecule (5, 53, 59). When potent inflammatory molecules are released in the environment, inhibitors are often physiologically generated, like it occurs for the primary inflammatory cytokines, TNFα and IL-1β. The identification of putative HMGB1 inhibitors requires further study. The development of analytical techniques to discriminate among the various forms of HMGB1 might allow to dissect the actual HMGB1 involvement in the various facets of vascular inflammation: effective repair of injured vessels, angiogenesis, persistent inflammation with extensive remodeling, aneurysm formation, development of atherosclerotic lesions, complications associated with their disruption, etc. (89).

Leukocytoclasia (i.e., the presence of of uncleared leukocyte debris within and around the vessel wall), small-vessel thrombosis, necrosis, and hemorrhage in target organs (mainly the skin, the kidneys, and the airways) are hallmarks of small-vessel vasculitis. Immune complexes play a major role in eliciting vascular inflammation during some small-vessel vasculitis (IgA vasculitis or cryoglobulinemia, for example), and immunoglobulin and complement deposition at the site of vascular injury accompanies in these patients' leukocytoclasia. In contrast, a "pauci-immune" inflammation, without local immunoglobulin or complement deposition, characterizes vasculitis syndromes associated with antineutrophil cytoplasmic antibodies (ANCA-associated vasculitis). Elevated levels of plasmatic HMGB1 have been found in patients with small-vessel vasculitis, including IgA vasculitis, Kawasaki's disease, and ANCA-associated vasculitis (90–93). The concentration of plasmatic HMGB1 is elevated in the active phase of systemic vasculitis, and the concentration of HMGB1 is higher in patients with granulomatosis with polyangiitis with a predominantly granulomatous disease (94). In contrast, conventional markers of inflammation or the validated disease activity score, BVAS, fail to discriminate between the two groups of patients (94). The result well fits the preferential expression of the HMGB1 in the granulomatous tissue (94) and suggests that systemic levels might actually reflect local *in situ* production. HMGB1 levels are also been described to be higher in patients with active renal involvement, a threatening manifestation of the disease (90). Levels of HMGB1 are still elevated in patients with a quiescent nephritis, possibly indicating a persistent low-grade inflammation that persists in the subclinical phases of the disease (90). Urinary levels of HMGB1 represent a robust biomarker of active glomerulonephritis in patients with ANCA-associated vasculitis (42, 95). Actually, urinary HMGB1 might represent a more solid biomarker of kidney involvement in ANCA-associated vasculitis than serum HMGB1 (96).

The preferential involvement of HMGB1 in ANCA-associated vasculitis might be related to its ability to regulate the activation

state and function of neutrophils, which are the key cells in the pathogenesis of these diseases. Of interest, HMGB1 could contribute as inflammatory priming of neutrophils in circulation, inducing translocation of ANCA antigens at cell membrane, providing the substrate of antigen–antibody interactions (97).

The recognition of extracellular HMGB1 dramatically influences several characteristics of neutrophils, a key population in ANCA-associated small-vessel vasculitis. It induces a swift redistribution of intracellular vesicles, an event that might be associated with the ability to activate neutrophil autophagy (5) through the putative HMGB1 receptor, the receptor for advanced glycation endproducts (RAGE) (**Figures 2** and **4**).

The redistribution of the granule in response to primary inflammatory stimuli allows the exposure of ANCA antigens (namely, myeloperoxidase and proteinase 3) on the neutrophil plasma membrane, where they become accessible for interaction with the ANCA autoantibodies because of a preferential location at lipid rafts that also contain β2 integrins, signalling molecules, cross-linked Fcgamma receptors, and NADPH oxidase. ANCA, in turn, amplify the activation of the neutrophil, which is transmigrating, favouring a vigorous and untimely response, with oxidative burst and premature degranulation (97, 98). HMGB1 might, thus, act on neutrophils favouring the exposure of ANCA antigens and facilitating the further neutrophil activation caused by the antigen recognition by ANCAs. Of importance, HMGB1 has been recently shown to potentiate the NETs formation induced in the presence of ANCAs (99).

HMGB1 AND DIABETIC VASCULOPATHY

High mobility group box 1 elevation appears as a relatively shared feature in patients with an inflammatory vascular involvement. This applies not only to other primary vasculitides (91, 100, 101) but also to other systemic diseases characterized by extensive inflammatory vessel involvement (102). Diabetes mellitus represents a privileged scenario for the study of the role in vascular inflammation of HMGB1 and of the RAGE receptor. The systemic HMGB1 concentration is consistently elevated in diabetic patients and in animal models of the disease (103, 104). HMGB1 might contribute to the accelerated atherosclerosis, which is a hallmark of diabetes mellitus (105–107). Hyperglycemia is an effective stimulus leading the release of HMGB1, which in turn might play

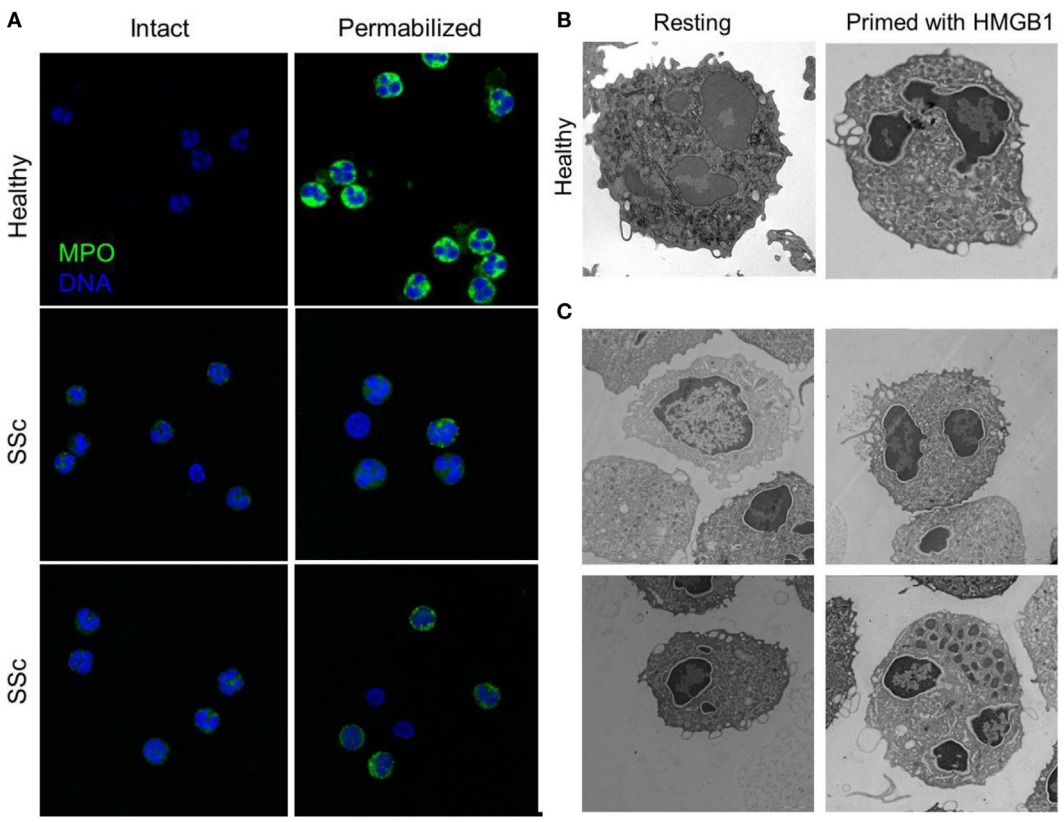

FIGURE 4 | **Peripheral granules distribution characterized neutrophils of patients with SSc**. **(A)** The expression of MPO (green) in the blood neutrophils of patients with SSc and of matched controls has been analyzed by confocal microscopy before and after permeabilization of the plasma membrane, to allow the access of the mAb. MPO intracellular expression is substantially lower in SSc patients and appears to cluster at the plasma membrane of intact, non permeabilized neutrophils. **(B,C)** Representative images by electron microscopy of neutrophils from a healthy control, untreated or treated with HMGB1 **(B)** or of untreated neutrophils from four SSc patients **(C)** showing the extensive remodeling of intracellular granules, most of which acquire a pericellular distribution, characterizes SSc neutrophils and healthy neutrophils treated with HMGB1. Images originally published by Maugeri et al. (5).

a role in the failure of tolerance in diabetes mellitus type 1 (108, 109) and in the early rejection of transplanted islets (110, 111). Furthermore, glycated albumin is recognized by RAGE inducing the neutrophil activation and release of NETs (20).

HMGB1 AND SYSTEMIC SCLEROSIS

Systemic sclerosis is an immune-mediated multisystem disease, characterized by a diffuse obliterative microvasculopathy and by fibrosis of the skin and of visceral organs. The abnormal generation of ROS observed in patients with SSc contributes fostering autoimmunity, fibrosis, and vascular inflammation. Recently, the presence of an increased concentration of platelet-derived microparticles (PDμP) bearing HMGB1, P-selectin expressing platelets (5, 27), the redistribution of the content of primary granules, and the transactivation of β2 integrins leukocytes was observed in blood cells of SSc patients (**Figure 4**). P-selectin (purified or expressed on activated platelets) induces the ROS generation by neutrophils, which in turn cause the oxidation of the HMGB1 expressed by PDμP. Oxidation amplifies the ability of HMGB1-expressing PDμP to activate neutrophils, favoring the redistribution of molecules present in the neutrophil primary granules to the plasma membrane and the transactivation of β2 integrins. Leukocyte activation caused by oxidized extracellular HMGB1 abates in the presence of inhibitors of HMGB1 or of catalase, which catalyzes the dismutation of hydrogen peroxide into water and molecular oxygen. Neutrophils from healthy donors challenged with HMGB1-expressing PDμP purified from SSc patients, but not those purified from control subjects, reproduce the phenotype of neutrophils of SSc patients, whereas HMGB1 inhibitors reverse the effects of microparticles (5, 27). These results suggest that HMGB1 might represent a crucial signal in the cross talk between platelets and leukocytes in SSs, thus sustaining microvascular inflammation. Its ability to promote epithelial and endothelial to mesechimal transition might further link vascular inflammation to the other prominent feature of SSc, fibrosis (112).

HMGB1: A PLAYER IN ANGIOGENESIS AND THROMBOSIS

Platelet-derived HMGB1 appears as a crucial signal in the cross talk between platelets and leukocytes with potent and specific effects in the regulation of the ability of neutrophils to generate NETs and to activate the autophagic flux (20).

Neutrophil extracellular traps have a well-characterized role in thrombosis, and HMGB1 appears as a player in coronary thrombi formation in patients with acute myocardial infarction (20). A primary role of platelet-derived HMGB1 in thrombosis induction has been confirmed in an elegant genetic model relying on transgenic mice in which the molecule has been specifically ablated (56).

Mechanical or immune-mediated injury of vessels and ischemia/reperfusion cause HMGB1 release (113–117). HMGB1 blockade substantially improves the clinical outcome in several such models, indicating that HMGB1 broadcasts news of ongoing tissue injury and is involved in the ensuing inflammatory response. HMGB1 acts on virtually all cell populations involved in vascular inflammation. It is produced by injured endothelial cells and attracts endothelial cell precursors, which favor neovascularization. HMGB1 overexpression activates a pro-angiogenic program in endothelial cells, mediated *via* the increased activity of matrix metalloproteinases, of intregrin receptors, and the activation of the NF-κB pathway (118).

Thus, HMGB1 might represent a crucial event to switch the homeostatic inflammatory response to acute vessel injury to self-sustaining vasculitis (3). DCs play a critical role in the establishment of small vessel vasculitis (85, 119–121) and in the vessel wall inflammation, which characterize large vessel vasculitis (122, 123). HMGB1 prompts its own autocrine/paracrine release, enforcing a vicious circle, which is further amplified by other cytokines known to elicit HMGB1 release, including IL-1β and TNF-α (55, 82, 83). Finally, the ability of HMGB1 to prompt angiogenesis [see above and Ref. (124)] and to attract vessel-associated stem cells (125) might contribute to intimal hyperplasia/neo-angiogenesis, typical of vessel remodeling during large artery vasculitis.

The events that are implicated in this amplificatory loop associated are not completely characterized. For example, pericellular myeloperoxidase distribution could directly implement the HMGB1/RAGE pathway (5), since the myeloperoxidase system of human neutrophils generates *N″*-(carboxymethyl) lysine, a highly reactive advanced glycated end product and RAGE–ligand, at sites of inflammation (126).

Thrombosis is a common and often underestimated complication of ANCA-associated vasculitis (127, 128). Thrombosis occurs as a clinically apparent event and can often in active lesions biopsies be identified at the microscopic levels (129). A study comparing platelet and neutrophil activation of patients with acute coronary syndromes and autoimmune diseases demonstrated that the average of neutrophil myeloperoxidase content in patients with ANCA-associated vasculitis is similar to the one observed in patients with no segment T elevation myocardial infarction or unstable angina, while the fraction of neutrophil expressing the activated isoform of Mac-1 and platelets expressing P-selectin is similar to all acute coronary syndromes studied (26). Enhanced concentrations in the blood of markers of platelet activation, soluble P-selectin, and CD154 directly correlate with disease activity in large cohort of patients with granulomatosis with polyangiitis (130) [see also Ref. (26)]. P-selectin and CD154 are both involved in the physical interaction and mutual activation of platelets and neutrophils. Their increased turnover in patients with ANCA-associated vasculitis might reflect the link between vascular damage and thrombosis (3). Disrupted endothelial layers recruit and activate platelets, with ensuing activation of the coagulation system cascade. Moreover, platelet P-selectin expression compensates for the lack of endothelial P-selectin, making neutrophil rolling and extravasation possible [discussed in Ref. (7, 131)]. Neutrophil activation implies the release of protease, which contribute to platelet P-selectin and CD154 cleavage, whose circulating levels consequently increase.

High mobility group box 1 acts as a prototypic agonist for a variety of innate receptors, including RAGE, TLR2, TLR4, TLR9, TREM1, and Mac-1. *Via* these receptors, HMGB1 in pathological conditions perturbs vessel integrity and contributes to maintain the vicious cycle by which the inflamed endothelium increases the adhesion and the transmigration of leukocytes, and leukocytes, in

turn, sustain the activation of endothelial cells, eventually leading to cell death and to the activation of programs that might sustain further vessel injury and thrombosis, such as the generation of NETs (132–134).

ENDOTHELIAL RESPONSE TO HMGB1

Endothelial cells express are exquisitely sensitive to extracellular HMGB1. They express an array of HMGB1 receptors, which comprises RAGE, TLR2, TLR4, TREM1, proteoglycans, and thrombomodulin. The outcome of HMGB1 recognition by endothelial cells dramatically differs depending on which receptors are activated in the various conditions (135). A net activatory effects apparently ensues TLR2 or TLR4 activation, as assessed by the upregulation of adhesion molecules, by the production of cytokines, by the increased vascular permeability, by the activation of the coagulation system, resulting in certain conditions in microvascular thrombosis. HMGB1 not only *per se* activates endothelial cells (136, 137) but also behaves as a general adaptor of the ability of the endothelia to response to various sterile noxious signals. For example, uric acid recruits a complex series of events, including the enhanced expression of the HMGB1-mRNA, the acetylation of HMGB1, its translocation to the cytoplasm, and eventual release. In turn, HMGB1 recognition activates a positive feedback loop causing further HMGB1 expression and release (117).

The outcome of HMGB1 recognition by endothelial cells is finely regulated: this is expected, given the abundance of the molecule and the relatively easy access to the extracellular environment in case of cell activation or death. This might imply the recruitment of pathways that protect the host against the inflammatory action of endogenous components, in particular the CD24–Siglec pathway (138, 139) or the thrombomodulin-dependent pathway. Thrombomodulin is an evolutionary conserved glycosylated type I transmembrane protein with multiple functional domains, which is expressed by endothelial cells, endowed with anticoagulant actions. The thrombomudulin/thrombin complex activates protein C and in the presence of protein S interferes with factors VIIIa and Va and quenches thrombin generation (140). Thrombomulin ensures vessel homeostasis under stress and a rapid and localized inflammatory response to injury. Indeed, besides thrombin, thrombomodulin interacts *via* independent domains with various other molecules, including fibrinolysis inhibitors, complement components, and HMGB1. The interaction of HMGB1 with the lectin-like domain of thrombomodulin attenuates inflammation. This might be due to interactions with intermediary proteins that quench the endothelial cell activation (140, 141). Moreover, the binding to the lectin-like domain of thrombomodulin might limit HMGB1 binding to RAGE, thus impairing NF-κB activation (141). Thrombomodulin also enhances thrombin-mediated proteolytic degradation of HMGB1, reducing its pro-inflammatory activity (142). Since HMGB1 has been linked to the pathogenesis and/or progression of a large range of clinical disorders characterized by endothelial dysfunction, including sepsis and autoimmune diseases, the identification of thrombomodulin as a natural inhibitor of HMGB1 is of clinical importance (140, 142, 143) (**Table 1**).

TABLE 1 | Comparison of some features of vascular inflammation in sepsis and ANCA-associated small-vessel systemic vasculitis.

	Sepsis	Vasculitis
Platelet count	Frequently low	Normal
Neutrophil count	High	Normal
Platelet activation	Yes	Yes
Neutrophil activation	Yes	Yes
Apoptotic neutrophils in circulation	Yes	No
Endothelial activation	Yes	Yes
Plasma thrombomodulin level	Low	Normal or high
HMGB1	High	High
NETs	High	Not documented in all types of vasculitis

During acute phases of vessel inflammation, thrombomodulin expression on the endothelial surface decreases because of at least two mechanisms: (i) internalization by endocytosis (144) or (ii) cleavage by enzymes like neutrophil elastase or cathepsin G (145, 146). Indeed, high levels of plasma thrombomodulin charaterize patients with systemic vasculitis [e.g., see Ref. (147–149)]. Whether the soluble cleaved thrombomodulin maintains the ability to bind to HMGB1 and whether the complex retains biological activities remain to be established.

Several other mechanisms possibly contribute to quench the inflammatory and thrombogenic actions of HMGB1 in the blood. For example, the vagus nerve is a part of a reflex that prevents or neutralizes excessive inflammation in response to tissue injury and infection. Sepsis is a prototypical condition in which an early unrestrained production of cytokines initiates a systemic response involving chemokines, amines, and activation of the complement and of the coagulatory systems eventually leading to disrupted vascular integrity, hypotension, and shock. HMGB1 is a recognized player in the late phases of sepsis. Pioneering studies have shown that stimulation of the vagus nerve or administration of cholinergic agents or selective agonists of the alpha7 nicotinic acetylcholine receptor abate HMGB1 systemic levels and improve animal survival in endotoxaemia or upon cecal ligation and puncture, a standardized model of septic peritonitis. Activation of the alpha7 nicotinic acetylcholine receptors represents a key event in the anti-inflammatory reflex, since it could be responsible for NF-κB nuclear translocation inhibition and thus for the restoration of homeostasis *via* suppression of pro-inflammatory cytokines generation and release (150, 151).

Of importance, this homeostatic system is activated even in sterile conditions, limiting the tissue damaging actions of NF-κB activation and HMGB1 expression in response to heart or hepatic ischemia–reperfusion injury (152, 153). The involvement of this pathway in systemic vasculitis has not been studied extensively so far. However, the accumulating evidence on the role that HMGB1 plays in persisting vascular inflammation (see above) and the possibility to pharmacologically exploit its anti-inflammatory actions (154) suggest that these

studies might underpin the development of novel and effective therapeutic strategies.

CONCLUDING REMARKS

High mobility group box 1 is the best characterized alarmin, and its recognition plays a role that is more and more appreciated in several apparently unrelated conditions, in which inflammation does not abate with the original noxa but *per se* causes self-sustaining cell and tissue damage. Many factors contribute to make systemic vasculitis a particularly attractive scenario to dissect the complex biology of HMGB1. These include the characteristics of the pathogenesis of vasculitis, which stem from a deregulated interaction between leukocytes, endothelial cells, and vessel wall cells, and the increasing understanding of the mechanisms that physiologically regulate the homeostatic response of vessels to injury. Increasing knowledge of the immunobiology of HMGB1 will form a foundation for novel targeted immune strategies aimed at specifically targeting the early events in the natural history of vasculitis.

AUTHOR CONTRIBUTIONS

NM, PR-Q, and AM selected the bibliography and wrote the manuscript.

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Vascular endothelium as a target of immune response in renal transplant rejection

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This review of clinical and experimental studies aims at analyzing the interplay between graft endothelium and host immune system in renal transplantation, and how it affects the survival of the graft. Graft endothelium is indeed the first barrier between self and non-self that is encountered by host lymphocytes upon reperfusion of vascularized solid transplants. Endothelial cells (EC) express all the major sets of antigens (Ag) that elicit host immune response, and therefore represent a preferential target in organ rejection. Some of the Ag expressed by EC are target of the antibody-mediated response, such as the AB0 blood group system, the human leukocyte antigens (HLA), and MHC class I related chain A antigens (MICA) systems, and the endothelial cell-restricted Ag; for each of these systems, the mechanisms of interaction and damage of both preformed and *de novo* donor-specific antibodies are reviewed along with their impact on renal graft survival. Moreover, the rejection process can force injured EC to expose cryptic self-Ag, toward which an autoimmune response mounts, overlapping to the allo-immune response in the damaging of the graft. Not only are EC a passive target of the host immune response but also an active player in lymphocyte activation; therefore, their interaction with allogenic T-cells is analyzed on the basis of experimental *in vitro* and *in vivo* studies, according to the patterns of expression of the HLA class I and II and the co-stimulatory molecules specific for cytotoxic and helper T-cells. Finally, as the response that follows transplantation has proven to be not necessarily destructive, the factors that foster graft endothelium functioning in spite of rejection, and how they could be therapeutically harnessed to promote long-term graft acceptance, are described: accommodation that is resistance of EC to donor-specific antibodies, and endothelial cell ability to induce Foxp3+ regulatory T-cells, that are crucial mediators of tolerance.

Keywords: endothelial cell antigens, angiotensin II type 1 receptor, vimentin, accommodation, regulatory T-cells, renal transplantation, antibody-mediated rejection, mTOR inhibitors

INTRODUCTION

Over the last few decades, the practice of kidney transplantation has improved in many areas up to becoming the optimal treatment for end-stage renal disease (1). However, despite brilliantly achieving a 95% 1-year survival, long-term outcomes have not benefited from such improvements and remain unsatisfying (2). One of the major causes of late graft loss is occurrence of antibody-mediated rejection (ABMR), which current immunosuppressive regimens have mostly proven to be unable to cure (3). Another issue is the development of accelerated cardiovascular disease, which is due to the side effects inherent in the immunosuppressive drugs and, along with opportunistic infections and malignancies, represents the first cause of recipient death (4).

Allograft endothelium is the first barrier between self and non-self in vascularized solid-organ transplantation, and preservation of its integrity and functions is mandatory to ensure a prolonged survival of the graft (5). As endothelial cells (EC) express a number of antigens (Ag) that are visible by the immune system of a genetically disparate individual, donor endothelium is invariably recognized by the host immune system, and therefore, it is the first and preferential target of the allo-immune response that follows organ transplantation without an adequate immunosuppression (6).

Both naturally occurring and induced allo-antibodies directed to the Ag expressed on the membrane of EC are commonly found in renal recipients, and such antibodies, being capable of fixing the complement and damaging the tissues, are detrimental for the correct functioning of the endothelium (7). Moreover EC, besides being target of antibody-mediated response, can directly interact with allogenic T-cells by displaying not only the major histocompatibility complex (MHC) antigens but also adequate co-stimulatory molecules and adhesion proteins on their surface (8).

Nevertheless, the host immune response that follows recognition of EC allo-Ag is not necessarily destructive, in spite of graft rejection, accommodation, where not active tolerance, may operationally establish, thus fostering the endothelium to fulfill its functions (5). Endothelial regulation of blood flow and vessel permeability is paramount not only for the survival of any vascularized allograft but also for the specific depurative activities of the kidneys.

This review aims at analyzing the interplay between allograft endothelium and host immune system, and how differential unfolding of this interplay may ultimately affect the fate of the graft. A particular attention will be paid to the factors that, at the endothelial level, contribute to tipping the balance in favor of graft acceptance rather than rejection.

DISCUSSION

ANTIBODY-MEDIATED IMMUNE RESPONSE TOWARD ALLOGRAFT ENDOTHELIAL CELLS

The importance of donor-specific antibodies in causing allograft rejection has progressively been uncovered, to the extent that a humoral theory of transplantation has been formulated in juxtaposition to the cellular one (9). Here, all the sets of Ag expressed on human EC that are relevant for kidney transplantation will be discussed (**Table 1**), along with the mechanisms of damage and accommodation.

ENDOTHELIAL CELL ANTIGENS TARGET OF ALLO-ANTIBODIES

AB0 blood group antigens

Endothelial cells highly express the AB0 blood group antigens on their surface (10). Such Ag are carbohydrates linked to glycoproteins and glycolipids and are targets of specific Ab (isoagglutinins), which occur naturally in people lacking the A and/or B antigens (11). Isoagglutinins, upon binding to A/B incompatible EC, cause hyperacute or accelerated acute graft rejection (12). AB0 compatibility has, therefore, been required for successful cadaveric transplantation; however, being the allelic variability of this system little, it does not represent a barrier for allocation of deceased donor organs. On the other hand, due to the allelic frequencies, any two individuals have roughly a 35% probability of being AB0 incompatible (AB0i), and this is an actual limitation to living renal donations (11). A first breach to the absolute requirement for AB0 compatibility emerged soon after the recognition of AB0 Ag as a barrier for solid-organ transplantation; the analysis of the outcomes showed that acceptable results were only obtained when renal grafts from A2 donors had been transplanted to non-A, i.e., 0 or B, recipients (12, 13). This donor-recipient combination proved somehow permissive because of the scarce expression of A2 Ag on EC, and the consequent low titers of anti-A2 Ab in non-A2 recipients (13). A second breach was the good results reached with AB0i heart transplants in children, who are known to express lower amount of A/B Ag and produce less Ab compared to adults (14). Finally, in 1981, the report of a successful rescue treatment for a mistakenly performed AB0i renal transplantation was published (15); the concept of removal of the isoagglutinins with plasmapheresis in order to avoid hyperacute rejection laid the basis to current practice that aims at reducing anti-A/B Ab titers before and soon after transplantation as a strategy to overcome AB0 barrier (16). Since then, kidney transplantation from AB0i living donor has become a routine practice in many transplantation centers (17).

Human leukocyte antigens

Major histocompatibility complex antigens, also known as human leukocyte antigens (HLA) in human beings, are highly polymorphic surface proteins whose principal function is to display Ag to T-cells for recognition and activation. Two different classes of HLA molecules exist: class I molecules are constitutively expressed by all cell types and present Ag to CD8+ cytotoxic T-cells; class II molecules, that present Ag to CD4+ helper T-cells, are commonly restricted to professional antigen-presenting cells (APC) such as dendritic cells, but, upon stimulation, can be induced onto other cell types. Human EC highly express class I and, albeit at lesser extent, also class II HLA molecules, which can be further enhanced by appropriate stimuli of inflammatory and immunologic origin (6, 7).

Apart from presenting Ag to lymphocytes, HLA molecules can themselves be recognized by an allogenic immune system, and anti-HLA Ab are produced following immunizing events like pregnancies, blood transfusions, and organ transplantation (18). Preformed anti-HLA donor-specific Ab (DSA), i.e., DSA present prior to transplantation, have long been known to be responsible for hyperacute or accelerated acute graft rejection, which is determined by mechanisms similar to those for AB0i transplantation performed without an adequate desensitization (19). Nevertheless, due to the extreme polymorphism of HLA genes, donor-recipient matching is an exceptional occurrence; therefore, unlike bone marrow transplantation, most of the kidney transplants are performed across the HLA barrier, for which a profound immunosuppression is lifelong required. In order to eliminate the risk of hyperacute rejection, laboratory cross-matching techniques have been developed, and routinely applied, to identify preformed anti-HLA Ab in the serum of recipients before transplantation; a

Table 1 | Antibody-mediated immune response toward allograft endothelial cells.

Type of immunity	Target Ag on EC	Preformed Ab	*De novo* Ab	C^1 fixing Ab	Hyper- or Accel.-AR	Acute rejection	Chronic rejection	Detected by current XM	Reference
Allo-Ab	AB0	Yes	Yes	Yes	Yes	Yes	Yes	–	(10, 12, 16, 17)
	HLA	Yes	Yes	Yes	Yes	Yes	Yes	Yes	(6, 7, 18–26)
	MICA	Yes	Yes	Yes	Yes	Yes	Yes	No	(28–33)
	ECA	Likely yes	Yes	Likely yes	Likely yes	Yes	Yes	No	(35–51)
Auto-Ab	ATR1	No	Yes	No	No	Yes	Yes	No	(56, 57)
	Vimentin	No	Yes	No	No	No	Yes	No	(59, 63)

Ag, antigens; EC, endothelial cells; Ab, antibody; C^1, complement; Accel., accelerated; AR, acute rejection; XM, cross-match; ECA, endothelial cell-restricted antigens; ATR1, angiotensin II type 1 receptor.

positive donor-recipient HLA cross-match would currently halt the organ allocation in the absence of adequate desensitization (20). Moreover, anti-HLA DSA can occur *de novo* after transplantation mainly as a consequence of suboptimal immunosuppression or scarce adherence to the therapy (21). *De novo* DSA, particularly if complement-fixing Ab, is pathogenic for both acute and chronic rejection of the allograft (22); and the presence of such Ab in recipient's serum has prospectively been linked to graft failure in several studies (23). The EC of graft peritubular capillaries (PTC) are the preferred targets of DSA so much so that microvascular inflammation is a required criterion for histopathologic diagnosis of ABMR (24), which is further underpinned by the presence of deposits of the complement fragment C4d on PTC endothelium (25, 26), and of circulating DSA (24).

MHC class I related chain A antigens

The description of sporadic cases of hyperacute or accelerated acute rejection of non-AB0i kidneys in recipients lacking anti-HLA DSA have urged researchers to investigate further sets of allo-antigens that might be relevant for transplantation (27). MHC class I related chain A antigens (MICA) are surface glycoproteins encoded by highly polymorphic genes located on chromosome 6 within the region of MHC genes (28). MICA, whose function is related to immune surveillance, are expressed by different types of cells including EC, but importantly neither T nor B lymphocytes; thus, current standard cross-match procedures are unable to detect anti-MICA antibodies (28). MICA have proven to be the target of complement-fixing allo-Ab that can cause hyperacute, acute, and chronic ABMR (28–32); the presence of anti-MICA DSA negatively impact short-term and long-term graft survival, albeit at lesser extent compared to the effect of anti-HLA DSA (30). Endothelium damage, microvascular inflammation, and C4d deposition on PTC endothelium are the hallmark also of ABMR mediated through anti-MICA DSA (33).

Non-HLA nor-MICA endothelium-restricted antigens

Along with MICA, other non-HLA systems are thought to add to the gamut of the traditional transplantation Ag (34). Indeed, ABMR may exceptionally occur following renal transplantation between non-AB0i HLA-identical siblings, who usually also share MICA, being them in *linkage disequilibrium* with the HLA genes (28).

Between 1997 and 2005, endothelium-restricted antigens (EA), expressed neither by lymphocytes nor by monocytes, were proposed as possible targets of pathogenic Ab in renal recipients who had experienced acute ABMR without any obvious DSA (35–40). Following these results, the suggestion to adopt newer cross-matching techniques that would investigate the presence of these anti-endothelial cell antibodies (AECA) in the recipient's serum before transplantation has become stronger (41–46). Meanwhile, the first studies have come out and shown an association between circulating AECA and acute rejection, chronic rejection, poor renal graft survival, and transplant glomerulopathy (47–51).

As for the identity of EA, it is still ill defined, despite the application of proteomic, protein microarrays, and transcriptome measures (52). The most relevant information we have is that EA are expressed only by activated or damaged EC (53). This observation has lead some authors to hypothesize that the EA, or at least some of them, might actually be self-molecules rather than allo-antigens, and AECA would be auto-Ab that arise following the exposure of these cryptic self-Ag on EC primarily hit by host immune response, and they would cooperate to graft destruction with allo-immunity (54, 55). Two examples of self-Ag displayed on EC and targeted by host immune response following transplantation are angiotensin II type 1 receptor and vimentin.

Angiotensin II type 1 receptor. In 2005, Dragun et al. linked the presence of anti-angiotensin II type 1 receptor (AT1R) Ab to acute rejection of non-AB0i kidneys in 16 recipients without anti-HLA or anti-MICA DSA (56). Anti-AT1R Ab were studied because malignant hypertension was part of the clinical picture in all the rejecting patients, thus somehow resembling preeclampsia, a condition the researchers had already linked to the presence of such Abs (57). AT1R is present on EC and vascular smooth muscle cells and, upon ligation of angiotensin II, elicits transduction of secondary signals that contribute to the regulation of body liquids and blood pressure. Anti-AT1R Ab are agonistic non-complement-fixing immunoglobulins that promote malignant hypertension by over-activating AT1R (47); moreover, anti-AT1R Ab can induce microvascular inflammation and coagulation by stimulating NF-kB pathway and tissue factor expression by EC (58). In accordance with these data, graft biopsies from the renal recipients in Dragun's study lacked C4d deposition, but revealed the presence of endoarteritic lesions, fibrinoid necrosis, and thrombi. The authors finally provided evidence of the pathogenic role of anti-AT1R Ab as their removal with plasmapheresis and selective blockade of AT1R with losartan significantly improved graft survival (56). The origin of such Abs is not clear yet, but, as no polymorphism of the AT1R gene has been identified, they could be auto-Ab occurring due to molecular mimicry or to anomalous presentation of over-expressed AT1R on damaged EC (58).

Vimentin. Vimentin is a cytoskeleton intermediate filament protein present within the cytosol of cells of mesenchymal origin, such as EC, fibroblasts, and leukocytes. Anti-vimentin auto-Abs (AVA) are described in a number of autoimmune diseases (59). In organ transplantation, exposition of vimentin isoforms on apoptotic EC, irreparably damaged by the allo-immune response, results in break of self-tolerance, emergence of active vimentin-specific CD8+ T-cells, and production of AVA (59, 60). AVA have been found in heart as well as kidney transplantation (61); albeit capable of fixing the complement and activating platelets (62), AVA alone are not pathogenic, they instead contribute with the allo-immune response to cause the vascular lesions typical of chronic rejection, and to accelerate the progression of atherosclerotic lesions (63).

FOCUS ON THE MECHANISMS OF REJECTION TOWARD GRAFT ENDOTHELIAL CELLS

Nature and abundance of the allo-Ag expressed on EC, and type and titers of the DSA are the determinants of the intensity of host antibody-mediated immune response toward the allograft (**Figure 1**). This evidence has both theoretical and practice consequences. First, AB0 Ag, carbohydrates in nature, are less immunogenic than HLA molecules that are proteins, and indeed

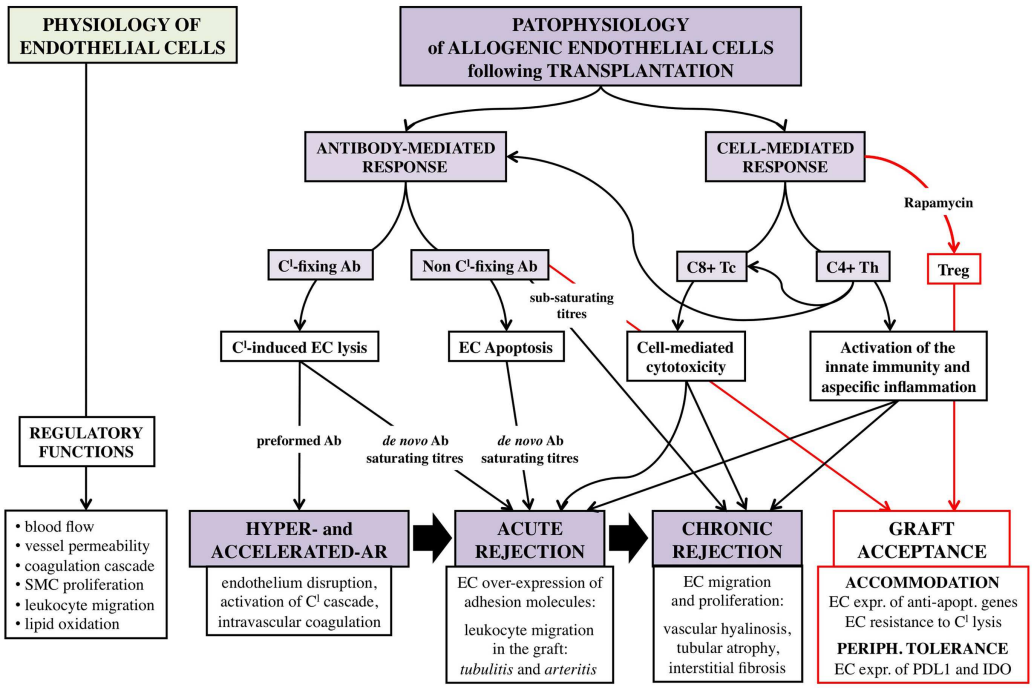

FIGURE 1 | Schematic representation of the complex interaction between allogenic endothelial cells and host immune system following vascularized solid-organ transplantation. EC, endothelial cells; Tc, CD8+ cytotoxic T-cells; Th, CD4+ helper T-cells; Treg, CD4+ CD25+ Foxp3+ regulatory T-cells; SMC, smooth muscle cells; AR, acute rejection; Ab, antibody; C', complement; expr., expression; apopt., apoptotic.

desensitization procedures for AB0i recipients are less intense and obtain better outcomes than those for anti-HLA immunized patients (16, 20). Second, A1 Ag elicit a more powerful response in kidney recipients of non-A blood group compared to A2 Ag due to minor expression of the latter on EC (13). Third, preformed DSA cause a more acute and severe rejection than *de novo* DSA, because they are more rapidly and abundantly produced by memory B-cells without any need of helper T-cells, and are more harmful for the graft (64). Fourth, complement-fixing DSA are associated with a poorer prognosis compared to non-fixing Abs (65). Finally, DSA concentration is critical for survival of both AB0i and HLAi renal transplants, with higher titers linked to more rejection episodes and shorter graft survival (20, 66).

Preformed DSA can cause hyperacute or accelerated acute rejection within minutes from the revascularization by binding to EC and fixing the complement; antibody-mediated complement activation extensively damages the endothelium integrity and initiates intravascular coagulation cascade that results in vessel thrombosis and tissue infarction (67).

Acute ABMR is a severe, albeit less catastrophic, event characterized by deposition of complement-fixing Abs on graft endothelium, mainly on the PTC endothelium, without initial activation of the coagulation (3). Upon DSA binding, activated EC increase the display on their surface membrane of MHC molecules, which further amplify the allo-immune specific response (65). Moreover, anti-HLA Ab ligation forces targeted EC to release prothrombotic mediators, like von Willebrand Factor (VWF), and to express more adhesion molecules, which foster platelets aggregation and leukocytes invasion of the graft (68). Graft invasion by T-cells is sustained by the enhanced expression on EC of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and endothelium leukocyte adhesion molecule-1 (ELAM-1) (69), adhesion molecules, which can be induced by the inflammatory mediators interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α), and interferon-γ (INF-γ), produced by the lured leukocytes (70–72). The invasion by host lymphocytes and inflammatory cells of functional structures of the graft, such as the *tubuli* and the PTC, and the development of small vessel occlusion due to thrombi formation and cell accumulation determine acute deterioration of graft function (3).

Alternatively, acute episodes self-limit and relapse thereafter repeatedly, thus resulting in chronic injuries that lead to transplant atherosclerosis, tubular atrophy, and interstitial fibrosis, the hallmark of chronic rejection (24). Transplant atherosclerosis in particular is sustained by the acquisition by EC of proliferative capabilities. Upon DSA binding to HLA molecule, EC are driven to express growth factor receptors, such as the fibroblast growth factor receptor (FGF-R) (73), and to re-arrange filaments of the cytoskeleton, such as forming stress fibers (74) and recruiting integrin-ß4 (75). Cytoskeleton rearrangements confer EC the capability of reacting to appropriate stimuli by proliferating. The transduction of integrin-ß4-dependent signals activates several cytoplasmatic kinases that ultimately result in the stimulation of the mechanistic target of rapamycin (mTOR) pathway (76); mTOR activation promotes progression of cell cycle from G1 phase to S phase and ultimately induces EC division, such a proliferation is reinforced by the susceptibility to FGF of EC expressing FGF-R (73).

ACCOMMODATION

Accommodation was originally the term used to describe the acquisition by EC of resistance to ABMR of AB0i renal grafts following reappearance of anti-A/B donor Ab (77). Nowadays, much interest is dedicated at understanding the mechanisms of accommodation and whether they could also be therapeutically harnessed to prevent transplant damage from anti-HLA DSA, that unlike anti-A/B Ab, are currently thought to invariably lead to allograft ABMR (78).

A small but interesting study investigated the behavior of EC in hyper-immune desensitized recipients who experienced reappearance of anti-HLA Abs following renal transplantation (79). None of the seven enrolled patients had hyperacute ABMR but three lost the graft due to various immunological damages, and three of the four remaining patients suffered curable acute rejection or transplant glomerulopathy. Analysis of the graft biopsies revealed increased expression of anti-apoptotic Bcl-xl gene in glomerular and peritubular capillary EC. Furthermore, *in vitro* incubation of human EC with sub-saturating concentrations of anti-HLA Ab eluted from the patients decreased ICAM-1 expression and provided resistance to complement-mediated cell lysis (79).

These preliminary results have been corroborated by further studies according to which sub-saturating anti-HLA Ab can induce EC expression of the anti-apoptotic genes Bcl-2 and Bcl-xL, whereas saturating titers induce EC apoptosis (**Figure 1**) (80–82).

Despite these encouraging results, a full knowledge of the accommodation process is far from been achieved and many more studies are needed in order to establish adequate protocol to desensitize hyper-immune recipients and safely perform transplantation in such population.

CELL-MEDIATED IMMUNE RESPONSE TOWARD ALLOGRAFT ENDOTHELIAL CELLS

As said, EC have all the properties required to drive direct activation of allogenic T-cells that is the pivotal step in all the forms of rejection non-mediated by preformed DSA (**Figure 1**) (8).

The rejection process has long been thought to be induced, at least initially, by donor professional APC that, upon migration into host secondary lymphoid organs, would present allo-Ag to T-cells. Nevertheless, human EC, which are not professional APC, have proven to be able to directly activate T-cells (**Table 2**); human EC provide "signal 1" as, unlike porcine and rodent EC, they robustly express HLA molecules, and in particular small vessel and capillary human EC constitutively express both class I and II HLA molecules (8). They also provide "signal 2" by expressing the co-stimulatory molecules required for an effective Ag presentation (8). Direct activation of T-cells by EC is of particular importance because while donor professional APC are destined to fade over time, EC, whose survival is linked to that of the allograft, can potentially ignite acute rejection at any time following transplantation (83).

ENDOTHELIAL CELLS AND CD8+ CYTOTOXIC T-CELLS

In vitro mixed lymphocyte reaction (MLR) experiments using EC as stimulators of allogenic CD8+ sorted naive T-cells have confirmed that EC are able to behave as professional APC (84).

CD8+ cytotoxic T-cells (Tc), so co-cultured with EC, respond proliferating and acquiring an effector phenotype defined by higher expression of perforin and production of IL-2 and INF-γ, which in turn enhance EC expression of HLA class I and II molecules (84).

With the use of blocking monoclonal Ab (mAb), the essential signals for Tc activation have been identified in the HLA-A and B class I molecules on EC that are target of the T-cell receptor (TCR) and CD8 co-receptor, as well as the co-stimulatory molecule CD80 (B7-I) on EC and its ligand CD28 on T-cells (85). *In vivo* experiments have confirmed that CD8+ T-cell direct activation by non-hematopoietic cells, such as EC, leads to graft rejection in a murine model of class I restricted heart allo-grafts transplanted into CD4-depleted recipients devoided of secondary lymphoid organs (86). Finally, the finding of vimentin-specific autoreactive CD8+ T-cells in heart recipients have shown that transplantation cellular response, as for the humoral response, may spread from being directed to allo-antigens to autoimmunity (60).

ENDOTHELIAL CELLS AND CD4+ HELPER T-CELLS

Accumulating evidence has convincingly clarified that human EC of microvascular origin can directly activate CD4+ helper T-cells (Th) (8, 87–89). Th are central mediators of allo-immunity as they provide help for allo-Ab production, they arm cytotoxic T-cells,

Table 2 | Cell-mediated immune response toward allograft endothelial cells.

T-cell types	Defining TF	Direct activation by EC	Co-stimulation (T-cell vs EC)	Outcome upon activation	Mechanisms of action	Effects on EC	Reference
CD8+ cytotoxic	–	Yes, through HLA class I	CD28 vs CD80	Graft rejection	Cytotoxicity	Enhancement of HLA expression	(8, 59, 84–86)
CD4+ helper	T-bet (Th1) GATA3 (Th2) RORγt (Th17)	Yes, through HLA class II	LFA1, LFA2 vs ICAM-1, LFA3	Graft rejection	Provision of help to B- and T-cells, guidance of innate immunity	Enhancement of HLA and adhesion molecules expression	(8, 83, 94–100)
CD4+ CD25+ Treg	Foxp3	Yes, through HLA class II	–	Tx tolerance	Disarming of APC, recruitment of new cohorts of Treg	VCAM-1 and IL-6 red. CD62E and CD62P red. PDL-1 and IDO induct.	(95–100)

TF, transcription factor; EC, endothelial cells; Th, helper T-cell; Treg, regulatory T-cell; Tx, transplantation; APC, antigen-presenting cells; red., reduction; induct., induction.

and drive innate unspecific inflammatory response (8). Human EC constitutively express MHC class II molecules, HLA-DR, DP, and DQ, albeit at lesser extent compared to class I molecules. These class II molecules, which are recognized by TCR and bound with the help of CD4 co-receptor, are "signal 1" for Th direct activation. EC can also provide "signal 2" specific for Th; they indeed display ICAM-1 (CD54) and lymphocyte function-associated antigen-3 (LFA3) (CD58), which are bound by LFA1 (CD11a/18) and LFA2 (CD2) on T-cells. As shown *in vitro*, following EC provision of signals 1 and 2, Th start to proliferate and acquire an effector phenotype characterized by the induction of the co-stimulatory molecule CD40L (90) and of the adhesion molecules that favor trans-endothelium migration (91). Depending on local cytokine microenvironment, resting CD4+ T-cells differentiate into different Th subsets. Along with Th1 and Th2 subsets, whose ability to mediate rejection is well known (92), also Th17, which are implicated in a number of autoimmune diseases, can emerge guided by activated EC that provide the critical cytokine IL-6 in the presence, under inflammatory conditions, of transforming growth factor-ß (TGF-ß) (93). This is of particular interest as not only Th17 have recently proven capable of causing allograft rejection (94) but also they and CD4+ CD25+ Foxp3+ regulatory T-cells (Treg) seem to keep reciprocally at bay. Since Treg, that are crucial cells for transplantation tolerance, require TGF-ß but not IL-6 for their induction, it has been hypothesized that EC could also mediate the induction of peripheral Treg.

ENDOTHELIAL CELLS AND REGULATORY T-CELLS

CD4+ CD25+ Treg are a well-defined subset of CD4+ T-cells identified by the expression of the master transcription factor Foxp3. They are crucial regulators of the immune response; natural Treg of thymic origin prevent autoimmune diseases, while peripherally induced Treg actively regulate transplantation tolerance (95). Treg act at a tissue level where they influence APC ability of presenting Ag to conventional T-cells (Tconv), which in turn become either anergic or regulatory cells themselves. Therefore, empowering Treg at the expense of Tconv can induce a state of local immune privilege that promotes long-term graft survival (95).

A few papers have investigated the ability of EC to interact with Treg. INF-γ-stimulated EC have proven to be capable of inducing Treg when co-cultured with allogenic CD4+ T-cells (96); but more importantly, similar results have been obtained when EC were pre-treated with the clinically available immunosuppressant rapamycin (Rapa), which exerts its functions by inhibiting the mTOR pathway (97). Expansion of Treg results from the conversion of naive CD4+ cells into Foxp3+ cells, and depends on cell–cell contact and the local microenvironment (98); not only Rapa reduces the display of VCAM-1 in EC (99) but also forces the expression of the inhibitory molecules programed death ligand-1 (PDL-1) and indoleamine 2,3-dioxygenase (IDO), which are crucial for Treg induction (96–98). Finally, EC pre-treated with Rapa produce less IL-6 (97), which is instead required for the expansion of Th17 but not that of Treg (93). EC-induced Treg are functionally active as they can effectively suppress the proliferative response of Ag-stimulated CD8+ T-cells. On the other side, Treg can influence EC behavior; Treg release TGF-ß that downregulate the expression on EC of the adhesion molecules CD62E and CD62P (respectively, E- and P-selectin), thus limiting transmigration of Tc and reducing local inflammation (100).

CONCLUSION

Graft endothelium is the first barrier between self and non-self in transplantation of vascularized solid organs, such as kidney transplants. Indeed, upon organ reperfusion, host lymphocytes initially encounter graft EC, which express all the most relevant antigens in transplantation immunobiology. Such antigens are invariably recognized by the host immune system, and toward them antibody-mediated and cell-mediated immune responses mount. Moreover, not only are EC a passive target of the host immune system but they also are an active player in the recruitment and activation of allogenic lymphocytes, and in the invasion of graft tissues by them. The ability of EC to directly activate allogenic naive T-cells deserves to be highlighted as EC represent a long-term source of allo-antigens and can potentially induce graft rejection at any time post-transplantation. Therefore, the fate of renal transplants largely depends on how such interplay between graft endothelium and host immune system unfolds: on one side, the activation of the immune response may lead to all the forms of graft rejection, from hyperacute to chronic, and to the deterioration of graft function primarily because of damages to the endothelium integrity. On the other side, even in the presence of circulating donor-specific antibodies directed to antigens expressed on donor EC, graft endothelium may thrive and fulfill its functions of regulating blood flow and vessel permeability, which are crucial not only for graft survival but also for the depurative activities of the kidney.

Current immunosuppressive drugs have been developed with the aim of targeting host immune response, be it depleting or blocking T-cells and B-cells or halting the complement cascade; however, given the central role of EC in modulating the alloimmune response, graft endothelium may represent a preferential target of newer immunosuppressive protocols aimed at promoting long-term graft acceptance by reducing EC immunogenicity and antigen presentation, while favoring their survival. The class of immunosuppressants mTOR inhibitors has been more and more utilized in the clinic for its pro-tolerogenic and anti-neoplastic activities; the pro-tolerogenic effects of the mTOR inhibitor rapamycin have proven to rely at least in part on its ability to condition antigen presentation by EC so that regulatory T-cells emerge at the expense of rejecting conventional T-cells. Although scientific data lack, it is conceivable that also the latest immunosuppressant introduced in the clinic, belatacept, may prevent graft rejection by limiting EC ability to activate T-cells (101); belatacept is a CTLA-4 fusion protein with the human IgG Fc, which blocks "signal 2," by binding to the co-stimulatory molecules CD80 and CD86 that are expressed not only on the APC but also on the surface of EC.

As therapeutically blocking EC from presenting antigens prevents direct activation of T-cells and, in turn, induction of *de novo* donor-specific antibodies, developing such immunosuppressive strategy could become the answer to the issue of late ABMR, toward which current immunosuppressants are blunt, and could contribute to the improvement of long-term transplantation outcomes.

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T cell–macrophage interactions and granuloma formation in vasculitis

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Granuloma formation, bringing into close proximity highly activated macrophages and T cells, is a typical event in inflammatory blood vessel diseases, and is noted in the name of several of the vasculitides. It is not known whether specific properties of the microenvironment in the blood vessel wall or the immediate surroundings of blood vessels contribute to granuloma formation and, in some cases, generation of multinucleated giant cells. Granulomas provide a specialized niche to optimize macrophage–T cell interactions, strongly activating both cell types. This is mirrored by the intensity of the systemic inflammation encountered in patients with vasculitis, often presenting with malaise, weight loss, fever, and strongly upregulated acute phase responses. As a sophisticated and highly organized structure, granulomas can serve as an ideal site to induce differentiation and maturation of T cells. The granulomas possibly seed aberrant Th1 and Th17 cells into the circulation, which are known to be the main pathogenic cells in vasculitis. Through the induction of memory T cells, aberrant innate immune responses can imprint the host immune system for decades to come and promote chronicity of the disease process. Improved understanding of T cell–macrophage interactions will redefine pathogenic models in the vasculitides and provide new avenues for immunomodulatory therapy.

Keywords: macrophage, dendritic cell, T cell, granuloma, vasculitis

INTRODUCTION

Protecting the host from infection and maintaining tissue integrity relies on two highly complex and evolutionary distinguished systems, the innate and adaptive immune system. The two arms of the immune system have developed a sophisticated and efficient crosstalk to defend the host. Monocytes that come into contact with penetrating pathogens differentiate into specialized antigen-presenting cells (APC), such as macrophages and dendritic cells (DC) (1). After phagocytosis and degradation of the pathogen, proteins are presented to specialized cells of the adaptive immune system, specifically T cells. Interactions between macrophages and T cells are critical in the communication between innate and adaptive immunity. Errors in this interaction may result in immunodeficiency, failure to destroy invading pathogens, or damage to host–tissues in the form of autoimmunity. Although the principal function of macrophages was recognized a long time ago (2), the precise mechanisms of macrophage physiology are only now beginning to be unraveled.

Chronic (aberrant) macrophage–T cell interaction leads to the formation of organized lymphoid organ structures, such as granulomas. Granulomas are typically formed during infection, especially when the host has difficulties to eliminate the infectious organism. Classic examples are granulomas induced by *Mycobacterium tuberculosis* infection, often considered a mechanism to contain the infectious organism (3). Granuloma formation is equally important in non-infectious disease states, such as inflammatory blood vessel disease. In giant-cell arteritis (GCA; formerly known as temporal arteritis), granulomas are an almost obligatory part of the disease process. In granulomatosis with polyangiitis (GPA; formerly known as Wegener's granulomatosis), granuloma formation is captured in the disease name. An important issue in granulomatous diseases is whether the highly activated macrophages building the granulomatous structures have primarily a protective function or whether they are key drivers of tissue damage and disease propagation (4).

In the current review, we compare and contrast the interaction of macrophages and/or DC with T cells in the context of granuloma formation and vasculitis and focus on GCA and GPA as quintessential model systems of how the interface between innate and adaptive immunity contributes to disease pathogenesis.

MACROPHAGES AND DENDRITIC CELLS INFLUENCE T CELLS

Monocytes relocate to inflammatory lesions upon sensing a chemokine gradient (5) and can differentiate into distinct types of APC on site. A discussion of the similarities and differences between DC and macrophages is beyond the scope of this review (6). Macrophage subtypes form two main groups: M1 or classically activated macrophages (CAM) and M2 or alternatively activated macrophages (AAM). M1 generally specialize in amplifying inflammatory reactions and produce high levels of TNFα, IL-6, and IL-1β. In contrast, M2 are primarily active in tissue repair and their product profile includes IL-10, TGF-β, and growth factors. An active TGF-β pathway results in suppression of inducible nitric oxide synthase (iNOS) expression and NO secretion in macrophages, deviating the cells away from M1 differentiation (7). M1 have been described as "fighting" or "soldier" cells and M2

as "fixing" or "repair" cells (8, 9). The M2 or AAM subtype is not as well defined and much debated (4). It is plausible that monocytes can differentiate into macrophage subtypes positioned somewhere on the M1–M2 or CAM–AAM continuum and are endowed with varying adaptability and plasticity (8, 10).

ANTIGEN RECOGNITION AND PRESENTATION

Macrophages recognize pathogens through so-called pathogen associated molecular patterns, which are detected through Toll-like receptors (TLR) (11, 12), thus distinguishing between self and non-self. As critical recognition structures, TLR enable the build-up of a defensive immune response, they also participate in shaping immune responses underlying autoimmunity (13, 14). To orchestrate tissue cleanup and repair, macrophages must be able to recognize and remove modified host proteins and lipids, e.g., oxidized proteins and lipids. Such products are often described as danger-associated molecular patterns and require competent TLR as recognition structures (15). Oxidation of host proteins, lipids, and nucleic acids results from the action of reactive oxygen species (ROS), often derived from activated macrophages themselves. The latter process has been implicated in the development and propagation of atherosclerosis (16). Importantly, T cells also express TLR, but it is currently unknown what the precise role of these receptors is in modulating T cell function (14, 17).

MACROPHAGE-INDUCED POLARIZATION OF T CELL DIFFERENTIATION

Macrophages are principal regulators of immunity by processing and presenting antigens to T cells (18), which are charged with distinguishing self from non-self (19). Antigen recognition by T cells involves the highly polymorphic major histocompatibility complex (MHC) molecules classes I and II (20, 21), which selectively bind antigen peptides and present them on the surface of APC. While T cell receptors bind to HLA–peptide complexes, costimulatory molecules such as CD28 are co-ligated, a mechanism that is mandatory for a more powerful induction of T cell activation (22–24). After entering the T cell activation cascade, T cells differentiate into distinct functional lineages. Some of them become effector cells; others specialize as memory T cells and position themselves in lymphoid storage sites (25). The fate of individual T cells is ultimately shaped by the microenvironment, composed of cytokines, chemokines, and tissue-specific signals (26). The exact mechanism by which macrophages induce activation, proliferation, and differentiation of T cells is incompletely understood (27). Antigen dose, the type of APC and the contact between APC and T cell are all important variables (28). It has been proposed that DC are more powerful partners of naïve T cells and preferentially interact with T cells in organized lymphoid tissue. Conversely, macrophages function as APC for naïve and memory T cells, encountering them in peripheral tissue lesions (29). DC are thought to skew $CD4^+$ cells toward Th1 differentiation in an IL-12 dependent manner (30). Other studies have demonstrated a similar effect of macrophages on $CD4^+$ cells (31). DC that have been activated by inflammatory mediators can stimulate Th1 proliferation *in vitro*, but these same DC could not do so *in vivo*, possibly due to lacking pathogen contact, resulting in diminished IL-12p40 production (32).

Importantly, one study found inflammatory DC to be more potent inducers of Th17 cells when compared to inflammatory macrophages. The authors concluded that this difference was reflective of differential IL-23 production, which was observed in inflammatory DC but not in macrophages (33). In contrast, other DC subsets have been implicated in inducing regulatory T cells by virtue of producing TGF-β or expressing PD-L1 (34). Suppression of T cell function and proliferation may also result from the local action of IL-10 and TGF-β, typically secreted by M2 macrophages. Gut-residing macrophages have been implicated in inducing regulatory T cells, whereas DC were found to induce Th17 cells by secreting IL-6 combined with TGF-β and possibly IL-23 (35).

MACROPHAGE-INDUCED INHIBITION OF T CELLS

Generally, macrophages inhibit the proliferation of T cells via cell–cell contact. Abundantly studied inhibitory mechanisms in T cells are the programed death (PD1) and cytotoxic T lymphocyte antigen (CTLA) pathways. PD1 and CTLA-4 are found on T cells and mediate inhibitory signals when engaged by their respective ligands expressed on the surface of interacting macrophages (36). Malfunctioning of these inhibitory signals, e.g., due to polymorphisms, increases susceptibility for autoimmunity (37–39). Both DC and macrophages express membrane-integrated ligands for PD-1 and CTLA-4 (24). Blockade of PD-L1 on DC is a powerful mechanism to enhance T cell proliferation and cytokine release (34, 40). Besides polymorphisms in *PD-1* and *CTLA-4*, a series of gene polymorphisms, including genes relevant for cytoplasmic signaling pathways, have been associated with susceptibility for autoimmunity (41, 42). As minor variations in threshold settings of cytoplasmic signaling cascades have the potential to bias immune interactions profoundly, it is likely that they impact macrophage–T cell interactions both by accelerating as well as downregulating immune responses.

Another concept has been that resting macrophages preferentially dampen immune responses. Accordingly, resting macrophages have been reported to induce allogeneic T cell anergy, partly by enhancing regulatory T cells. In one study, T cells proliferated when co-cultured with immature DC, became anergic when in a second co-culture with macrophages and proliferated when co-cultured with mature DC in a third co-culture, finally producing IL-2 and IFN-γ. Immature and mature DC both expressed high levels of MHC class II (HLA-DR), but macrophages did not. The costimulatory molecules CD80 and CD86 were present at higher density on mature DC than on immature DC or on macrophages (43). These studies support the notion that APC functions of macrophages and DC are fundamentally distinct.

In some infectious settings, specifically in filarial and yeast infections, the pathogen undermines protective immune responses by inducing macrophages and DC that are able to suppress T cell activation (44, 45). This mechanism is dependent on the production of TGF-β and/or IL-10 in combination with a lack of IL-6. Also, co-culturing allogeneic naïve $CD4^+$ T cells with immature DC has been reported to lead to the expansion of IL-10 producing T cells, whereas mature DC promote the proliferation of Th1 cells (46).

Under hypoxic conditions, macrophages were found to suppress the proliferation of T cells via hypoxia-inducible factor

1α (HIF-1α). More HIF-1α knockout macrophages were necessary to suppress T cells as compared to wild-type macrophages (47). HIF-1α may enhance the production of nitric oxide species, which directly suppress T cell proliferation. In contrast, under hypoxic conditions, which enrich the environment for macrophage-derived oxidative species, T cells preferentially differentiated into Th17 cells. HIF-1α-dependent proteosomal degradation of the transcription factor Foxp3 and enhancement of IL-17 expression via RORγt and Stat3 have been proposed as the underlying mechanism (48). The HIF-1α dependent processes have been shown to play an important role in rheumatoid arthritis and may be of importance in other autoimmune diseases (49).

T CELLS REGULATE THE MATURATION OF MACROPHAGES AND DENDRITIC CELLS

The differentiation of monocytes into macrophages is a rapid process controlled by cytokines in the environment and cell–cell interactions (10). Thus, neighboring cells can effectively regulate the induction, functional differentiation, and the survival of macrophages (1, 50). It is believed that the polarization of monocytes into M1 or M2 can occur in the absence of T cells (51). However, generally it is assumed that Th1 cells skew monocytes toward M1 whereas Th2 cells skew monocytes toward M2 (4). The lineage commitment of M1 and M2 cells has been associated with the induction of distinct arginine metabolical pathways (52). How T cells regulate this process, however, is not understood.

IL-17 was shown to induce macrophages to produce high levels of IL-6, IL-1β, and TNFα, as well as lower levels of IL-10, IL-12, and PGE_2 (53), suggesting that Th17 cells bias monocytes toward an M1-like phenotype. Another study demonstrated that pretreatment of monocytes with IFN-γ (in addition to IL-10 and glucocorticoids) prevented the differentiation of monocytes into M2c cells. Instead, IFN-γ-treated monocytes had a higher expression of Fas and were apoptosis susceptible. Interestingly, monocytes treated with IL-17 (in addition to IL-10 and glucocorticoids) differentiated into M2c and had enhanced phagocytic capacity (54). In essence, Th17 cells may regulate phagocytic effector functions. *In vivo*, however, the source of IL-17 can be heterogeneous since neutrophils, DC, and macrophages are all capable to produce IL-17, although in low amounts (55, 56).

In mice, $CD4^+CD25^+$ regulatory T cells exert a potent suppressive effect on splenic APC, which cannot be overcome by preactivation (57). In humans, $CD4^+CD25^+Foxp3^+$ regulatory T cells were found to direct monocytes toward M2; characterized by high surface expression of CD206 and CD163 but low levels of HLA-DR (58). Also, human $CD4^+CD25^+$ T cells decrease the production of TNFα and IL-6 and increase the production of IL-10 in co-cultured monocytes (59). Murine $CD4^+CD25^-Foxp3^-$ cells can temper the production of proinflammatory cytokines in macrophages via close cell–cell contact. Biologic relevance of this mechanism is suggested by the observation that in the absence of $CD4^+$ T cells, innate immune responses are so vigorous that they cause a cytokine storm and death (60). A more recent murine study has demonstrated that both memory and effector $CD4^+$ T cells decrease IL-1β production in bone marrow-derived macrophages without affecting TNFα or IL-6 production, possibly by selective inhibition of the inflammasomes NLRP3 and NLRP1 (61).

In summary, selected macrophages and DC can shape T cell differentiation through the secretion of IL-1β, IL-6, and TNFα, whereas other macrophages and DC can suppress T cells via cell–cell contact and the secretion of IL-10 and TGF-β (**Table 1**). Vice versa, Th1 and Th17 activate macrophages via IFN-γ and IL-17, respectively. Regulatory T cells can suppress the activity of macrophages by secreting IL-10, thus driving them toward the M2 phenotype (**Table 2**).

Table 1 | Summary of macrophage products in relation to possible effects on T cells.

	Producer	Giant-cell arteritis	Granulomatosis with polyangiitis	Effect
IL-1β	M1	++	+++	Proinflammatory
IL-6	M1	++	+	Proinflammatory
IL-8	M1	+/−	+	Proinflammatory
IL-18	M1/M2	unknown	+	Neutrophil attractant and primer
TNFα	M1	+	+	Proinflammatory; granuloma formation
IL-23	M1	+	+	Th17 sustaining
CCL2	M1	+	+	Monocyte attractant
PGE_2	M1	unknown	+	Phagocytosis
IL-15	M1/M2	unknown	+	T cell and NK-cell activation; vitamin D pathway
Vitamin D	M1/M2	unknown	+	Anti-inflammatory
Osteopontin	M2	unknown	++	Monocyte and neutrophil chemoattractant
VEGF	M2	++	+	Neoangiogenesis
PDGF	M2	++	+	Tissue remodeling and repair
TGF-β	M2	+	++	Anti-inflammatory; Th17 inducing
IL-10	M2	+	+	Anti-inflammatory; Treg inducing

Macrophage products involved in vasculitis.

Table 2 | Summary of T cell products in relation to possible effects on macrophages.

	Producer	Giant-cell arteritis	Granulomatosis with polyangiitis	Effect
IL-17	Th17	++	++	Proinflammatory; M1 supporting
IL-21	Tfh; Th17	++	+	Proinflammatory
IL-22	Th22	unknown	unknown	Proinflammatory
GM-CSF	Th17	+	unknown	M1 supporting
IFN-γ	Th1	++	++	Proinflammatory; M1 supporting; drives multinucleation
IL-27	Th1	+	unknown	Proinflammatory
IL-32	Th1	+	+	Proinflammatory
IL-10	Treg	+/−	+	Anti-inflammatory; M2 supporting

T cell products involved in vasculitis and granulomatous microenvironments.

GRANULOMA FORMATION

Designed to protect the host from infection and cancer, the adaptive immune system displays complex microarchitectures to optimize immune responses. One of these lymphoid microstructures has been named granuloma and typically consists of a sphere of highly activated macrophages, surrounded by a shell, i.e., a peripheral layer of T lymphocytes (**Figure 1**) (62). The current paradigm holds that antigens that are difficult-to-eliminate are prone to elicit granuloma formation. Persistent particulate substances, such as silica, beryllium, or zirconium, but also suture material, are often considered as typical triggers of non-infectious granulomas (63). Difficult-to-eliminate antigens eventually induce the palisading of monocytes/macrophages, which depend on activating signals from other cell populations to form the sophisticated structure of a granuloma. Lymphocytes, especially $CD4^+$ T cells, and DC consistently participate in granulomatous infiltrates, but neutrophils, eosinophils, and B cells have also been described (64). Cells are attracted to granulomas by chemokines, cytokines such as interleukins and complement breakdown products. Over time granulomas mature, resulting in the formation of multinucleated giant-cells and epithelioid cells. The precise composition of granulomas may differ according to the inciting agent or pathogen, but the overall architecture of granulomas is usually maintained.

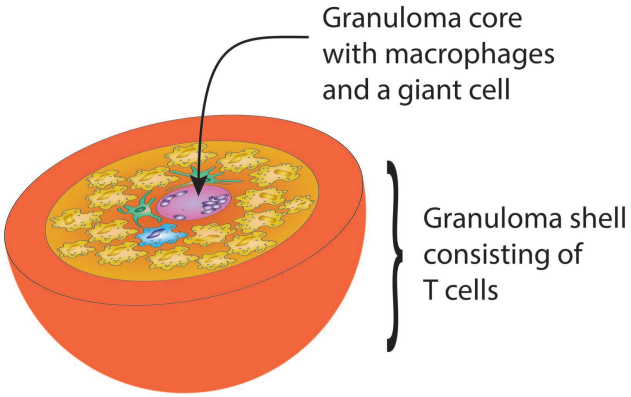

FIGURE 1 | Schematic drawing of the gross architecture of a granuloma with macrophages, dendritic cells, and multinucleate giant-cells forming the core of the sphere, surrounded by a shell of lymphocytes. By clustering innate and adaptive immune cells, the granuloma has great potential to induce and perpetuate immune responses, but is equally powerful in causing damage to surrounding tissues.

Much of the knowledge on granulomas originates from studying the model system of *M. tuberculosis* infection (3), which remains one of the most prevalent and lethal infectious diseases on the planet (65). It is believed that tuberculous granuloma formation reflects a strategy of the immune system to encapsulate the infection and prevent spreading throughout the body. Tuberculous granulomas have been a rich source of information on the bi-directional interaction between macrophages and T cells (66, 67). One of the hallmark events on the side of macrophages and DC are cell–cell fusions, resulting in multinucleated giant cells. Why and under what circumstances these phagocytes fuse is incompletely understood. Culturing monocytes with IFN-γ *in vitro* reliably results in multinucleation, emphasizing the role of Th1 cells in the formation of giant cells (68). Th1 cells are critical drivers in the M1 differentiation of macrophages and M1 cells have been implicated in the formation and/or maintenance of granulomas (69). One study has suggested that DC fusion takes place under the influence of autocrine IL-17 and exogenous IFN-γ (56), proposing that the concerted action of several cytokines is necessary to promote the optimal granuloma function. In sarcoidosis, believed to be a Th1-dependent disease, granulomas are more inflammatory than suture granulomas or fungal granulomas, based on significantly higher production of IL-6, CCL2, IFN-γ, and Nox2 (70).

The role of TNFα in the formation of granulomas is debated. In tuberculosis, some studies have shown that TNFα induces the formation of granulomas (71) and that TNFα deficient mice have a more severe *M. tuberculosis* infection, possibly due to deficient granuloma formation and thus inability to contain the infection (72). Administering anti-TNFα medication in humans, however, does not appear to suppress granulomatous inflammation (73) or cause disassembly of existing granulomas more effectively than corticosteroids alone (74).

Increased numbers of IL-17 positive cells have been found in granulomas in patients with sarcoidosis (75), suggesting that Th17 cells may participate in the assembly of sterile granulomas. Alternatively, the cytokine milieu of granulomas may provide ideal conditions to polarize peripheral T cells toward the Th17 lineage.

Over time, granulomas can become necrotic or fibrotic. It is unknown which factors regulate the progression of the granulomatous reaction. Differences in the architecture, in the tissue distribution, and in the persistence of granulomas strongly support the notion that granuloma formation is distinct in different disease states. As a common rule, granulomatous infiltrates reflect an intense immune response, associated with marked inflammation and potential of tissue damage.

EFFEROCYTOSIS

Besides being a site of concentrated cytokine production, granulomas have a critical function in debris removal, including the removal of apoptotic cells; a process named efferocytosis (76). It is conceivable that the inefficiency of efferocytosis could lead to the persistence of the granulomatous reaction and this could be particularly important in disease states characterized by sterile granulomas. The notion of ineffective phagocytosis in granulomatous disease was suggested in the past (77). Impaired efferocytosis has been described in chronic granulomatous disease, typically associated with a hyperinflammatory state (78). The deficiency of efferocytosis has been related to a defect in phosphatidylserine (PS) signaling and has been reversed by treating macrophages with IL-4, essentially skewing them toward M2.

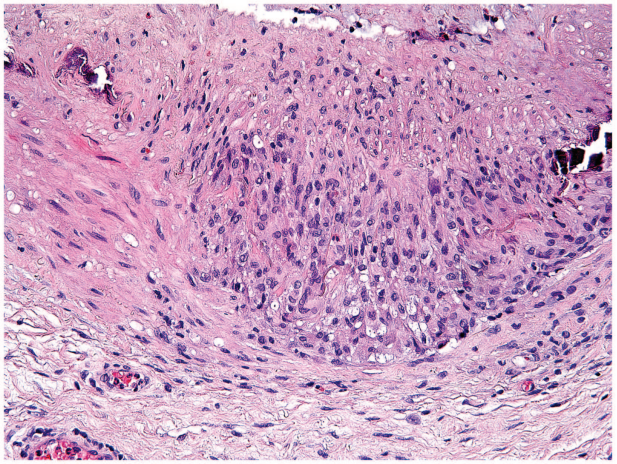

FIGURE 2 | Histological section showing a granulomatous infiltrate in the temporal artery of a 77-year-old patient with giant-cell arteritis. The granulomatous reaction is localized at the media-intima border and includes fragments of the lamina elastica externa. Lymphocytes are surrounding highly activated macrophages and giant cells (hematoxylin and eosin staining).

GRANULOMA FORMATION AND HYPERVITAMINOSIS D

Patients with granulomatous disease can present with hypercalcemia, considered a result of hypervitaminosis D. This excess vitamin D originates from the granulomas, most likely from the macrophages within. In sarcoidosis, macrophages can produce vitamin D and this production is increased upon stimulation with IFN-γ (79). In line with these findings, a TLR-mediated microbicidal pathway has been reported to upregulate the vitamin D receptor (VDR). The enzyme converting 25-hydroxyvitamin D into the active form of vitamin D, 1,25-hydroxyvitamin D, known as CYP27b1, is also upregulated upon activation of the above mentioned microbicidal pathway (80). In tuberculosis, IFN-γ can increase the production of autocrine IL-15, upregulating CYP27b1 and expression of the VDR. Upon inhibiting the VDR on monocytes, a reduction in autophagy as measured by LC3-positive vesicles was noted. The authors, therefore, suggest that enhanced production of vitamin D and the upregulation of the VDR in monocytes after IFN-γ stimulation result in maturation of the phagosome (81). It is unknown whether the vitamin D pathway is impaired or hyperactive in patients with sterile granulomatous disease.

Interestingly, in GPA and in sarcoidosis variations of disease prevalence patterns according to hours of sunlight per year (and thus dermal vitamin D production) have been discussed. Also, less exposure to sunlight and/or low levels of vitamin D increases the risk for developing GPA or a disease relapse (82, 83).

GIANT-CELL ARTERITIS

Giant-cell arteritis is a medium- to large-vessel vasculitis almost exclusively diagnosed in patients older than 50 years (84). In brief, presenting symptoms are such of an intense acute phase response; e.g., fever, malaise, weight loss, and laboratory abnormalities of systemic inflammation combined with manifestations of tissue ischemia. It mainly affects extracranial and upper extremity branches of the aorta (e.g., temporal arteries, axillary arteries) and the aorta itself (85). The gold standard for diagnosis remains the biopsy of the temporal artery (86). Typical histopathological findings are granulomatous lesions and/or lymphomonocytic infiltrations in the vessel-wall layers, often containing multinucleated giant cells (87) (**Figure 2**). In contrast to tuberculous granulomas and granulomas found in Crohn's disease [the latter assumed to result from a host–commensal bacteria homeostasis gone awry (88, 89)], granulomatous lesions in GCA have not been connected to an infectious agent (90). Reported associations between GCA and pre-existing infection with parvovirus B19, *Chlamydia trachomatis* and *Mycoplasma pneumonia* have raised the possibility of an infectious trigger, but subsequent studies could not always confirm these associations (91). Nevertheless, there is strong evidence for antigen-driven chronic T cell responses (92), particularly Th1 and Th17 responses (93). The induction of Th1 cells in GCA may originate from excessive IL-12 production, which has been found increased in GCA lesions during active disease and during remission (94). The source of IL-12 in GCA remains obscure, but DC have recently been described to produce high levels of IL-12 (95).

Dendritic cells and macrophages are obligatory components of the granulomatous infiltrates in the wall of GCA-affected arteries (96). DC are thought to act as gate-keepers of the vasculitis by inducing T cell activation, and display a phenotype of strong immune-stimulatory APC (92). It has been demonstrated that vessel-wall residing DC are specifically activated via TLR4 or TLR5 (97), enabling them to activate p38 MAPK, and activate downstream TNFα and IL-1β (98).

Accordingly, serum IL-6 and IL-1β levels have been found strongly increased in patients with active GCA (74, 99) and associated with disease activity (100, 101). While the precise cellular source of the excess IL-6 and IL-1β has not been determined,

highly activated macrophages and DC in the vasculitic lesions emerge as the likely producers (102). There is evidence that the granulomatous infiltrates in GCA contribute to IL-6 secretion (103), together with production of TGF-β, another well-known product of activated macrophages (104). IL-6 levels in GCA patients decrease upon treatment with corticosteroids, but remain higher in some patients when compared to healthy controls, indicating a more chronic course of disease (99).

Recently, IL-33 was reported to be increased in vessel-wall lesions of patients with GCA (105). IL-33 is secreted by endothelial cells when under stress or becoming apoptotic. This stress possibly results from high levels of circulating IL-6. Importantly, IL-33 has been found to favor M2 polarization of macrophages, implicating this macrophage subset in the pathogenesis of GCA (106).

Giant-cell arteritis is considered an antigen-dependent disease in which Th1 cytokines dominate and Th2 cytokines are generally absent. Moreover, during early and untreated disease, Th1 and Th17 cells co-exist in the vasculitic lesions (93) (**Figure 2**). The granulomatous inflammation may present the platform that allows differentiation of entering naïve T cells toward Th1 and Th17 under the influence of macrophage and DC products (**Table 1**). By seeding Th1 and Th17 cells into the periphery, granulomas could have a major impact on the composition of the overall immune system. Since differentiated memory cells are long-lived cells, even a temporary granulomatous reaction could permanently remodel the immune system and have long-term implications for the host. During corticosteroid treatment, Th17 cells decrease, but Th1 cells appear to persist (93). In line with these findings, increased serum levels of IL-12, IL-17, IL-21, IL-23, IL-27, IL-32, and IFN-γ have been observed in patients with active GCA (74, 107). Granulomas may represent an important source of IL-17, with elevated protein levels and mRNA observed within the granulomatous vessel-wall infiltrates (108, 109). It is important to consider that the overall frequencies of Th17 cells are low, outnumbered by Th1 cells by a factor of 10 or higher. This may be particularly relevant during the chronic phase of GCA, when persistent inflammation relies on Th1 cells and their major product, IFN-γ. Other Th1 products produced in the inflamed vessel wall include IL-27 and IL-32. Both have been reported to induce M1 cells (107).

Since the differentiation of T cells into distinct lineages results from the exposure of non-committed T cells to antigen plus polarizing cytokine environment, it is highly likely that macrophages and DC residing in the vessel wall ultimately shape vasculitogenic T cell responses (**Figure 3**). Differentiation of Th17 cells depends on the combined action of IL-6, TGF-β, and IL-23 (110, 111). Recent data have given rise to the concept that Th17 cells are plastic and, dependent on environmental signals, can be redirected to acquire regulatory T cell (Treg) functions (112). There is some evidence that Tregs may be underrepresented in GCA patients, perhaps explaining the inability of affected patients to clear granulomatous lesions (109).

Granulocyte-macrophage colony-stimulating factor (GM-CSF) is distinctly elevated in the serum of patients with GCA. Terrier et al. have reported that GM-CSF is essential for the production of IL-6 and IL-23 by DC; thus indirectly promoting the generation of Th17 cells (113). DC in GM-CSF$^{-/-}$ mice produce lower levels of IL-6, resulting in deficient proliferation of T cells in general and reduced Th17 differentiation. More recently, it has been reported that Th17 cells produce GM-CSF when stimulated with IL-23 and that this production is upregulated by IL-1β (114).

Given the central role of granulomatous infiltrates in GCA, disrupting granuloma formation should be a valuable therapeutic target. Surprisingly, anti-TNF therapy has failed to reduce steroid requirements or prevent disease flares in GCA patients when combined with corticosteroids (115).

With the knowledge of macrophages and DC forming the basis of granuloma formation, inhibiting these cells directly may prove beneficial in treating GCA; bearing in mind, however, the risk of infectious complications associated with a deficient innate immune system. As mentioned earlier, TLR play an important role in the activation of APC in infectious and in sterile inflammatory settings. TLR may, therefore, represent a therapeutic target to treat sterile granulomatous inflammation by inhibiting granuloma formation and reducing tissue damage (116). Other receptors and APC markers are being studied for their suitability in new therapeutic interventions. Targeting CD14 with anti-CD14 antibodies has been attempted in septic models, but not in autoimmune settings (117). In murine sepsis, blocking the innate immune system results in less inflammation, less intense cytokine storms, and a higher survival rate.

GRANULOMATOSIS WITH POLYANGIITIS

Small-vessel vasculitides associated with the production of autoantibodies reactive to proteinase-3 (PR3) or myeloperoxidase (MPO) are collectively called anti-neutrophil cytoplasmic antibodies (ANCA)-associated vasculitides (AAV). The group of AAV encompasses GPA, microscopic polyangiitis, and eosinophilic GPA (118). The pathogenesis of these vasculitides is incompletely understood, but great progress has been made in diagnosis and therapy of these autoimmune diseases. Yearly incidence rates are estimated at 20 cases per million individuals (119). A high mortality and (co)morbidity unfortunately persists despite improvement of therapy and knowledge of the disease process (120–122). In GPA, granulomas are typically found in the ear, nose, and throat region, in the lungs, periglomerularly in the kidneys, and more rarely in other organs (123) (**Figure 4**). The autoantigens recognized by the autoantibodies in AAV are intracellular enzymes produced by neutrophils and monocytes. In both cell types, PR3 and MPO are expressed on the cell membrane in low levels in healthy controls and in aberrantly high levels in AAV patients, especially during active disease (124, 125). Over 75% of GPA patients are PR3-ANCA positive with the remainder being either MPO-ANCA positive or ANCA-negative, especially in patients of Caucasian descent (126). Interestingly, when monocytes differentiate into macrophages they lose expression of PR3 and MPO on their cell membrane. Thus, monocytes can be activated by ANCA (127), whereas mature macrophages cannot (128), placing antibody-dependent disease mechanisms early into the pathogenic immune response (**Figure 5**).

The neutrophil, playing a central role in the pathogenesis of GPA, degranulates once ANCA bind to surface PR3 or MPO (129). Neutrophils then become apoptotic and are cleared by macrophages. While dying, neutrophils excrete so-called neutrophil extracellular traps (NETs), physiologically meant to "trap"

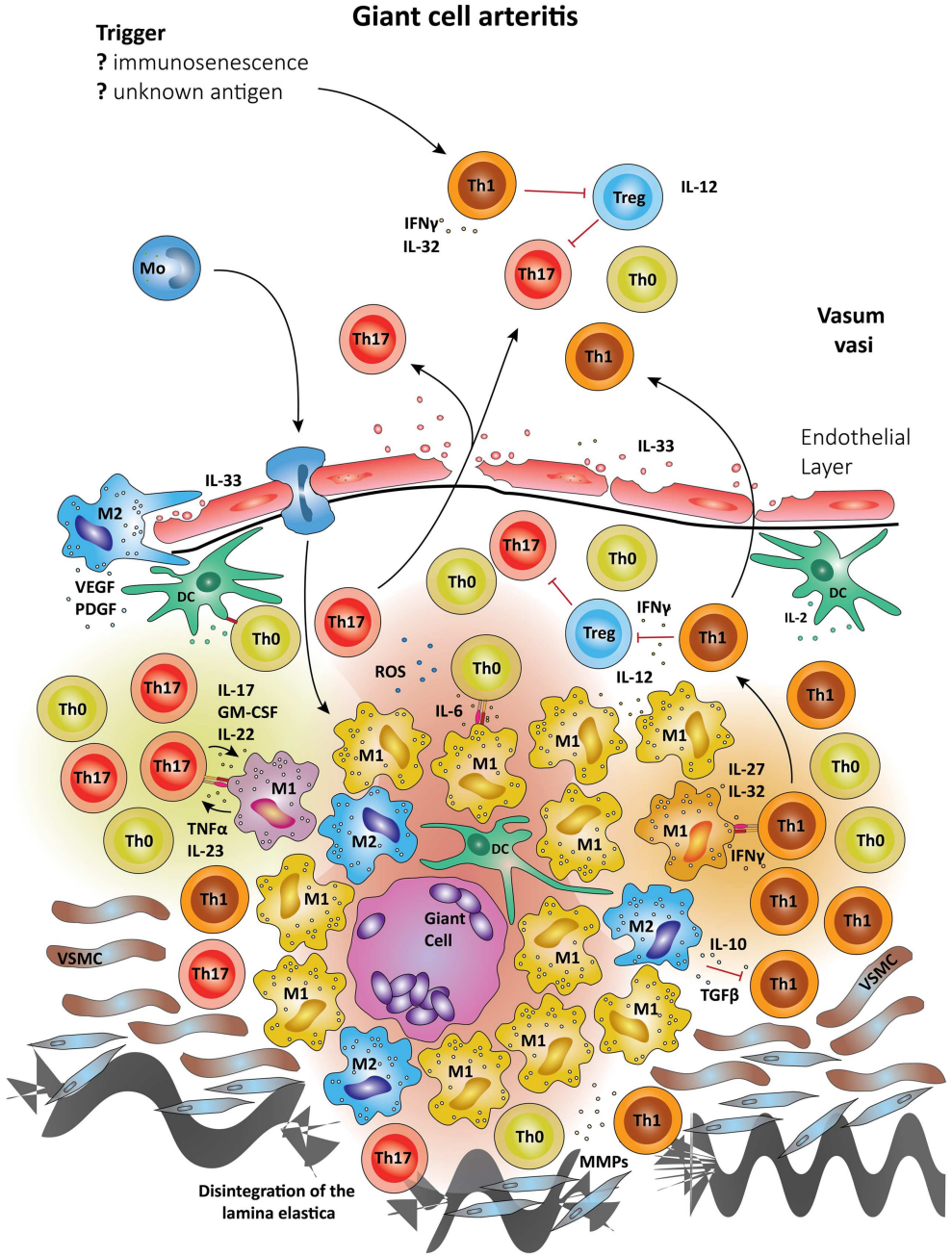

FIGURE 3 | Schematic summary of the pathogenic pathways implicated in granulomatous lesions in giant-cell arteritis. The upper panel represents pathogenic events in GCA. All immune recognition events and tissue damage occur within the vessel wall, not the perivascular tissue. Wall-resident DC, so-called vasDC, coordinate the recruitment and the differentiation of macrophages and T cells. IL-12 is instrumental in biasing T cells toward the Th1 lineage; IL-6, TGF-β, and IL-23 provide signals for Th17 differentiation. Dependent on their positioning in the vessel-wall monocytes commit to distinct functional profiles; e.g., metalloproteinases production, release of ROS, secretion of cytokines. Treg are underrepresented, partially due to inhibitory effects from IFN-γ. The lower panel shows the typical localization of granulomatous infiltrates on the adventitia side of the vessel wall. Monocytes (Mo) enter these lesions via the vasa vasorum and mature in the lesions under the influence of specific microenvironments. Different M1 cells are presumed to influence Th0 cells into Th1 and Th17 cells via cell–cell contact and the secretion of cytokines. Activated macrophages and DC may fuse and form multinucleate giant cells, a hallmark of GCA. The granulomatous infiltrate is a highly inflammatory microenvironment, which promotes the differentiation of Th0 to Th1 and Th17, which are then seeded into the circulation. Due to their localization, granulomatous infiltrates in GCA influence vascular smooth muscle cells (VSMCs) and myofibroblasts. The latter expand in number, migrate and result in concentric media hypertrophy. Also, the external lamina elastica disintegrates as a result of damage from ROS and matrix metalloproteases (MMPs). Although M1 are assumed the most frequent in granulomatous infiltrates, M2 cells have been proposed as a counter-mechanism and implicated in supporting tissue repair. Vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF) have been described in the vasculitic lesions of GCA and networks of neoangiogenic microvessels typically accompany the process of intimal hyperplasia (167, 168).

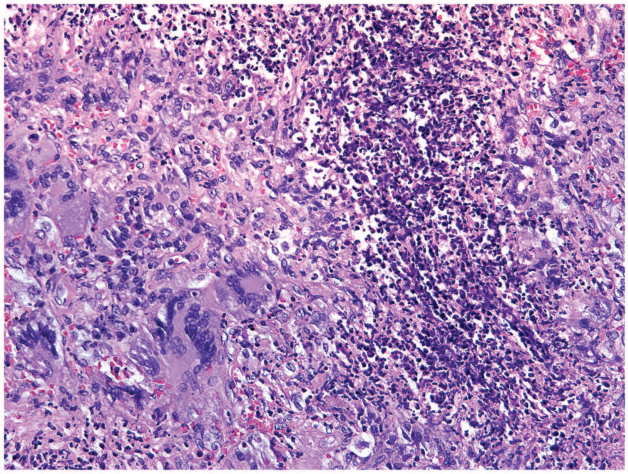

FIGURE 4 | Histological section of a granulomatous lesion in the lung of a 56-year-old patient with granulomatosis with polyangiitis. The core of the granuloma consists of multinucleate giant cells, macrophages, and neutrophils and is encircled by a rim of lymphocytes (hematoxylin and eosin staining).

pathogens in a network of decondensed chromatin, MPO, PR3, and other enzymes, but causing damage in the case of GPA by releasing more autoantigen into the granulomatous lesion (130). The clearance of apoptotic neutrophils by macrophages has been termed efferocytosis (see above) and is associated with secretion of a variety of cytokines and chemokines, including TNFα and prostaglandin E_2 (PGE_2) (131). Strongly activated macrophages have been localized within and around glomeruli affected by pauci-immune necrotizing crescentic glomerulonephritis, the pathognomonic lesion of AAV (132). These macrophages have been found to produce IL-18, thus attracting more neutrophils to the granulomatous inflammation (133). Such, activated macrophages express high levels of HLA class II molecules, enabling them to act as highly efficient APC (132). Of the leukocytes infiltrating the glomeruli and the tubulointerstitium, macrophages are the most prevalent, followed by granulocytes (134), differentiating GPA from GCA, in which granulocytes essentially do not participate. In renal biopsies from patients with active GPA, DC, and macrophages are localized in glomeruli and in periglomerular infiltrates, whereas DC and macrophages are absent from normal renal tissue. DC and T cells appear to interact within the periglomerular infiltrates (135), consistent with antigen recognition events orchestrating the tissue-damaging immune responses.

Moderately elevated levels of serum IL-1β and IL-6 have been reported in GPA patients during active disease, in line with persistent activation of macrophages in the tissue lesions (136–138). IL-1β and IL-6 are produced by M1 (138), but also by damaged endothelial cells (139). Serum levels of IL-1β, TNFα, and sIL-2R have been correlated with the presence of corresponding mRNA in tissue lesions, suggesting that mononuclear cells in vasculitic lesions are the origin of these proinflammatory mediators (140). In a mouse model of small-vessel vasculitis, IL-1β produced by monocytes has been associated with glomerulonephritis and blocking the IL-1β receptor with anakinra has resulted in a decrease of cellular crescents and hematuria (141).

The enzyme PR3 may play a central role in granuloma formation since it was found to be capable of cleaving IL-32, thereby enhancing its activity. IL-32 is produced by Th1 cells and its active form results in macrophage activation, leading to TNFα and IL-8 production (142). In addition, PR3 can cleave the protease-activated receptor 2 located on the cell surface of macrophages, leading to downstream inflammation, particularly IL-18, CXCL2, and IL-8 (142, 143). IL-18 is a known neutrophil attractant (**Figure 5**).

In search for the mechanisms through which autoantibodies mediate pathology in GPA, PR3-ANCA have been shown to induce upregulation of TLR2, 3, 4, 7, and 9, as well as NOD-1 and NOD-2 (144). Nucleotide-binding oligomerization domain-containing protein 2 (NOD-2) is an intracellular pattern-recognition molecule enabling macrophages to recognize bacterial molecules that contain muramyl dipeptide (MDP). In contrast to Crohn's disease, where mutations in *NOD2* have been implicated as disease risk factors (145), no such mutations were found in GPA patients (146). Interestingly, however, another study showed an association between mutations in the *TLR9* gene and PR3-ANCA positivity as opposed to MPO-ANCA positivity (147). In mice, ligands for TLR2 and TLR9 have both been implicated in kicking off autoimmunity. Notably, ligands for TLR2 have led to an expansion of Th17 cells, whereas ligands for TLR9 preferentially facilitate the expansion of Th1 cells (148). A possible role of TLR2 ligands in the pathogenesis of GPA is supportive of an involvement of *Staphylococcus aureus* in the development (83) as well as the risk for relapse in GPA (149), as *S. aureus* is a known ligand for TLR2 (150). In support of this notion, monocytes from patients with GPA have been found to express higher levels of TLR2 on the cell surface (151). Genetic factors are likely to be associated with the process of granuloma formation: these factors remain difficult to establish. One study found an association between the PTPN22 R620W polymorphism and the presence of granulomatous lesions (especially in the ENT region) specifically in patients with GPA, as opposed to patients with MPA or EGPA (152).

A macrophage product previously associated with granuloma formation in tuberculosis and silicosis is osteopontin (153). Interestingly, osteopontin is elevated in patients with active GPA (154) and has been detected in crescentic lesions in glomeruli (155). Osteopontin production has been associated with macrophage activation and it has been proposed that this monokine functions as a monocyte chemoattractant, securing the influx of fresh monocytes into the granulomatous lesions. Importantly, osteopontin synthesis is stimulated by active vitamin D, the latter, as mentioned above, a signifying product of active granulomas (156). There is evidence that osteopontin may play a role in TLR4-mediated IL-10 production in T cells, which suppress macrophages (157), delineating a negative feedback loop via which T cells can minimize granuloma-associated tissue damage.

Considering the destructive consequences of granuloma formation in GPA, it would be advantageous to treat GPA patients with therapies inhibiting this process. Studies on the use of anti-TNFα treatment in GPA patients have suggested that it may have a place in induction therapy (158), but patients remained at a higher

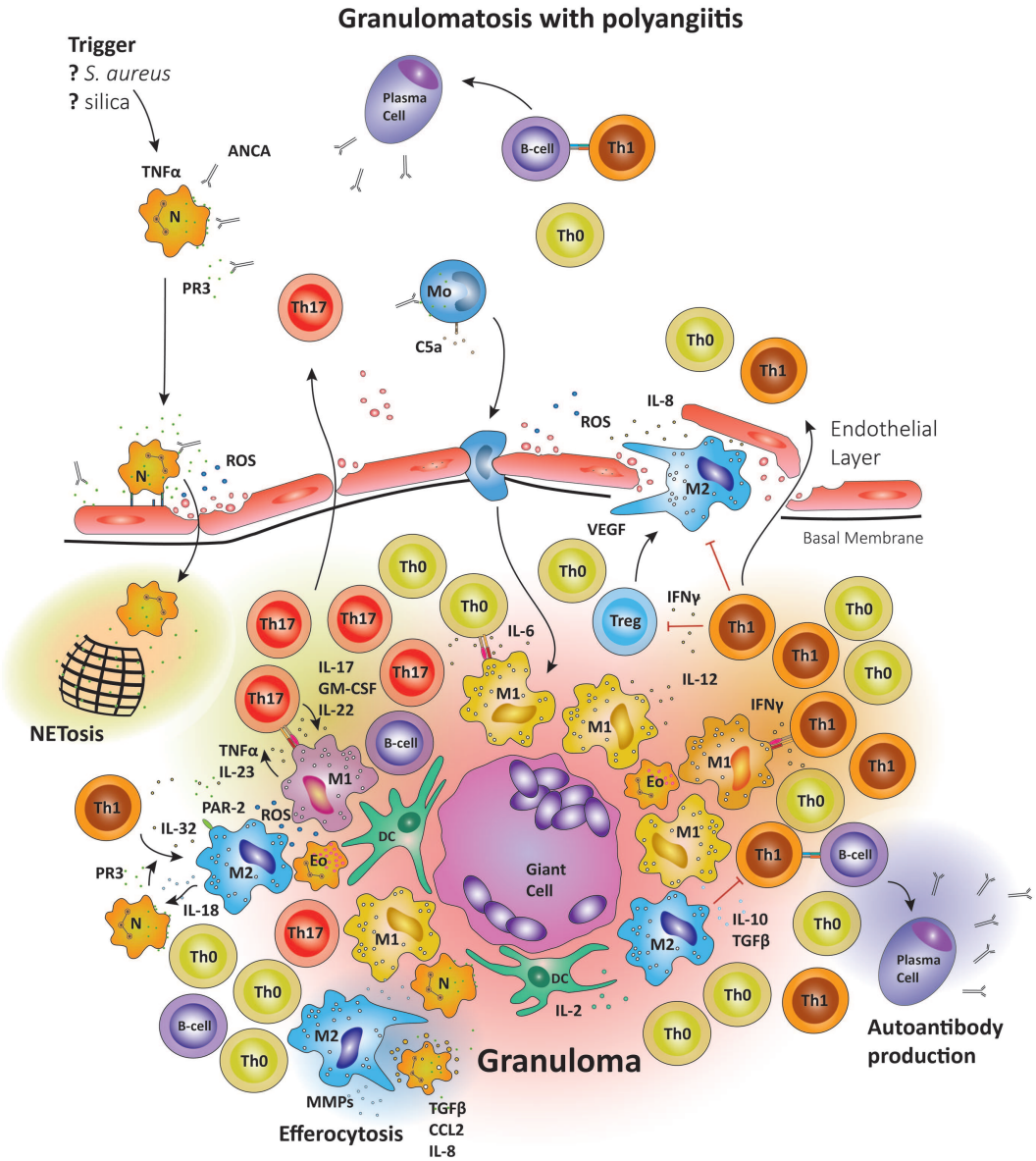

FIGURE 5 | Schematic summary of the pathogenic pathways implicated in granuloma formation in granulomatosis with polyangiitis. The upper panel represents the pathogenesis of granulomatosis with polyangiitis (GPA). Upon priming neutrophils bring to their surface high levels of proteinase-3 (PR3) and myeloperoxidase (MPO), the autoantigens recognized by ANCA. The priming process is believed to be mediated by TNFα. TNFα production may be induced by a variety of triggers; e.g., *S. aureus*, silica, etc. Anti-PR3 or anti-MPO antibodies are then able to bind these enzymes on the cell surface, causing neutrophils to degranulate, bind to endothelial cells, enter the perivascular tissue, and release ROS; thereby damaging the vessel wall. In the tissue, neutrophils releases so-called neutrophil extracellular traps (NETs), which are networks of fibers and DNA to trap pathogens. Many of the highly activated neutrophils become apoptotic and are then phagocytosed by resident M2 macrophages, a process that has been named efferocytosis and that may be deficient in GPA. During efferocytosis, macrophages release TGF-β, IL-8, and CCL2. Monocytes may also be activated by circulating ANCA, enhancing their chemotactic responsiveness and enabling them to participate in granuloma formation. The lower panel represents the formation of a sterile granuloma in extravascular tissue. Monocytes and $CD4^+$ cells enter the granuloma following a gradient of chemokines and cytokines; a process sufficient to transform monocytes into macrophages. Commitment to the M1 or M2 lineage is dependent on the specific cytokine environment. Neutrophils and eosinophils are commonly found in granulomas in GPA. Also, B cells have been reported in the surroundings of granulomas, where they may undergo further maturation. Multinucleate giant cells are present within the granulomas, resulting from the fusion of either macrophages or dendritic cells. The organized arrangement of the granuloma provides an ideal platform for macrophage–T cell interaction. $CD4^+$ cells coming into contact with IL-6 and TGF-β producing M1 cells are skewed toward the Th17 lineage. M1 also secrete IL-23; sustaining the Th17 population. In turn, Th17 cells can secrete granulocyte and GM-CSF in addition to IL-17 and IL-22, thus stabilizing M1 differentiation. However, M2 cells are equally represented in granulomas and secrete IL-10 and TGF-β. M2 cells are a source of vascular endothelial growth factor (VEGF) and support the outgrowth of microvessels, critically important as the granuloma grows in size.

risk for relapse (159). Depletion of B cells by anti-CD20 antibodies provides effective immunosuppression in patients with GPA (160). Whether this therapeutic approach functions by depressing autoantibody-dependent mechanisms or whether B cells provide other disease relevant functions, such as cytokine production and antigen presentation, remains speculative. It is conceivable that B cells are critically involved in toning the immune system and that their depletion impairs innate as well as adaptive immunity. Directly targeting macrophages may hold promise in immunosuppressing patients with GPA, although loss of macrophage function may further weaken their ability to mount protective immunity, especially against microbial pathogens.

FUTURE DIRECTIONS

It is currently unknown whether macrophages trapped in granulomas can be easily assigned to a functional lineage, e.g., M1 and M2, or whether residence in a granuloma directs macrophages into a separate differentiation program. Circulating cytokines in granulomatous disease favors the concept that the majority of macrophages may be M1. However, M2 have been localized in granulomatous lesions. This may simply be a negative feedback mechanism to temper inflammation in order to prevent excessive tissue damage. Indeed, one study found both M1 and M2 in the vessel lesions in GCA patients (105). A distinguishing feature of granulomas is the high cell turnover, giving rise to the need to clear apoptotic short-lived cells, such as neutrophils and macrophages. Effective removal of apoptotic bodies relies on the process of efferocytosis, again placing macrophages at a center stage. The current paradigm suggests that mainly M2 are responsible for effective efferocytosis. The intactness of these mechanisms in granulomatous vasculitis is unknown, but it could be hypothesized that a major defect lies in the inability of the patients to turn down M1 macrophage activation and bring to bear M2 macrophages.

Interestingly, GCA and GPA are distinct diseases affecting different sizes of blood vessels, but they are both characterized by granulomatous lesions. The granulomatous infiltrates in GCA are predominantly located in the vessel wall, where monocytes arrive via the vasa vasorum (87). In contrast, the granulomatous lesions in GPA are more often extravascular and, in the case of renal involvement, periglomerular (161). The classic granuloma with palisading as described by Godman and Churg in 1954 (162), can be found in GPA but not in GCA. The overall architecture of granulomas, characterized by an inner core of macrophages and DC surrounded by T cells, however, is present in both vasculitides. Importantly, the cellular composition of GCA- and GPA-associated granulomas seems to be different. Due to the intramural localization of the granulomatous lesion in GCA, vascular smooth muscle cells (VSMCs) and myofibroblasts are in intimate relationship to the granuloma-forming immune cells. Neutrophils are found exclusively in granulomas of GPA patients, as well as eosinophils. Also, B cells are absent from lesions in GCA (163) but they can be found in granulomas of GPA patients (164), where they may be able to mature and contribute to B cell dependent pathology. It has been proposed that the granuloma in GPA may participate in autoantibody production (165).

Granulomatous vasculitides reflect abnormalities in both, the innate and adaptive arm of the immune system. Activation products of innate cells, in particular cytokines, have attracted much attention as potential biomarkers of disease and effort has been invested to test whether they can help in quantifying disease burden. Similarities in the abnormal immune reactions of distinct vasculitides make it unlikely that a single cytokine will emerge as a disease-specific biomarker. However, cocktails of cytokines may have value in assessing how active the disease is in individual patients. Quantifying adaptive immunity beyond antibody formation has been challenging. In AAV, autoantibodies against PR3 and MPO have served an important diagnostic role, as they can help in rapidly reducing differential diagnosis in acutely sick patients. There is currently insufficient evidence that the titer of these autoantibodies is a good marker of disease activity. During the chronic course of AAV, autoantibody titers have limited use in helping make therapeutic decisions (166).

The possibility remains that granulomas will guide the search for the disease inducing antigens; as such antigens should be enriched in these tissue sites. Understanding the mechanisms of granuloma formation and the role of these lymphoid microstructures in perpetuating pathology could greatly enhance the spectrum of therapeutic targets. In all inflammatory vasculopathies, corticosteroids remain a cornerstone of therapy. Their therapeutic benefit may mainly result from their ability to suppress macrophage function. Temporary suppression of macrophage function, while effective in reducing acute phase responses, has little impact on the long-lived cells of the adaptive immune system, and thus fails to induce durable remission.

There remain considerable challenges in optimizing the management of patients with inflammatory blood vessel disease, despite enormous progress in deciphering processes of innate and adaptive immunity. The initial triggers derailing host protective immunity are undetermined. Given the importance of granuloma formation in protective and pathogenic immunity, speculations about infectious agents setting off vasculitis have held steady over decades. Hopes for the identification of such a disease inducer have not been met with success. Clustering of risk in geographic regions and populations have nurtured the belief that genetic risk factors are important, but could equally well support the role of environmental determinants. Most of the vasculitides are HLA associated diseases, providing further support for a critical contribution of antigen recognition and adaptive immunity in pathogenesis. Granulomas remain fascinating structures that bring together innate and adaptive immune cells and may ultimately hold the key to understanding why the power of immune protection is misused to harm the host.

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T cells in vascular inflammatory diseases

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Inflammation of the human vasculature is a manifestation of many different diseases ranging from systemic autoimmune diseases to chronic inflammatory diseases, in which multiple types of immune cells are involved. For both autoimmune diseases and chronic inflammatory diseases several observations support a key role for T lymphocytes in these disease pathologies, but the underlying mechanisms are poorly understood. Previous studies in several autoimmune diseases have demonstrated a significant role for a specific subset of $CD4^+$ T cells termed effector memory T (T_{EM}) cells. This expanded population of T_{EM} cells may contribute to tissue injury and disease progression. These cells exert multiple pro-inflammatory functions through the release of effector cytokines. Many of these cytokines have been detected in the inflammatory lesions and participate in the vasculitic reaction, contributing to recruitment of macrophages, neutrophils, dendritic cells, natural killer cells, B cells, and T cells. In addition, functional impairment of regulatory T cells paralyzes anti-inflammatory effects in vasculitic disorders. Interestingly, activation of T_{EM} cells is uniquely dependent on the voltage-gated potassium Kv1.3 channel providing an anchor for specific drug targeting. In this review, we focus on the $CD4^+$ T cells in the context of vascular inflammation and describe the evidence supporting the role of different T cell subsets in vascular inflammation. Selective targeting of pathogenic T_{EM} cells might enable a more tailored therapeutic approach that avoids unwanted adverse side effects of generalized immunosuppression by modulating the effector functions of T cell responses to inhibit the development of vascular inflammation.

Keywords: vascular inflammation, ANCA-associated vasculitis, atherosclerosis, T lymphocytes, effector memory T cells, Kv1.3 channels

INTRODUCTION

Vasculitides comprises a group of rare diseases, characterized by inflammation of the blood vessel walls. The clinical manifestations are dependent upon the localization, the type of vessel involved as well as the nature of the inflammatory process. Vasculitis constitutes, in most cases, as a primary autoimmune disorder, but can also be secondary to other conditions. The underlying conditions to secondary vasculitis are infectious diseases, connective tissue disorders, or hypersensitivity disorders. In general, primary vasculitides are systemic diseases with variable clinical manifestations making it difficult to classify. According to the latest Chapel Hill Consensus Conference, primary systemic vasculitides can be divided into seven main entities of which three are most common; large vessel vasculitis, medium vessel vasculitis (MVV), and small vessel vasculitis (SVV) (1). The group of large vessel vasculitides (LVV) affects the aorta and its major branches. The two major variants of LVV are giant cell arteritis (GCA) and Takayasu's arteritis (TA). MVV is vasculitis that predominantly affects medium arteries defined as the main visceral arteries and their branches. The two major categories are polyarteritis nodosa (PAN) and Kawasaki disease (KD). SVV is divided into anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) and immune complex SVV (2). AAV is characterized by necrotizing vasculitis with few or no immune deposits that predominantly affects small vessels, which lead to systemic organ damage. AAV are associated with the presence of circulating ANCA that are directed against proteinase-3 (PR3) or myeloperoxidase (MPO), proteins in the cytoplasmic granules of neutrophils. This group of systemic vasculitis includes granulomatosis with polyangiitis (GPA), primarily associated with antibodies to PR3–ANCA and microscopic polyangiitis (MPA) and eosinophilic granulomatosis with polyangiitis (EGPA), both principally associated with antibodies to MPO–ANCA.

Besides autoimmune disorders related to vascular inflammation, a more common chronic vascular inflammatory disease is atherosclerosis. Clinical evidence indicates that patients suffering from large and medium-sized vessel vasculitis show accelerated atherosclerosis (3). In SVV, this relation is less well defined. However, many patients with SVV carry several risk factors (e.g., impaired renal function, persistent proteinuria, and increased level of C-reactive protein) that contribute to the acceleration of the atherosclerotic process (3, 4). Enhanced oxidation processes, persistently activated T cells and reduced numbers of regulatory T (T_{REG}) cells are among the many pathophysiological factors that play a role in the acceleration of atherogenesis (5). Both vasculitis and atherosclerosis, although in nature different forms of chronic conditions, reveal similarities in T cell repertoire that occur within the process of vascular inflammation.

This review provides an overview of the role of adaptive immune mechanisms in vascular inflammation focusing on the T lymphocytes in particular. The main emphasis will be on the role of effector memory T (T_{EM}) cells in vasculitis (i.e., AAV and atherosclerosis) and the potential therapeutic interventions for modulating the activity of these cells.

T LYMPHOCYTES: KEY PARTICIPANTS IN VASCULAR INFLAMMATION

T cells are recruited to the vessel wall in conjunction with macrophages, but in lesser quantity. In the blood vessel wall or tissues, T cell responses are initiated by signals generated via the association of TCR complexes with specific peptide–MHC protein complexes on the surface of antigen-presenting cells (APCs) and through signals provided by co-stimulators expressed on APCs. The responses to antigen and co-stimulators include synthesis of pro-inflammatory mediators (e.g., IFN-γ) cellular proliferation, differentiation into effector and memory cells, and performance of effector functions. These initial events further amplify the inflammatory response, aggravating disease progression. Different T cell subsets exist that can influence vascular inflammation in various ways. In the last decade, substantial progress has been made in the characterization of T cell mediated responses in vascular inflammation.

T CELL INVOLVEMENT IN AAV AND ATHEROSCLEROSIS

In AAV it has been postulated that ANCA *in vivo* bind to surface expressed auto-antigens (PR3 or MPO) on primed neutrophils, which subsequently activates the neutrophils (6). These activated neutrophils enhance neutrophil degranulation and the release of cytotoxic products that promote endothelial cells damage leading to vascular inflammation and injury (6). This initial inflammatory response mediated by the innate immune system creates a pro-inflammatory (micro)environment to attract cells from the adaptive immune system. In the case of autoimmune mediated vascular pathologies, like AAV, loss of self-tolerance, and continuous antigen presentation also contributes to the involvement of the adaptive immune system. The contribution of T cell mediated immune responses in vascular inflammation is most likely because infiltrating T cells are detected in inflammatory lesions observed in the microvascular bed of kidney, lung, and in nasal biopsies from AAV patients (7–11). In accordance with these findings, soluble T cell activation markers [soluble interleukin-2-receptor (sIL-2R) and soluble CD30] are elevated in plasma or serum and have been shown to be associated with disease activity in AAV (12–15). Also, ANCA antigen specific T cells have been detected in AAV (16, 17). Moreover, the IgG subclass distribution of ANCA, predominantly consisting of IgG1 and IgG4 implies isotype switching of ANCA for which T cells are required (18). Importantly, Ruth et al. demonstrated a pivotal role of T cells in the expression of crescentic glomerulonephritis (19). They induced experimental anti-MPO-associated crescentic glomerulonephritis by immunizing C57BL/6 mice with human MPO followed by subsequent challenge with anti-glomerular basement membrane (anti-GBM) antibodies. Mice depleted of T cells at the time of administration of anti-GBM antibodies developed significantly less glomerular crescent formation and displayed less cell influx in glomeruli compared with control mice. Interestingly, specific T cell depleting therapies with anti-CD52 antibodies (Alemtuzumab) or anti-thymocyte globulin can induce remission in refractory AAV patients (20, 21).

Atherosclerosis is considered a chronic inflammatory disease, characterized by a slowly progressing passive lipid accumulation in large and medium-sized blood vessels that ultimately leads to the formation of plaques. Both innate and adaptive immunity are involved in this process. Ait-Oufella et al. recently reviewed the role of the adaptive immune response in atherosclerosis and discussed the role of dendritic cells (DCs) in the control of T cell involvement in atherosclerosis (5). Classically, DCs accumulate in the atherosclerotic plaque through direct chemokine mediated recruitment. DCs take up (atherosclerotic-specific) antigens such as ApoB100 and LDL and become activated and mature. Subsequently, DCs migrate to draining lymph nodes, where they can present antigens to naïve T cells. After activation, these T cells develop into effector cells, clonally expand and enter the bloodstream. When effector T cells are recruited into atherosclerotic plaques they are reactivated by antigens presented by local macrophages and DCs, boosting the immune response. In human atherosclerotic lesions, the ratio of macrophages to T cell has been reported to be approximately 10:1, thus T cells are not as abundant as macrophages. However, because T cells are activated in the lesions resulting in the production of pro-atherogenic mediators, they can importantly contribute to lesion growth and disease aggravation. The first evidence of T cell involvement in atherosclerosis came with the demonstration that MHC class II positive cells and T cell cytokines (e.g., IFN-γ) are expressed in human atherosclerotic plaques (22). Later, the presence of T cells was observed in atherosclerotic plaques in humans (23, 24) and mice (25, 26). These observations only demonstrated the association of T cell with atherosclerosis but did not revealed the role of T cells in atherogenesis. However, Zhou et al. demonstrated a specific role of T cells in atherogenesis using an animal model of atherosclerosis. They showed that transfer of $CD4^+$ T cells into $ApoE^{-/-}$ mice crossed with immunodeficient mice (scid/scid mice) fully reversed the atheroprotection provided by T and B cell deficiency (27).

Taken together, these observations indicate that T cell mediated immunity is an important contributor to the pathogenesis of vascular diseases such as AAV and atherosclerosis. In line with this, different T cell populations have been identified in vascular inflammation as will be discussed below. **Figure 1** presents a proposed mechanism of the T cell mediated vascular inflammatory process.

T HELPER CELLS IN VASCULAR INFLAMMATION

Aberrant T helper (T_H) cell polarization has been described in patients with vascular diseases. The involvement of different T_H cells subsets in the pathogenesis of vascular disease has been suggested to depend on disease activity/stage and whether the disease is localized or systemic.

In AAV, analysis of patients sera for soluble markers associated with either T_H1 cells (IFN-γ, sCD26) or T_H2 cells (IL-4, IL-5, IL-10, IL-13, sCD23, and sCD30) revealed a shift toward a T_H2-type response in patients with active generalized disease, whereas a T_H1-type response is predominant in patients with localized

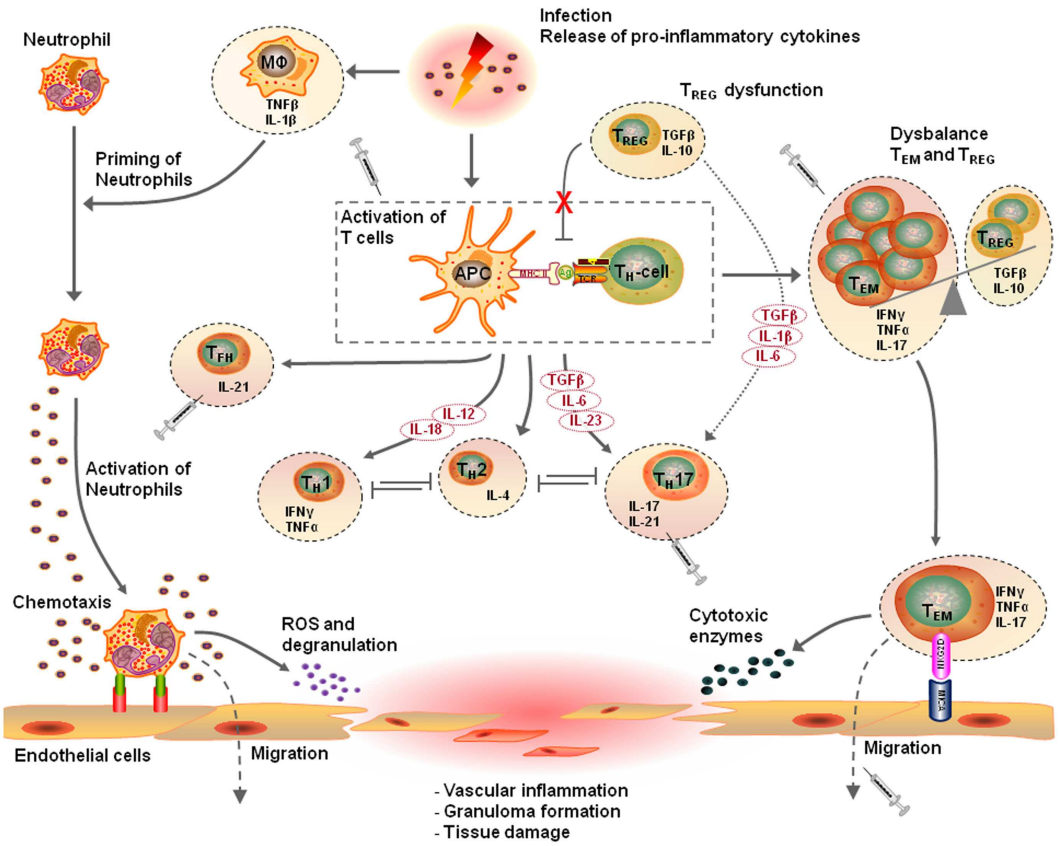

FIGURE 1 | Proposed pathophysiological mechanism of T cell mediated vascular inflammation. Vascular inflammation is initiated by a pro-inflammatory trigger such as an infection. Release of pro-inflammatory cytokines causes priming of neutrophils, up-regulation of adhesion molecules on endothelial cells, and an expansion of circulation effector T cells. Activation of primed neutrophils enhances vessel wall adherence and the transmigration capacity of the neutrophils. Production of reactive oxygen species and degranulation of fully activated primed neutrophils causes damage to vascular endothelial cells. This acute injury together with pro-inflammatory triggers elicits an innate inflammatory response that recruits T lymphocytes, which replace the neutrophils and either resolves or mediate the development of vasculitis. In this pro-inflammatory environment, the innate immune system with antigen-presenting cells (APCs) and T cells start to mediate the inflammatory response. Distinct cytokine patterns in combination with a defect in regulatory T (T_{REG}) cell function or frequency results in expansion of effector memory T (T_{EM}) cells. The dysbalance in the homeostasis of T_{REG} cells and T_{EM} cells, results in additional releases of pro-inflammatory cytokines promoting neutrophil priming and persistent activation of T_{EM} cells. Expanded circulating T_{EM} cells upregulate their killer immunoglobulin-like receptor (NKG2D) and interact with their ligand major histocompatibility complex class-I chain-related molecule A (MICA) on vascular endothelial cells. This event results in the migration of T_{EM} cells into target tissues, drive granuloma formation leading to tissues destruction in a perforin-dependent, and granzyme-dependent way, ending up in vasculitis. The T cell driven vascular inflammatory response is a multistep process and has different therapeutic possibilities. For this purpose, selective T_{EM} cell modulation might be beneficial to regulate the T_{EM} cell activity, proliferation, and migration. Other therapeutic options are modulation of T cell activation by interfering with co-stimulatory molecules, depletion of T cells, inhibition of T cell migration, or neutralizing secreted pro-inflammatory cytokines (This figure was created using Visi ScienceSlides® Software).

disease (28, 29). Consistent with these observations, analysis of nasal granulomatous lesions from AAV patients demonstrated a relative increase of cells expressing T_H1-associated markers such as IFN-γ and CD26 during localized disease, whereas the T_H2-associated marker IL-4 was found in generalized AAV (11). In addition, Lamprecht et al. compared chemokine receptors on peripheral blood-derived T cells. The inducible inflammatory T_H1-type chemokine receptor CCR5 was more prominent in the granulomatous lesions of AAV patients (30).

Similar to AAV, a study on cytokine expression in advanced human atherosclerotic plaques confirmed the dominance of pro-inflammatory T_H1 cytokines (IFN-γ, TNF-α, and IL-2) (31). Genetic deficiency in IFN-γ or its receptor in $ApoE^{-/-}$ mice reduced atherosclerotic lesion formation and enhanced plaque stability (32), whereas exogenously administered IFN-γ enhanced atherosclerosis in $ApoE^{-/-}$ mice (33). Intriguingly, it seems that the protective effect of IFN-γ deficiency is restricted to male $ApoE^{-/-}$ mice (34). In addition, several studies revealed that intervention in IL-12 or IL-18 gene, or receptor function was found to reduce plaque development in mouse models of atherosclerosis (35–37). Furthermore, administration of these cytokines accelerated disease progression (38, 39). Collectively, these data point toward a pro-inflammatory T_H1 response in atherosclerosis. However, the role of T_H2 immune responses in atherosclerosis is

controversial. IL-4, the signature cytokine of the T_H2 lineage, is not frequently observed in human atherosclerotic plaques (31). Moreover, experimental studies examining the involvement of T_H2 cells are contradictory, some showing pro-atherosclerotic effects (36, 40), whereas others show no or athero-protective effects (41, 42).

Overall, the balance between T_H1 and T_H2 cells plays a key role in the development of vascular inflammation. Interestingly, in the last decade T_H17 cells have emerged as a new $CD4^+$ T cell subset characterized by secretion of IL-17A and other cytokines including IL-17F, IL-21, and IL-22. These cells are considered another major pathogenic effector subset involved in the development of inflammatory and autoimmune diseases (43).

IL-17 has been reported to promote the release of the pro-inflammatory cytokines IL-1β and TNF-α from macrophages (44), which are essential for priming and activation of neutrophils. Furthermore, this pro-inflammatory milieu induces CXC chemokine release (45) and up-regulation of endothelial adhesion molecules (46) responsible for the recruitment of neutrophils to the site of inflammation (47). These pro-inflammatory events suggest that IL-17 may directly contribute to the acute vascular inflammatory response in AAV. Convincing experimental evidence that support this notion comes from several studies. Hoshino et al. demonstrated that neutrophils produce IL-17A and IL-23 in response to MPO–ANCA creating local conditions to promote T_H17-mediated autoimmunity (48). In addition, Gan et al. showed that immunization of C57BL/6 mice with murine MPO resulted in MPO-specific dermal delayed type hypersensitivity and systemic IL-17A production (49). Upon injection of low-dose anti-GBM antibodies these mice developed glomerulonephritis. In contrast, IL-17A deficient mice were nearly completely protected from disease induction due to reduced neutrophil recruitment and MPO deposition (49). Consistent with this finding, Odobasic et al. demonstrated that IL-17A contributes to early glomerular injury, but it paradoxically, attenuates the severity of fully established crescentic disease by limiting the T_H1 responses (50). They used a mouse model of crescentic anti-GBM glomerulonephritis assessing the renal injury and immune responses in IL-$17A^{-/-}$ and in wild-type (WT) mice. Crescentic glomerulonephritis was enhanced in IL-$17A^{-/-}$ mice, with increased glomerular T cell accumulation and augmented T_H1 responses (50). In contrast, mice lacking IL-12(p35), the key T_H1-promoting cytokine, had decreased T_H1 responses and increased T_H17 responses and developed less severe crescentic glomerulonephritis than WT animals (50). Thus, they provided evidence that T_H1 responses mediate severe crescentic injury and that T_H1 and T_H17 cells counter regulate each other during disease development in this model. In line with the *in vivo* observation, our group observed a skewing toward T_H17 cells following *in vitro* stimulation of peripheral blood samples of AAV patients (51). Moreover, it has been shown that $CD4^+CD45RC^{low}$ cells (T cells with a memory phenotype) are a source of IL-17 in AAV patients (52). These observations were corroborated by Nogueira et al., demonstrating significant elevated levels of serum IL-17A and its associated upstream cytokine IL-23 in acute AAV patients (53). Additionally, auto-antigen-specific IL-17 producing cells were significantly elevated in patients during disease convalescence compared to healthy controls (53). Moreover, increased frequencies of circulating T_H17 cells have been observed in various forms of vasculitis (GCA, EGPA, and Behçet disease) and correlated with disease activity (54–58).

A possible explanation for the involvement of T_H17 cells in AAV lies within the major physiological role of T_H17 cells. Physiologically, T_H17 cells are important in the defense against fungi and bacterial infections [e.g., *Staphylococcus aureus (S.aureus)* infections] by activating neutrophils through the production of IL-17 and IL-17F. It has been shown that peptidoglycans and superantigens of *S. aureus* might have an immunomodulatory effect on DCs by imprinting of a strong T_H17 polarization capacity (59). Furthermore, *S. aureus* α-toxin was shown to induce IL-17A secretion in $CD4^+$ T cells (60). In addition, Zielinski et al. demonstrated that *S. aureus* specific T_H17 cells produced IL-17 and surprisingly could produce IL-10 upon restimulation (61). Intriguingly, chronic nasal carriage of *S. aureus* has been found to be an important risk factor for disease relapse in AAV patients (62). This suggests that carriage of *S. aureus* may drive the T_H17 responses in AAV.

The role of T_H17 cells in atherosclerosis remains controversial. It has been demonstrated that T_H17 cells and IL-17 accumulate in atherosclerotic lesion of both mice and humans, but both atherogenic as well as athero-protective effects of IL-17 have been reported (63–67). Studies in $ApoE^{-/-}$ mice genetically deficient for IL-17 or treated with anti-IL-17A antibodies demonstrated that absence or depletion of IL-17 attenuated development of atherosclerosis (65, 68). Also, $Ldlr^{-/-}$ mice transplanted with bone marrow from mice deficient in IL-17 receptor showed smaller atherosclerotic lesions (63). In patients, IL-17A expressing T cells were detected in atherosclerotic lesions and increased IL-17 expression in these lesions has been shown to be associated with increased inflammation and plaque vulnerability (67). In contrast to the pathogenic role of T_H17 cells, Taleb et al. found a protective role for T_H17 cells in atherosclerosis. Using $Ldlr^{-/-}$ mice deficient for suppressor of cytokine signaling 3 (SOCS3), a suppressor of signaling from IL-17, showed less disease development (64). In the same study, administration of an anti-IL-17A antibody accelerated atherosclerosis, indicating a protective role for T_H17 cells (64).

A possible explanation for these contradictory observations may be that IL-17 is not only produced by T cells. The presence of different IL-17 isoforms (IL-17A, -E, and -F) in human atherosclerotic plaques revealed that the IL-17 family cytokines were expressed by various cells of the immune system (e.g., neutrophils) depending on the stage of the atherosclerotic plaque (66). Furthermore, not only immune cell are targets of IL-17. It has been demonstrated that endothelial cells and smooth muscle cells are likely to be IL-17E-responsive, given the expression of IL-17 receptor components on these cells and transient activation of ERK1/2 upon stimulation with recombinant IL-17E (66). Thus, this indicates a complex contribution of IL-17 in atherogenesis depending on the isoform and phases of atherosclerosis.

Beside IL-17, T_H17 cells can also produce IL-21, a cytokine that is produced primarily by T follicular helper (T_{FH}) cells. IL-21 is required for B cell class switching, antibody production (69), and induces differentiation of B cells toward plasma cells by synergizing with B cell activating factor (BAFF) (70, 71). The role of IL-21 has been demonstrated in Behçet disease, a form of variable vessel vasculitis. Geri et al. demonstrated increased serum levels of IL-21 that correlated with the disease activity in

patient with Behçet disease (58). In addition, they showed that IL-21 producing central memory $CD4^+$ T cells positively correlated with T_H17 responses and negatively correlated with FoxP3 T_{REG} cells. Conversely, blockade of IL-21 with IL-21R-Fc fusion protein resorted the balance between T_{REG} cells and T_H17 cells by suppressing IL-17A production and increasing FoxP3 expression by $CD4^+$ T cells (58). Interestingly, a significant increased population of IL-21 producing T_{FH} cells was observed in the circulation of AAV patients (72). In addition, IL-21 was shown to enhance the production of cytotoxic products such as granzyme B and perforin, by $CD8^+$ T cells and natural killer (NK) cells (73). Based on the studies in various forms of vasculitis it is therefore conceivable that IL-21 together with IL-17 plays a critical role in the pathogenesis of AAV.

In atherosclerosis, no major studies have been conducted to date to investigate the role of T_{FH} cells, but IL-21 may be involved in tissue damage. There is evidence that IL-21 acts directly on gut epithelial cells to induce the production of macrophage inflammatory protein-3α (MIP-3α), a chemokine that attracts both T_H1 and T_H17 cells to inflamed tissues (74). Given that endothelial cells are known to produce MIP-3α, it is possible that IL-21 enhances the migration and accumulation of T_H1 and T_H17 cells into the vascular wall in both vasculitis and atherosclerosis resulting in inflammation.

REGULATORY T CELLS IN VASCULAR INFLAMMATION

The actions of T_H cells can be balanced by T_{REG} cells, a subpopulation that is characterized by their ability to suppress a variety of physiological and pathological immune responses and prevent autoimmunity (75). T_{REG} cells are characterized by their expression of forkhead/winged helix transcription factor (FoxP3) that is required for their development and function (76). Defects in T_{REG} function or reduced numbers of T_{REG} cells have been described in several autoimmune disorders and chronic inflammatory disorders associated with vascular inflammation (77). To date different research groups reported controversial results regarding the frequency of T_{REG} cells in AAV patients compared to healthy controls. However, a consistent finding has been that these studies all reported impaired functionality of circulating T_{REG} cells (78–80). It has been found that the suppressive function of T_{REG} cells was defective in GPA patients compared to healthy controls (78). However, the GPA patients showed a significant increase of memory $FoxP3^+CD25^{high}$ T_{REG} cells. Consistent with this finding, Klapa et al. demonstrated an increased number of $FoxP3^+$ T cells as well as phenotypical and functional alteration of T_{REG} cells in GPA patients (79). They reported an increased number of interferon receptor I-positive T_{REG} cells in the peripheral blood of GPA patients. In addition, they showed that IFN-α exaggerates functional T_{REG} impairment *ex vivo* in response to the auto-antigen PR3 (79). Furthermore, Morgan et al. also reported altered T_{REG} function in GPA patients (80). They observed that T_{REG} cells from healthy controls and from ANCA-negative patients were able to suppress T cell proliferation to PR3, whereas T_{REG} cells from PR3-ANCA-positive patients failed to suppress this antigen specific response (80). Dysfunction of T_{REG} cells is thus believed to play a role in the development of GPA. In contrast, T_{REG} function in MPA patients was comparable to that in healthy controls although FoxP3 levels were diminished, suggesting that in MPA a numerical deficiency of T_{REG} cells exists (81). Additionally, Saito et al. demonstrated that the proportion of T_{REG} cells in the peripheral blood reflects the relapse or remission status of EGPA patients. They observed that FoxP3-expressing cells and IL-10 producing T_{REG} cells were detected in lower frequencies in patients with a relapse compared to patient in remission (82). However, the suppressive function of T_{REG} cells in EGPA patients still needs to be investigated. All together there are some inconsistent observations regarding the number and/or frequencies of T_{REG} cells in AAV patients. These differences might be due to variations in the methodology and gating strategies for the T_{REG} cells between the different studies. However, in all studies impaired functionality of the T_{REG} subset has been demonstrated indicating that T_{REG} cells from AAV patients are not able to suppress proliferation of other T_H cell subsets.

The terminally differentiated T_{REG} cells are not defined entirely by FoxP3 expression, and the $FoxP3^+$ T cell population is heterogeneous, consisting of a committed T_{REG} lineage and an uncommitted subpopulation with developmental plasticity (83). It has been reported that human T_{REG} cells can convert into pro-inflammatory IL-17 producing T cells depending on a specific cytokine environment (81–83). Both T_H cell subsets (i.e. T_{REG} and T_H17 cells) may develop from the same precursors under distinct cytokine conditions, and a subset of IL-17-producing $CD4^+FoxP3^+$ T_{REG} cells can be generated upon polarization by pro-inflammatory cytokines such as IL-6, which is crucial in orchestrating the balance of T_{REG} and T_H17 cells (84–86). It has been shown that the combination of transforming growth factor-beta (TGF-β) and IL-6 treatment can synergistically promote FoxP3 degradation (87), and induce the transcription of ROR-γt, which in turn participates in the induction of IL-17 expression and mediates the skewing toward a T_H17 cell phenotype.

Besides the local cytokine environment that orchestrates the balance of T_{REG} and T_H17 cells, the functional stability of FoxP3 might influence the developmental pathway. Post-translational modifications can transiently alter the functionality of transcription factors, and there is evidence that FoxP3 can be regulated via acetylation. For example, hyperacetylation of FoxP3 increases the stability of FoxP3 and treatment with histone deacetylases inhibitors results in increased numbers and functional T_{REG} cells (88). Indeed, Koenen et al. demonstrated that histone deacetylases inhibitors suppresses the conversion from T_{REG} to T_H17 cells (89). In addition, different isoforms of FoxP3 have been investigated in human T_{REG} that have been shown to affect T_{REG} function and lineage commitment. More specifically, the full length isoform FoxP3 interacts with ROR-γt and inhibits the expression of genes that define the T_H17 lineage, whereas the isoform lacking exon 2, FoxP3Δ2 fails to inhibit ROR-γt. Upon stimulation in an inflammatory environment these non-functional T_{REG} convert into IL-17 producing effector T cells. Based on these findings, our previous described non-functional T_{REG} cells in AAV patients may lack their suppressive function due to the up-regulation of FoxP3Δ2 that fails to inhibit ROR-γt mediated IL-17 transcription. Indeed, Free et al., demonstrated that T_{REG} cells from patients with active AAV disproportionately used FoxP3Δ2, which might alter T_{REG} cell function (90).

In atherosclerosis, several studies have demonstrated a protective effect of T_{REG} cells. FoxP3$^+$ T cells have been found in atherosclerotic plaques of humans, although in low numbers (91). In mice, the T_{REG} cytokine products IL-10 and TGF-β, have been demonstrated to induce potent anti-atherosclerotic activities. Genetic inactivation or blockade of IL-10 and TGF-β with neutralizing antibodies aggravated atherosclerosis in mice (92, 93). Depletion of T_{REG} cells directly addressed the protective role of these cells in atherosclerosis. Significant aggravation of atherosclerosis was observed in Ldlr$^{-/-}$ mice with reduced T_{REG} cell numbers, achieved either by deletion of CD80/86 or CD28, inducible T cell co-stimulators, or upon treatment with CD25-depleting antibodies (94, 95).

Thus, the interplay and imbalances between different T_H cells are important in the pathogenesis of vascular inflammatory diseases (**Table 1**). An imbalance in T_H1/T_H2 toward the T_H1 response promotes the development of vascular inflammation, whereas skewing toward prominent T_H2 and T_{REG} responses is anti-inflammatory and results in a reduction of vascular inflammation. In AAV, T_H17 cells are considered to be pathogenic but how T_H17 cell affect inflammation in atherosclerosis still needs to be determined.

INVOLVEMENT OF CD4$^+$ T EFFECTOR MEMORY CELLS

As mentioned above, several observations support the involvement of CD4$^+$ T_H cells in the pathogenesis of vascular inflammatory diseases like AAV and atherosclerosis. In line with these observations, an expanded population of CD4$^+$ T cells lacking the co-stimulatory molecule CD28 was observed in peripheral blood and in inflammatory lesions of AAV patients (10, 96). Furthermore, these CD4$^+$CD28$^-$ T cells are a major source of IFN-γ and TNF-α, display up-regulation of the T cell differentiation marker CD57 and show cytoplasmic perforin expression, indicating the cytotoxic potential of these cells (10).

Consistent with these findings, our group observed a significant increase in the frequency of circulating CD4$^+$ T_{EM} cells in the peripheral blood of AAV patients in remission (97). Subsequently, it was found that the number of these circulating CD4$^+$ T_{EM} cells decrease during active disease when compared with the number during complete remission (97). We proposed that these CD4$^+$ T_{EM} cells migrate toward inflamed tissues (97). In accordance, infiltrating T cells found in granulomas within lung and/or kidney tissues resemble mainly the CD4$^+$ T cell memory phenotype. A remarkable increase in CD4$^+$ T_{EM} cells in the urinary sediment with a concomitant decrease of circulating CD4$^+$ T_{EM} cells in patients with active renal involvement strongly suggests migration of CD4$^+$ T_{EM} cells during active renal disease into the affected organs (98). This finding might reflect the role of CD4$^+$ T_{EM} cells in renal injury during active disease. In line with these findings, is the observation that CD4$^+$ T_{EM} cells expressing CD134 are expanded in peripheral blood of AAV patients (99). It has been reported that the ligand for CD134 (CD134L) is expressed on endothelial cells (100) and ligation of CD134 contributes to T cell migration and tissue infiltration through its interaction with

Table 1 | T cell subsets associated with vascular pathologies.

T cell subset	Key characteristic	Finding in vascular pathology	Reference
T_H1 cell	Production of IFNγ	Skewing toward T_H1 in localized GPA	(11, 28–30)
	Enhances cellular immune responses	Dominant T_H1 cytokine prolife in human atherosclerotic plaques	(31)
T_H2 cell	Production of IL-4	Skewing toward T_H2 in active generalized GPA, and in EGPA	(28, 29)
	Promote humoral immune response		
T_H17 cell	Production of IL-17	Skewing toward T_H17 in GPA during quiescent disease, and in EGPA and Behçet disease during active disease	(51, 57, 58)
	Defense against fungi and bacterial infections		
	Mediates pathogenic responses in autoimmune diseases	Increased frequencies of T_H17 cells in GCA	(54–56)
		Contradictory observations of T_H17 cell function in atherosclerosis, IL-17A expressing T cells are present in human atherosclerotic lesions and associated with increased inflammation and plaque vulnerability. However, mouse models reveal a protective effect of T_H17 cells	(63–67)
T_{FH} cell	Production of IL-21	Increased T_{FH} population in GPA	(72)
	IL-21 required for B cell class switching	T_{FH} cytokine IL-21 correlates with disease activity in Behçet disease	(58)
T_{REG} cell	Production of IL-10	Contradictory observations regarding the frequency of T_{REG} cells in GPA patients, however all studies report an impaired function of T_{REG} cells in GPA	(78–80, 90)
	Regulation of other T cell subsets		
	Maintain peripheral tolerance to self antigens		
		Numerical defect of T_{REG} cells in MPA	(81)
		T_{REG} cells reflect relapse and remission status in EGPA	(82)
		Reduced frequencies of T_{REG} cells in GCA	(55, 56)
		Low number of T_{REG} cells in human atherosclerotic plaques, mouse models reveal a protective effect of T_{REG} cells	(91–93)

GPA, granulomatosis with polyangiitis; EGPA, eosinophilic granulomatosis with polyangiitis; GCA, giant cell arteritis; MPA, microscopic polyangiitis.

CD134L on vascular endothelial cells (101). Furthermore, CD134 expressing T cells have been detected in the inflammatory lesions of AAV patients (99), supporting the hypothesis that $CD4^+$ T_{EM} migrate to inflamed areas. The fact that $CD4^+$ T_{EM} cells migrate to the kidney during active disease suggests that a specific stimulus is being expressed by the (micro)vascular bed of the kidney, which attracts these cells.

In atherosclerotic disease, analysis of $CD4^+$ T cell subsets (i.e., naïve T cells, T_{CM}, and T_{EM} cells) revealed that $CD4^+$ T_{EM} cells are key players in disease pathogenesis (102). It has been shown that the frequency of circulating T_{EM} cells was significantly increased in $Ldlr^{-/-}$ and $ApoE^{-/-}$ mice compared to control C57BL/6 mice and also correlated with the extent of atherosclerotic lesions (102). In line with these observations, Almanzar et al. demonstrated that T cells isolated from early atherosclerotic lesions are mostly $CD4^+$ T_{EM} cells (24). Subsequently, the intralesional atherosclerotic $CD4^+$ T cells produce high amounts of the pro-inflammatory cytokines IFN-γ and IL-17 (24), which suggest that memory T cells in atherosclerotic plaques are in a state of activation reflecting the pro-inflammatory cytokine production.

At the functional level, $CD4^+$ T_{EM} cells have been shown to mimic features of NK cell including surface expression of the NK group 2 member D (NKG2D) and cytotoxic potential (103, 104). NKG2D is an activating C-type lectin-like receptor, which differs from other NKG2 family members as it apparently lacks an antagonist and substitutes for CD28-mediated co-stimulatory signaling in $CD28^-$ T_{EM} cells (104). One of the NKG2D ligands in human is the major histocompatibility complex class-I chain-related molecule A (MICA). MICA is usually absent on normal cells, but expressed upon cellular stress on target cells such as fibroblasts, epithelial cells, and endothelial cells (104). The expression of MICA on the surface of the endothelium makes this polymorphic molecule a potential target in vasculitis. In rheumatoid arthritis (RA), an other chronic and systemic autoimmune disorder, an unusual $CD4^+CD28^-NKG2D^+$ population was detected in peripheral blood and synovial tissue (105). Furthermore, the NKG2D ligand MICA is dramatically upregulated in RA synoviocytes and is capable of activating autoreactive T cells in an NKG2D-dependent manner (105). In addition, it has been shown in patients with Crohn's disease, a chronic inflammatory disorder, that $CD4^+NKG2D^+$ T_{EM} cells can kill target cells that express MICA via NKG2D–MICA interaction (106). These findings in RA and Crohn's disease translate very well to AAV. In AAV, it has been found that NKG2D was anomalously expressed on circulating $CD4^+CD28^-$ T_{EM} cells (107). Furthermore, it has been demonstrated that NKG2D, MICA, and IL-15 are simultaneously expressed in granulomatous lesions in AAV patients. Importantly, it was reported that survival, expansion, and cytotoxic properties of $CD4^+NKG2D^+$ T cells were dependent on IL-15 signaling in AAV (108). Therefore, it is tempting to speculate that combined IL-15 and MICA expression contributes to the killing mechanisms of $CD4^+NKG2D^+$ T cells in vessel inflammation and disease progression in AAV.

In atherosclerosis, Xia et al. reported that immune activation resulting from NKG2D–ligand interaction promotes atherosclerosis (109). They observed soluble MICA in sera and upregulated MICA expression in atherosclerotic plaques of patients with type 2 diabetes mellitus. Moreover, they investigated the role of NKG2D in atherosclerosis using $ApoE^{-/-}$ mice genetically deficient for NKG2D or treated with anti-NKG2D antibodies. Preventing NKG2D–ligand interaction resulted in a dramatic reduction in plaque formation and suppressed systemic and local inflammation mediated by multiple immune cell types. Since this is the only study reported on NKG2D in relation with atherosclerosis development, further studies are needed to fully elucidate the role of NKG2D in the pathogenesis of atherosclerosis.

T CELL DIRECTED THERAPEUTIC INTERVENTIONS

As described in this review, T cells and T cell migration are pivotal in vascular inflammatory diseases such as vasculitides and atherosclerosis. Therefore interfering with T cell activation, proliferation, and migration might be a beneficial approach to dampen the inflammatory response and cell-based therapy to modulate the T cell compartment may be a therapeutic option.

Regulatory T expansion may be of benefit to counterbalance persistent T cell activation in AAV. In this respect, treatment with low-dose IL-2 has been found to promote T_{REG} recovery and clinical improvement in patients with autoimmune vasculitis (110). Interestingly, tocilizumab, a humanized anti-IL-6 receptor antibody used in the treatment of RA demonstrated that blocking IL-6 function affects the balance between T_H17 and T_{REG} cells favoring a more anti-inflammatory response (111). In addition, control of T cell activation might be an attractive therapeutic possibility. In this regard, blockage of the co-stimulatory pathway CD28/CD80 using CTLA-4 fusion proteins has successfully been used in RA (112) and shown to be well tolerated in a small open-label trial in GPA patients (113), which suggests this treatment as a possible therapeutic opportunity in AAV.

Modulation of other T cell subsets is also considered as a means for future therapies. For example, since T_H17 cells contribute to inflammation and granuloma formation, this T_H cell subset could be a novel therapeutic target for AAV. Depletion of T_H17 cells by targeting specific surface proteins may be difficult as T_H17 cells share many surface markers with other T cell subsets. Another therapeutic approach could be to specifically target its signature cytokine, IL-17, which would probably be more feasible. Indeed, neutralizing IL-17 by IL-17A specific antibodies or administration of soluble IL-17 receptors reduces inflammation in animal models of atherosclerosis (49, 65, 68, 114). Several IL-17A blockers, including the anti-IL-17A monoclonal antibodies secukinumab and ixekizumab, and the anti-IL-17 receptor subunit A monoclonal antibody brodalumab have been evaluated in clinical trials (115–117), and shown to induce clinically relevant responses in patients. Besides IL-17, IL-21 also seems an interesting target in the treatment of autoimmune mediated vascular inflammation. Manipulation of IL-21 levels may have desirable therapeutic consequences as it might reduce the recruitment of inflammatory T_H1 and T_H17 cells to inflammatory lesions preventing tissue damage and inhibit expansion of autoreactive B cells. Recently, phase I clinical trials using an IL-21-specific monoclonal antibody have been completed for RA (NCT01208506 and EudraCT-2011-005376-42, www.clinicaltrial.gov) but terminated for SLE (NCT01689025, www.clinicaltrial.gov). Neutralization of IL-17 or IL-21 could therefore represent also novel therapeutic

approaches for patients with AAV. However, experiments using animal models of AAV and clinical trials need to elucidate the therapeutic potential of neutralizing IL-17 and IL-21 in AAV. It is important to note that interfering with the cytokine environment might also cause disturbances in the developmental pathways of the different T cell lineages. Since there is a tight interplay between different T cell lineages such as the T_{REG} and T_H17 cells, one should be cautious in modulating the cytokine environments. Besides targeting pro-inflammatory cytokines like IL-17 or IL-21, one can also consider to target effector T cells, which are predominantly responsible for the production of these cytokines.

EFFECTOR MEMORY CD4$^+$ T CELLS AS THERAPEUTIC TARGETS

According to aforementioned evidence in AAV and atherosclerosis, CD4$^+$ T_{EM} cells are considered to play a pivotal role in the pathogenesis of vascular inflammation and therefore, may serve as a potential therapeutic target. Selective targeting of CD4$^+$ T_{EM} cells without impairing other parts of the humoral or cellular immune system could be a major step forward in the treatment of chronic and/or autoimmune mediated vascular inflammation disorders.

The capacity of CD4$^+$ T_{EM} cells to interact with target cells via NKG2D–MICA interaction and attack them by releasing cytolytic enzymes has been demonstrated (104, 106). Therefore, NKG2D expressed on pathogenic T_{EM} cells could be an interesting target to inhibit the pathogenic effects of T_{EM} cells. Interestingly, interference with NKG2D signaling using anti-NKG2D antibodies has shown beneficial effects when administered early in two different mouse models. Blockade of NKG2D prevented autoimmune diabetes in non-obese diabetic mice (118) and attenuated transfer-induced colitis in SCID mice (119). However, no clinical trials on the blockade of NKG2D or its ligands in autoimmune diseases have been conducted.

Besides interfering with the NKG2D–MICA interaction, biophysical analyses revealed that ion channels expressed by immune cells perform functions vital for cellular homeostasis and T cell activation [reviewed by Ref. (120)]. In particular, human T cells express two types of potassium channels (voltage-gate potassium Kv1.3 channel; Kv1.3 and Ca^{2+}-activated potassium KCa3.1 channel; KCa3.1) that play a major role in their activation. Interestingly, Kv1.3 channels and the KCa3.1 channels are expressed on T cells in a distinct pattern that depends on the state of activation as well as on the state of differentiation of the given T lymphocyte subset (121). It has been shown that Kv1.3 channels are highly expressed in CD4$^+$ T_{EM} cells (~1500 channels per cell), whereas naïve and T_{CM} cells express lower levels of Kv1.3 channels (~250 channels per cell) (122). Therefore, Kv1.3 channels may serve as an attractive target for specific immunomodulation in T_{EM} cell mediated chronic or autoimmune diseases. Hu et al. have demonstrated that genetic silencing of Kv1.3 in human CD4$^+$ T cells results in selective expansion of T_{CM} cells and the disappearance of T_{EM} cells after multiple rounds of stimulation with anti-CD3/CD28 *in vitro*, suggesting that Kv1.3 is essential for maintaining the T_{EM} pool (123). Indeed, selective blocking of Kv1.3 channels inhibits Ca^{2+} signaling, pro-inflammatory cytokine production, and proliferation of CD4$^+$ T_{EM} cells *in vitro*, with little or no effects on CD4$^+$ naïve and T_{CM} cells (124). Furthermore, it has been shown that specific Kv1.3 blockade suppressed T_{EM} cell motility in inflamed tissues, but had no effect on homing to or motility in lymph nodes of naïve and T_{CM} *in vivo* (125). In addition, Kv1.3 blockers ameliorate disease development in animal models of multiple sclerosis, RA, T1DM, and contact dermatitis without compromising the protective immune responses to acute infections (124, 126, 127). Noteworthy, Gocke et al. demonstrated that genetic deletion of Kv1.3 biases T cells toward an immunoregulatory phenotype and renders mice resistant to experimental autoimmune encephalomyelitis (128). They showed that Kv1.3 is required for expression of pro-inflammatory cytokines IFN-γ and IL-17, whereas its absence led to increased IL-10 production (128). Thus, it is tempting to speculate that pharmacological blockade or genetic suppression of Kv1.3 channels can be employed as a means to skew CD4$^+$ T cell differentiation toward a regulatory phenotype, which might be beneficial for autoimmune mediated vascular diseases in general. Importantly, Kv1.3 blockers have a good safety prolife in rodents and primates and do not compromise the protective immune response to acute viral (Influenza) or bacterial (*Chlamydia*) infections (124, 125, 127).

Taken together, these studies demonstrated that specific blockade of Kv1.3 channels on T_{EM} cells suppresses the pathogenicity of T_{EM} cells by inhibition of their activation, proliferation and migration. Recently, it has been shown that expression of T cell Kv1.3 channels correlated with disease activity in ulcerative colitis (129). Therefore, Kv1.3 channels on CD4$^+$ T_{EM} cells in chronic or autoimmune inflammatory diseases could constitute a novel pharmacological target in immunomodulation therapies and at the same time may serve as a marker for disease activity.

CONCLUSION

Vascular inflammation can be driven by chronic inflammatory disorders or by autoimmune mediated diseases. In this inflammation process, there is a tight interplay between the innate and adaptive immune system. However, as described in this review, substantial evidence point to an important role for cell mediated adaptive immune responses in the pathogenesis of vascular inflammation. In particular, T cells in chronic or autoimmune mediated vascular inflammation show several functional abnormalities. The T cell compartment shows dysregulation in T_H subsets, including an imbalance between T_H1 and T_H2 cells, skewing toward T_H17 response and defective functions of T_{REG} cells. However, the importance of T_H2 and T_H17 cells in atherogenesis is controversial. The underlying mechanisms responsible for orchestrating the aberrations within the T cell compartment are not completely understood. However, multiple studies indicate an interplay between T_{REG} cells and T_H17 cells. It has been suggested that in the context of an inflammatory environment T_{REG} cells convert into IL-17 producing cells. Furthermore, T_{REG} cells clearly have a protective effect in experimental models of atherosclerosis. In AAV, T_{REG} cells are often quantitatively or functional defective. It has been suggested that a defect in T_{REG} cell function, may also contribute to the expansion of CD4$^+$ T_{EM} cell population and migration of these cells to inflamed sites. Indeed, observations in AAV and atherosclerosis support T_{EM} cell involvement in vascular inflammation that in part contributes to tissue damage. The persistent activation of T_{EM} cells results in a selective up-regulation of Kv1.3 channels. These potassium channels

play a key role in T_{EM} cell homeostasis, proliferation, and activation. Therefore, they seem to be a highly interesting target for immunomodulation of T_{EM} cells without compromising other subsets of the T cell compartment.

Currently efforts are being made to develop biological agents that can modulate the different T cell compartments specifically. In the case of autoimmune mediated vascular diseases such strategies could first be used for the prevention of disease flares. Considering that expansion of T_{REG} cells might be inadequate to control inflammatory responses, regulating T_{EM} cells would probably be the most effective approach. This approach may diminish effector responses and convert these into a more disease regulating response. To develop such therapeutic strategies further studies on the basic immunological properties of T_{EM} and T_{REG} cells in especially humans are needed. Investigation of the functional characteristics of T_{EM} cells in the pathogenesis of vascular inflammatory diseases and selective targeting of these cells will enable their application for the treatment of T_{EM} cell mediated vascular diseases.

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Pathogenesis of systemic sclerosis

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Systemic scleroderma (SSc) is one of the most complex systemic autoimmune diseases. It targets the vasculature, connective tissue-producing cells (namely fibroblasts/myofibroblasts), and components of the innate and adaptive immune systems. Clinical and pathologic manifestations of SSc are the result of: (1) innate/adaptive immune system abnormalities leading to production of autoantibodies and cell-mediated autoimmunity, (2) microvascular endothelial cell/small vessel fibroproliferative vasculopathy, and (3) fibroblast dysfunction generating excessive accumulation of collagen and other matrix components in skin and internal organs. All three of these processes interact and affect each other. The disease is heterogeneous in its clinical presentation that likely reflects different genetic or triggering factor (i.e., infection or environmental toxin) influences on the immune system, vasculature, and connective tissue cells. The roles played by other ubiquitous molecular entities (such as lysophospholipids, endocannabinoids, and their diverse receptors and vitamin D) in influencing the immune system, vasculature, and connective tissue cells are just beginning to be realized and studied and may provide insights into new therapeutic approaches to treat SSc.

Keywords: systemic sclerosis, scleroderma, innate immunity, adaptive immunity, vasculopathy, fibrosis, animal models

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Introduction

Systemic sclerosis (SSc, scleroderma) is a complex connective tissue disease of unknown etiology with multiorgan involvement and heterogeneous clinical manifestations. The clinical and pathologic manifestations of the disease are the result of three distinct processes: (1) innate and adaptive immune system abnormalities leading to production of autoantibodies and cell-mediated autoimmunity, (2) microvascular endothelial cells (MVEC) and fibroproliferative vasculopathy of small vessels, and (3) fibroblast dysfunction leading to excessive collagen (CI) and other matrix components accumulation in skin, blood vessels, and internal organs (1, 2).

The incidence of SSc is about 20 cases per million populations per year and the prevalence is more than 250 patients per million populations in USA (3). Major organ involvement leads to decreased survival in SSc. Pulmonary fibrosis [interstitial lung disease (ILD)] and pulmonary arterial hypertension (PAH) cause more than half of all SSc-related deaths (3). However, patients with SSc live longer and cardiac deaths are increasing.

Progressive thickening and fibrosis of skin secondary to excessive CI accumulation is the most evident and universal finding and can be associated with varying degrees of fibrosis of internal organs. Vascular dysfunction and abnormalities are often seen, and can precede organ involvement by several years (4).

Disease manifestations vary from limited skin involvement with minimal systemic involvement [limited cutaneous (lc) SSc] to widespread skin involvement accompanied by internal organ involvement [diffuse cutaneous (dc) SSc]. These two forms differ mainly in regards to extent of skin involvement, autoantibody association, and the pattern of organ involvement (**Table 1**) (5). Given the heterogeneity of clinical symptoms and signs, American College of Rheumatology (ACR)/EULAR recently developed new classification criteria (6). The new classification criteria would improve sensitivity, which would lead to earlier diagnosis, and it also incorporates the autoantibodies that are commonly used for diagnostic purposes.

It is widely believed that SSc develops in an individual with a "permissive" genetic makeup. Genetic associations of SSc are

Abbreviations: 2-AG, 2-arachidonoyl glycerol; α2AP, α2-antiplasmin; AA, African American; ACA, anticentromere antibodies; Acea, eicosatetraenamide; ACR, American College of Rheumatology; ADCC, antibody-dependent cellular cytotoxicity; AEA, anandamide; AFA, anti-fibrillarin antibody; AIF1, allograft inflammatory factor-1; AKT, protein kinase b; ALAS2, delta-aminolevulinate synthase 2 protein; ALK, activin receptor-like kinase; ANA, antinuclear antibodies; APC, antigen-presenting cells; ARA, anti-U3 ribonucleoprotein; αSMA, α smooth muscle cell actin; ATA, anti-DNA topoisomerase I antibody; ATGL, adipose triglyceride lipase; aTregs, activated Tregs; αZAP, α zinc-finger alpha protein gene; BANK1, B-cell scaffold protein with ankyrin repeats 1; bFGF, basic fibroblasts growth factor; BiP, immunoglobulin-heavy-chain binding protein; BLM, bleomycin; BMP, bone morphogenic protein; BP, binding protein; Bregs, B regulatory cells; Cav-1, caveolin-1; CB1/CB2, cannabinoid receptors 1 and CB2 ditto; CBP, CREB-binding protein; CCR1, C–C chemokine receptor type 1 protein or gene; CD40L, CD40 ligand; CGRP, calcitonin G-related peptide; CGS, candidate gene study; cGVHD, chronic graft-versus-host disease; CI, type I collagen; CII, type II collagen; CIII, type III collagen; COX-2, cyclooxygenase 2 (i.e., prostaglandin synthase 2); CpG, C phosphate G; cScl-GVHD, chronic scleroderma graft-versus-host disease; CSK, C-src tyrosine kinase; CTGF, connective tissue growth factor; CV, type V collagen; CXCL4, angiostatin, platelet factor-4; DAMPs, damage-associated molecular patterns; dcSSc, diffuse cutaneous systemic scleroderma; DNAM-1, DNAX accessory molecule-1 (i.e., CD226); ECM, extracellular matrix; EC(s), endocannabinoid(s); ECS, endocannabinoid system; EDNR-A or -B, gene that encodes endothelin receptor type A or B; Egr-1, early response gene 1; eNOS, endothelial nitric oxide synthase; ERK, extracellular signal-regulated kinase; ET-1, endothelin-1; EVI, evenness interrupted, in reference to cell surface multipass transmembrane protein; FAAH, fatty acid amide hydrolase; FasL, Fas ligand; FGF, fibroblast growth factor; FH-1, human fetal lung fibroblast line; Fli(1), friend leukemia integration; Fra-2, Fos-related antigen-2; FTY720, fingolimod; GATA-3, GATA binding protein-3; GMCSF, granulocyte monocyte-colony stimulating factor; GPCRs, G-protein-coupled receptors; GVHD, graft-versus-host disease; GWAS, genome-wide association studies; hCMV, human cytomegalovirus; HGF, hepatocyte growth factor; HLA, human leukocyte antigen; HMGB-1, high-mobility group box-1 protein; HMW-MAA, high molecular weight melanoma-associated antigen; HSP, heat-shock protein; IC, intracellular; ICAM-1, intercellular adhesion molecule-1; IFIT1, IFIT2, and IFITM1, interferon-regulated genes; IFN, interferon; IFNRI, type 1 interferon receptor; IGF, insulin-like growth factor; IGF-BP-5, insulin-like growth factor binding protein-5; ILCs, innate lymphoid cells; ILD, interstitial lung disease; IP, interferon-inducible protein; IPAH, idiopathic PAH; IPF, idiopathic pulmonary fibrosis; IRAK1, interleukin-1 receptor associated kinase 1; IRF, interferon regulatory factors; IRFSNP, interferon regulator factor, SNP (see entry); ITGAM, integrin αM; ITGAX, integrin αX; JAMs, junctional adhesion molecules; JNK, c-Jun N-terminal kinase; KCNA5, potassium voltage-gated channel, shaker-related subfamily, member 5 gene; kDA, kilo Dalton, measurement of; LAP, latency-associated peptides; lcSSc, limited cutaneous systemic scleroderma; LLC, large latent complex; LPA, lysophosphatidic acid; LPS, lipopolysaccharides; LTBP, latent TGF-β1-binding protein; MAGL, monoacylglycerol lipase; MAPK, mitogen-activated protein kinase; MCP-3, monocyte chemoattractant protein-3; MCR1, melanocortin receptor 1, gene found in yeast; MECP2, methyl-CpG-binding protein 2 gene; MHC, major histocompatibility complex; MIF, macrophage migration inhibitory factor; MIP, macrophage inflammatory protein; miRNA, micro RNA (miR); MMP, matrix metalloproteinase; mPGES-1, microsomal prostaglandin E2 synthase 1; mRNA, messenger RNA; MRSS, modified Rodnan skin score; MS, multiple sclerosis; MVEC, microvascular endothelial cells; MW, molecular weight; NAG-2, MVEC surface protein tetraspan novel antigen-2; NLR, a pattern recognition system (NOD)-like receptor family; NO, nitric oxide; NOD, nucleotide-binding and oligomerization domain; NOTCH, neurogenic locus notch homolog 4 protein encoded by the NOTCH4 gene, and an evolutionarily conserved pathway in multicellular organisms that regulates cell-fate determination during development and maintains adult tissue homeostasis; NSIP, non-specific interstitial pneumonia; PAI, plasminogen activator inhibitor; PAH, pulmonary arterial hypertension; PAMPs, pathogen-associated molecular patterns; PAR, protease-activated receptor; PASMC, pulmonary artery smooth muscle cells; PBMC, peripheral blood mononuclear cell; PDGF/PDGFR, platelet-derived growth factor/receptor; PECAM, platelet/endothelial cell adhesion molecule; PG, prostaglandin; PGF, placental growth factor; PG-Gs, microsomal prostaglandins; PI-3, phosphatidylinositol 3; PLD4, phospholipase D family member 4; PPARγ, peroxisome proliferation-activated receptor gamma; PRR, a pattern recognition receptor system in cell cytoplasm; PSD3, pleckstrin and Sec7 domain containing 3 gene; PTEN, phosphatase and tensin homolog; PTPN22, protein tyrosine phosphatase non-receptor type 22; PXK, *paraxylene–orthoxylene* (phox homology) domain containing serine/threonine kinase; RA, rheumatoid arthritis; RANTES, regulated upon activation, normal T-cell expressed and secreted; RGS-5, regulator of G protein signaling 5; RLRs, RIG-I-like receptors; RORγt, RAR-related orphan nuclear receptor gamma transcription factor; RP, Raynaud's phenomenon; R-Smads, receptor-regulated Smads; rTregs, resting Tregs; S1P/S1P_1, sphingosine 1-phosphate (type 1 receptor); scl GVHD, sclerodermatoses GVHD; Serpine 1, plasminogen activator inhibitor; Siglec-1, CD169, sialoadhesin; siRNA, small interfering RNA; SLE, systemic lupus erythematosus; Smad, small mother against decapentaplegic family of transcription factors; Snail-1, a protein of the C2H2-type zinc-finger family that regulates transcription; SNPs, single nucleotide polymorphisms; sRAGE, soluble advanced glycation end products; SSc, systemic scleroderma; STAT, signal transducer and activator of transcription; TG, transgenic; TGF-β, transforming growth factor beta; TIMP-1 or -2, tissue inhibition of metalloproteinase-1 or -2; TLRs, toll-like receptors; TNFAIP3, tumor necrosis factor alpha-induced protein-3; TNFSF4, tumor necrosis factor superfamily member 4 gene; TNIP1, TNFAIP3 interacting protein-1; tPA, tissue t plasminogen activator; Tregs, T regulatory cells; TRPV, transient receptor potential vanilloid; Tsk-1 (Tsk-1/+), tight skin; TSP-1 or TSP-2, thrombospondin-1 or -2; UPAR, urokinase-type plasminogen activator receptor; VDR, vitamin D receptor; VE, vascular endothelial; VEGF, vascular endothelial growth factor; VitD, Vitamin D; VW, von Willebrand Factor; Wnt, proteins that form a family of highly conserved secreted signaling molecules that regulate cell-to-cell interactions during embryogenesis; WT, wild-type.

TABLE 1 | Important differences between limited cutaneous systemic sclerosis (lcSSc) and diffuse cutaneous systemic sclerosis (dcSSc).

Features	lcSSc	dcSSc
Skin	Skin thickening occurs late, limited to the distal part of upper and lower extremities, face, neck, and upper chest. Telangiectasias and calcinosis are common. Tendon friction rub not seen	Skin thickening occurs early, moves up to proximal part of extremities and trunk. Telangiectasias and calcinosis may occur late in disease. Tendon friction rub present
GI	Esophageal dysmotility is more common than small and large intestine involvement	Esophageal dysmotility is frequently seen. Small and large intestinal involvement is more common
Pulmonary	Pulmonary fibrosis is less frequent and less severe. Frequent and severe pulmonary hypertension is more common	Pulmonary fibrosis is more common and severe. Pulmonary hypertension is less frequent
Kidney	Renal crisis uncommon	Renal crisis is more frequent
Autoantibody association	Anticentromere antibodies (ACA) are predominant	Anti-DNA topoisomerase I antibody (ATA) (Anti-Scl-70) antibody is predominant Anti-RNA polymerase antibody is more common

summarized below. A triggering event such as an infection or environmental toxin has been implicated as starting the processes that lead eventually to SSc in individuals with a permissive genetic background. The realization that an "interferon (IFN) signature" exists in most patients with SSc implies activation of the innate immune system and lends validity to the long-held suspicion that infections (such as with cytomegalovirus, Epstein-Barr virus, and more recently *Toxoplasma gondii*) could be SSc triggers in receiving more attention and a re-examination (7, 8). There is mounting evidence that the microbiota may play a role in development of autoimmunity, an area that is unexplored in SSc (9). Analysis of skin transcriptome has identified high levels of *Rhodotorula* sequences in dcSSc patients (10).

No animal model develops SSc that faithfully replicates human SSc, and this has impeded our understanding of the disease. There are many unresolved questions related to the etiopathogenesis of SSc. For example, it is unclear whether the innate/adaptive immune system abnormalities, vasculopathy, and fibroblast dysfunctions are separate, unrelated processes or are mechanistically linked, which of the three processes is of utmost importance and how interaction among the three processes leads to the development of the disease. These three processes will be discussed.

We first review evidence for genetic abnormalities in SSc since they can influence responses of the innate and adaptive immune systems, vascular function, connective tissue metabolism, and fibroblast function. Since the innate and adaptive immune systems are the first to respond to environmental triggers, be they infections or toxins in nature, and through generations of cytokines, chemokines, and growth factors that can affect function of vascular and connective tissue cells, we discuss them next. The vascular abnormalities and fibrosis in SSc are then discussed. The endocannabinoid system (ECS) (which influences functions of the immune system, vasculature, and fibroblasts) may be dysregulated in SSc as suggested by recent studies of SSc dermal fibroblasts. We have included a discussion of this important system with special emphasis on potential ECS targets that might offer new therapeutic approaches for management of SSc. Lysophospholipids [lysophosphatidic acid (LPA) and sphingosine 1-phosphate (S1P)] and their different receptors (which regulate immunity, vascular physiology, and fibrosis) are dysregulated in SSc and likely contribute to the pathogenesis of the disease. Vitamin D (VitD) status also impacts function of most cell types and likely influences pathogenesis and clinical features of SSc. An overall scheme of SSc pathogenesis is illustrated in **Figure 1**.

Genetics of SSC

Genetic influences have long been suspected to impact SSc. In families with a history of SSc, the incidence of disease can range from 1.5 to 1.7% (11). Having a family history of SSc increases the risk of developing disease 15–19-fold in siblings and 13–15-fold in first-degree relatives (11–13). Over the last decade, candidate gene study (CGS) approach and genome-wide association studies (GWAS) have been used to identify genetic associations that confer susceptibility to SSc. CGS and GWAS have allowed for the identification of genetic variations [single nucleotide polymorphisms (SNPs)] that are likely to be involved with the pathogenesis of scleroderma. CGS analyses SNPs to determine if the gene has association with a disease or a disease trait. The SNPs being studied have been selected based on their known association with other autoimmune diseases or on their possible functional relevance in the disease pathogenesis. GWAS arrays on the other hand, use tagSNPs to scan the entire genome to identify millions of SNPs. It takes into consideration the haplotype structure of the population being studied. Unlike CGS, GWAS identifies SNPs in a non-hypotheses-driven manner and allows for the identification of newly identifiable genes that were not previously identified in the disease. As regards to SSc, GWAS has confirmed major histocompatibility complex (MHC) II region as being most significant in this disease. Both CGS and GWAS have identified multiple genes that have been found to have firm associations in the pathogenesis of SSc.

Performing a GWAS can be very costly. Recently, the immunochip consortium was developed and implemented the immunochip analysis assay. The immunochip array provides high-density mapping of autoimmune diseases-associated loci using a custom SNP genotyping array (14). It was designed to increase efficiency of mapping autoimmunity risk loci and to reduce the cost of mapping (15). The immunochip uses variants from across 186 known autoimmunity risk loci and places them on an Illumina Infinium array platform. The platform contains 196,524 different variants of autoimmunity risk loci that may have functional significant effects in diseases like SSc. It also identifies variants with lower penetrance using a cost efficient strategy (14). Many of these genes have been

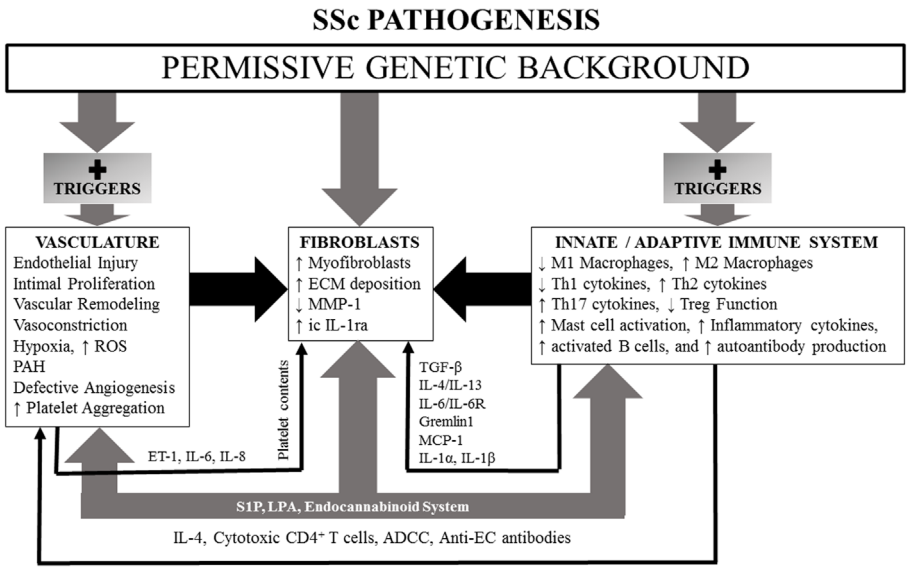

FIGURE 1 | A simplified schematic of SSc pathogenesis, illustrating influences of a permissive genetic background and lysophospholipids and endocannabinoid system participation which have the capacity, if dysregulated, to effect changes in vasculature, fibroblasts, and innate and adaptive immune systems. See text for details.

firmly established in the pathogenesis of SSc. In this review, we will focus on genetic associations in MHC – human leukocyte antigen (HLA, **Table 2**), non-HLA genetic SNP (Table 3), and microRNAs (miRNAs) (**Table 4**). We will focus on the most relevant associations first and then discuss others that may have modest effects on SSc.

HLA Association with SSc

The HLA-1 complexes HLA-A, -B, -C, and -G and HLA class-II complexes HLA-DP, -DQ, and -DR have all been identified in SSc susceptibility (11, 17, 108, 109) (**Table 2**). HLA class-II is the most significant region associated with SSc (14). *HLA-DRB1*01*, *HLA-DRB1*11*, *HLA-A*30*, and *HLA-A*32* have SSc susceptibility, while *HLA-DRB1*07*, *HLA-B*57*, and *HLA-Cw*14* are protective against SSc (17). *HLA alleles DRB1*0802* and *DQA1*0501* are associated with increased mortality (110). Clinical features of disease, disease phenotype, and SSc-specific autoantibodies have been distinguished based on HLA subtypes (**Table 1**). In a GWAS study that included 5471 SSc patients of European ancestry, *HLA-DQB1* locus was associated with anticentromere antibodies (ACA), *HLA-DPA1/B1* loci with anti-DNA topoisomerase I antibody (ATA), and neurogenic locus notch homolog 4 (NOTCH4) with ACA and ATA (24). In another study that included SSc patients of African American (AA) and Hispanic descent, *DRB1*1104*, *DQA1*0501*, *DQB1*0301*, and *DQB1* had strong positive association in SSc patients of Hispanic and of European ancestry (24, 31). *DRB1*0404*, *DRB1*11*, and *DQB1*03* alleles are associated with anti-U3 ribonucleoprotein (ARA) in this subpopulation (24). In this same subpopulation, *DRB1*0701*, *DQA1*0201*, *DQB1*0202*, and *DRB1*1501* had a negative or protective association against SSc (27). These studies have also identified *DRB1*11* with association with ATA and *DRB1*01*, *DRB1*04*, and *DRB1*0501* have association with ACA (31). *HLA-DPB1* and *HLA-DPB2* SNPs rs7763822/rs7764491 and rs3117230/rs3128965 have strong association with ATA or ACA positivity (25). In AA patients with SSc, *DRB1*0804*, *DQA1*0501*, and *DQB1*0301* are associated with SSc (28), and have a higher frequency of ARA or anti-fibrillarin antibody (AFA) positivity (111).

*HLA-DRB1*1101*, **1104*, **1501*, and **0802* (commonly associated with the dcSSc subset) show the amino acid sequence ^{67}FLEDR^{71} in their β chain, whereas *HLA-D Q β1* alleles **D301*, **0302*, **0401*, **0402*, **0601*, and **0602* (commonly associated with SSc) show a ^{71}TRAELDT^{77} motif on their β chain (29). In a study in French SSc patients with European ancestry, both FLEDR and, to a lesser degree, TRAELDT were associated with dcSSc (29). Addition of a tyrosine at position 30 strengthened the TRAELDT association with dcSSc (29). Further analysis showed that the FLEDR motif had the highest association with SSc patients who were ATA positive, while TRAELDT had lesser association in this subset (29). The TRAELDT association with ATA positivity and dcSSc were not dependent entirely on FLEDR (29). The authors concluded that double dose of the shared epitope, as well as compound heterozygosity, may confer a higher risk for development of SSc.

HLA-DPB1 and *-DPB2* are reported to have strong susceptibility with SSc in the Korean and Chinese populations (25). Subtypes *DPB1*1301* and *DPB1*0901* were most common in Korean patients with SSc, while *DPB1*03:01*, *DPB1*13:01*, *DQB1*03:03*, *DQB1*05:01*, and *DQB1*06:11* were significantly increased in the Chinese SSc patient population (26). Those who carried the *DPB1*03:01* had a higher chance of developing pulmonary fibrosis verses those who carried *DPB1*04*, and those SSc patients were more likely to be ACA positive (112). *DQB1*03:03* and *DQB1*05:01* were strongly associated with

TABLE 2 | HLA genes associated with SSc.

HLA-associated genes	Population (n = SSc)	Disease phenotype and/or clinical features	Autoantibody association	Reference
HLA CLASS I GENES ASSOCIATED WITH SSc				
A*30	Brazil (141)	Pulmonary fibrosis	ATA	(16, 17), (16, 18–22), (17), (17),
B*13	European ancestry (95)	PAH		(16), (18), (17), (23)
B*35	Brazil	PAH		
	Native Indian	dcSSc		
	Hispanic			
B*62	European ancestry (95)	dcSSc and high skin scores, pulmonary fibrosis		
B*65		PAH		
C*04	Brazil	PAH		
Cw4	Native Indian	Pulmonary fibrosis	dcSSc pulmonary fibrosis ATA	
Cw*0602	European ancestry (95)	Pulmonary fibrosis	ARA	
G	European ancestry	Lower vascular cutaneous	dcSSc	
	African	ulcers telangiectasias		
	Brazilian	arthropathy		
HLA CLASS-II GENES ASSOCIATED WITH SSc				
DPA1/B1	European ancestry (5,471)	Pulmonary fibrosis	ATA	(24–26)
DPB1*1301	Korean Chinese (338)		ACA, ATA, and dcSSc	
*0901			ATA	
DPB1/B2 SNPs				
rs3128930				
rs7763822/rs7764491	European ancestry (1,107)		ACA	
rs3117230/rs3128965				
rs3117230	Caucasian			
rs7763822	African American		ACA	
rs7764491	Hispanic		ATA	
rs3117230				
rs312965				
rs3128965A				
DPA1/B1				
DQA1	African American	Pulmonary fibrosis	ATA/ACA	(16, 18, 19, 26–30)
*0501	Native American		dcSSc	
DQB1			ACA	
*03:01	European ancestry		Anti-U1RNP/	
*03:03	Korean/Chinese		ATA	
*04:00	Hispanic			
*05:01	Italian			
*06:11	Brazilian		ATA	
*26:00	Native American			
[71]TRAELDT[77]	French (282)		dcSSc ATA	
DRB1	European ancestry			(17–19, 29–34)
	Hispanic			
	Italian			
*01			ACA	
*0404			ACA	
*05			ACA	
*07				
*0804	African American (278)		AFA/ARA	
*11		Increased skin score, pulmonary fibrosis	ATA	
*1104			ACA	
			ATA	
*1502	Thai (50)	No association PF, DU, sclerodactyly, myositis, SICCA	ATA	
*1602	Native American (32)	dcSSc PF	ATA	
*0407	European ancestry	Renal crisis dcSSc		
	African American			
	Hispanic (1,517)			
*1304	European ancestry			
	African American			
	Hispanic (1,517)			
DRB5*01:02	Thai (50)		ATA	
[67]FLEDR[71]	French (282)	dcSSc	ATA	

(Continued)

TABLE 2 | Continued

HLA-associated genes	Population (n = SSc)	Disease phenotype and/or clinical features	Autoantibody association	Reference
NOTCH4	European ancestry (5,471)		ACA ATA	(24)
GENES WITH NEGATIVE ASSOCIATION FOR SSc				
C*03	Brazilian Spanish	PAH	ATAq	(16, 27, 30, 33)
DQA1	European ancestry (1,300)			
*0201	Hispanic			
*0501	Italian Spanish (940)	dcSSc		
DQB1	European ancestry			
*0202	Hispanic			
*04	Brazilian	dcSSc		
DRB1	Brazilian			
*01	Spanish			
*04	Thai			
*0701				
*1501				

TABLE 3 | Non-HLA genes associated with SSc listed from largest to smallest SSc population analyzed.

No.	Non-HLA-associated genes	Other diseases identified	Study type	Population (n = SSc)	Polymorphism/SNPs (OR)	Disease association	Proposed function	Reference
1	*BANK1*	SLE	Case–control meta-analysis cohort	European ancestry (21,568)	rs10516487*G (1.12), rs17266594*T (1.14), rs3733197* A (0.73), AA (0.41), AG (0.85)	dcSSc ATA+	Mobilization of calcium from intracellular stores in B-cell receptor	(35–37)
2	*IRF5*	SLE, RA, UC	GWAS meta-analysis immunochip array case–control cohorts	European and Asian ancestry (15,251)	rs2004640 (0.84), rs2004640*TT (1.56), rs10488631 (1.63), rs4728142 (1.22)	dcSSc, lcSSc, ATA, ACA, interstitial lung, longer survival mild PF	Regulate IFN-gene expression and inflammatory cytokine production. Stimulate TLR expression	(38–42), (42–44), (14, 45, 46)
3	*PXK*	SLE	Immunochip pan-meta-analysis GWAS	European ancestry (12,685)	rs2176082 (1.21), rs4681851 (1.58)	SSc, ACA	Degradation and trafficking of epidermal growth factor	(14, 47)
4	*STAT4*	SLE, RA, primary biliary cirrhosis	GWAS immunochip meta-analysis cohort, case–control cohorts	Multi-ethnic ancestry (10,696)	rs7574865 (1.29), additive effect with IRF5 rs2004640 (1.72-2.752), rs11889341 (1.33), rs7574865*, TT vs. GG (0.49), TT vs. TG (0.48), TT + TG vs. GG (0.74), T vs. G (0.72), rs10168266*, CC (0.69), T (1.44)	lcSSc, ACA, fibrosing alveolitis, increased in patients who carry TBX21 CC genotype, dcSSc, ATA, pulmonary fibrosis	T-cell signaling and differentiation; signaling IFN1; regulate cytokine signals	(38, 48–54)
5	*PTPN22*	DM-1, RA, SLE	Meta-analysis case–control cohorts	Multi-ethnic and European ancestry (10,204)	PTPN22 CT/TT (2.21) higher risk of SSc than PTPN22 CC (1.70), PTPN22, C1858T [rs2476601* T (1.15)], [1858 T (1.147)], 1858 C	ATA and ACA, ACA, protective	T-cell receptor signaling	(55–58)
6	*TNFSF4*	SLE	Meta-analysis cohort	European ancestry (10,093)	rs1234314 (1.15), rs12039904 (1.18), rs2205960*AA (1.33), rs844648 (1.10), rs844644 (0.91)	dcSSc, lcSSc and ACA+, protective in all sub-groups of SSc except ARA+	B-cell proliferation and differentiation T-cell stimulation and survival	(48, 59–61)
7	*BLK*	SLE	Case–control meta-analysis	European and Asian ancestry (9,305)	rs2736340 (1.27), rs13277113 (1.16), *C8orf13-BLK*, and *BANK1* additive effect, *FAM167-BLK*	dcSSc, dcSSc, dcSSc, lcSSc	Disruption in B-cell gene expression and abnormal NFκB signaling	(48, 62–64)

(Continued)

TABLE 3 | Continued

No.	Non-HLA-associated genes	Other diseases identified	Study type	Population (*n* = SSc)	Polymorphism/SNPs (OR)	Disease association	Proposed function	Reference
8	*IL-2*, *IL-2RA*	SSc	Case–control cohort	European (7,516)	IL-2: rs6822844 (0.86), rs907715 (0.91), rs2069762*A- (1.06), rs6822844*T- (0.86), rs683545*G (0.93), rs907715*T (0.91); IL-2RA genes: rs11594656, rs2104286 (1.30), rs12722495	SSc, lcSSc, dcSSc, and lcSSc when ACA+	T-cell proliferation and turning off T-cell response	(65, 66)
9	*DNASE1L3*	SLE, RA	Immunochip	European ancestry (7,169)	rs35677470 (2.03)	ACA	Defective apoptotic DNA breakdown	(14, 46)
10	*JAZF1*	SLE	GWAS pan-meta-analysis	European ancestry (6,835)	rs1635852 (1.13)	SSc	Repress transcription	(47)
11	*KIAA0319L*	SLE, dyslexia	GWAS pan-meta-analysis	European ancestry (6,835)	rs2275247 (1.49)	lcSSc	Protein coding	(47)
12	*IL-12Rβ2*	Psoriasis, Behcet's disease, primary biliary cirrhosis	GWAS	European ancestry (6,250)	rs3790567 (1.17), rs2305743* A/G (0.81), rs8109496* C/G (0.82), rs436857* A/G (0.81), rs11668601* C/T (0.84)	SSc	Stimulates IFN production and TH1 differentiation	(67)
13	*IRF8*	SLE	Cohort meta-analysis	European and Asian ancestry (6,201)	rs11642873 (0.75), rs2280381 (1.36)	lcSSc	Regulate IFN-gene expression and inflammatory cytokine production. Stimulate TLR expression	(24, 68)
14	*CD247*	SLE	GWAS	European ancestry (6,080)	rs2056626 (0.78)	SSc-G minor allele protective effect	T-cell signaling and activation	(49, 69)
15	*ATG5*	SLE	Immunochip	European ancestry (5,850)	Intron rs9373839* G (1.19)	SSc	Autophagy vesicle formation	(14)
16	*IL-12A-SCHIP*	Primary biliary cirrhosis, idiopathic pulmonary fibrosis	Immunochip	European ancestry (5,850)	rs77583790 (2.81), intergenic between SCHIP1 and IL-12A	LcSSc	Stimulates IFN production and TH1 differentiation	(14)
17	*CSK*	SLE, RA	GWAS	European ancestry (5,270)	rs1378942 (1.2)	SSc	Cell regulation, differentiation, migration, and immune response	(70)
18	*PSD3*	Hepato-cellular carcinoma	GWAS	European ancestry (5,270)	rs10096702 (1.18)	SSc	Binding protein for signal transduction	(66)
19	*IL-12Rβ1*	Psoriasis, Behcet's disease, primary biliary cirrhosis	Immunochip	European ancestry (5,052)	rs2305743 (0.81), rs436857	SSc	Stimulates IFN production and TH1 differentiation	(71)
20	*IRAK1*	SLE	Cohort meta-analysis	European ancestry (4,873)	rs1059702*TT (1.43)	dcSSc, ATA, SSc-related fibrosis alveolitis	Influence T-cell receptor signaling and TLR activation. Regulator of NFκB by way of X chromosome	(42, 48, 72)
21	*IL-21*	RA, SLE, DM-type 1, Graves' disease, celiac disease	Case–control	European ancestry (4,493)	rs6822844 (0.86), rs907715 (0.91), allelic combination: rs2069762* A- (1.06), rs6822844* T- (0.97), rs6835457*G- (0.93), rs907715* T (0.91), rs6822844* T (0.84)	lcSSc, ACA+, global SSc, dcSSc, lcSSc, protection: SSc, lcSSc, and ACA+	B-cell differentiation and regulates TH17 development	(65)

(Continued)

TABLE 3 | Continued

No.	Non-HLA-associated genes	Other diseases identified	Study type	Population (*n* = SSc)	Polymorphism/SNPs (OR)	Disease association	Proposed function	Reference
22	*TNIP1*	RA, SLE, psoriatic arthritis	GWAS cohort	European ancestry (4,389)	rs9275224 (0.69), rs6457617 (0.69), rs9275245, rs3130573 (1.12) located in *PSORC1C1* gene, rs2233287 (1.19), rs4958881 (1.19), rs3792783 (1.19)	Global SSc, global SSc except ACA+: dependent on HLA II, global SSc	Negative regulatory of NFκB	(48, 73, 74)
23	*MIF*	SLE, psoriatic arthritis, Lofgren's syndrome, inflammatory bowel disease	Cohort	European ancestry (4,286)	MIF-173 (1.10)	dcSSc	Activates innate immunity and sustains cellular response	(60, 75)
24	*NFkβ1*	Behcet's disease, Grave's disease, Hashimoto thyroiditis	GWAS	European ancestry (4,156)	rs1598859 (1.19)	SSc	Controls inflammation, transcription regulator	(66, 76)
25	*CD226*	SLE, DM-type 1, multiple sclerosis	Cohort	European ancestry (4,131)	rs763361* T (1.02), rs34794968 (0.90), rs727088 (1.02)	dcSSc, ATA+, ILD, pulmonary fibrosis	Co-stimulator of T cells and T-cell adhesion	(77–80)
26	*PPARγ*	RA, psoriatic arthritis, DM-type 1	GWAS	European ancestry (3,989)	rs310746 (1.28)	SSc	Blocks TGF-β, mediated fibrosis	(81–83)
27	*ITGAM*	SLE	Case–control cohort meta-analysis	European ancestry (3,735)	rs1143679* A (1.12)	SSc	Regulates neutrophil and monocyte cell activation and adhesion. Phagocytosis of complement-coated particles	(47, 84, 85)
28	*TNFAIP3*	SLE, RA, celiac disease, multiple sclerosis	Cohorts case–control	European and Asian ancestry (3,365)	rs5029939* G (2.08), rs6932056 (1.69), rs117480515* A (3.20), rs117480515* A (3.94)	dcSSc, fibrosing alveolitis, PAH, SSc, polyautoimmune subset	Regulate immune system signaling by regulating ubiquitin	(43, 48, 68, 86)
29	*IRF7*	SLE	Case–control	European ancestry (2,316)	rs1131665 (0.87)	ACA	Regulate IFN-gene expression and inflammatory cytokine production. Stimulate TLR expression	(87)
30	*IL-23R*	Inflammatory bowel disease, psoriasis, AS	Case–control	US – multi-ethnic and European ancestry (2,134)	rs11209026*GG (0.81), (Arg381 Gln), rs11465804*TT (0.83)	ATA, protection against PAH, dcSSc, ATA, protection against PAH	Promotes TH17 expansion	(28, 88, 89)
31	*TLR2*	Inflammatory bowel disease, multiple infections	Cohort	European ancestry (1,170)	rs5743704 (2.24), (Pro63 His)	dcSSc, ATA+, PAH	Pathogen recognition and direct immune response	(90)
32	*CD87(UPAR)*	Vascular disease, paranasal disease	Cohort	European ancestry (732)	rs344781*GG (1.96)	SSc-associated digital ulceration, PAH, ACA, dcSSc, lcSSc	Promotes extracellular matrix and vascular remodeling	(91)
33	*PLD4*	RA	Cohort	Japanese (730)	rs2841277 (1.29), rs2841280* G (1.29), (minor)	lcSSc, dcSSc, protective SSc	Phagocytosis of microglia	(68)
34	*MMP-12*	Kidney disease, skin diseases, arthro-sclerosis	Cohort	Italian ancestry (250)	rs2276109*AA (2.44)	dcSSc, lcSSc, ATA+, pulmonary fibrosis	Inhibits endothelial cell proliferation and angiogenesis	(92)

ACA, while *DQB1*06:11* was associated with ATA positivity and a marginal association with pulmonary fibrosis. *DQB1*03:01* had an increase frequency of anti-U1RNP positivity in Chinese patients with SSc (26).

The role of HLA II in Italian and Spanish SSc patients has also been examined. *HLA-DRB1*1104*, *DQA1*0501*, and *DQB1*0301* haplotypes are overexpressed in this patient population (30). Carrying the *HLA-DQB1*03* and *HLA-DRB1*11* alleles are risk factors for developing SSc in this subset of patients. Having the *HLA-DRB1*0701* allele was protective (30). *HLA-DRB1*1104* allele has association with ATA, while *HLA-DQB1*0501* in ATA patients is protective (30). ACA-positive patients expressed *HLA-DRB1*01* and *-DQB1*05*. Patients who had pulmonary fibrosis were found to have an association with *DRB1*11* (32).

*HLA-A*30* and *-DQB1*04* alleles were found to relate to SSc susceptibility in a subset of Brazilian patients (16). In patients who had PAH, *HLA-B*35*, and *C*04* were associated as risk genes for this complication, while *C*03* was protective (16). *HLA-DRB1*15:02* and *DRB5*01:02* are associated with ATA positivity in SSc Thai patients. There were no associations seen between these genes and other clinical manifestations of disease including pulmonary fibrosis, digital pits, sclerodactyly, myositis, or SICCA symptoms. *DRB1*04* was protective in this patient population (33).

In a population of French SSc patients of European ancestry, amino acid sequences ^{67}FLEDR^{71} shared by *HLA-DRB* was associated with ATA positive and dcSSc. Amino acid sequence ^{71}TRAELDT^{77} shared by *HLA-DQB1* showed weak association in dcSSc patients with positive ATA (29). A higher prevalence of SSc has been identified in the Choctaw Indian population in comparison to non-full-blooded Choctaws, other Native Americans, as well as the general population (18, 19). Multiple genetic loci located on chromosome 6 near the HLA complex have been identified and may contribute to the high prevalence of disease (19). *HLA-B35*, *Cw4*, *DRB1*1602*, *DQA1*0501*, and *DQB1*0301* are strongly associated with SSc in the Choctaw Indian population who present clinically with dcSSc, pulmonary fibrosis, and ATA positivity (18).

*HLA-B*62* and *HLA-DRB1*07* correlate with diffuse skin involvement while high skin scores correlate with *HLA-DRB1*11* (17). *HLA-B*62* and *HLA-Cw*0602* has association with pulmonary fibrosis, while *HLA-B*13* and *HLA-B*65* with PAH (17). *HLA-B*35* is associated with a high risk of developing PAH in systemic sclerosis by influencing the production of endothelin-1 (ET-1) and decreasing endothelial nitric oxide synthase (eNOS) (18, 20–22). HLA-G is expressed in skin of patients with systemic sclerosis. Its presence is associated with having lower vascular cutaneous ulcers, telangiectasias, and inflammatory arthropathy (23).

Non-HLA-Associated Genes

Multiple studies including GWAS, meta-analysis, and recently immunochip array analysis have repeatedly shown that modifications in *CD247*, interferon regulatory factor 5 (*IRF5)*, and signal transducer and activator of transcription protein 4 (*STAT4*) genes are associated with SSc susceptibility (**Table 3**). Many autoimmune disorders share a common genetic background. Both systemic lupus erythematosus (SLE) and SSc share many clinical features and genetic components. Disease sample size and lack of statistical power limits the ability to determine which genes may contribute to autoimmunity. Combined analysis of different autoimmune diseases increase sample size and allows for statistical power to identify genetic variants that effect disease. Using a GWAS pan-meta-analysis approach allows for the detection of new genetic susceptibility loci, as determined by Martin et al. (47). In the Martin et al. study, GWAS pan-meta-analysis approach for SSc and SLE identified and validated three new susceptibility genes for SSc [*KIAA0319L*, paraxylene–orthoxylene domain containing serine/threonine kinase (*PXK*), and *JAZF1*] (47). Genes related to cellular response to IFNγ and the nervous system was overrepresented in both SLE and SSc. In SSc, genes related to cell signaling, migration, and adhesion were over-represented (47). In this section of the review, we will discuss Non-HLA-associated genes reported to be associated with SSc. In **Table 3**, we have listed the non-HLA SSc-associated genes in order of decreasing SSc sample size analyzed.

Autophagy Protein-5

Autophagy protein-5 (ATG5) is an ubiquitin ligase protein that assists in autophagosomal elongation that mediates pathogen clearance; allowing for the degradation of unwanted cytoplasmic material. It has a role in the development of both the innate and adaptive immune system (14). Variations in *ATG5* are associated with susceptibility in SLE and childhood and adult asthma (14). Variants located within *ATG5* intron rs9373839 G minor allele have been identified as SSc susceptibilities (14). The location of this variant may suggest that distant genes may affect downstream the function of *ATG5*.

B-Cell Scaffold Protein with Ankyrin Repeats 1

B-cell scaffold protein with ankyrin repeats 1 (BANK1) exerts influence in B-cell receptor-induced calcium mobilization from intracellular (IC) stores. It has been identified in SLE as a susceptibility gene. There is an increased risk for developing SSc with *BANK1* haplotype G–C compared to A–T haplotype (35). *BANK1* variants rs3733197 G alleles, rs10516487, rs10516487*G, and rs17266594*T are strongly associated with diffuse dcSSc and ATA autoantibodies (36).

B-Lymphocyte Kinase

B-lymphocyte kinase (BLK) encodes B-cell signal transducer and functional variant *C8orf13-BLK*. Disruption in *BLK* may result in abnormal B-cell gene expression and altered NFκB signaling (48). *C8orf13-BLK* has been identified in multiple studies as a risk gene for SSc (62–64). *C8orf13-BLK* variant rs2736340 and rs13277113 are associated with SSc and dcSSc (63). An additive effect between *C8orf13-BLK* and *BANK1* increases susceptibility to dcSSc (62). Two haplotype blocks (*FAM167A* and *BLK*) have also been identified. Allele rs13277113*A in the *BLK* block is significantly associated with SSc (64). This association was observed despite autoantibody profile or disease classification (dcSSc or lcSSc) (64).

CD247

CD247 encodes T-cell receptor zeta (CD3ζ), which functions in the assembly of TCR–CD3 complex and its transport to the cell surface, thereby playing a crucial role in cell signaling (49). Variants of *CD247* may lead to impaired immune response and dysregulation of T-cell activation. *CD247* has been associated with susceptibility to SLE. *CD247* rs2056626 (in addition to *IRF5*, MHC, and *STAT4*) were identified as susceptibility genes for SSc in multiple studies. The G minor allele of this variant has a protective effect (49, 69). This variant was not found to have an association with SSc or disease subtypes in a Hans Chinese cohort, suggesting that the association may be ethnicity-dependent (113).

c-SRC Tyrosine Kinase

c-SRC tyrosine kinase (CSK) is important for cell regulation, differentiation, migration, and immune response. *CSK* inactivates src kinases by phosphorylating tyrosine at the C-terminus. In fibrosis, srk kinases regulate FAK needed for integrin signaling and fibroblast adhesion to extracellular matrix (ECM). Incubating fibroblasts with inhibitors of *CSK* decreases COLIAI and COLIA2. Polymorphisms in *CSK* prevent or inhibit the phosphorylation of src leading to fibrosis (70). Polymorphism in the intron of the *CSK* gene is associated with SSc. Variant rs1378942 is associated with overall SSc (70).

Deoxyribonuclease 1-Like 3

A member of the human DNase 1 family, deoxyribonuclease 1-like 3 (DNASE1L3) is secreted by macrophages and is found in the liver and spleen (14). During apoptosis, *DNASE1L3* has a role in the fragmentation of DNA. It also generates double-strand breaks in immunoglobulin-encoding genes. In regards to autoimmune susceptibility, *DNASE1L3* is found to be associated with susceptibility to SLE and rheumatoid arthritis (RA). Using the immunochip array, *DNASE1L3* SNP rs35677470 was identified as a risk for SSc and ACA positivity. These authors identified a substitution in amino acid Arg to Cys at position 206 on exon 8 of *DNASE1L3* protein resulted in the loss of a hydrogen bond. The amino acid substitution in this position may cause the protein to become inactive suggesting a potential role for SNP rs35677470 in autoimmunity due to defective apoptotic DNA breakdown (14).

Interferon-Regulated Genes and PAH

Multiple studies using GWAS, meta-analysis, and immunochip analysis assays have confirmed the involvement of IFN in SSc susceptibility. The identification of multiple variants in IFN genes in association with SSc, SSc lung disease, and SSc mortality highlights the significance of the IFN pathway in the development and progression of SSc. IFN modulate differentiation, survival, proliferation, and cytokine production by T and B cells and dendritic cells. IFN stimulate the expression of toll-like receptors (TLRs) 3, 7, and 9. IFN genes were overexpressed in peripheral blood mononuclear cells (PBMCs) from patients with SSc and SLE. Higher IFN scores correlated with ATA, anti-U1RNP, lymphopenia, and IFNα/IFNβ receptor 2 (*IFNAR2*) missense mutation rs7279064 GG or GT (114). Other variants in the IFN pathway have also been well established in SSc. Polymorphisms in *IRF5*, *IRF7*, and *IRF8* have been identified. *IRF5* mediates IFN activity and is an important inflammatory signaling pathway. Polymorphisms in IRF5 are associated with SLE, RA, ulcerative colitis, and others. Regulation in immune reaction to infections by *IRF5* is activated by TLRs 7 and 9. In SLE, *IRF5-transportin-3* gene (*TPO*) rs4728142 correlates with *IRF5* expression leading to increased binding of zinc-finger BD 3 (ZBTB3) affecting both RNA transcription and DNA binding (115). In SSc, *IRF5* rs200460 is associated with dcSSc, lcSSc, ATA, and ACA. The strongest association is with ATA and ILD (38). It is linked to overall mortality independent of disease type or serology (39). A Han Chinese cohort of 424 SSc patients identified rs2004640*TT genotype as being significant in this population. This variant is associated with pulmonary fibrosis and ATA positivity (40). *IRF5* rs4728142 is predictive of longer survival and milder pulmonary fibrosis. The association is independent of age of disease onset, autoantibody profile, or disease type (41). *IRF7*: Interferon regulatory factor 7 (IRF7) activates type IFN genes in response to DNA/RNA immune complexes and viral infections. *IRF7* associates with susceptibility to SLE. Multiple variants in the *IRF7* genes confer susceptibility to SSc. *IRF7* rs1131665 is associated with SSc-associated ACA positivity. The variants identified were replicated in a Spanish cohort (87). *IRF8:* Multiple studies have identified *IRF8* association with SSc and rs11642873 with lcSSc (24). *IRF8* rs2280381 has been identified as SSc susceptible gene in a Japanese cohort (68).

Attention has focused on the possible contribution of the immune system to pathogenetic processes in PAH, especially innate immunity and IFNs (116, 117). Type I IFNs are implicated by the association of use of IFNα in the treatment of hepatitis and of IFNβ in the treatment of multiple sclerosis (MS) with development of PAH (118, 119). Diseases in which there is an "IFN signature" (such as SLE, SSc, and infection with HIV) are associated with development of PAH (120–124). Furthermore, IFNα and IFNγ added to cultures of human pulmonary artery smooth muscle cells (PASMC) primed with TNFα or to cultures of human lung MVEC or human lung fibroblasts, cause release of the potent vasoconstrictor, ET-1, and of IFN-inducible protein-10 (IP-10) (117). In a series of 128 SSc patients with PAH and 35 patients with no PAH, the SSc patients with PAH had higher levels of IP-10 and ET-1 in their sera compared to SSc patients without PAH or compared to healthy controls. More SSc patients with PAH had detectable levels of IFNα and IFNγ in their sera than SSc patients without PAH (117). In this series of SSc patients, levels of TNFα, IL-12p70, IL-6, IL-1α, and IL-8 were significantly higher in sera in SSc patients with PAH when compared to SSc patients without PAH (117). Additional studies of this patient group revealed that serum levels of IP-10 in the SSc-PAH patients correlated with pulmonary vascular resistance, and levels of brain natriuretic peptide in serum, and serum IP-10 levels in the SSc-PAH patients inversely correlated with cardiac index and 6-min walks test (117). Sections of lung from patients with idiopathic PAH (IPAH) or with SSc-PAH expressed higher levels of type I interferon receptor 1 (IFNR1) in endothelium, smooth muscle layer, vascular interstitium, and in intravascular inflammatory cells as assessed by immunohistochemistry and Western blotting (117). While the above studies strongly implicated type I

IFN as playing a pathogenic role in SSc-PAH and IPAH, further evidence was substantiated in the type I interferon α receptor 1 knockout mouse which was found to be resistant to experimental hypoxic PAH induction. These mice did not have elevated serum levels of ET-1 when compared to wild-type (WT) control mice (117). Analysis of PBMC from patients with SSc revealed CD169/sialoadhesin (*Siglec-1)* and other IFN-regulated genes were overexpressed in patients with dcSSc, whereas patients with lcSSc with PAH overexpressed *IL-13RA1*, intercellular adhesion molecule-1 (*ICAM-1*), *C–C chemokine receptor type 1 protein or gene* (*CCR1*), *JAK2*, and *melanocortin receptor 1 (MCR1)* (123, 125, 126). IL-13 was also elevated to higher levels in sera of patients with lcSSc with PAH, and *MCR1* was induced on $CD14^+$ monocytes suggesting monocytes are activated in lcSSc patients with PAH of an alternative (i.e., IL-4/IL-13) rather than classical [i.e., IFNγ/lipopolysaccharides (LPS)] pathway (123). The identification of multiple IFN genes having association in SSc, SSc lung disease, and mortality highlights the significance of the IFN pathway in the development and progression of SSc.

Interleukin-1 Receptor Associated Kinase 1

Interleukin-1 receptor associated kinase 1 (*IRAK1*) gene is located on the Xq28 and is in the same haplotypic block with methyl-CpG-binding protein 2 gene (*MECP2*). *IRAK1* encodes a serine/threonine protein kinase that regulates NFκB through T-cell receptor signaling and TLRs/IL-1R activation. It also plays a role in IFN induction. IRAK1 has been identified in SLE as a susceptibility gene (42, 72). In SSc, *IRAK1* rs1059702*TT is associated with dcSSc, SSc-related fibrosing alveolitis, and ATA positivity (42, 72). The presence of the T allele may contribute to disease severity, and presence of *MECP2* rs17435 may explain the association of *IRAK1* variant rs1059702 with this subset (42, 72).

IL-2/IL-12 Genetic Susceptibility

Variants in interleukin-2 receptor α (IL-2A), *IL-12R* (*IL-12R*β1 or *IL-12Rβ2*) have been reported to be associated with SSc. *IL-2* plays a role in immune system homeostasis and self-tolerance. It facilitates B-cell immunoglobulin production and induces natural killer cell proliferation and differentiation (65). The binding of *IL-12* to its receptors stimulates IFN production and promotes TH1 differentiation. IL-12 signals through STAT pathway and a defect in either *STAT4* or *IL-12R* could influence SSc pathogenesis. Variant SNP rs77583790 found in the intergenic region between *SCHIP1* and *IL-12A* was found to be associated with lcSSc (14). *IL-12Rβ1* and *IL-12Rβ2* recruit tyrosine kinases and activate transcription of other genes. Polymorphisms in IL-*12Rβ1* and *IL-12Rβ2* have been identified in psoriasis, Behcet's disease, and primary biliary cirrhosis (67). Two studies were conducted to investigate the role of *IL-2* in SSc. ILR2 gene variants: rs11594656, rs2104286, and rs12722495 were associated with SSc, lcSSc, and ACA positivity. The associations are strongly dependent on ACA since removal of ACA from the analysis resulted in loss of association, and the strongest association with ACA positivity was with rs2104286, with associations of the other IL-2 RA gene variants being lost after conditioning to rs2104286 (66). Polymorphism in rs2104286 has the strongest association with ACA while rs6822844 and rs907715 have association with SSc and lcSSc (66). *IL-12Rβ1* rs2305743 and rs436857 were found to be associated with SSc (71). Polymorphisms in these receptors may affect the binding of transcription factors decreasing the expression of IL-12. *IL-12Rβ2* rs3790567 is associated with SSc. *IL-12Rβ2* gene maps close to the *IL-23* coding region, the association between rs3790567 was not found to be dependent on *IL-23* (67). *IL-2/IL-21: IL-21* affects the innate and adaptive immune response playing a role in the differentiation of B cells into plasma cells and regulation of TH17 development (65). Polymorphism in the *IL-2/IL-21* region is associated with lcSSc and global SSc. *IL-2/IL-21* variant rs682284 is strongly associated with multiple autoimmune diseases and is considered an autoimmune susceptibility locus (127). The rs907715 minor allele and rs682284 have association with SSc. Variant rs6822844 influences lcSSc and ACA positivity (65). The allelic combination of rs2069762*A–rs6822844*T–rs6835457G–rs907715*T is associated with dcSSc and lcSSc (65). The T allele for rs6822844 acts as a protective for lcSSc and ACA positivity.

IL-23

IL-23 promotes the expansion of TH17. *IL-17* and *IL-23* are elevated in the plasma of SSc patients (28). Polymorphism in *IL-23R* is associated with SSc and ATA positivity. *IL-23R* variant rs11209026*GG (Arg381 Gln variant) has association with ATA positivity and rs11465804*TT is associated with dcSSc and ATA positivity. The major alleles rs11209026*G and 11465804*T were decreased in patients with PAH, suggesting that the major allele is protective against PAH (28).

Integrin αM

Integrin αM (ITGAM) β2 is a leukocyte-specific integrin that regulates neutrophil and monocyte cell activation and adhesion. It allows for phagocytosis of complemented-coated particles. Deficiency in ITGAM results in increased IL-6 production by antigen-presenting cells (APC) (128). Pooled meta-analysis, subsequent independent meta-analysis, and GWAS looking at shared risk polymorphisms for SLE and SSc confirmed *ITGAM* variant rs1143679 were associated with susceptibility to SSc (47, 84, 128).

Juxtaposed with Another Zinc-Finger 1

Juxtaposed with another zinc-finger 1 (*JAZF1*) encodes a nuclear protein with zinc-fingers that functions to repress transcription. It has been associated with bone morphogenesis and CI deposition (47). *JAZF1* has been identified as an SLE-associated locus, and a recent GWAS pan-meta-analysis has confirmed *JAZF1* rs1635852 association with SSc (47).

KIAA03192L

KIAA03192L has been identified in polycystic kidney disease and dyslexia as a disease susceptibility gene. It is expressed in macrophages and natural killer cells in mice and in $CD33^+$ myeloid cells and $CD14^+$ monocytes in humans. *KIAA03192L* is overexpressed in PBMCs of SLE patients. In SSc, *KIAA03192L* variant rs2275247 is associated with lcSSc (47).

Protein Tyrosine Phosphatase Non-Receptor Type 22

Protein tyrosine phosphatase non-receptor type 22 (*PTPN22*) plays a critical role as a gatekeeper for T-cell receptor signaling. It encodes the protein tyrosine phosphatase lymphoid tyrosine phosphatase in T-cells and acts to inhibit T-cell signaling through dephosphorylation of substrates. Polymorphism in *PTPN22* has been associated with type 1 DM, RA, and SLE. Earlier studies looking at the relationship between *PTPN22* and SSc failed to show an association between *PTPN22* and SSc (129, 130). Larger studies in SSc patients showed association with *PTPN22* Ct/TT genotypes with both ATA and ACA positivity. The T allele associated with ATA positivity and the CC genotype with both ACA and ATA positivity (55). Meta-analysis confirmed *PTPN22* rs2476601*T and the minor allele 1858T are associated with SSc and ACA positivity (56, 57). Haplotype 1858C allele was protective in a French cohort (58).

Paraxylene–Orthoxylene (Phox Homology) Domain Containing Serine/Threonine Kinase

Paraxylene–Orthoxylene domain containing serine/threonine kinase is a protein that plays a role in the ligand-induced internalization, degradation, and trafficking of epidermal growth factors. Variation in PXK is association with SLE susceptibility where it is found to alter B-cell receptor internalization (131). *PXK* rs2176082 and rs4681851 are associated with SSc and rs2176082 has association for ACA positivity. The association of rs2176082 is related to *DNASE1L3* (14, 47).

Signal Transducer and Activator of Transcription Protein 4

Signal transducer and activator of transcription protein 4 is critical for T-cell signaling and differentiation (132–134). *STAT4* is involved in effecting a Th1 cytokine response by transmitting signals from IL-2, IL-12, and IL-23 receptors and in signaling after type 1-IFN engages its receptor (135, 136). The role of *STAT4* in fibrosis was assessed in scleroderma mouse models. To assess the contribution of *STAT4* to bleomycin (BLM)-induced skin fibrosis and fibrosis of skin in (tight skin) Tsk-1/+ mice, BLM was injected for 3 weeks into $STAT4^{-/-}$ and $STAT4^{+/+}$ mice. $STAT4^{-/-}$ mice were crossed with Tsk-1/+ mice, and skin fibrosis was assessed (137). The deletion of *STAT4* significantly reduced skin fibrosis in the BLM model but not in the Tsk-1/+ model (137). In the BLM model, it was noted that there were decreased numbers of inflammatory cells including T cells and proliferating T cells and decreased quantity of IL-6, IL-2, TNFα, and IFNγ in lesional skin of $STAT4^{-/-}$ vs. $STAT4^{+/+}$ mice (137).

Signal transducer and activator of transcription protein 4 is considered an autoimmunity loci since its association has been firmly confirmed in SLE, RA, primary biliary cirrhosis, and SSc (48). SNP rs7574865 is associated susceptibility to lcSSc and ACA positivity (50, 51). SNP rs7574865 and rs10168266 were associated with dcSSc, ATA positivity, and pulmonary fibrosis in a Chinese cohort (52). Variant rs7574865*T allele has an additive effect with IRF5 rs2004640 seen in fibrosing alveolitis (38). Gene–gene interactions between *STAT4* and polymorphism in the transcription factor T-bet show increased susceptibility to SSc. Transcription factor *T-bet* [(T-box expressed in T cells) (*TBX21*)] is an important transcriptional activator of Th1 differentiation effecting Th1/Th2 balance. Polymorphisms in *TBX21* have associations with RA, asthma, and type 1 DM. TT genotype of *TBX21* variant rs11650354 confers susceptibility to SSc in a recessive manner while *STAT4* variant rs11889341 A allele is associated with an increased risk of SSc in a dominant pattern. *STAT4* genotype increased the SSc risk in the presence of *TBX21 CC* genotype (53). Plasma levels of circulating IL-6 and TNF were increased in SSc patients who carry the *TBX21 CC* genotype where as those who carry the TT genotype show increased circulating IL-2 and IL-5 suggesting that patients who carry the CC genotype have a prominent pro-inflammatory cytokine profile (53). Gene expression profile from whole blood RNA of SSc patients suggest a role for type 1-IFN and pro-inflammatory cytokines in the CC genotype and of the T-cell pathway in the TT group (53).

Tumor Necrosis Factor Alpha-Induced Protein-3

Tumor necrosis factor alpha-induced protein-3 (*TNFAIP3*) encodes ubiquitin-modifying protein A20 and has a critical role in the regulation of immune signaling pathways.

Polymorphism in *TNFAIP3* is associated with SLE, RA, and celiac disease. *TNFAIP3* rs117480515, rs5029939*G allele, and rs6932056 carry an increase of susceptibility to SSc (43, 68). TNFAIP3 SNP and rs5029939*G is associated with dcSSc, fibrosing alveolitis, and PAH (43). The rs117480515*A allele is associated with SSc polyautoimmune subset (86).

Tumor Necrosis Factor Superfamily Member 4 Gene

Tumor necrosis factor superfamily member 4 gene (*TNFSF4*) encodes for the T-cell co-stimulatory molecule, OX40 ligand. *TNFSF4* has a role in B-cell proliferation and differentiation and T-cell proliferation. Ox40–OX40L promotes generation of Th2 cytokines. It has been identified as a susceptibility gene for SLE. TNFSF4 SNPs variant rs1234314, rs2205960, rs844648, rs12039904, rs1234317, and rs10912580 have been identified as susceptibility genes in SSc and are associated with lcSSc- and ACA-positive SSc patients in multiple French European studies (120–122). The minor allele rs1234314 has association for lcSSc, ACA, and ATA, while rs844648 confirmed association with dcSSc and ARA. Variant rs844648 was found to be protective in all SSc sub-groups except ARA+. In women, rs2205960*TT/GT and rs844648*AA associates with increased risk for SSc (59). These studies suggest *TNFSF4* as a susceptibility gene for SSc.

TNFAIP3 Interacting Protein-1

TNFAIP3 interacting protein-1 *(TNIP1)* gene interacts with A20 binding protein (BP) and inhibits TNF-induced NFκB-dependent gene expression; thereby negatively regulating NFκB. Mutations in this gene have been associated with RA, SLE, and psoriatic arthritis. *TNIP1* gene and protein expression was reduced in lesional skin tissue and cultured fibroblasts from SSc patients. *In vitro*, *TNIP1* had inhibitory effects on inflammatory cytokine-induced CI production (73). *TN1P1* SNP rs2233287, rs4958881, and rs3792783 are associated with global SSc (74). A two-staged GWAS showed strong linkage disequilibrium in the *HLA-DQB1*

gene: rs9275224, rs6457617, and rs9275245. Within the MHC region, there was association with rs3130573 located in the *PSORC1C1* gene. *PSORS1C1* also show susceptibility in global SSc except for ACA positivity patients but this association is dependent on HLA class-II (74).

CD87 (UPAR)

Urokinase-type plasminogen activator receptor (UPAR) promotes ECM and vascular remodeling. It regulates growth factor activation and is responsible for cell adhesion, migration, and proliferation (91). *UPAR* rs344781*G allele is associated with SSc-related digital ulcers, pulmonary artery hypertension, ACA positivity, and lcSSc (91). Genotype rs344781*GG is identified as an independent risk factor for SSc-related digital ulcers and PAH (91). *CD226*: acts as a co-stimulator of T cells and plays a role in T-cell adhesion. It is expressed on NK cells, monocytes, platelets, and B and T cells (77). It has been correlated with susceptibility to SLE, type 1 diabetes, thyroid disease, and MS (78–80). In SSc, the *CD226* T allele of rs763361 may contribute to disease severity due to its association with multiple SSc subsets including dcSSc, ATA positivity, and ILD (80). *CD226* haplotype SNP rs763361, rs34794968, and rs727088 correlates with pulmonary fibrosis (77). *MIF*: Macrophage migration inhibitory factor (MIF)-173 acts upstream, activates innate immunity, and sustains cellular and inflammatory responses. MIF induces endothelial adhesion and induces fibroblast proliferation that may contribute to vasculopathy (135). MIF-173 is lower in lcSSc. *In vitro*, *C7 MIF* encoded fibroblasts produced more MIF than non-stimulated fibroblasts (75). In an American and European study that included 3,800 SSc patients, MIF was found to have higher association with dcSSc compared to controls and lcSSc (75, 138). *MMP-12*: matrix metalloproteinase-1 (MMP-1) rs2276109*AA genotype has significant association in dcSSc, lcSSc, ATA positivity, and pulmonary fibrosis in an Italian SSc population (92). *NFkB1* gene SNP rs1598859 is associated with overall SSc disease (70). *PLD4:* phospholipase D family member 4 (PLD4) was identified as a susceptibility gene for SSc in Japanese (68). *PPARγ*: peroxisome proliferation-activated receptor gamma (*PPAR-γ*) when engaged by ligands of different types blocks transforming growth factor (TGF)-β mediated fibrotic responses *in vitro* in cultured fibroblasts and in various fibrotic animal models *in vivo* (81, 82). PPARG rs310746 is associated with SSc (83). *PSD3*: involved in signal transduction pathways and IC signaling. Polymorphism in the *PSD3* gene rs10096702 is associated with overall SSc (70). *TLR2*: subcutaneous injections of TLR ligands into the skin of SSc results in a significant inflammatory reaction resulting in SSc skin changes (90). *TLR2 pro63* His is associated with dcSSc, PAH, and ATA positivity (90). *TLR5* and 10 expression were increased in SSc fibroblasts *in vitro* and *in vivo* (139).

Vascular Related Genes

Endothelin-1 is one of three isoforms and is synthesized by vascular endothelial (VE) cells, fibroblasts, bone marrow mast cells, neutrophils, macrophages, and cardiac myocytes (140). Various triggers induce synthesis of ET-1 including TGF-β and other growth factors, cold exposure, low shear stress, hypoxia, and angiotensin II (140); but its synthesis is reduced by nitric oxide (NO), natriuretic peptides, increased blood flow, and prostacyclin (141). ET-1 is also degraded by MMP-1, which is reduced in SSc (140). Two types of receptors for ET-1 (ETα and ETβ) are variably expressed on endothelial cells, vascular smooth muscle cells, adventitial fibroblasts, tissue fibroblasts, neutrophils, mast cells; and monocytes and ET receptor engagement on these cells triggers a variety of pro-inflammatory or fibrotic response, including vasoconstriction of vasculature (140). ET-1 increases surface expression of ICAM-1 on fibroblasts, stimulates CI synthesis, promotes formation of myofibroblasts, and facilitates binding of T cells to fibroblasts (140, 142). ET-1 acts as a downstream mediator of TGF-β, and its induction by TGF-β in fibroblasts is via small mother against decapentaplegic (Smad)-independent signaling that involves c-Jun N-terminal kinase (JNK) and activin receptor-like kinase (ALK)5 pathways (143). Polymorphisms of ET-1 receptors are associated with SSc. For example, there is an association of *EDNRB* polymorphisms and dcSSc and *EDNR-A* polymorphism with anti-RNA polymerase autoantibodies in SSc (140). Polymorphisms were also described in the promoter of the NOS2 gene that confers susceptibility to PAH in SSc (144). Potassium voltage-gated channel shaker-related subfamily 5 (*KCNA5*) has a role in the regulation of vascular tone. It is inhibited by hypoxic conditions leading to vasoconstriction. *KCNA5* may have a protective role against PAH-associated SSc, this protective role was identified with variant rs10744676 (145).

MicroRNAs

MicroRNAs are translational regulators of gene expression and also destabilize messenger RNAs (mRNAs) of target genes (146). MiRNAs are tissue- and cell type-specific short, single-stranded non-coding RNAs that function to modulate gene expression (**Table 4**). MiRNA bind to the 3′ untranslated region of mRNA of the target gene and mediate post-transcriptional regulation. Once bound, they either cause translational repression of the target gene or induce the degradation of the gene (147–149). In SSc, several miRNAs are associated with TGF-β and CI expression. In comparison to normal skin tissue, Zhu et al. (93, 147) found that skin from patients with lc and dc SSc expressed miR-21, miR-31, miR-146, miR-503, miR-145, and miR-29b. In these patients, miR-21 was increased in both tissue and fibroblasts whereas miR-145 and -29b were decreased. These miRNAs targeted the TGF-β pathway – including Smad7, Smad3, and COL1A1. TGF-β stimulation resulted in increased miR-21 expression and decreased expression of Smad7, while the upregulation of miR-145 was associated with a downregulation of Smad3 message. These same authors found that overexpressing miR-21 in fibroblasts decreased Smad7 but knocking down the expression of miR-21 increased Smad7 expression (93). miR-21 was also found to have increased expression in BLM-induced skin fibrosis. Reporter gene assay analyses revealed that the target gene for miR-21 is Smad7, while the target gene for miR-145 is *Smad3* (93, 94).

Ninety-five miRNAs were analyzed in the sera of SSc patients and healthy controls. This analysis revealed that miR-30b was significantly downregulated in SSc patients and the modified Rodnan skin score (MRSS) inversely correlated with the level of miR-30b (95). Downregulation was also seen in the skin of scleroderma patients and BLM-treated sclerotic skin (95). Transfection studies

TABLE 4 | MicroRNAs in SSc.

Micro RNA (miR)	Implications for SSc pathogenesis	Reference
miR-21 ↑ in SSc skin and fibroblasts	↑ by TGF-β, ↓ Smad7	(93, 94)
miR-145 ↓ in SSc skin and fibroblasts	Smad3 is its target gene	(93, 94)
miR-29b ↓ in SSc skin and fibroblasts	Suppressor of fibrosis	(94)
miR-30b ↓ in SSc skin and fibroblasts	Suppressor of PDGFR-β	(95)
miR-29a ↓ in SSc sera and skin	Suppressor of CI and CIII synthesis by fibroblasts, miR-29a is reduced by TGF-β, PDGF-β, and IL-4. Lower serum miR-29a associates with PAH	(96–98)
miR-196a ↓ in dcSSc sera	Expression of miR-196a reduced by TGF-β. May regulate CI synthesis. ↓ miR-196 = ↑ MRSS, ↑ digital pitting, and scars	(99, 100)
miR-150 ↓ in SSc fibroblasts and sera	Reduces fibroblast CI, pSmad3, and integrin expression	(101)
miR-7 ↓ in localized scleroderma skin and fibroblasts	Reduces CI synthesis by fibroblasts. Regulated by TSP2	(102, 103)
miR-let-7a ↓ in SSc and localized scleroderma skin and sera	Reduces CI synthesis by fibroblasts	(104)
miR-129-5p ↓ in SSc	Suppressor of COL1A1 expression in fibroblasts	(105)
miR-142-3p ↑ SSc in sera	May regulate αV integrin, which may recruit and activate small latent complex that regulates autocrine TGF-β activity	(106)
miR-92a ↑ in SSc sera and fibroblasts	May downregulate MMP-1	(107)
miR-21 ↑ SSc in fibroblasts	miR-21 is upregulated by TGF-β and decreases expression by Smad7	(93, 94)

showed that miR-30b affects platelet-derived growth factor/receptor (PDGFR)-β expression by suppressing this receptor (95). In their evaluation of 15 SSc patients and 15 normal subjects, Koba et al. (150) found that miR-206 and miR-21 were useful in distinguishing patients with SSc from normal subjects (150).

miRNA-Targeting CI

The expression of miR-196a was investigated in SSc both *in vitro* and *in vivo*. *In vivo* miR-196a was detected in the serum of SSc patients. Patients who had measurable lower levels of miR-196a had dcSSc compared to lcSSc. Lower levels of miR-196a was also associated with higher prevalence of pitting digital scars and more fibrotic skin as measured by MRSS (99). *In vitro*, the expression of miR-196a was normalized by TGF-β small interfering RNA (siRNA) in SSc fibroblasts, and the addition of miR-196a inhibitor to these fibroblasts resulted in the downregulation of CI. When the inhibitor was added to normal fibroblasts, there was an overexpression of CI (99). These results suggest that miR-196a may regulate CI expression.

Micro-RNA-29 (miR-29) is a TGF-β associated miRNA and is linked to fibrosis likely by interaction with several extracellular genes including ELN, FBN1, COL1A, COL1A2, and COL3A1 (151, 152). TGF-β/Smad3 signaling appears to negatively regulate miR-29 (153). Support for this relationship was the finding that in BLM pulmonary fibrosis mouse model, Smad3 was upregulated while miR-29 was downregulated in contrast to results with $Smad3^{-/-}$ mice, which were protected from BLN pulmonary fibrosis and miR-29 was upregulated (153). In addition, therapeutic delivery of miR-29 to mice using Sleeping Beauty transposon-mediated gene transfer protected mice from developing BLM-induced lung fibrosis (153). MiR-29a has the ability to bind to the *3′UTR of COL1A1* and *COL1A2* (96, 154). Maurer et al. (97) found that miR-29a was strongly downregulated in SSc fibroblasts and skin sections when compared to healthy controls (97). SSc fibroblasts, in which miR-29 was overexpressed, exhibited decreased expression and protein levels of CI and CIII, while knockdown of miR-29 in normal fibroblasts increased CI production. Levels of miR-29 were reduced in normal fibroblasts when these fibroblasts were cultured with TGF-β, PDGF-β, or IL-4 (97). These studies confirm that miR-29a directly regulates CI. Serum levels of miR-29a were investigated to determine its potential role as a biomarker in SSc. In 61 patients with SSc, approximately 40% of which had dcSSc, miR-29a was found to be upregulated and not downregulated as expected in the serum of these patients. Patients with scleroderma spectrum disorder (SSD) are those who did not fulfill the ACR diagnosis criteria for SSc but who may develop scleroderma in the future. In these patients, miR-29a was downregulated compared to healthy controls, dcSSc, and lcSSc patients (96). Decreased serum levels of miR-29a may also be associated with higher right ventricular systolic pressure and PAH (96).

MiR-150 expression is decreased in SSc fibroblasts and sera. Normal fibroblasts that were transfected with miR-150 inhibitor had induced expression of type 1 CI, pSmad3, and integrin (101). Forced expression of miR-150 in SSc fibroblasts resulted in downregulation of CI, pSmad3, and integrin (101). In patient sera, lower expression of miR-150 correlated with severe clinical disease (101).

Skin and fibroblasts from localized scleroderma showed decreased levels of miR-7 compared to keloid skin and normal skin *in vivo* and *in vitro* (102). Normal fibroblasts that were transfected with miR-7 inhibitor exhibited upregulation of COL1A2 (102).

Skin and sera from SSc and localized scleroderma patients showed a downregulation of miR let-7a when compared to normal and keloid skin (104). CI was reduced by the overexpression and inhibition of miR let-7a in human and mouse skin fibroblasts (104). Intermittent overexpression of miR let-7a by intraperitoneal injections reduced dermal fibrosis in the BLM skin model (104).

MiR-129-5p is a regulator of *COL1A1* (154) and is downregulated in SSc (105). Nakashima et al. (105) found that, in their 20 patients with SSc, IL-17A expression was increased in the involved skin and sera, but IL-17R type A was decreased in SSc fibroblasts when compared to normal (105). IL-17A reduced protein expression of type I CI α1 chain [α1(I)] and connective tissue growth factor (CTGF). IL-17A also induced the expression of miR-129-5p (105). In the presence of IL-17A, miR-129-5p is increased with α1(I) and CTGF. The authors suggest that since SSc fibroblasts have intrinsic activation of TGF-β, TGF-β suppresses IL-17A, in addition to miR-129-5p with resultant CI accumulation (105).

MicroRNA-29a and miRNA-196a are low in SSc fibroblasts and can suppress CI gene expression, suggesting the low-level expression of the miRNAs permit CI to be upregulated by TGF-β and other mediators in SSc fibrogenesis (97, 99). Levels of other miRNAs have been found to differ in patients with SSc compared to healthy controls as follows: serum miR-142-3p was higher in SSc patients than healthy controls (106); levels of miR-21 were increased, whereas levels of miR-145 and miR-29b were decreased in SSc lesional fibroblasts (94); miR-92a is more elevated in sera and SSc lesional fibroblasts than in normal healthy controls and may downregulate MMP-1 (107); and levels of miRNA-7 were found to be reduced in sera and lesional fibroblasts from patients with localized scleroderma and may regulate CI expression (102). MiR-150 regulates β3 integrin expression and was found to be downregulated in lesional SSc dermal fibroblasts compared to healthy donor fibroblasts (101); miR let-7a was found to be decreased in sera and lesional fibroblasts from patients with SSc or localized scleroderma (104); and miR-21 was found to be upregulated in SSc lesional dermal fibroblasts (93).

Discoidin domain receptor 2 (DDR2) and thrombospondin-2 (TSP2) were both found to be decreased in SSc dermal fibroblasts (103, 104). In SSc dermal fibroblasts, *DDR2* mRNA and protein levels were suppressed, but the knockdown of TGF-β in these fibroblasts resulted in increased expression of *DDR2* (104). In normal fibroblasts, *DDR2* knockdown increased miR-196a expression with resultant decrease in CI. This was not seen when *DDR2* was knocked-down in SSc fibroblasts (104). In SSc, fibroblasts, knocking down *DDR2* did not affect TGF-β signaling or miR-196a expression, suggesting that intrinsic expression of TGF-β causes the downregulation of *DDR2* in SSc fibroblasts (104).

Thrombospondin 2 mRNA expression and protein levels are decreased in SSc fibroblasts when compared to controls but were upregulated in conditioned medium from SSc fibroblasts (103). Knockdown of *TSP2* in dermal fibroblasts caused decreased expression of CI and increased miR-7 expression (103). SSc dermal fibroblasts show an increased expression of miR-7 (103) suggesting that a negative feedback mechanism may exist between *TSP2* and miR-7 (103).

Matrix metalloproteinase-1 was downregulated when normal dermal fibroblasts were overexpressed with miR-92a (107). In 61 patients with SSc, medium serum levels of miR-92a were elevated. This upregulation was constitutively also found in SSc dermal fibroblast, but when these fibroblasts were transfected with siRNA of TGF-β, the expression of miR-92a was decreased (107). These studies suggest that miR-92a ability to affect *MMP-1* suggest that miR-92a may be a target for *MMP-1*.

Hair miRNA

MicroRNA from the hair shaft and roots was studied. Hair-miR-196a was found to be significantly decreased in SSc patients (100). Hair miR-29a was obtained from 20 SSc patients, 5 dermatomyositis, and 13 controls to determine its usefulness as a biomarker. Hair miR-29a was significantly lower in SSc patients, and the decreased levels were associated with a higher prevalence of phalangeal contractures (98). We may see more studies using hair miRNAs to assess biomarkers and disease phenotypes.

Immune System in SSc Pathogenesis

Role of Innate Immune System

Engagement of the innate immune system depends on 13 different TLRs, which are not antigen-specific but instead recognize patterns and which segregate on the basis of the nature of the ligands they encounter such as distinct molecular patterns in particular pathogens, in endogenous cellular constituents, or in cellular products of the host [reviewed in Ref. (155)]. Considerable evidence suggests that *TLR2* and *TLR4* expressed on cells and IC *TLR3*, *7*, *8*, and *9* have particular relevance to SSc pathogenesis. For example, a rare functional polymorphism (Pro^{631} His) in TLR2 (which has bacterial peptidoglycan, lipoprotein, and lipoteichoic acid and yeast-derived zymosan as natural ligands) is associated with ATA positivity and enhanced IL-6 production by dendritic cells when engaged by a TLR2 ligand (90, 155). TLR4 endogenous ligands [including fibronectin, hyaluronan fragments, heat-shock protein (HSP) 70, HSP9, high-mobility group box-1 (HMGB-1), and S100A proteins] could engage TLR4 (which is increased in SSc skin and lungs) and synergize with TGF-β to increase fibroblast CI production (155–160). Importantly, HSP70, HMGB-1, and hyaluronan are elevated in SSc sera or tissues (161–163). Of interest, elevated HMGB-1 and soluble advanced glycation end products (sRAGE) levels in sera of patients with SSc correlated with more internal organ involvement, immunological abnormalities, and total MRSS but correlated negatively with lung function (161). Double-stranded RNA is recognized by TLR3, single-stranded RNA, and imidazoquinoline compounds by TLR7 and TLR8, whereas unmethylated CpG oligonucleotide sequences are recognized by TLR9 and some of these ligands are present in SSc (164, 165).

Siglec-1 (CD169, *sialoadhesin)* is a marker for macrophage activation and its expression was found to be increased $CD14^+$ monocytes in peripheral blood and on macrophages in dermis of lesional skin of a subset of patients with SSc (125). Furthermore, Siglec-1 was induced in peripheral blood $CD14^+$ monocytes from normal donors when cultured with IFNα, TLR3, 7, or 9 agonists but not by TLR2 or 4 (125). In the skin, activated macrophages expressing Siglec-1 may also release cytokines or growth factors that are able to stimulate fibroblasts or myofibroblasts to synthesize CIs and other matrix components (125). In addition, sera containing autoantibodies from patients with SSc induce high levels of IFNα in normal monocytes that is inhibited by pretreatment of the sera with bafilomycin and RNA-degrading enzymes, suggesting that the immune complexes in SSc sera contain RNA that can bind IC TLRs (166). While other agents (e.g., IL-4, LSP, IFNβ, IFNγ) might also induce Siglec-1 expression on monocytes/macrophages in SSc, these findings are compatible with the notion that generations of IFNα by activation of IC TLRs 3, 7, or 9 agonists might be ongoing in a subset of SSc patients (125, 167).

Interferon Signature in SSc

Interferons are multifunctional cytokines that are responsible for inducing cellular resistance to viruses. IFN-α, -β, and -ω are type 1-IFNs. There is evidence for a prominent IFN signature in SSc. For example, peripheral whole blood cells in 50% of SSc patients have increased expression of IFN-regulated genes and lung tissues

from SSc patients with ILD have increased IFN and IFN-regulated gene expression (168, 169). It appears that the IFN signature in SSc discussed below may arise from activation of TLRs expressed on the surface of cells by infectious agents or by endogenous proteins, RNA, DNA, and other cellular products that can trigger IC TLRs summarized above. IFN regulatory factors (IRF) coordinate the expression of IFN and IFN-inducible genes that help regulate the innate and adaptive immune responses (169, 170). Thus far, *IRF5*, *IRF7*, and *IRF8* appear to be relevant to SSc (169) (see **Table 3**).

IRFSNP Associations

IFN regulatory factor 5, a major regulator of type 1-IFN, induces the transcription of IFN-α and other pro-inflammatory cytokines, is involved in TLR signaling, and is critical for activation of IFN-associated genes (109, 169) (see **Table 3**). *IRF5* has association with SLE (171–173), and multiple studies have shown SNPs of *IRF5* are associated with SSc susceptibility. *IRF5* rs2004640*TT was found to have a strong association with dcSSc, fibrosing alveolitis, antinuclear antibody (ANA), and ATA positivity in a French cohort (38). In addition to rs2004640, these same authors found an association between rs3757385 and rs10954213 variants and SSc (43). In this study, *IRF5* haplotype "R" was identified as a risk while haplotype "P" was protective (43). A Japanese case–control association study with 281 SSc and 477 controls found that rs2004640, rs10954213, and rs2280714 were all significantly associated with SSc, with rs2280714 having the strongest association with SSc, and these SNPs were significantly enriched in dcSSc and ATA-positive patients (45). Carmona et al. found that SNPs rs10488631, rs2004640, and rs4728142 showed strong associations in SSc global disease, and that association of rs20004640 was dependent on rs4728142 (174). rs728142*A-rs2004640*T haplotype explained this association suggesting that all three haplotypes provide an additive effect (174). In another study, *IRF5* SNP rs4728142 was found to be predictive of longer survival in SSc patients with ILD (41). *IRF7* is upregulated in peripheral blood cells from patients with early SSc and is associated with ACA-positive SSc (175). *IRF8* is induced by IFNγ and modulates TLR signaling (24). Polymorphism rs11642873 in the *IRF8* gene was found to be associated with lcSSc (24). *IRF8* SNP rs2280381 was found to have association with SSc in a Japanese population consisting of 415 SSc and 16,891 controls with a replication study consisting of 315 SSc (68). While associations of the above variations in IRF genes with certain manifestations do not establish cause and effect, they suggest genes that regulate IFN expression and downstream effects may play a central role in determining disease severity and specific organ involvement.

Inflammasome and SSc

The cytoplasm of cells also contains another pattern recognition receptor (PRR) system called the nucleotide-binding and oligomerization domain (NOD)-like receptor (NLR) family that recognize IC motifs and, when activated via the "inflammasome" involves NFκB and mitogen-activated protein kinase (MAPK), which in turn stimulates production of pro-inflammatory cytokines IL-1B and IL-18. Polymorphisms of one of the NOD family members, *NLRP1*, are associated with ILD and ATA positivity in patients with SSc (176). Relevance of the NOD family to SSc was further evidenced by studies showing inhibition of inflammatory activation-reduced IL-1β and CI production by SSc lesional fibroblasts and studies in *NALP3* null mice showing they were resistant to lung fibrosis (177, 178). *NLRP3* and pro-inflammatory cytokines (IL-1β and IL-18) were found to be increased in skin biopsies of patients with dcSSc or lcSSc compared to age-matched control and correlated with MRSS (179).

Transitioning from Innate to Adaptive Immunity

Rather than two separate and mutually exclusive immune systems, it is being realized that there is likely an ongoing interplay between the innate and adaptive immune systems (180). Attention has focused on innate lymphoid cells (ILCs) that are involved not only in immediate immune host defense but also in maintaining homeostasis of mucosal and lymphoid tissue (180, 181). Three different types of ILCs have been described to-date: *ILC1*, *ILC2*, and *ILC3* (181). These ILCs do not express somatically rearranged antigen receptors, but express MHC Class-II and possess transcription factors and cytokine profiles reminiscent of Th cells (181, 182). ILC1s, like Th1 cells, utilize T-bet and produce IFNγ; ILC2s, like Th2 cells, utilize GATA-binding protein-3 (GATA-3) and produce IL-5, IL-9, and IL-13; and ILC3s, like Th17 cells, utilize RAR-related orphan nuclear receptor gamma transcription factor (RORγt) and produce IL-17A and IL-22 (181). ILCs express TLRs and IL-1 receptor, and ILC2s and ILC3s can act as APC similar to dendritic cells (181, 183, 184). In mouse models, ILC3s were shown to promote antigen-specific $CD4^+$T cells and antigen-specific T-cell-dependent B-cell antibody production (181). What role ILCs play in innate and adaptive immunity in SSc remains to be defined and ongoing research should eventually better elucidate how ILC effect transition from innate to adaptive immunity.

Dendritic cells by using surfaces and IC PRRs play key roles in linking innate immune response to adaptive immune responses by identifying antigens from pathogen-associated or damage-associated molecular patterns (PAMPS or DAMPS) by using TLRs, NLRs, RIG-I-like receptors (RLRs), and receptors for advanced glycation end products (RAGE) (185). The identified antigens are then processed and the information is presented to T cells in the context of MHC-II/antigen complex binding the T-cell receptor, CD86/CD80 costimulation of T-cell CD28, followed by release of cytokines from dendritic cells that affect T-cell differentiation and effect Th1, Th2, Th17, and T regulatory (Treg) cell differentiation (185, 186).

Adaptive Immunity in SSc

A number of observations over several decades strongly implicate a major role for the adaptive immune system in SSc pathogenesis. These include the development of features of SSc in chronic graft-versus-host disease (cGVHD) in humans, which is largely mediated by donor T cells and reversal of fibrosis and vasculopathy after autologous hematopoietic $CD34^+$ stem cell treatment of patients with SSc (187, 188).

Immunohistochemical analysis of skin of patients with SSc shows perivascular and tissue accumulations of activated *$CD4^+$ T cells*, *monocytes*, and *$CD4^+CD8^+$ double positive T cells* that express high levels of IL-4 (189, 190). DNAX accessory molecule-1 (DNAM-1) modulates adhesion; co-stimulates

T lymphocytes; expresses on most $CD4^+$ and $CD8^+$ T cells, NK cells, monocytes, platelets, and some B cells; and is found to be expressed on inflammatory cells in biopsies of lesional skin of patients with SSc (191).

Autoantigens Recognized by SSc T Cells

Of particular significance is the finding in lesional SSc skin sites of *Vdelta1*$^+$*/gamma/delta T cells* that express HLA-DR and CD49d, suggesting that they have homed to these locations and expanded (192). Furthermore, analysis of T-cell repertoire in different skin locations from the same patient is compatible with clonal expansion of T cells to a widely distributed and persistent antigen (193). A variety of autoantigens that elicit T-cell responses in patients with SSc are widely distributed in tissues, have been described, and include types I, II, and V CIs (CI, CII, CV); laminin; low molecular weight (MW) *N*-sulfated heparin sulfate; 3500 MW RNA antigen; elastin; and DNA topoisomerase I (189, 194–198). Of potential relevance is the finding that the CI-specific $CD25^+CD4^+$ T cells isolated from SSc PBMC have a memory ($CD45R^+$) phenotype (195). Most patients with SSc have production of IFNγ by their PBMCs when cultured with CI or constituent α1 and α2 chains, which can be reduced by inducing immune tolerance via chronic administration in a dose-dependent manner by oral bovine CI (199, 200). In a double-blind, randomized clinical trial of daily oral bovine CI or placebo for 12 months, patients with dcSSc ≥3 years duration, patients receiving oral bovine CI had a significant improvement in MRSS compared to the placebo-treated patients (201). These studies suggest CI might be a widely distributed relevant antigen in SSc.

Microchimerism in SSc

Fetal–maternal and maternal–fetal microchimerisms have been proposed as mechanisms triggering autoimmunity in SSc and other autoimmune diseases (202–204). This microchimerism, in susceptible individuals, could initiate a type of cGVHD producing SSc with the microchimeric cells acting as effectors or as targets of an immune response (204). It is noteworthy that, in women with SSc who have given birth to male children, male offspring Th2-oriented T cells that express high levels of IL-4 are found in these women's skin and blood (205).

$CD4^+$ Regulatory T Cells and $CD4^+$ Th17 T Cells in SSc

The dysregulation in SSc of Th17 and/or Tregs (mostly $CD4^+CD25^+Foxp3^+$) has been reported by several groups. Different (and contradictory) results have been reported that seem to be dependent to some extent on how Tregs are defined by flow cytometry. Tregs have been found to be increased in the blood of SSc patients but have defective suppressive function (206). Papp et al. (207) reported decreased percentages and suppressive function of $CD4^+CD25^+$Tregs but increased percentage of Th17 cells in blood of SSc patients (207). Klein et al. (208) reported SSc patients had elevated $CD4^+D24^+Foxp3^+$Tregs in lesional skin but normal percentages in the peripheral blood (208). Slobodin et al. reported an increased number of Tregs in the blood of SSc patients but no concomitant increase in TGF-β or IL-10 production by $CD4^+$T cells (209). Fenoglio et al. found SSc patients had reduced frequency in blood and reduced suppressive function of $CD4^+CD25^+$Tregs and increased Th17 cell expansion after polyclonal or antigen-specific stimulation of SSc PBMC (210). Finally, Mathian et al. analyzed circulating activated (a)Tregs ($CD4^+CD45RA^-CD25^{bright}$ T cells) and resting (r)Tregs ($CD4^+CD45RA^+CD25^+$ T cells) in controls and SSc and found decreased frequency but normal suppressive function of both types of Tregs and in the lesional skin found no $CD4^+$Foxp3 mRNA in SSc compared to normal donor skin (211).

Abnormalities in Treg numbers or function could facilitate development of adaptive immune responses to autoantigens in SSc. Mast cells and S1P which are increased in SSc are two potential antagonists for proper development and function of Treg cells, as both have the capacity to inhibit Tregs (212–214). Furthermore, both S1P and mast cells enhance generation of Th17 cells (213, 215). The field of Tregs is still evolving and future studies with better markers for Treg subsets will need to be performed to better characterize this role in SSc.

Possible Influence of Vitamin D Deficiency and Lysophospholipids on Immune Dysregulation in SSc

Vitamin D insufficiency/deficiency has been implicated in triggering and enhancing a number of autoimmune diseases. Low serum 25(OH)D concentrations have been reported to be more common in patients with SSc than in healthy controls. Furthermore, 25(OH)D levels have been reported to negatively correlate with several laboratory and clinical parameters in European Disease Activity Score, Raynaud's phenomenon (RP), erythrocyte sedimentation rate, systolic pulmonary artery pressure, MRSS, and positively correlate with carbon monoxide diffusion lung capacity (216–218). A number of effects of $1,25(OH)_2D3$ on immune cells have been reported that could explain its ability to decrease autoimmunity and, conversely, how VitD deficiency contributes to increased autoimmunity [these are summarized in Ref. (219)]. For example, effects of $1,25(OH)_2D3$ on APC include: (1) downregulation of MHC class-II molecule expression in APC; (2) downregulation of surface expression of co-stimulatory receptors (CD40, CD80, and CD86) and other maturation-induced proteins (CD1a, CD83); (3) inhibition of dendritic cell maturation, induction of tolerogenic DC that are able to induce Treg cells; (4) inhibition of IL-12 p70 release from DC; and (5) inhibition of pro-inflammatory cytokines in monocytes and macrophages (219). Effects of $1,25(OH)_2D3$ on T cells include: (1) inhibition of antigen-specific and lectin-stimulated T-cell activation and progression from G1a to G1b proliferation; (2) inhibition of IL-12, IFNγ, IL-2 release; (3) stimulation of IL-4, IL-5, and IL-10 production; and (4) inhibition of Fas ligand (FasL) expression by activated T cells (219). The effect of $1,25(OH)_2D3$ on B cells is to inhibit production of IgA, IgE, IgG, and IgM and in NK cells to inhibit IFNγ production (219, 220).

Administration of VitD3 in escalating daily doses of 2000 U (2000 U for the first month, then 4000 U for the second month, and 8000 U for the third month) to healthy VitD-deficient individuals induced increased frequencies of $CD38^+$ B cells and reduced frequencies of $CD4^+IFN\gamma^+$ and $CD4^+IL\text{-}17^+$ T-helper cells (221). Treatment of SLE patients with hypovitaminosis D with 100,000 U of VitD3 weekly for 4 weeks and then monthly

for 6 months resulted in an increase in naïve $CD4^+$ T cells and $CD3^+CD4^+CD25^{hi}CD127^-Foxp3^+$Tregs and decreases in $CD19^+$ B cells, anti-ds DNA antibody titers, and proteinuria (222). Similar studies with high-dose VitD supplementation have not been reported in patients with SSc, but the above studies in SLE and normal hypovitaminosis individuals demonstrate the potential for immune modulation by high-dose VitD supplementation that might decrease autoimmunity in patients with SSc.

Lysophosphatidic acid and *S1P* levels are increased in sera of patients with SSc, suggesting they may play a role in different aspects of the disease (214) [reviewed in Ref. (223)]. Platelets, macrophages, dendritic cells, mast cells, and endothelial cells are sources of LPA and S1P, and these cells (plus T cells and B cells), NK cells, fibroblasts, and other cells express various types of LPA and S1P G-protein-coupled receptors (GPCRs) [reviewed in Ref. (223)]. *PPARγ*, which resides intracellularly and counters TGF-β fibrogenesis, is also an additional receptor for LPA (224). In addition to S1P being able to "disarm" Foxp3 Tregs mentioned above, S1P and LPA regulate the function, migration, and trafficking of all lymphoid cells and monocyte/macrophage/dendritic cells with S1P also being able to sequester T cells in the thymus and peripheral lymphoid organs, resulting in some instances in lymphopenia, which is frequently found in patients with SSc (225–227). By acting on APC, S1P and LPA each can suppress development of Th1 T-helper cells, but they have different effects on Th2 T-helper cells in that S1P suppresses their development while LPA fosters their development (228). Th2 T-helper cell predominance is a feature of some patients with SSc with production of IL-4 and IL-13, which facilitate development and expansion of B cells and autoantibodies that are common features of SSc. Lysophospholipids need further study in SSc, given the potential to regulate immunity.

Vascular Abnormalities in SSc

Vascular dysfunctions and abnormalities leading to RP, digital ulcers, and nail-fold capillary abnormalities usually are among the earliest and key manifestations of SSc. The various vascular abnormalities are summarized in **Table 5**. Postmortem examination reveals the vascular changes in SSc are more typical of a vasculopathy than of a vasculitic process – given the paucity of inflammation in the vessel wall with widespread systemic intimal proliferation in the pulmonary, coronary, and the renal arteries (229). Patients with SSc who develop PAH and renal crisis exhibit vascular lesions characterized by classic concentric intimal proliferation, marked luminal obstruction, lymphocyte infiltration, and relative paucity of plexiform lesions (230–233).

Earliest signs of vascular dysfunction include impaired vascular tone and vascular permeability (234). Impaired balance of vasoconstrictor substances (e.g., ET) and vasodilator substances (e.g., NO), plays important roles in vascular dysfunction. Platelet activation and enhanced coagulation with reduced fibrinolysis also contribute to the vasculopathy in SSc. Abnormalities in the vascular system can be seen in clinically normal skin of SSc patients (235). Large gaps between endothelial cells, vacuolization of endothelial cell cytoplasm, and loss of membrane-bound storage vesicles are some of the earliest detectable changes in the endothelial cells (235–237). In a 20-year follow-up study, sequential changes can be seen in capillaries (4) in skin, which include capillary enlargement, capillary loss, and telangiectasia. Further morphologic changes in vessel wall occur including fibrosis. Such capillary changes are wide spread in internal organs (e.g., lungs, heart, kidneys, and muscles) (238). Intimal proliferation and accumulation of proteoglycans in the arterioles and small arteries are also common (239, 240). The operative mechanisms that lead to this widespread vasculopathy in SSc of unknown, but animal models and *in vitro* studies have provided some clues.

TABLE 5 | Key vascular abnormalities of SSc.

Presence of proliferative vasculopathy with intimal proliferation in peripheral, pulmonary, coronary, and renal arteries in the absence of inflammation is a hallmark feature of scleroderma
Endothelial cell damage is a key and early process. It precedes fibrosis and particularly involves the arterioles
Early detectable changes in the endothelial cells include disappearance of membrane-bound vesicles, vacuolization of endothelial cell cytoplasm, and gaps between endothelial cells
Defective angiogenesis is an early event in the form of drop out of capillaries and abnormal capillary architecture without a compensatory process
There are conflicting reports regarding the presence and role of circulating endothelial progenitor cells in SSc
There is dysregulation of coagulation and fibrinolysis process
Platelets show enhanced aggregability to various triggers such as type I collagen and adenosine etc…, and are activated throughout the clinical course of SSc
LPA and S1P could potentially contribute to the vasculopathy via endothelial cell activation, neointimal formation, vascular leakiness, increased vasoconstriction, cardiac fibrosis, and hypertension

Mechanism of Vascular and Endothelial Cell Injury in SSc

The etiology of the initial vascular damage in SSc is not known and is a topic of speculation. Infectious agents, cytotoxic T cells, NO-related free radicals, and autoantibodies against endothelial cells have all been implicated (234). Endothelial cell dysfunction, neural abnormalities, and various other intravascular defects likely contribute to the impaired vascular flow (241).

Endothelial Cell Injury

Evidence suggests that endothelial cell injury is an early and central event in the pathogenesis of SSc vasculopathy, and viral agents [especially human cytomegalovirus (hCMV)], cytotoxic T cells, antibody-dependent cellular cytotoxicity (ADCC), anti-endothelial cell antibodies, and ischemia-reperfusion injury are all suggested mechanisms for endothelial cell damage (234, 242). Levels of antibodies to hCMV are increased in patients with SSc which is reminiscent of the association of hCMV antibodies with vascular intimal proliferation and vasculopathy in patients with graft rejection and coronary artery bypass restenosis (243). In addition, there is evidence of binding of some ATAs to an epitope in hCMV-derived UL94 protein which happens to also show homology to MVEC surface protein tetraspan novel antigen-2 (NAG-2) (243). Apoptosis of MVEC can be effected by purified anti-UL94 peptide antibodies (244). Cytotoxic $CD4^+$ T cells induce MVEC apoptosis via *in vitro* Fas-related pathway in contrast to $CD8^+$

T cells, NK, and LAK cells which utilize the granzyme/perforin system (243). ADCC to MVEC is operative in many patients with SSc (243). Anti-endothelial cell antibodies are commonly found in sera from patients with SSc and are capable of inducing MVEC apoptosis directly *in vitro* (245). Ischemia and reperfusion injury (especially associated with attacks of RP) is accompanied by upregulation of expression of junctional adhesion molecules (JAMs). This upregulation indicates endothelial dysfunction and allows attachment of platelets and neutrophils to the endothelium that is thought to lead to MVEC injury through production of superoxide radicals (which limit release of vasodilation substances such as NO and prostacyclin) (243, 246, 247). The major evidence for the presence of the endothelial injury in SSc is high serum levels of circulating von Willebrand (VW) factor, ET-1, increased levels of circulating viable and dead endothelial cells, and soluble JAM-A and JAM-C (234, 247–251). Subendothelial tissue forms a nidus for platelets to aggregate and initiates fibrin deposition and intravascular thrombus formation (1). The role of endothelial apoptosis is not clear. Sgonc et al. (252) demonstrated endothelial cell apoptosis in the University of California at Davis chicken lines 200/206, which spontaneously develop an SSc-like disease (252). Apoptotic endothelial cells may contribute to tissue injury when engulfed by immature dendritic cells and macrophages, which subsequently present cellular antigens to $CD8^+$ T cells, causing further tissue injury (253). These apoptotic endothelial cells can also activate the alternate complement pathway and coagulant pathway leading to vasculopathy (254, 255). Proof that there is ongoing endothelial apoptosis in SSc is thus far lacking, and Fleming and Wanless (256) failed to detect apoptotic endothelial cells in their study, although they did demonstrate loss of VE-cadherin, which regulates endothelial barrier function and found evidence of IFNα signaling (256). IFNα signaling suggests endoplasmic reticulum stress and the unfolded protein response in these cells (257, 258).

Defective Angiogenesis

The remarkable loss of capillaries and small vessels in patients with SSc suggests a defect in the process of angiogenesis. Tissue ischemia usually leads to the expression of angiogenic growth factors [e.g., vascular endothelial growth factor (VEGF)], which causes vasodilatation, proliferation, and migration of endothelial cells and stabilization of the lumina to form new vessels (259). Plasma levels of VEGF are elevated in SSc, and this could stimulate angiogenesis (260). Levels of other proangiogenic factors [e.g., PDGF, placental growth factor (PGF), and fibroblast growth factor 2 (FGF-2)] are also considerably elevated in the plasma of SSc patients (261). Expression of VEGF and its receptors, *VEGFR1* and *VEGFR2*, are increased in skin of SSc patients (260, 262, 263). In addition to elevated level of VEGF, other proangiogenic mediators (such as ET-1, adhesion molecules, and chemokines) are found in the circulation of SSc patients (264). Elevated levels of antiangiogenic factors such as *angiostatin*, *platelet factor-4* (also called CXCL4), thrombospondin-1 (*TSP-1*), and *IL-4* have been described in patients with SSc (264, 265).

Defective Vasculogenesis

The role of vasculogenesis in SSc is not clear, and there are conflicting reports regarding the presence and role of circulating endothelial progenitor cells in SSc (266). Increased levels of circulating endothelial progenitor cells have been demonstrated which supports their mobilization from bone marrow (267). However, in another study, there were substantially reduced numbers of bone marrow-derived circulating endothelial precursors compared to healthy subjects or patients with RA. The lowest number of these cells was observed in SSc patients with active fingertip ulcers, and this may suggest inadequate recruitment of these precursor cells and impaired vascular repair mechanisms (268). Atorvastatin can be effective in RP – perhaps by increasing the number of circulating endothelial progenitor cells, which suggests a role of endothelial progenitor cells in vascular dysfunction (269). Apoptosis of endothelial progenitor cells by a circulating factor has been implicated as the potential mechanism for the reduced number of circulating precursor cells in SSc (270). Mesenchymal stem cells might be another source of endothelial progenitor cells. In SSc, the angiogenic potential of these cells is reduced (271). This suggests that endothelial repair may be affected by unknown SSc disease effects on the bone marrow.

Pericytes mediate vascular maturation and stabilization during angiogenesis (272). They can further differentiate into vascular smooth muscle cells, fibroblasts, and myofibroblasts (273–275). Pericytes express *PDGFR*-β, and high molecular weight melanoma-associated antigen (HMW-MAA) in vascular lesions in SSc patients with associated RP and ANA (276). Another marker of angiogenic pericytes is regulator of G protein signaling (RGS-5), which is highly expressed in SSc vasculature (277). The exact role of RGS-5 is not clear, but it can negatively regulate vessel maturation (278). Pericytes proliferate and contribute to increased vascular wall thickness, which is characteristic of SSc vasculopathy (279).

Endothelial to Mesenchymal Cell Transition in the Pathogenesis of SSc Vasculopathy

There is subendothelial accumulation of activated fibroblasts or myofibroblasts and production of excessive CI and ECM components in blood vessels of SSc patients (1). During this process, endothelial cells lose their specific markers such as *VE-cadherin* and *VW factor* and acquire a mesenchymal phenotype expressing α smooth muscle actin (αSMA), Vimentin, and CI. It is postulated that endothelial cells might transform into mesenchymal cells induced by local growth factors and cytokines (1). The exact molecular mechanism and the cytokines involved are not known, but TGF-β has been implicated. There are recent reports of TGF-β being involved in various disease processes such as endothelial to mesenchymal transformation (280–284). Li and Jimenez (285) further examined the role of TGF-β in the transformation process and the signaling pathways involved (285) in a murine pulmonary endothelial cell model. They concluded that TGF-β could lead to mesenchymal transformation of the endothelial cells. They further demonstrated that the transformation is associated with strong upregulation of transcriptional repressor snail-1 and is mediated by the c-abl kinase and protein kinase C-δ. Snail-1 is a zinc-finger transcription factor that forms a complex with Smad3/Smad4 (1). Snail-1 induces numerous transcriptional events that could lead to expression of a mesenchymal phenotype. Besides this, Wnt signaling as well as NOTCH signaling pathways might be involved in this endothelial–mesenchymal transformation process (1).

Other potential mediators of this transformative process include PDGF (286), VEGF (287), insulin-derived growth factor (288), CTGF (289), ET-1 (290), and miRNAs (291, 292). Endothelial to mesenchymal cell transition is an interesting concept but needs further study to determine what role, if any, it plays in SSc vasculopathy.

Circulating Mediators of Vasculopathy

Higher levels of *ET-1* have been observed in patients with scleroderma renal crisis, lung fibrosis, PAH, and RP (293). Increased ET-1 expression is associated with increased ET-1B receptor in the skin and lung tissue of SSc patients (294).

In SSc, there is a reduction in *eNOS* gene expression and *NO release* in SSc and MVEC derived from lesional and non-lesional skin biopsies in the steady-state and after shear stress (295). This is probably associated with deficient endothelium-dependent relaxation in SSc (296). Impaired NO results in alteration of vascular tone, enhancement of platelet aggregation, and increased susceptibility of endothelial cells to oxidative injury. NO also limits cytokine-induced endothelial cell activation and monocyte adhesion and inhibits the endothelial cell release of IL-6 and IL-8, which are important inflammatory cytokines (297). Further, NO inhibits vascular smooth muscle cell proliferation through elevation of cyclic GMP and inhibition of mitogenic proteins, TGF-β and PDGF. Therefore, impaired NO production in SSc may contribute to the pathogenesis of arteriolar intimal proliferation and may have a prominent role in pathophysiology of the disease.

Coagulopathy in Systemic Sclerosis

Coagulation and fibrinolysis processes are dysregulated as evidenced by presence of microvascular thrombosis and enhanced fibrin deposition frequently seen in the vasculature of SSc patients. The loss of balance between fibrinolysis and coagulation contributes to vessel engulfment with fibrin and breakdown of vessel patency (298). The authors demonstrated impairment of fibrinolysis and activation of the coagulation pathway in a study of 29 patients (298). Activation of the coagulation system, as well as elevated levels of fibrinogen and VW factor, has been demonstrated in patients with SSc (299–302). Reduction of fibrinolysis, expressed as defective tissue t plasminogen activator (tPA) antigen release and/or elevated tPA inhibitor (PAI) antigen, supports existence of heterogeneous hypofibrinolytic pattern in SSc (303).

Plasmin has both pro-fibrotic and anti-fibrotic properties [pro-fibrotic by activating TGF-β and anti-fibrotic by activating both hepatocyte growth factor (HGF) and MMPs] (304, 305). Plasmin is inactivated via formation of a complex with α2-antiplasmin (α2AP), and elevated levels of plasmin-α2AP are associated with several fibrotic conditions including SSc (306). α2AP promotes fibrosis by activating phospholipase A_2 by binding to adipose triglyceride lipase (ATGL) to generate $PGF_2\alpha$, which in turn stimulates production of TGF-β (307). Levels of α2AP are elevated in lesional BLM skin in mice, which is induced by CTGF via extracellular signal-regulated kinase 1/2 (ERK 1/2) and JNK pathways (308). α2AP induces αSMA^+ myofibroblasts *in vitro* and mice with deletion of α zinc-finger alpha protein gene (αZAP) exhibit less infiltration of myofibroblasts at the site of BLM injections in the skin (308). Plasmin increases ECM degradation, and inhibition of plasmin of α2AP decreases ECM degradation, which could be another mechanism by which α2AP could promote fibrosis.

Platelet Abnormalities in SSc

Chronic activation of platelets and their released products could contribute to the vascular, immunologic, and connective tissue pathology of SSc (309). SSc platelets show *enhanced aggregation* to various triggers [e.g., CI, adenosine diphosphates, 5-hydroxytryptamine (309–311), ET-1, S1P, and LPA (223)]. ET-1 and S1P cause vasoconstriction by engaging $S1P_2$ and $S1P_3$ receptors (312). In the human fetal lung fibroblast line (FH-1), S1P utilizes $S1P_1$ receptors to inhibit TGF-β1-induced αSMA expression while utilizing $S1P_3$ receptors to stimulate αSMA expression (313). Sera from patients with SSc have elevated levels of arachidonoyl-LPA and S1P (214). LPA induces platelet aggregation, vascular smooth muscle proliferation, and neointima formation, which can induce vasospasm and RP (314–317).

The various platelet-derived factors include: *inflammatory mediators* [NO, serotonin, thromboxane A_2, prostaglandin (PG) D_2, PGE_2, PGF_2, 12-hydroxyeicosatetraeonic acid, β thromboglobulin, neutrophil-activating peptide-2, platelet factor-4, platelet activating factor, adenosine, histamine, P-selectin, CD40 ligand (CD40L), dinucleoside polyphosphates, 2-arachidonyl glyceride, MMP-27], *chemokines* [macrophage inflammatory protein (MIP-1α); monocyte chemoattractant protein-3 (MCP-3); IL-8; and regulated upon activation, normal T-cell expressed and secreted (RANTES)], *cytokines* [IL-1β and granulocyte monocyte-colony stimulating factor (GMCSF)], and *growth factors* [(PDGF) A, B, C, D, TGF-β1 and 2, epidermal growth factor, VEGF-A and C, brain-derived neurotrophic factor, insulin-like growth factor-1 (IGF-1), basic fibroblasts growth factor (bFGF), HGF, and CTGF] (309). Platelets from scleroderma patients overexpress a specific non-integrin *65-kDa receptor for CI*, phosphatidylinositol (PI)-3 secondary to increased nitrotyrosylation and increased protein kinase B (Akt) activity (309, 318). Overexpression of these mediators is induced by cytokines produced by T cells and monocytes activated by autoantigen such as CI that (in turn) changes the phenotype of megakaryocytes (318). The platelets store numerous *fibrogenic mediators* and contribute to chronic tissue fibrosis in SSc by release into tissue of TGF-β1, TGF-β2, PDGF-A, B, C, D, LPA, S1P, adenosine, bFGF, CTGF, and IGF-1. These aforementioned mediators have many biological properties and effects on a host of cells that could also facilitate and contribute to autoimmunity and fibrosis (5).

Animal Models Resembling SSc Vasculopathy

Animal studies in mice recapitulate some of the vasculopathy of SSc. Mice with a conditional deletion of *Fli1* develop systemic vascular lesions characterized by capillary dilation, vascular fragility, stenosis of arterioles, increased vascular permeability, micro-aneurysms, decreased expression of platelet/endothelial cell adhesion molecule (PECAM)-1, PDGF-β, and S1P type I receptor ($S1P_1$) and increased endothelial cell MMP-9 expression (319).

Caveolin-1 (cav-1) is one of three membrane proteins that coat caveolae which are plasma membrane invaginations important in clustering together of receptors that can influence signal

transmission of the specific receptor ligand (320). *Cav-1* is involved in internalization and degradation of TGF-β receptors, thereby reducing signaling by TGF-β (321, 322). There is decreased expression of *cav-1* in lesional skin and lungs of patients with SSc and in lungs of patients with idiopathic pulmonary fibrosis (IPF) (323, 324). *Cav-1* null mice develop PAH and right and left ventricular enlargement and failure (325). However, in contrast to *cav-1* null mice with PAH, in human IPAH, there is an apparent increase in cav-1 expression in the PASMC compared to healthy controls and that the over expression of *cav-1* increases capacitive Ca^{++} entry and DNA synthesis in PASMC (326). The cav-1 null mice also develop pulmonary fibrosis, raising questions regarding the etiology of the PAH in this model which is yet to be clearly defined. In a French and Italian SSc population, *Cav-1* rs959173C showed protective association with SSc and lcSSc (327). The rs959173C protective allele is associated with increased CAV-1 protein expression (327).

Fos-related antigen-2 (Fra-2) transgenic (TG) mice develop microvascular and proliferative vasculopathy and express *Fra-2* in vascular structures (endothelial cells and vascular smooth muscle cells) similar to its expression in skin of SSc patients (328). An early event in the *Fra-2* TG model is apoptosis of endothelial cells (328). The *Fra-2* TG mice also developed pulmonary vascular lesions resembling SSc-associated PAH and later developed dermal and pulmonary fibrosis resembling the "non-specific interstitial pneumonia" (NSIP) (328). These results suggest *Fra-2* might be involved in pathogenesis of SSc vasculopathy and to-date this is the only mouse model that manifests both vasculopathy and fibrosis with features shared by the human SSc disease.

Pulmonary Arterial Hypertension

Cellular Stress in SSc-PAH

Patients with lcSSc, who also have PAH, have the highest expression of the endoplasmic reticulum stress/unfolded protein response genes, *Activating Transcription Factor-4al-b*, a spliced form of X-box BP, and immunoglobulin-heavy-chain BP (257). In PBMC of the lcSSc patients, HSP gene (*DNAJB1*), and IFN-regulated genes (*IFIT1*, *IFIT2*, and *IFITM1*) were upregulated, but *IRF4* was downregulated compared to healthy controls (257). Further analysis showed that the severity of PAH (as reflected in pulmonary artery pressure) positively correlated with level of *DNAJB1* expression, while endoplasmic reticulum stress marker correlated with IL-6 levels in the whole lcSSc population (257).

Interferon Signature in SSc-PAH

Type I IFNs are implicated by the association of use of IFNα in the treatment of hepatitis and of IFNβ in the treatment of MS with development of PAH (118, 119). Diseases in which there is an "IFN signature" (such as SLE, SSc, and infection with HIV) are associated with development of PAH (120–124). Furthermore, IFNα and IFNγ (added to cultures of human PASMC primed with TNFα or to cultures of human lung MVEC or human lung fibroblasts) cause release of the potent vasoconstrictor, *ET-1*, and of *IP-10* (117). In a series of 128 SSc patients with PAH and 35 patients with no PAH, the SSc patients with PAH had higher levels of IP-10 and ET-1 in their sera compared to SSc patients without PAH or compared to healthy controls; more SSc patients with PAH had detectable levels of IFNα and IFNγ in their sera than SSc patients without PAH (117). In this series of SSc patients, levels of TNFα, IL-12p70, IL-6, IL-1α, and IL-8 were significantly higher in sera in SSc patients with PAH when compared to SSc patients without PAH (117). Additional studies of this patient group revealed that serum levels of IP-10 in the SSc-PAH patients correlated with pulmonary vascular resistance, and levels of brain natriuretic peptide in serum, and serum IP-10 levels in the SSc-PAH patients inversely correlated with cardiac index and 6-min walks test (117). Sections of lung from patients with IPAH or with SSc-PAH expressed higher levels of IFNR1 in endothelium, smooth muscle layer, vascular interstitium, and in intravascular inflammatory cells as assessed by immunohistochemistry and Western blotting (117). While the above studies strongly implicated type I IFN as playing a pathogenic role in SSc-PAH and IPAH, further evidence was substantiated in the *type I IFN α receptor 1* knockout mouse which was found to be resistant to experimental hypoxic PAH induction. These mice did not have elevated serum levels of ET-1 when compared to WT control mice (117). Analysis of PBMC from patients with SSc revealed *Siglec-1* and other IFN-regulated genes were overexpressed in patients with dcSSc, whereas patients with lcSSc with PAH overexpressed *IL-13RA1*, *ICAM-1*, *CCR1*, *JAK2*, and *MCR1* (123, 125, 126). IL-13 was also elevated to higher levels in sera of patients with lcSSc with PAH, and *MCR1* was induced on $CD14^+$ monocytes suggesting monocytes are activated in lcSSc patients with PAH of an alternative (i.e., IL-4/IL-13) rather than classical (i.e., IFNγ/LPS) pathway (123).

Other Mediators and Gene Polymorphisms in SSc-PAH

Polymorphisms were described in the promoter of the *NOS2* gene that confers susceptibility to PAH in SSc (144).

In another report, patients with lcSSc with PAH, had higher levels of circulating monocyte-related cytokine mediators (TNFα, IL-1β, IL-6, and ICAM-1) and vascular injury markers (VEGF, VCAM-1, and VW Factor), and their PBMCs exhibited increased expression of mRNA for ICAM-1, IL-1β, JAK2, IFNGR1, IL-13Rα1, tissue inhibitor of metalloproteinase (TIMP)-2, delta-aminolevulinate synthase 2 protein (ALAS2), CCR1, and AIF1akt (126).

Urokinase-type plasminogen activator receptor, *CD87*: (discussed under "Genetics of SSc") SNP, *UPAR* rs344781G allele, is associated with SSc-related digital ulcers, pulmonary artery hypertension, ACA positivity, and lcSSc (91).

Sphingosine 1-phosphate and *LPA* may have effects on the vasculature in SSc that contribute to some of the abnormalities observed in the disease. For example, there is overexpression of VE-cadherin, IFNα signaling, and *Rgs-5*, which is associated with an antiangiogenic phenotype (188). Overexpression of *Rgs-5* may reduce signaling via $S1P_1$ receptor and increase S1P signaling through other S1P receptors that could reduce endothelial eNOS, increase vasoconstriction, increase vascular leakiness, and reduce angiogenesis [reviewed in Ref. (223)]. Furthermore, S1P may contribute to PAH by constricting pulmonary arteries while LPA may contribute to systemic hypertension, cardiac fibrosis, endothelial cell activation, and neointima formation (via PPARγ) [reviewed in Ref. (223)].

Lysophosphatidic acid, S1P, and other chemoattractants (such as TGF-β1, TGF-β2, IL-8, MCP-3, and other mediators released from aggregated/activated platelets adhering to damaged microvascular endothelium and diffusing into perivascular tissue) could establish chemotactic gradients that would promote outward transversal migration of monocytes, dendritic cells T and B lymphocytes, and NK cells resulting in perivascular accumulation of these cells to set the stage permitting innate and adaptive immune responses that lead to autoimmunity and fibrosis (223).

Fibrosis in SSc

Links to the Innate and Adaptive Immune Systems

Over three decades ago, it was recognized that human lymphocytes and monocytes (when stimulated by antigen or T-cell mitogen *in vitro*) elaborate soluble mediators (lymphokines, monokines, growth factors, chemokines, and cytokines) that induced fibroblast chemotaxis or (when added to cultures of human fibroblasts) induce fibroblast growth and synthesis of collagenase (MMP-1) and CI (329–340). These studies provided tangible evidence that immune cells are fully capable of modulating chemotaxis and growth of fibroblasts, as well as regulating synthesis of CI and CIII and the major CI degradative enzyme, MMP-1, by fibroblasts.

Later studies conducted with purified recombinant or natural cytokines, chemokines, and growth factors known to be synthesized by cells of the innate and adaptive immune system have allowed fibroblasts specific functions to be assigned to certain ones. *TGF-β1*, which is produced by most cell types but also by $CD4^+CD25^+$Foxp3 Tregs, monocytes/macrophages, mast cells, and platelets and *IL-4*, which is produced by Th2 cells, and mast cells received early attention as being potent stimulators of CI synthesis and chemotaxis by fibroblasts (341–345).

Cells of the innate and adaptive immune system elaborate a variety of cytokines and chemokines in addition to TGF-β and IL-4 (such as *IL-6*, *PDGF*, *IL-1*, *IL-13*, *IL-17*, *IL-5*, *MCP-1*, and *CTGF*) that have been found to be increased in serum or in tissues in which excess connective tissue matrix is accumulating in SSc. These cytokines/chemokines are at the interface between the immune system and fibroblasts.

Signal transducer and activator of transcription protein 4 is critical for T-cell signaling and differentiation (132–134). *STAT4* is involved in effecting a Th1 cytokine response by transmitting signals from IL-2, IL-12, and IL-23 receptors and in signaling after type 1 IFN engages its receptor (135, 136). The role of *STAT4* in fibrosis was assessed in scleroderma mouse models. The deletion of *STAT4* significantly reduced skin fibrosis in the BLM model but not in the Tsk-1/+ model (137). In the BLM model, it was noted that there were decreased numbers of inflammatory cells including T cells and proliferating T cells and decreased quantity of IL-6, IL-2, TNFα, and IFNγ in lesional skin of $STAT4^{-/-}$ vs. $STAT4^{+/+}$ mice (137). In addition to having a role in SLE and RA susceptibility, *STAT4* has been identified as a susceptibility gene in SSc (50) (see **Table 3**).

Macrophage migration inhibitory factor-173 acts upstream and activates innate immunity. It plays a role in sustaining cellular and inflammatory response. It causes fibroblasts proliferation and acts as an antiapoptotic (135).

Links to Vascular Damage

Endothelin-1 is one of three isoforms and is synthesized by VE cells, fibroblasts, bone marrow mast cells, neutrophils, macrophages, and cardiac myocytes (140) (See discussion under "Genetics of SSc"). ET-1 is overexpressed in skin biopsies of patients with dcSSc (179).

Fos-related antigen-2, reviewed above, appears to have both vasculopathic and fibrogenic properties and may be a contributor to these processes in patients with SSc.

SSc Fibroblast Phenotype and Myofibroblasts

Earlier studies indicated that normal human dermal fibroblasts (grown for prolonged periods of time *in vitro* in the presence of culture medium supplemented with culture supernatants obtained by activating normal human donor peripheral blood lymphocytes and monocytes with T-cell mitogen *in vitro*) acquired a "scleroderma-like phenotype" that resembled cultured lesional SSc skin fibroblasts at the ultrastructure level with respect to excessive production of glycosaminoglycans (346). A phenotypic characteristic of cultured SSc lesional skin fibroblasts is that they produce reduced levels of *MMP-1*, an enzyme necessary for degradation of triple helical CI and CIII (347). Some SSc lesional fibroblasts regain production of MMP-1 after several subpassages, and when these fibroblasts lines are then cultured for 3 weeks with *IL-13* or *PDGF-BB*, then cultured in plain medium before TNFα stimulation, the production of MMP-1 in response to TNF-α stimulation is markedly reduced compared to normal donor fibroblasts similarly treated with IL-13 or PDGF-BB (348). These studies suggest that *in vivo* chronic exposure of SSc fibroblasts to certain cytokines, derived from activated lymphocytes and monocytes either in circulation or from lymphocytes/monocytes infiltrating SSc lesional skin, can induce an SSc fibroblast phenotype that persists in the absence of the cytokines for some period of time. Platelets that are being chronically activated/aggregated in patients with SSc may also contribute some cytokines/growth factors (e.g., PDGF-BB) that could contribute to induction of the scleroderma fibroblast phenotype (309).

Fibroblasts cultured from lesional skin biopsies of patients with SSc contain increased numbers of myofibroblasts and synthesize increased amounts of CI and *TIMP-1*, in contrast to fibroblasts grown from non-lesional SSc skin or skin of healthy controls (349). This increased CI production phenotype reverts toward normal as the SSc lesional fibroblasts in culture are passaged, as shown by LeRoy (350). The myofibroblasts in SSc lesional skin contain αSMA and fibronectin ED-A splice variant, the latter being a requirement for TGF-β1 to induce myofibroblast formation (275, 351). In normal wound healing, myofibroblasts contract the newly formed ECM, and their development and function are modulated by mechanical forces and stiffness of the ECM microenvironment (352). The origin of myofibroblasts in SSc lesional skin is not completely understood, but likely candidates include resident connective tissue fibroblasts, epithelial cells, pericytes, and circulating fibrocytes. Myofibroblasts are induced by a number of cytokines, growth factors, and other agents present in SSc tissue or serum, including: *TGF-β1*, *TGF-β3*, *IL-4*, *TNFα*, *IL-6*, *GMCSF*, *thrombin*,

bradykinin, *histamine*, *tryptase*, *oncostatin M*, *IL-13*, *PDGF*-β, *ET-1*, *TLR 2/1 ligands*, and the *lysophospholipids*, *S1P* and *LPA* (5, 353–355).

Levels of *IL-1*α are elevated in sera of patients with SSc, and SSc monocytes produces more. IL-1 than normal monocytes when stimulated *in vitro* (356, 357). IL-1α and -β stimulate proliferation of human dermal fibroblasts and upregulate production of CI, TIMP, PGE_2, MMP-1, and hyaluronan (358, 359). IL-1α and -β were observed to promote viability of cultured SSc lesional skin fibroblasts and myofibroblasts *in vitro* in the presence or absence of serum and directly induced expression of αSMA and N-cadherin (360). This suggests that IL-1 may contribute to the longevity of myofibroblasts in SSc skin.

Fibroblasts grown from SSc lesional skin biopsies constitutively overexpress IC IL-1α; and after stimulation *in vitro* with TNFα or IL-1β, both *icIL-1*α and *icIL-1* receptor protein antagonist (*icIL-1ra*) are markedly upregulated compared to normal donor fibroblasts (361). Overexpression of icIL-1α in normal skin fibroblasts also induces expression of icIL-1ra (361). When icIL-1ra is overexpressed in cultures in normal human skin fibroblasts via transfection with a viral vector (pLXSNicIL-1ra type 1), it induces a myofibroblast phenotype characterized by increased expression of α*SMA* and *PAI-1* (362).

Treatment of SSc lesional fibroblasts with IL-1α siRNA resulted in decreased proliferation and production of IL-6 and CI, whereas stably transfecting with icIL-1α induced proliferation and IL-6 and CI synthesis (363).

TGF-β Receptor-Smad Signaling in Fibroblasts

A great deal of effort has elucidated the complex receptor engagement and signaling of TGF-β and its 1, 2, and 3 isotypes that occur in mammals and which have been the topic of several recent reviews (364–367). TGF-β1, 2, and 3 are synthesized as *inactive propeptides* which have to be cleaved intracellularly by the protease, *farin*, to generate active 25 kDa MW, active TGF-β1, 2, or 3. The active TGF-β is bound by the cleaved amino terminal peptide called "latency-associated peptide" (LAP) and, in connective tissue, the latent TGF-β1-LAP complex is bound to latent TGF-β1-binding protein (LTBP), which is termed "large latent complex" (LLC) (365, 368). Latent TGF-β can be activated by interaction with *integrins* and by *several proteases* such as thrombin, plasma transglutaminase, cathepsin D, and plasmin (369). There are three classes of TGF-β receptors. *TGF-*β *receptor 1* has two forms: *ALK1* (found mainly in endothelial cells) and *ALK5* (which is present in most cells) (367). *TGF-*β *receptor 2* forms a heteromeric complex with type 1 receptors and phosphorylates it, setting in motion IC signaling via receptor-regulated Smads (R-Smads) which are type 1 receptor specific [i.e., ALK1 causes Smad1/5/8 phosphorylation while the predominant ALK5 causes Smad2/3 phosphorylation (367)]. The phosphorylated R-Smads complex with Smad4 and in the nucleus interact with co-activators [e.g., CREB-binding protein (CBP)/p300] and co-expressors (e.g., Ski/Sno) to transcriptionally activate or repress target genes (367). Inhibitory Smads (Smad 6 and 7) can bind to TGF-β type 1 receptors and to Smad4 or effect ubiquitination and proteasomal degradation (367). A coreceptor called *endoglin*, of which there are two spliced variants called short and long forms, can (under different conditions by interacting with ALK1 or ALK5) decrease or enhance TGF-β signaling, respectively (367). *Betaglyan* ("type 3" TGF-β receptor) can also act as a coreceptor by facilitating TGF-β binding/interaction with type 1 and 2 TG-β receptors (370). *CTGF* can also interact with TGF-β type 1 and 2 receptors and facilitate Smad3 signaling, which has a pro-fibrotic effect (367). Other members of the TGF-β superfamily including *Activin* (*A*, *B*, and *AB*), bone morphogenic proteins (BMPs), and growth differentiation factors utilize components of the TGF-β receptor complex (366). In addition to the canonical Smad-dependent pathway described above, TGF-β can signal through non-canonical Smad-independent Wnt, MAPK, phosphatidylinositol-3-kinase/AKT, and Rho-like GTPase pathways (366). Activating transcription factor 3 (ATF3), which regulates oxidation and cellular stress, is upregulated in SSc dermal fibroblasts by TGF-β; and ATF3 suppresses TGFG-β-induced proliferative effects via interaction with Smad3 in a c-Jun-dependent manner (371).

Recently, it was reported that the fibrogenic effect of IL-6 in fibroblasts is brought about by binding of IL-6 to soluble IL-6 receptor (IL-6R) by a JAK1 and STAT3-dependent mechanism that is mediated through *Gremlin-1*, which utilizes TGF-β type 1 and 2 receptors and the TGF-β signaling pathway dependent on Smad3 that leads to CI gene expression, but is not dependent on TGF-β protein (372).

Transforming growth factor-β induces the early response gene (*Egr-1*), via a Smad-independent pathway via MEK1/2/ERK signaling (373). Overexpression of *Egr-1* induces CI gene upregulation (374). In addition, IL-13 and insulin-like growth factor-binding protein-5 (IGF-BP-5) have been shown to induce *Egr-1* expression by MAPK signaling pathway (375). Other extracellular signals which are relevant to SSc [such as PDGF, hypoxia, HGF, or LPS (bacterial LPS), oxidative stress, thrombin, LPA, ultraviolet light, cigarette smoke, mechanical strength, ischemia-reperfusion, and T-cell receptor ligature] have been shown to increase *Egr-1* expression (373). TGF-β also induces *Egr-3* by canonical Smad3 signaling, and *Erg-3* overexpression stimulates CI gene expression (376).

Antifibrotic Mediators

Bone morphogenic protein-7, although a member of the TGF-β superfamily, stimulates fibroblast chemotaxis like TGF-β1, but does not induce CI, fibronectin, hyaluronan, or TIMP synthesis (377). BMP-7 also inhibits fibrogenic properties of TGF-β1 (378) and signals through a receptor complex structurally different from that of TGF-β and utilizes SMAD1/5/8 (365). *IL-10* inhibits both proliferation and CI synthesis by fibroblasts (379). Certain IL-10 genotypes have been associated with development of SSc in Caucasian and Japanese subjects (380). *TNF*α inhibits CI, stimulates MMP-1 synthesis by fibroblasts, and is a potent chemoattractant for these cells (381). *IFN*γ is a potent inhibitor of expression of CI and CIII mRNA and protein by cultured SSc fibroblasts *in vitro* (382). To what extent BMP-7, TNFα, IL-10, IFNγ, or other antifibrotic mediators or mechanisms try to counter the drivers of fibrosis such as TGF-β, IL-4/IL-13, IL-6/IL-6R-Gremlin-1 in SSc is unknown but provides candidates to be the focus of future studies.

Effect of blocking *TNF*α with etanercept was assessed in the BLM scleroderma mouse model. Compared to vehicle-treated mice, the etanercept-treated mice had less dermal fibrosis and lower serum levels of TGF-β1 than controls not treated with etanercept (383). Etanercept has not been efficacious in ameliorating dermal fibrosis in patients with SSc (384) (see **Table 3**).

Peroxisome proliferation-activated receptor gamma-γ, when engaged by ligands of different types, blocks TGF-β-mediated fibrotic responses *in vitro* in cultured fibroblasts and in various fibrotic animal models *in vivo* (81, 82). PPARG rs310746 is associated with SSc (83).

In the *cGVHD* murine model of scleroderma induced by transferring splenocytes from B10.D2 donor mice into BALB/c recipients, tolerizing the recipient BALB/c mice *by oral administration of protein extract of BALB/c spleens* for 11 days after transfer of B10.D2 splenocytes was associated by upregulation of IL-10 and downregulation of IFNγ production by T cells from the BALB/c recipients and protected the recipient BALB/c mice from dermal fibrosis and other manifestations of cGVHD (385). IL-10 was likely produced by Tregs induced by oral tolerance induction by the BALB/c spleen extract and was likely responsible for suppression development of fibrosis (379).

Genome-Wide Gene Expression of Skin

The fibrogenic role of *TGF*-β, *IL-13/IL-4*, and *Egr-1* in patients with SSc has been assessed by performing genome-wide gene expression studies on lesional and non-lesional skin biopsies from patients with dcSSc, lcSSc, morphea, and healthy controls. These studies show four intrinsic subsets of gene expression termed "diffuse proliferation" (further divided into diffuse1 and diffuse2) and containing only dcSSc patients; inflammatory group containing dcSSc, lcSSc, and morphea; limited group containing lcSSc; and a normal-like group containing normal, dcSSc, and lcSSc patients (386). Further comparisons were made subjecting TGF-β, IL-13/IL-4, and Egr-1-stimulated normal dermal fibroblasts in culture to gene expression microarray analysis and comparing these fibroblast microarrays to gene expression arrays of biopsies of skin from SSc, morphea, and normal donors. TGF-β responsive gene signature was found in 10 out of 17 patients with dcSSc (59%) and none of 7 lcSSc, none of 3 morphea, and none of 6 healthy controls (387). The dcSSc patients with the TGF-β-responsive signature had higher MRSS and likelihood of having ILD (387). The TGF-β signature-positive dcSSc patients also were in the diffuse-proliferation subset; however, one in the diffuse-proliferation subset did not have the TGF-β signature. This suggests that only a subset (and not all) SSc patients have the TGF-β signature. The fibroblast *Egr-1*-responsive gene signature was present in the skin biopsies from diffuse-proliferation subset of dcSSc patients, but was not present in biopsies of patients with lcSSc, morphea, or healthy controls (388). The *IL-4* response signature overlapped approximately 60% with the IL-13 response signature, which were both enriched in the SSc inflammatory subset (389). Expression in skin biopsies from SSc patients of the IL-13 pathway activation [as well as transcripts of IL-13 receptor components (*IL-13RA1* and *IL-4RA*)] correlated with MRSS (389). Expression of *CCL2* (MCP-1) transcripts also correlated with MRSS and *IL-13RA1* (389). This study also assessed gene expression profiling in skin of a sclerodermatous graft-versus-host disease (scl GVHD) model in *Rag2*$^{-/-}$ mice, which were found to also exhibit the IL-13 pathway activation resembling that in SSc patients of inflammatory subset (389). This observation is interesting, given that it has been hypothesized that fetal–maternal or maternal–fetal microchimerism might induce a cGVHD state in some patients with SSc as described above. Since IL-6 and IL-6R induces Gremlin-1 protein (which then signals through the canonical Smad-dependent pathway), it raises the question as to whether some of the TGF-β signature in the dcSSc diffuse-proliferation subset (discussed above) is actually due to *Gremlin-1*. Further studies would need to be done comparing Gremlin-1-induced gene signature in dermal fibroblasts with that of TGF-β1 to sort this out.

A more extensive genome-wide expression profiling skin biopsies involving analysis of additional pathway-specific gene signature for *PDGF*, *S1P*, *PPAR*-γ, *TNF*α, *IFN*α, *NFκB*, *IL-13*, *IL-4*, *poly (I-C)*, and *inomycin-phorbol 12-myristate 13-acetate (inomycin-PMA)* was recently conducted by this group (390). Results showed IFNα signaling was strongly associated with early disease, compatible with the notion that innate immune response may be a feature in early disease which was contrasted with TGF-β signaling being a feature of later disease with worse MRSS (390). Surprisingly, PDGF signaling was most strongly associated with the fibroproliferative subset (more so than TGF-β), and the inflammatory subset exhibited strong activation of innate immune pathways including enrichment of IL-4, S1P, NFκB, LPS, poly(I-C), and TGF-β gene signatures (390). The findings support an earlier hypothesis by Gabrielli et al. that a stepwise process of SSc development begins with inflammatory (e.g., IFNα signaling) and continues with fibrosis (e.g., PDGF and TGF-β signaling) and ends in atrophy (391). IL-4 pathway was significantly enriched in the inflammatory subset more than IL-13, and suggests a T_H2 enhancement of immune response in patients within the inflammatory subset (390).

Most patients with dcSSc have some resolution with the passage of time of dermal fibrosis after the onset of their disease. This has been observed in several different studies clinically as decreases in the MRSS. In a large, single SSc center in the UK, 131 patients with dcSSc had MRSS measured repeatedly up to 36 months after onset of their disease (392). Three patterns were discernable as follows: those with high baseline MRSS that did not improve over 36 months from baseline (38%); those with high baseline MRSS that improved over 36 months from baseline (21%); and those with low baseline MRSS that improved over 36 months from baseline (35%). The reason for these three clinical trajectories of change in MRSS over time is not apparent, but could be a function of different genetic backgrounds, different triggers, or other environmental modifications that either ameliorate or contribute to perpetuation of the disease. The patients received different medications; however, clinical MRSS response or survival could not be attributed to any of the medications (392). The fact that most of these patients with dcSSc had improvement in their MRSS suggests that the myofibroblast phenotype responsible for excessive ECM deposition does not persist, that the fibrotic skin can revert toward normal, and that a normal-like homeostasis can be re-established in such patients. This study suggests that

those dcSSc patients with persistently high MRSS likely have a continuous presence of a driver of dermal fibrosis that constantly stimulates the fibroblasts to maintain the myofibroblasts phenotype with maintenance of increased ECM in their dermis. Application of the genome-wide gene expression studies of skin biopsies, in a cohort such as this one in which patients have skin biopsied repeatedly over several years, may shed light on the mechanisms responsible for the three different MRSS trajectories over time, and would answer the question whether the inflammatory subset morphs into the fibroproliferative subset.

Vitamin D and Fibrosis

Vitamin D has a variety of antifibrotic actions. Studies *in vitro* have demonstrated *1,25(OH)$_2$D3* inhibits growth of murine fibroblasts (393–397), inhibits fibroblast-mediated contraction of CI gels (largely a TGF-β-stimulated function) (398), inhibits fibroblast synthesis of IL-6 and IL-8 (399, 400), and inhibits production of plasminogen activator (401). It was also observed that 1,25(OH)$_2$D3 *in vitro* inhibited CI and CIII synthesis by fibroblasts grown from different human tissues including bone marrow, lung, and skin (402, 403). In mice, *in vivo* administration of 1,25(OH)$_2$D3 has been shown to ameliorate renal interstitial fibrosis, glomerulosclerosis in rats, and reduce conversion of adipose tissue to fibrous tissue in mouse skin exposed to chronic UV irradiation (404, 405).

The cutaneous formation and metabolism of VitD in patients with SSc has been reported to be normal (406–408). In one report, fibroblasts grown from biopsies of lesional skin from patients with SSc and from healthy volunteers were inhibited in proliferation and CI synthesis to a similar extent by 1,25(OH)$_2$D3 addition to the fibroblast culture (409). The VitD receptor (VDR) in SSc lesional skin fibroblasts is reported to be decreased, likely due to TGF-β's ability to downregulate the VDR (410).

Studies using a mouse mesenchymal multipotent cell line revealed that 1,25(OH)$_2$D3 promoted increased expression and nuclear translocation of the VDR; decreased expression of TGF-β1 and plasminogen activator inhibitor (SERPINE 1); decreased expression of CI I, III, and other CI isoforms; and increased expression of several other antifibrotic factors including *BMP-7*, *MMP-8*, and *follistatin* [an inhibitor of the pro-fibrotic factor, myostatin (411)]. Studies in rat interstitial myofibroblasts showed that 1,25(OH)$_2$D3 inhibited in a dose-dependent manner (10^{-9}–10^{-6} M) TGF-β1-induced *de novo* αSMA expression and suppressed CI and TSP-1 expression induced by TGF-β1, which was shown to be mediated by upregulated HGF (412).

Slominski et al. have discovered the skin and other tissues in humans synthesize other VitD derivatives [including *20(OH)D3*, *20,23(OH)$_2$D3*, and *17,20(OH)$_2$pD*] that, also like 1,20(OH)$_2$D3, *in vitro* inhibit CI and hyaluronan synthesis by fibroblasts grown from normal or SSc lesion skin (144). Unlike VitD3, 25(OH)D3, or 1,25(OH)$_2$D3, these novel endogenously produced VitD analogs are non-calcemic when given in high doses to mice. 20(OH)D3 also suppressed development of dermal fibrosis in the BLM, scleroderma mouse model (413). These results suggest multiple endogenous forms of VitD3 have antifibrotic properties that may prove useful in SSc as therapeutic agents in the future.

Lysophospholipids and Fibrosis

Lysophosphatidic acid induces fibroblast chemotaxis and proliferation (414). LPA induces αvβ6 integrin-mediated TGF-β activation by engaging LPA$_2$ receptors on epithelial cells and makes fibroblasts resistant to apoptosis, which is a characteristic of SSc lesional fibroblasts that would prolong their survival (415, 416). Evidence that LPA is involved in myofibroblast formation in SSc lesional skin was suggested by the finding that fibroblasts cultured from skin of SSc patients exhibited increased LPA-activated chloride current, which is a hallmark of LPA-induced myofibroblasts (417). *AMO95*: a selective small molecule inhibitor of LPA$_1$ signaling (AMO95) protected mice from developing BLM-induced skin fibrosis and increased regression of established BLM-induced skin fibrosis (418). Contrary to the results in the BLM skin fibrosis model in which LPA$_2$ knockout did not affect dermal fibrosis, in the BLM lung fibrosis model, LPA$_2$ knockout mice exhibited reduced lung injury, fibrosis, and fibronectin deposition in BLM-treated lungs (419). *S1P* facilitates migration of fibroblasts in response to a chemotactic gradient of fibronectin in a S1P$_2$ receptor-dependent manner (420). S1P signals through the Smad pathway utilized by TGF-β1 in fibroblasts and other cell types and mimics TGF-β1 pro-fibrotic effects in that it decreases MMP-1 and increases TIMP and CI production by fibroblasts (421–423). SSc dermal fibroblasts express more S1P$_3$ receptors than control donor fibroblasts and exhibit an exaggerated pro-fibrotic response to TGF-β1 (421). Furthermore, S1P levels are elevated in sera of patients with SSc (214). As mentioned above, S1P gene signature is prominent in the inflammatory subset (390). Fingolimod (*FTY720*) has both agonist and antagonist effects in different S1P receptors and modulates lymphocyte trafficking, monocyte/macrophage biology, dendritic cell biology, and enhances Treg function at marginal zone B lymphocytes (144). When administered to chronic scleroderma graft-versus-host disease (cScl-GVHD) mice, FTY720 in either preventative or therapeutic protocols reduced fibrosis, expanded splenic myeloid suppressor cells, increased Tregs and B regulatory cells (Bregs), protected against vascular damage, reduced serum S1P and E-selectin levels, reduced numbers of inflammatory cells in skin, and reduced dermal expression of mRNA for TGF-β1, MCP-1, MIP-1α, RANTES, TNFα, IFNγ, IL-6, IL-10, and IL-17A (424). FTY720 also returned phosphatase and tensin homolog (PTEN) and Smad3 phosphorylation to normal levels in cScl-GVHD mice (424). Although FTY720 is approved to treat MS, its use in SSc clinical trials has not been reported.

The Endocannabinoid System and SSc

The ECS is an endogenous regulatory network made up of multiple GPCRs and a series of endogenous arachidonic acid derivatives, which act in an autocrine fashion and seem to play a homeostatic role affecting diverse key biologic and physiologic processes including angiogenesis, cell proliferation, apoptosis, differentiation, metabolism, immune function, and vascular tone that may have implications for SSc pathogenesis and potential therapeutic targets. The term "endocannabinoid" generally refers to the first two characterized endocannabinoids (ECs), anandamide (AEA) and 2-arachidonoyl glycerol (2-AG), though a number of endogenous cannabinoid receptor agonists have since been discovered.

AEA and 2-AG may be degraded into free arachidonic acid by fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), respectively, but may also be metabolized by lipoxygenases, cyclooxygenase 2 (COX-2), and P450 epoxygenases, and acyl transferases yielding a vast library of EC analogs with different actions and target receptors (425). Some of these metabolites engage EC receptors, while others have been demonstrated to modulate the activity of ECs via "entourage effects" at cannabinoid receptors 1 (CB1) and 2 (CB2), inhibition or potentiation of ECS degradation, activation of downstream targets such as *PPARγ* or metabolic interconversion (426).

Efforts to identify new cannabinoid receptors are ongoing; but CB1, CB2, and GPR55 are among the most extensively studied, and recent studies clearly demonstrate that all three of these receptors are activated by AEA and play a key role in transduction of EC signaling. Interestingly, CB1, CB2, and GPR55 have also been shown to modulate one another's activity via heteromerization, cross-antagonism, and other strategies (427, 428). GPR18 (also known as "abnormal cannabinoid receptor"), another candidate cannabinoid receptor, may play a role in EC-mediated central blood pressure control and peripheral vascular tone (429, 430). Unlike CB1, CB2, and GPR55; however, activation of this receptor requires FAAH-mediated metabolism of AEA or 2-AG to *N*-arachidonoyl-glycine (431). It is interesting to speculate that if there is reduced FAAH in tissue-expressing GPR18 in patients with SSc as has been found in SSc dermis, then this might contribute to vasoconstriction and hypertension (432). Substantial cross talk has also been established between the ECS and a network of non-selective action channels known as the transient receptor potential vanilloid (TRPV) family, which serve to integrate mechanical and environmental stimuli with local autocrine signals to effect a variety of cell processes (433, 434).

Endocannabinoid System as a Therapeutic Target in SSc

The ECS is an appealing potential target for treatment of SSc, as it modulates endothelial cell function, vascular tone (including pulmonary artery vasodilation), the innate immune response to injury, autoimmunity, and fibrogenesis (435–440).

Endocannabinoid Modulation of the Immune System

The general effect of cannabinoids on cells of the immune system is to act as immunosuppressive and anti-inflammatory agents. Although immune cells express more CB2 than CB1, both receptors and other non-CB receptors (such as PPARγ and GPR55) have been implicated in effecting immunomodulatory actions of cannabinoids (441, 442). CB1 mRNA and protein expression in/on immune cells is responsive to cellular activation signals, i.e., cell type, cannabinoid ligand type, and immune stimulus-dependent (443). Of relevance to SSc, IL-4 is specifically able via STAT5 pathway to induce CB1 mRNA in human T cells (444). Dendritic cells exposed to cannabinoids undergo NFκB-dependent apoptosis and reduce production of IL-12, which is important in priming Th0 cells to a Th1 orientation (445, 446). Cannabinoids induce apoptosis of T cells via CB1 and CB2 engagement and effect a Th2 polarization (e.g., increased IL-4 by T cells) while decreasing Th1 polarization (e.g., decreased IL-12 by DC) but also suppress activation, differentiation, and expansion of T cells (443, 447, 448). B cells are affected by cannabinoids in several ways, including direct effects on activation, differentiation, and proliferation but also via effects on T cells that provide help to B cells (449). The effect of cannabinoids on B cells to suppress IgM and enhance IgE production apparently is mostly via engagement of CB2 (450). Production of pro-inflammatory cytokines (including TNFα, IFNγ, IL-2, and IL-1β) is suppressed *in vitro* and *in vivo*, by engaging CB2 by cannabinoids or other CB2 agonists (451–453). Of particular relevance to SSc is that mast cell activation is inhibited by 2-AG (454). The CB2 agonist, Gp1a, was found to suppress clinical disease in the EAE mouse model with a reduction in Th1 and Th17 cells in peripheral lymphoid organs. Analysis of the $CD4^+$ cells *in vivo* in the periphery revealed Gp1a-treated mice had lower levels of expression of T but also RORγt (Th17 marker) and exhibited increased Foxp3 and GATA-3 expression (455). Under polarizing conditions *in vitro*, Gp1a suppressed Th1 and Th17 development of $CD4^+$ T cells (455). The role of the ECS in innate and adaptive immune dysregulation in SSc is an area for further investigation, and these results with this CD2 agonist suggest similar agents might decrease autoimmunity and autoantibody production in SSc.

Endocannabinoid Modulation of the Vasculature

The upregulation of *ICAM-1* and *VCAM-1* on the endothelium of human coronary arteries by treatment with TNFα or LPS is inhibited by the CB2 agonist drug, *JWH-133* (437). Engaging CB2 in rat coronary arteries by AEA induced the coronary arteries to dilate (456). Blocking CB1 on isolated human coronary smooth muscle cells by the CB1 antagonist, *rimonabant*, reduced ability of the smooth muscle cells to migrate and proliferate in response to PDGF (438). The ECs (*AEA* and *virodhamine*) were found to have a potent vasodilatory effect on preconstricted isolated human pulmonary artery rings that was endothelium-dependent and likely involved PGE_2 (436). The effect of AEA and virodhamine was CB1- and CB2-independent but involved a third receptor termed "endothelial cannabinoid receptor" (436). These studies suggest that a target for development of treatment for PAH in SSc might be based on virodhamine-like drugs that are agonist for the endothelial cannabinoid receptor.

Endocannabinoid Modulation of Fibrosis

C57BL/6 mice with either *TRPV1* receptor or *calcitonin G-related peptide (CGRP)* knocked out compared to WT mice developed enhanced dermal fibrosis after repeated subcutaneous injection of BLM (434). This suggests that *TRPV1* receptor and *CGRP* have antifibrotic effects and may have relevance to patients with RP and SSc since skin biopsies from patients with SSc have reduced numbers of *CGRP*-immunoreactive C fibers and would likely have reduced vasodilatation from *CGRP* in response to stressors that trigger RP (434). It is unclear whether other *TRPV1* receptor engagement or *CGRP* effects are operative to protect against fibrosis. *TRPV4* has been implicated as a mechanosensor in endothelial cells and fibroblasts, and has been shown to stimulate myofibroblast differentiation in rat cardiac fibroblasts via integration of mechanical and soluble (autocrine) signals, and pretreatment with the TRPV4 antagonist, *AB159908*, resulted in significant inhibition

of TGF-β1-induced myofibroblasts differentiation of cardiac fibroblasts (457). *5,6-EET* (generated by activation of *PAR-2* by mast cell tryptase or Factor Xa) has been implicated as the most likely autocrine mediator contributing to activation of *TRPV4*, though other autocrine signals may be involved as well, with the known *TRPV4* agonist *N*-acyl taurine being another possible candidate. It is worth noting that this system of channels seems to be dysregulated in dcSSc fibroblasts with profound downregulation of *TRPV2* (and possibly *TRPV1*) and overexpression of *TRPV4*. While the role of *TRPV1* and *TRPV2* is less clear, *TRPV4* is known to stimulate myofibroblast differentiation in response to activation by mechanical stress and arachidonic acid derivatives. In normal wound repair, release of the myofibroblast from this mechanical stress signal plays a role in inducing apoptosis or, alternatively, may help drive the myofibroblast back into a quiescent fibroblast. Given that TRPV4 remains overexpressed in the dcSSc fibroblast, this may suggest that certain autocrine signals are present, which alter the cellular milieu in favor of constitutive activation of *TRPV4*, thus rendering the myofibroblast incapable of responding appropriately to mechanostress signaling. AEA and 2-AG activation of *TRPV4* is indirect and requires hydrolysis of these compounds to free arachidonic acid, which is then converted into the potent *TRPV4* agonist 5,6-EET. The fatty acid amide *N*-acyl-taurine was recently discovered to be a potent agonist of TRPV4, as well, and is likely overexpressed in SSc owing to underexpression of FAAH (432). This compound was shown to be elevated 10-fold following experimental inactivation of FAAH, with highest levels noted in the lungs and kidneys (458). Furthermore, inaction of FAAH in mice increases dermal fibrosis in response to subcutaneous administration of BLM (432).

Serine proteases activate PARs, which have been associated with fibrosis of internal organs (459–461). *PAR-1* is expressed by keratinocytes, endothelial cells, and fibroblasts, while *PAR-2* is expressed in suprabasal keratinocytes in SSc lesional skin and in healthy donor skin (462). There is more expression of PAR-1 by fibroblasts in biopsies of SSc lesional skin than by fibroblasts in biopsies of normal donor skin, and PAR-2 was expressed only by SSc lesional skin fibroblasts and not by normal donor fibroblasts (462). A large portion of fibroblasts in samples from SSc lesional skin were myofibroblasts, staining positive for αSMA suggesting that PAR-1 and PAR-2 may be involved in fibrosis development in SSc (462). PAR-1 is increased on SSc-associated ILD myofibroblasts, and when it is inhibited by the direct thrombin inhibitor, dabigatran, there is abrogation of formation of myofibroblasts, αSMA, and production of CI (463). Agonists of PAR-2 include mast cell tryptase and Factor Xa, and mast cells have been demonstrated to stimulate human lung fibroblast proliferation via activation of PAR-2.

It is worth noting the importance of COX-2 in the metabolism of ECs – specifically regarding 2-AG, which is more readily metabolized by COX-2 than AEA and has also been shown to activate PPARγ via a mechanism that is COX-2 dependent. Recent studies suggest the COX-2 metabolite of 2-AG, 15-deoxy-PGJ2-G, activates PPARγ (464) and that inhibition of IL-2 secretion by AEA in murine splenocytes is attenuated by COX-2 inhibition and also partially antagonized by PPARγ inhibition (465). Further work should be directed at evaluating PPARγ as a downstream target of the oxygenated metabolites of AEA and 2-AG, as this nuclear receptor is known to modulate fibrogenesis (likely by being a transcriptional repressor of TGF-β), autoimmunity, and a wide range of other physiologic processes (82, 466). 2-AG is oxygenated by COX-2 and other PG synthases to produce several different glycerol-esters of the prostaglandins (PG-Gs). During the early stages of inflammation, in which microsomal prostaglandin E2 synthase (mPGES)-1 is high, one would expect to see higher levels of PGE_2-G. Similarly, during resolution, in which PGD_2 and its spontaneous degradation products predominate, one would expect to see higher levels of PGD_2-G and its metabolites. Interestingly, the EC analog of 2-AG, which is known as 15-deoxy-PGJ_2-G, also has been shown to activate PPARγ and this cyclopentanone-EC derivative may be the mediator of 2-AG's activation of PPARγ. Further characterization of the P450-derived epoxides of AEA and 2-AG is needed, especially given the importance of P450, epoxygenase in PAR-2-mediated sustained activation of TRPV4, which may play a role in perpetuating fibroblast activation.

The efficacy of several cannabinoid agonists in attenuation of fibrosis has been demonstrated in both the BLM and hypochlorite murine models of SSc. Additionally, these compounds have been shown to counter several behavioral abnormalities of SSc fibroblasts, including reversal of myofibroblast differentiation and decreased resistance of SSc myofibroblasts to apoptosis, with the ultimate effect of decreased ECM deposition and attenuation of fibrogenesis. The exact mechanism by which these cannabinoid agonists exert their antifibrotic action is still a matter of debate, but it seems to be mediated in part by activation of CB2 and PPARγ. CB1 and CB2 are both overexpressed in dcSSc fibroblasts. Twenty-four hour incubation with the CB1/CB2 agonist, *WIN55,212-2*, resulted in agonist-induced inhibition of both CB receptors, which was reversible after agonist withdrawal (439). After 10 μM WIN55,212-2 incubation, a reduction in CI mRNA and protein was observed in both dcSSc and healthy fibroblasts (439). TGF-β and CTGF mRNA expression, as well as IL-6 levels were also substantially decreased after exposure to WIN55,212-2 (439). Analysis for αSMA by Western blotting, RT-PCR, and immunocytochemistry showed that WIN55,212-2 induced reduction in αSMA expression by 43% and increased by twofold the number of apoptotic fibroblasts from patients with dcSSc but not in fibroblasts from healthy donors (439). Pre-incubation of dcSSc and healthy fibroblasts with synthetic cannabinoid receptor antagonists *AM281* (CB1 antagonist) and *AM630* (CB2 antagonist) did not significantly reverse the effects of WIN55,212-2 on CI neosynthesis, inhibition of IL-6, or fibroblast apoptosis, indicating that the antifibrotic actions of WIN55,212-2 are mediated, in part, by pathways not involving CB1 and CB2, perhaps as the authors suggest by transducing pathways involving p-ERK (439). Incubation of dcSSc fibroblasts and healthy controls with WIN55,212-2 was noted to result in decreased phospho-ERK-1/2 protein expression in both groups (439). WIN55,212-2 was found to prevent BLM-induced dermal fibrosis in DBA/2J mice *in vivo*. Levels of phospho-Smad2/3 were analyzed and found to be significantly lower after WIN55,212-2 exposure. Subcutaneous inflammatory cell infiltration, dermal thickness, and CI content were comparable to the control group (440). BALB/c mice injected daily for 6 weeks with PBS or hypochlorite were injected intraperitoneally

with PBS or with WIN55,212-2, an agonist of CB1 and CB2, or with JWH-133, a selective agonist of CB2. Both WIN55,212-2 and JWH-133 prevented development of skin and lung fibrosis, as well as fibroblast proliferation and formation of autoantibodies (467).

As mentioned above, FAAH levels are markedly reduced in biopsies of SSc lesional skin compared to skin from healthy donors, and mRNA for FAAH expression in cultured lesional skin fibroblasts from patients with SSc was also reduced from that expressed by cultured normal donor dermal fibroblasts (432). The induction of BLM skin fibrosis in *FAAH* null mice or in normal mice treated with the FAAH inhibitor, *JNJ1661010*, resulted in a marked increase in skin fibrosis at the BLM injection site (432). Furthermore, blocking CB1 receptor in BLM-treated mice with FAAH blocked by JNJ1661010 resulted in a marked increase in skin fibrosis at the BLM injection site (432). Additionally, blocking CB1 receptor in BLM-treated mice with FAAH blocked by JNJ1661010 prevented the enhanced fibrosis induced by BLM treatment, whereas blocking CB2 further enhanced skin fibrosis in BLM-treated mice with FAAH blocked by JNJ1661010, suggesting CB1 mediated fibrosis whereas CB2 dampened fibrosis as a result of increased EC present because of blocking FAAH (432).

The role of the CB1 receptors in the BLM skin fibrosis model and Tsk-1/+ mice model was assessed using *CB1*KO mice (468). WT and *CB1*KO mice were treated with BLM and with the CB1 selective agonist *N*(a-chloroethyl)-5Z, 8Z, 11Z, 14Z eicosatetraenamide (ACEA) (468). *CB1*KO mice were protected from developing BLM dermal fibrosis; however, crossing *CB1*KO mice with Tsk-1/+ mice did not prevent fibrosis (468). This suggested that fibrosis associated with inflammation was dependent on CB1 expression or leukocytes. Indeed, chimeric bone marrow studies revealed that CB1 on leukocytes was essential for leukocyte infiltration and fibrosis in the BLM skin fibrosis model induced by the CB1 agonist (468).

These preclinical studies of CB receptor agonists/antagonist provide useful information for translation of these or similar CB receptor active agents for treatment of SSc.

Conclusion

Systemic sclerosis is one of the most complex systemic autoimmune diseases that target the vasculature and connective tissue-producing cells (namely fibroblasts/myofibroblasts) and components of the innate and adaptive immune systems – all three of which themselves interact and affect each other. The disease is heterogeneous in its clinical presentation that likely reflects different genetic background or triggering factor influences on the vasculature, connective tissue cells, and immune system. The roles played by other ubiquitous molecular entities (such as lysophospholipids, ECs, and their diverse receptors) in influencing the vasculature, immune system, and connective tissue cells are just beginning to be realized and studied and may offer new therapeutic approaches to treat SSc.

Author Contributions

Statement pertaining to each author's contribution. Drs. DP and AP wrote the "Introduction," Dr. MB wrote the section on "Genetics and GWAS," Dr. AP wrote the sections on "Immune System in SSc Pathogenesis," Dr. DP wrote the section on "Vascular Abnormalities in SSc," Dr. AP wrote the section on "Fibrosis in SSc" and "Animal Models of SSc/Scleroderma." Dr. BP wrote the section on "The ECS and SSc." All authors reviewed the final manuscript.

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Eosinophilic granulomatosis with polyangiitis: an overview

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Eosinophilic granulomatosis with polyangiitis (EGPA) is a multisystemic disorder, belonging to the small vessel anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis, defined as an eosinophil-rich and necrotizing granulomatous inflammation often involving the respiratory tract, and necrotizing vasculitis predominantly affecting small to medium-sized vessels, associated with asthma and eosinophilia. EGPA pathogenesis is not well known: *HLA-DRB1*04* and **07*, *HLA-DRB4* and IL10.2 haplotype of the IL-10 promoter gene are the most studied genetic determinants. Among the acquired pathogenetic factors, the exposure to different allergens, infections, vaccinations, drugs, and silica exposure have been involved. Eosinophils are the most characteristic cells in EGPA and different studies have demonstrated their role as effector and immunoregulatory cells. EGPA is considered as a disease with a prevalent activation of the Th-2 cellular-mediated inflammatory response and also humoral immunity plays an important role. A link between B and T inflammatory responses may explain different disease features. EGPA typically develops into three sequential phases: the allergic phase, distinguished by the occurrence of asthma, allergic rhinitis, and sinusitis, the eosinophilic phase, in which the main pathological finding is the eosinophilic organ infiltrations (e.g., lungs, heart, and gastrointestinal system), and the vasculitic phase, characterized by purpura, peripheral neuropathy, and constitutional symptoms. ANCA (especially pANCA anti-myeloperoxidase) are present in 40–60% of the patients. An elevation of IgG4 is frequently found. Corticosteroids and cyclophosphamide are classically used for remission induction, while azathioprine and methotrexate are the therapeutic options for remission maintenance. B-cell depletion with rituximab has shown promising results for remission induction.

Keywords: eosinophilic granulomatosis with polyangiitis, vasculitis, eosinophils, vascular diseases, ANCA-associated vasculitis

INTRODUCTION AND EPIDEMIOLOGY

Eosinophilic granulomatosis with polyangiitis (EGPA) is a multisystemic disorder, belonging to the small vessel anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitides (AAVs). According to the 1994 Chapel Hill consensus conference (CHCC), EGPA is defined as an eosinophil-rich and granulomatous inflammation often involving the respiratory tract, and necrotizing vasculitis predominantly affecting small to medium-sized vessels, associated with asthma and eosinophilia. Formerly known as "Churg–Strauss syndrome," this eponym has been replaced during the 2012 Revised International CHCC, with the aim of focusing on the histopathology of the disease (1). Unlike in the 1990 American College of Rheumatology classification criteria and the former CHCC, the CHCC 2012 has reported for the first time that ANCA are found in EGPA, especially in patients with glomerulonephritis. This reflects some of the newest evidences of the distinction of two EGPA subsets, depending on the presence or the absence of ANCA (**Table 1**) (2).

Eosinophilic granulomatosis with polyangiitis incidence in Europe is 0.5–6.8 new cases/year per million populations, whereas its prevalence is 10.7–13 cases per million populations. It mostly affects subjects between 40 and 60 years old and the mean age at diagnosis is 48 years (3).

PATHOGENESIS

Eosinophilic granulomatosis with polyangiitis pathogenesis is not well known. The disease is probably the result of a complex interaction in which genetically and environmental factors lead to an inflammatory response whose principal players are eosinophils, T, and B lymphocytes (2) (**Figure 1**).

GENETIC DETERMINANTS

Eosinophilic granulomatosis with polyangiitis is an HLA-associated disease (4). It has been proven that it is associated with *HLA-DRB1*04* and **07* (5) and with *HLA-DRB4* (6). This contraction of the class II HLA repertoire suggests a strong $CD4^+$ T lymphocyte activation, possibly triggered by allergens or antigens.

It has been also investigated the presence of single nucleotide polymorphisms (SNP) of the gene, which encodes interleukin (IL)-10, an important molecule for the activation of the Th-2 pathway; EGPA ANCA-negative subset has been associated with the IL10.2 haplotype of the IL-10 promoter gene, a condition, which leads to an increased production of IL-10 (7). This is apparently in line with EGPA pathogenesis, which is characterized by an increased Th-2 response and an increase in IgG4 levels, both of which seem to be mediated by IL-10.

Table 1 | Diagnostic criteria, classification, and nomenclature of eosinophilic granulomatosis with polyangiitis during the last 20 years.

Lanham diagnostic criteria (1984)[a]	American College of Rheumatology classification criteria (1990)[b]	Revised International Chapel Hill consensus conference nomenclature of vasculitides (2012)
Asthma	Asthma	Eosinophil-rich and necrotizing granulomatous inflammation often involving the respiratory tract, and necrotizing vasculitis predominantly affecting small to medium vessel, and associated with asthma and eosinophilia. ANCA is more frequent when glomerulonephritis is present.
Blood eosinophilia >1500/mm^3 or >10% of total WBC	Eosinophilia (>10% of total WBC)	
Evidence of vasculitis involving two or more organs	Neuropathy	
	Pulmonary infiltrates non-fixed	
	Paranasal sinus abnormalities	
	Extravascular eosinophils	

[a]*All three criteria must be met for a diagnosis of EGPA.*

[b]*The presence of four or more of these six criteria yielded a sensitivity of 85% and a specificity of 99.7% for the classification of vasculitis as EGPA.*

WBC, white blood cells.

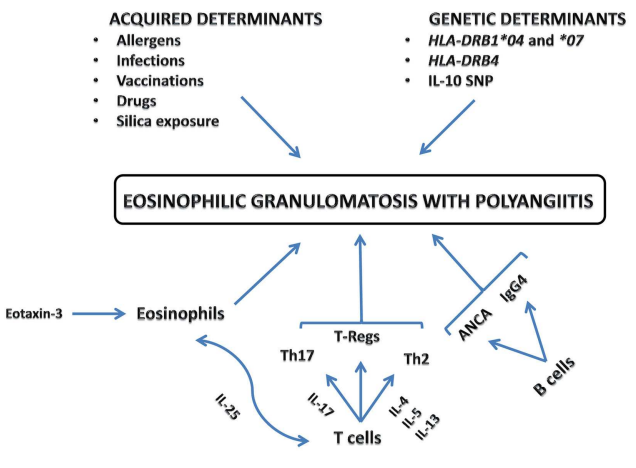

FIGURE 1 | Eosinophilic granulomatosis with polyangiitis pathogenesis.

ACQUIRED DETERMINANTS

Some environmental triggers have been identified: the exposure to different allergens, infections, vaccinations could trigger the disease. Drugs may also have a pathogenetic role and, among these, the leukotriene receptor antagonists are the most frequently involved more often used as steroid-sparing agents for asthma, their key role in triggering EGPA is still uncertain (8). More recently, also the recombinant anti-IgE monoclonal antibody omalizumab used in patient with asthma has been considered as an EGPA trigger (9–11). According to the most reliable hypotheses, both LTRA and anti-IgE antibody may be involved in EGPA pathogenesis simply unmasking the disease, due to the delayed use of steroids.

A recent review has shown the possible pathogenetic influence of silica exposure in AAVs, including EGPA (12).

EOSINOPHILS

The role of the eosinophils is still uncertain in EGPA but different studies have demonstrated the cytotoxic (13, 14) and pro-coagulant (15, 16) properties of this cell type, which may result in the development of cardiovascular and cerebrovascular complications in patients with any type of hypereosinophilic syndromes including EGPA. Although they are usually considered to be effector cells, they may act as immunoregulatory cells (2): indeed, a cross-talk between T-lymphocytes and eosinophils has been pointed out. In a recent study, high concentrations of IL-25 have been detected in the sera of EGPA patients; eosinophils are the main source of IL-25, which induces T-cells to produce cytokines that stimulate Th-2 and, at the same time, eosinophilic responses (17).

T-LYMPHOCYTES

It has been demonstrated that T-lymphocytes have an important role in the EGPA pathogenesis. T-cells are present in the most of the organ lesions and in some of them, like peripheral neuropathy, they represent the main component. Moreover, serum levels of T-cell activation markers, like IL-2r, are increased during the active phase of the disease (18). T-cells receptors show a restricted repertoire suggesting oligoclonal expansion (19), which is in line with the hypothesis of an antigen-driven disease. Clonal restricted effector CD8$^+$ lymphocytes with a proinflammatory profile have been recently described in patients with EGPA (20).

Specifically, EGPA is considered as a disease with a prevalent activation of the Th-2 pathway. In keeping with this view, it has been demonstrated that tissue infiltrates in patients with EGPA are rich in T-cells with Th-2 makers such as CD294. Furthermore, EGPA patients CD4$^+$ T-cells are able to produce, *in vitro*, high concentrations of IL-4, IL-5, and IL-13, molecules that hallmark the Th-2 immunoresponse.

High-blood concentrations of IL-17 have been found in patients with EGPA, a finding, which suggests that the involvement of Th17 lymphocytes into EGPA pathogenesis; indeed, these lymphocytes are involved in the pathogenesis of other autoimmune diseases (2).

Finally, reduced levels of regulatory CD4$^+$ T-cells (Tregs) have been discovered in EGPA patients (21, 22). Tregs usually have a protective role toward the development of autoimmune diseases. Lower numbers of Tregs were found in active EGPA patients than in patients with asthma or with chronic eosinophilic pneumonia; additionally, the percentages of circulating Tregs were lower in active than quiescent EGPA (2).

B-LYMPHOCYTES

The role of the humoral immunity in EGPA seems to be less relevant as compared to other autoimmune diseases. Despite this, EGPA patients often show an abnormal humoral response. ANCA

are found in about 40% of patients with EGPA, they are characterized by a perinuclear pattern (pANCA) at the immunofluorescence assay and are directed against the neutrophil myeloperoxidase (MPO), as revealed by ELISA. Their pathogenetic role and their potential harmful effect is still matter of debate. Even though animal models and *in vitro* studies have shown a pathogenic role of the anti-MPO antibodies (23), their role in causing organ damage in EGPA is still unknown.

A substantial number of patients show an increased IgG4 blood levels. In a recent analysis of 46 EGPA patients, IgG4 levels correlated with the number of disease manifestations and the Birmingham vasculitis activity score (BVAS). Furthermore, serum IgG4 levels paralleled the disease course as they normalized during remission. The skewed IgG4 response is likely due to the enhancing effects of the Th-2 cytokines IL-4, IL-5, and IL-13 (24).

CYTOKINES AND CHEMOKINES

Regarding chemotaxis, eotaxin-3 (CCL26), a chemokine, which attracts eosinophils in the sites of inflammation, apparently has a key role into EGPA pathogenesis. Two different studies reported that eotaxin-3 was highly elevated in serum samples of active EGPA patients and correlated highly significantly with eosinophil counts, total immunoglobulin E (IgE) levels, and acute-phase parameters. Immunohistochemical analysis revealed strong expression of eotaxin-3 in endothelial and inflammatory cells in affected tissues of active EGPA patients (25, 26).

Also, CCL17, another Th-2 chemokine, seems to be present into both tissues infiltrates and patients' sera (27).

Some recent studies have demonstrated that EGPA patients' T-cells produce, after stimulation *in vitro*, a large amount of interferon-γ (INF-γ), a cytokine, which boosts Th-1 immune response (28).

The hypothesis of a cross-talk between humoral and cell-mediated immunity and eosinophils is still the object of different pathogenetic studies.

CLINICAL FEATURES

Eosinophilic granulomatosis with polyangiitis mainly affects patients with asthma (often developed in the adult age), sinusitis, allergic rhinitis, and nasal polyposis (**Table 2**) (29).

Eosinophilic granulomatosis with polyangiitis typically develops into three sequential phases, marked by a progression of the main symptoms. The first phase, also called prodromic or allergic, is most common in the second or third decade and it is distinguished by the occurrence of asthma, allergic rhinitis, and sinusitis. Subsequently, the eosinophilic phase develops the main pathological findings of this phase are the raise in the peripheral eosinophilic count and the eosinophilic organ infiltrations, especially in lungs, heart, and gastrointestinal system. The third phase is the vasculitic one during this last phase, the patient suffers from the consequences of a necrotizing vasculitis (e.g., purpura, peripheral neuropathy), generally associated with vascular or extravascular granulomatosis and constitutional symptoms like fever, malaise, and weight loss (35).

Eosinophilic granulomatosis with polyangiitis is a multisystemic disease. One of the most frequently involved sites is the respiratory system asthma has a prevalence of about 95% (36). Pulmonary eosinophilic infiltrates may be present and their biopsy is often highly informative for the histopathologic diagnosis (37).

The otorhinolaryngoiatric system is also frequently involved nasal polyposis is one of the conditions, which lead patients to undergo repeat surgery. Allergic rhinosinusitis, epistaxis, and neurosensory hearing loss are other common features (32).

Cardiac involvement may be represents the most harmful manifestation of EGPA characterized by myocardial infarction, pericarditis, or congestive heart failure, it is the main cause of death (30, 36, 38).

Among the most frequent skin manifestations, subcutaneous nodules, and purpura (especially involving the legs) represent a clinical hallmark of the vasculitic phase, a skin biopsy of purpuric lesions generally shows a leukocytoclasic vasculitis (39, 40).

Table 2 | Main clinical features in eosinophilic granulomatosis with polyangiitis and their prevalences.

Clinical features	Prevalence (%)	Reference
Mean age at diagnosis (years)	50 ± 16	Comarmond et al. (30)
Asthma	91–100	Comarmond et al. (30); Sablé-Fourtassou et al. (31)
Ear, nose, and throat involvement	48–75	Comarmond et al. (30); Bacciu et al. (32)
Neuropathy	55–72	Comarmond et al. (30); Sablé-Fourtassou et al. (31)
Pulmonary involvement	65–91	Sablé-Fourtassou et al. (31); Comarmond et al. (30)
Cutaneous involvement	40–52	Comarmond et al. (30); Sablé-Fourtassou et al. (31)
Renal involvement	27	Sinico et al. (33)
Cardiac involvement	27–35	Comarmond et al. (30); Sablé-Fourtassou et al. (31)
Gastrointestinal involvement	23–32	Comarmond et al. (30); Sablé-Fourtassou et al. (31)
Central nervous system involvement	5–9	Comarmond et al. (30); Sablé-Fourtassou et al. (31)
ANCA positivity	38	Sinico et al. (34)
pANCA positivity	74 of all $ANCA^+$ patients	Sinico et al. (34)

ANCA, anti-neutrophil cytoplasmic antibody.

Although less frequent than the other two AAVs, renal involvement occurs in about 25% of the patients and the most typical expression is pauci-immune crescentic glomerulonephritis with a high range of clinical features, from isolated urinary abnormalities (proteinuria, hematuria) to rapidly progressive glomerulonephritis. Kidney involvement is a bad prognosis factor for patients with EGPA (33).

Peripheral neuropathy, either sensory or motor or sensory-motor, affects a large portion of the patients; mononeuritis multiplex, with axonal damage, usually unilateral and asymmetric, is the most characteristic manifestation of peripheral nervous system involvement. Patients report paresthesia and pain in the affected areas (peroneal, tibial, ulnar nerve), especially during the vasculitic stage of the disease (41).

In the gastrointestinal system, the vasculitic phase may be preceded by an eosinophilic gastroenteritis with abdominal pain, diarrhea, and intestinal bleeding (42).

In the 30–40% of the patient, there can be diffuse lymphadenopathy, frequently affecting axillary and cervical lymph nodes (43).

The most frequent laboratory findings in EGPA patients is marked hypereosinophilia, frequently between 5000 and 9000 eosinophils/μL [at least >1500 eosinophils/μL or >10% of the total white blood cells, according to Lanham criteria (44)], this is one of the most common signs of EGPA (36). An increase in non-specific inflammatory markers (ESR, CRP) is often found (36). The role of the complement is still uncertain. ANCA are present approximately in 40–60% of the patients; pANCA (perinuclear) is the prevalent pattern, with antibody specificity for MPO (33, 34, 45).

All these clinical manifestations and laboratory features could be frequently gathered into two patterns: the *vasculitic* and ANCA-positive phenotype, characterized by manifestations resulting from small and medium-sized vessel vasculitis (e.g., purpura, mononeuritis multiplex, glomerulonephritis) and the *eosinophilic*, ANCA-negative phenotype, in which the organ is damaged mainly by an eosinophilic infiltration (e.g., pulmonary infiltrates, cardiomyopathy) (2). These findings may have pathogenetic implications, as they suggest that ANCA, as observed in MPO-ANCA mouse models, mediate vasculitis in EGPA as well; however, there are no animal models of EGPA. In addition, the ANCA-positive and ANCA-negative subsets are not clearly separated, as overlapping manifestations occur very frequently.

HISTOPATHOLOGY

The main histological findings in EGPA are the extravascular granulomas, small and medium-sized vessels vasculitis, and the eosinophilic infiltrates.

Interstitial and vascular granulomas are composed by eosinophilic necrotic matrix surrounded by giant cells and palisading lymphocytes. The vasculitic process affects mainly small and medium vessels (especially small arteries) and is characterized by fibrinoid necrosis of the vessel wall associated or not with granuloma or eosinophilic infiltrates (46, 47). It is difficult to find all these features together, which makes the histological diagnosis sometimes challenging (48). In addition, specific disease manifestations often show specific histopathological features; for example, purpura is caused by a leucocytoclastic vasculitis (eosinophilic infiltration or fibrinoid necrosis is frequently absent) and alveolar hemorrhage depends on an alveolar capillaritis (without granuloma) (36). Furthermore, glomerulonephritis (33) and peripheral neuropathy frequently lack eosinophilic infiltrates. Gastrointestinal biopsies reveal eosinophilic tissue infiltration and histological signs of mesenteric vessel vasculitis, which may induce bowel ischemia (42).

Cardiac involvement may show coronary vasculitis, myocardial granuloma, eosinophilic endomyocarditis, and pericarditis (36).

DIFFERENTIAL DIAGNOSIS

Different conditions have to be considered in the differential diagnosis, mainly eosinophilic and vasculitic diseases.

Parasitic infections as well as hypersensitivity reactions (e.g., to drugs) must be excluded. The hypereosinophilic syndrome (HES) is characterized by persistent eosinophilia and organ involvement without a reason, which can explain hypeosinophilia. Cardiac and pulmonary manifestations are analog to those of EGPA patients but subjects with HES usually do not have asthma or vasculitic complication like purpura or glomerulonephritis; furthermore, ANCA are absent in HES (49). A recent revised classification of HESs has focused on the pathogenesis of many hypereosinophilic disorders: myeloproliferative and lymphocytic forms of HES should be excluded in all patients. Particularly, Fip1-like-1(FIP1L1)/platelet-derived growth factor receptor α (PDGFRA) fusion genes must be investigated (50).

Broncho-pulmonary allergic aspergillosis may mimic pulmonary involvement in EGPA: differential diagnosis is helped by finding *Aspergillus* spp at bronchoscopy lavage or dosing *Aspergillus fumigatus* specific serum IgE, which are pathognomonic of allergic aspergillosis (51).

Acute eosinophilic pneumonia is featured by pulmonary infiltrates and bronchoscopy lavage rich in eosinophils but usually originates as an acute illness with fever and dyspnea, without peripheral eosinophilia or other organ involvement.

Chronic eosinophilic pneumonia diagnosis is more insidious. Patients may present with asthma, peripheral eosinophilia, and constitutional symptoms. The absence of other organ manifestations and the negativity of ANCA may help to differentiate chronic eosinophilic pneumonia from EGPA (52).

Eosinophilic granulomatosis with polyangiitis must be distinguished from the other AAVs. Granulomatosis with polyangiitis (GPA) may mimic particular aspects of EGPA, especially in those patients, which present peripheral eosinophilia, the ANCA specificity (cANCA PR3-specific, in GPA) and the presence, in GPA, of pulmonary cavitated nodules associated with nasal crusting and nasal and paranasal sinuses erosion, allow clinicians to differentiate the two vasculitides.

Although microscopic polyangiitis (MPA) could be also characterized by pANCA with MPO specificity, it rarely shows peripheral eosinophilia, nodules, or eosinophilic pulmonary infiltrates (48).

Finally, EGPA must be differentiated from IgG4-related disease (IgG4-RD), which may present with allergic manifestations, blood eosinophilia, pulmonary infiltrates, and sinusitis. However, tissue biopsies in patients with IgG4-RD show fibrosis and obliterative phlebitis, without vasculitis or eosinophilic granulomas (53).

In our center experience, first level examinations include blood tests and, in particular, complete blood cell count, ESR, CRP, immunoglobulins with their subclasses (especially IgG subclasses), rheumatoid factor, ANCA, eosinophil cationic protein (ECP), serum B12 levels (elevated in myeloproliferative neoplasms), and a screening of renal function and urinalysis. Detection of FIP1L1/PDGFRA fusion genes and stool cultures for ova and parasite examination must be done in the early stages of diagnosis. ANCA are thought to be useful in the differential diagnosis between EGPA and other (especially infectious and hematological) eosinophilic disorders. Likewise, finding fusion genes clearly points toward a diagnosis of myeloproliferative HES. The differential diagnosis with lymphocytic forms of HES is more challenging, as most laboratories do not perform clonal analysis of circulating lymphocyte subsets or their intracellular cytokine production, which could be helpful in these conditions.

Second level examinations include imaging studies such as lung and facial computed tomography (CT), as well as functional studies such as electromyography.

Finally, kidney biopsy and a bronchoscopy with bronchoalveolar lavage are reserved for those patients with severe (and often rapidly progressive) clinical manifestations.

TREATMENT AND OUTCOME

Eosinophilic granulomatosis with polyangiitis treatment is a matter of debate because of the lack of large-scale, randomized controlled trials. The five factors score (FFS) may be a guide for clinicians, this score assigns one point to each of the following items, namely, gastrointestinal involvement, CNS involvement, cardiac involvement, proteinuria >1 g/24 h and serum creatinine >141 μmol/L (35). Patients with poor prognosis factors (FFS ≥ 1) are often treated with both glucocorticoids (classically prednisone at dosage of 1 mg/kg of total body weight/day with a maximum dosage of 75 mg/day, for 1 month and then tapered) and cyclophosphamide (CYC, 2 mg/kg of total body weight/day), while the typical approach for patients with a better prognosis (e.g., FFS of 0) is glucocorticoid therapy alone (54). Recently, a revised FFS has been proposed an age over 65 years, cardiac symptoms, gastrointestinal involvement, renal insufficiency (serum creatinine >150 μmol/L) and absence of ear, nose, and throat manifestations have been pointed out as predictors of 5-year mortality (55).

Classically, used therapies in EGPA remission maintenance are azathioprine or methotrexate (56).

Although primarily used for GPA, the BVAS, a clinical index of disease activity (57), might be useful to better decide when to stop therapy with CYC and introduce maintenance therapy like azathioprine or methotrexate.

Cyclophosphamide toxicity has long been known (58) and, based on our center experience, we recommend not to exceed the dose of 10–15 g of CYC (including both oral and pulse medications). On the other hand, too-short duration of CYC administration has been associated with more relapses (59).

Azathioprine too requires a constant monitoring of liver function, due to the drug-related hepatotoxicity (60).

B-cell depletion adjunct therapy with rituximab has shown promising results for remission induction (61–67).

Interleukin-5, a major survival factor for eosinophils, has been targeted in patients with EGPA using the monoclonal antibody mepolizumab. Use of mepolizumab in refractory cases (68, 69) and steroid-dependent patients (70) has given positive results but EGPA manifestations recurred on drug cessation.

On the assumption of its inhibitory effects on the eosinophil degranulation, interferon-alpha therapy has been tried with positive results in refractory patients, but the severe drug-related toxicity has greatly limited its use (71, 72).

Plasmapheresis may be an adjunctive therapy particularly in patients with rapidly progressive glomerulonephritis, peripheral neuropathy, or alveolar hemorrhage (2).

Eosinophilic granulomatosis with polyangiitis outcomes are well represented in a retrospective study of 383 EGPA patients in the French Vasculitis Study Group cohort. Vasculitis relapse occurred in 97 patients (25.3%), while 72 additional patients experienced asthma flares, sinusitis, and/or increased eosinophilia. Of the 383 patients, 45 (11.7%) died and the major cause of death was attributed to cardiac events. Five-year and 10-year survival rates were, respectively, 88.9 and 78.6%. Vasculitis relapse-free survival rate at 5 years was 64.8%, while at 10 years was 54.4%. ANCA positivity and cutaneous signs were independent predictors of relapse (30).

Another recent analysis of EGPA patients' long-term follow up has demonstrated that the outcome of EGPA is good with respect to mortality. According to the analysis of 118 patients with EGPA (enrolled in two prospective trials), 108 (91.5%) patients achieved remission (34 of the 108 achieved long-term remission without relapse) and 12 (10.2%) died (only 5 of them died for EGPA-related causes). During relapses, pulmonary symptoms predominated (81%), followed by ear nose and throat signs (38%) and mononeuritis multiplex (36%) (73).

Finally, in a German cohort of 150 EGPA patients, the analysis of the follow-up of 104 of them has evidenced that 70 patients (67.3%) attained remission after conventional therapies, 21 (14%) suffered from major relapses and 42 (28%) from minor relapses. Twelve patients died 94 ± 16 (mean $\pm$ SD) months after diagnosis (74).

PERSPECTIVE FUTURE

Despite the great levels of knowledge reached, more has to be done to clarify EGPA pathogenesis, a genome-wide association study (GWAS) will probably help to better understand the genetic determinants of the disease. Besides, the environmental factors like silica or any other occupational exposure (e.g., asbestos) must be studied in depth.

In the future, probably, the distinction between $ANCA^+$ and $ANCA^-$ small vessels vasculitides will lead to re-define the current classification criteria with a more simplistic view of all the AAVs.

Despite this, clinicians should keep in mind all the distinctive clinical features and differential diagnosis approaches that make EGPA one of the more characteristic and complex AAV.

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Non-invasive imaging of vascular inflammation

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In large-vessel vasculitides, inflammatory infiltrates may cause thickening of the involved arterial vessel wall leading to progressive stenosis and occlusion. Dilatation, aneurysm formation, and thrombosis may also ensue. Activated macrophages and T lymphocytes are fundamental elements in vascular inflammation. The amount and density of the inflammatory infiltrate is directly linked to local disease activity. Additionally, patients with autoimmune disorders have an increased cardiovascular (CV) risk compared with age-matched healthy individuals as a consequence of accelerated atherosclerosis. Molecular imaging techniques targeting activated macrophages, neovascularization, or increased cellular metabolic activity can represent effective means of non-invasive detection of vascular inflammation. In the present review, novel non-invasive imaging tools that have been successfully tested in humans will be presented. These include contrast-enhanced ultrasonography, which allows detection of neovessels within the wall of inflamed arteries; contrast-enhanced CV magnetic resonance that can detect increased thickness of the arterial wall, usually associated with edema, or mural enhancement using T2 and post-contrast T1-weighted sequences, respectively; and positron emission tomography associated with radio-tracers such as [^{18}F]-fluorodeoxyglucose and the new [^{11}C]-PK11195 in combination with computed tomography angiography to detect activated macrophages within the vessel wall. Imaging techniques are useful in the diagnostic work-up of large- and medium-vessel vasculitides, to monitor disease activity and the response to treatments. Finally, molecular imaging targets can provide new clues about the pathogenesis and evolution of immune-mediated disorders involving arterial vessels.

Keywords: vasculitis, non-invasive imaging, contrast-enhanced ultrasound, vascular inflammation, positron emission tomography, cardiovascular magnetic resonance

INTRODUCTION

Vasculitides are conditions defined by the presence of inflammation of the vessel wall, with progressive alteration of the lumen, including lumen stenosis, occlusion, or even aneurysmal dilation. They can be broadly divided into infectious vasculitides, characterized by direct invasion of pathogens in the vessel wall and non-infectious vasculitides. The latter, also known as primary vasculitides, encompass a heterogeneous group of immune-mediated disorders, classified, according to the size of vessels that are predominantly involved, into small-, medium-, and large-vessel vasculitides (LVV) (1). The diagnosis of small and medium vessels vasculitis is generally based on clinical findings, serological markers, and histological evaluation (2). LVV include giant cell arteritis (GCA), Takayasu arteritis (TAK), primary central nervous system vasculitis (PCNSV), and chronic periaortitis (CP) (3). The most common LVVs are GCA and TAK in which the aorta and its main branches are generally involved. GCA occurs more frequently in older adults, showing a predilection for the temporal artery and other extracranial vessels (4). Clinical manifestation of this disease ranges from ischemic symptoms and signs (such as jaws claudication) to aneurysmal rupture (4, 5). TAK is more prevalent in adolescent girls and young women, with a strong predilection for the aortic arch and its branches, in particular the subclavian arteries (up to 98%) and common carotid, although also pulmonary and coronary arteries (<10%) may be involved. The involvement of the major branches of the aorta is much more marked at their origin than distally, leading to clinical manifestations that ranges from arm claudication to myocardial infarction. Different classification criteria for LVV have been defined, and all of them are based upon clinical presentation, evidence of inflammation, and vascular abnormalities (6–8). However, they have proven largely unsatisfactory for diagnostic purposes (9, 10), frequently leading to delayed diagnosis (11). Even once the diagnosis is established, accurate monitoring of disease activity and response to therapy is not an easy task (5). Extensive clinical evaluation of the arterial tree is currently recommended (12), and imaging plays a major role in this setting. *In vivo* detection of inflammation within affected blood vessels may provide a reliable tool to assess disease activity, leading to better clinical management of the patient. Although imaging techniques are particularly useful to diagnose and monitor LVV, they can also play a role in the work-up of medium-vessel vasculitides, classically represented by polyarteritis nodosa (PAN) in

Table 1 | Features of main non-invasive imaging technique for vascular imaging.

Imaging technique	Form of energy	Spatial resolution (mm)	Availability
Ultrasonography	High frequency sound waves	0.1-1	Widespread
CT	X rays	0.3-1	Widespread
MRI	Radiofrequency waves	0.2	Large centers
PET	Photons annihilation	4-6	Large centers

PET: positron emission tomography; CT: computed tomography; MRI: magnetic resonance imaging.

adults and Kawasaki disease (KD) in children (13, 14). In contrast, current imaging techniques are unable to visualize small vessels, thus we will focus on the role of imaging studies in diagnosing and monitoring LVV, including some applications in medium-vessel vasculitides and autoimmune systemic disorders with potential vascular involvement such as Behcet's disease.

Inflammation of the vascular wall is characterized by different pathological changes, such as edema, vasa vasorum activation and proliferation (15, 16), alteration of endothelial homeostatic function, and immune cells infiltration (4, 17, 18), ultimately leading to anatomical remodeling with consequent functional alteration. Knowledge of the biological basis of these processes has led to the development of imaging strategies aimed at identifying them *in vivo*, mainly with the development of probes directed to key molecular targets (19). Imaging techniques allowing for molecular imaging include ultrasonography, positron emission tomography (PET), most often associated with computed tomography (CT), and magnetic resonance imaging (MRI), all of which will be discussed in this review. **Table 1** summarizes the main features of the imaging techniques used to study patients with vasculitides. Furthermore, inflammation of arterial vessels is a fundamental pathogenetic element in atherosclerosis and associated clinical manifestations. Compelling evidence of the link between atherogenesis and inflammation has built over the last decades, leading to the current hypothesis that atherosclerosis is not merely a disease due to passive lipid accumulation in the vascular wall but an active, immune-driven process (17, 18). Not surprisingly, many diseases associated with systemic inflammation due to immune alterations are associated with increased risk of cardiovascular (CV) morbidity and mortality due to atherosclerosis (20, 21) that cannot be fully explained by traditional CV risk factors, suggesting a role for immune activation (22). Therefore, characterization of inflammation within atherosclerotic plaques by means of molecular imaging may identify patients at risk for disease progression or acute clinical manifestations (23, 24).

ULTRASOUND

Ultrasound imaging is widely available, inexpensive, and repeatable and does not involve the use of ionizing radiation. It is generally performed by an experienced sonographer using high-quality Doppler ultrasound equipment and linear probes > 8 MHz (25, 26). Examination typically comprises B-mode ultrasonography, which depicts anatomy using a gray scale, and Duplex ultrasound, which combines color Doppler ultrasound and pulsed Doppler ultrasound to display information about blood flow and to estimate blood flow velocities (26). The main limitation of ultrasound imaging is that it cannot depict structures below bone or air. For this reason, it does not provide reliable information about the thoracic aorta, unless performed via a transesophageal approach. In addition, acquisition of ultrasound images is operator dependent, although studies of vascular ultrasound have shown high rates of interoperator agreement (27, 28). In the following sections, we will review the main ultrasonographic findings in blood vessel inflammation, which are summarized in **Tables 2** and **3**.

COLOR DOPPLER ULTRASOUND

Schmidt et al. pioneered the use of ultrasonography in LVV. They showed that inflamed temporal arteries in GCA were characterized by a dark hypoechoic circumferential wall thickening dubbed the halo sign, which appeared to be edema of the vessel (29). The investigators reported a sensitivity of 73% and a specificity of nearly 100%. Subsequently, temporal artery ultrasonography has increasingly been used to screen patients with suspected GCA. An early meta-analysis showed a sensitivity of 69% and a specificity of 82% for the halo sign using temporal artery biopsy as the reference standard (30). Subsequent meta-analysis showed a sensitivity of 69% and a specificity of 91% (31), and a sensitivity of 69% and a specificity of 89% (32), respectively, when American College of Rheumatology (ACR) criteria were used as reference standard. Interestingly, specificity increased to 100% when presence of the halo sign was bilateral (31). As expected, the quality of the equipment used to perform the examination as well as operator experience was shown to affect diagnostic power of the halo sign (30–32). The halo sign can also be found in inflamed large vessels, which have been shown to be involved in approximately one-third of patients affected by GCA (33, 34). Detection of the halo sign in large vessels further increases ultrasound sensitivity to nearly 100% (35, 36). The equivalent of the halo sign in TAK is called the macaroni sign, a circumferential midechoic thickening of the blood vessel wall (37). Both of these echographic findings have been shown to fade and disappear after initiation of steroid therapy during patients follow-up (38–40). Aside from edema, ultrasound evaluation of the vessel wall may show other vascular alterations. Evaluation of carotid artery wall thickness, i.e., carotid artery intima-media thickness (CIMT), in particular has proved a reliable surrogate measure of atherosclerotic burden and CV risk in the general population (41). CIMT (**Figure 1**) was shown to be significantly increased in patient affected by a variety of rheumatic diseases: this may represent the result of a multifactorial process in which age and other traditional CV risk factors (i.e., systolic blood pressure, low-density lipoprotein (LDL), cholesterol levels, and body mass index) make a continuous contribution, and interact with inflammation and immunological factors (42, 43). Clear depiction of blood vessel lumen allows detection of stenosis or aneurysmal dilation, which appear as alterations in wall profile as well as flow abnormalities, making ultrasound a valuable tool for monitoring vasculitides complications (3).

Table 2 | Summary of main ultrasonographic findings in inflamed blood vessel, together with their pathological correlate and clinical significance.

Sign	Alteration	Pathological correlate	Clinical significance	Vasculitis	Reference
Halo sign or Macaroni sign	Hypoechoic concentric thickening of blood vessel wall	Edema	High sensitivity and specificity for LVV diagnosis; potential role for follow-up	GCA (halo sign) and TAK (macaroni sign)	(29–32, 38–40)
Increased common carotid artery intima-media thickness (IMT)	Thickening of common carotid artery vessel wall	Vascular remodeling under pathological stimuli	Increased risk for CV events	All conditions associated with high CV risk, including vasculitis	(41–43)
Stenosis, occlusion or aneurysmal dilation	Reduction (stenosis and occlusion) or increase in vessel caliber; flow alterations	Advanced pathological remodeling of blood vessels	Cause of ischemic symptoms and signs; risk of aneurysmal rupture; patient follow-up	All	(3, 5)
Adventitial neovessels with contrast-enhanced ultrasound (CEUS)	Moving bright spots on the adventitial layer of the vessel wall after microbubbles administration	Neoangiogenesis due to inflammation*	May correlate with vasculitis activity	TAK and GCA	(48–50)

**Correlation with neovessel formation has been demonstrated for atherosclerosis. Pathological correlation studies for vasculitides have not yet been performed.*

CONTRAST-ENHANCED ULTRASOUND

Several different formulations of ultrasound contrast agents exist. They share the feature of being micro or nano sized, gas-filled particles, known as microbubbles, which remain in the vascular compartment. They generate a hyperechogenic signal because they resonate, i.e., cyclically expand and contract, or release gas when insonated at frequencies used by ultrasound imaging systems (44). When applied to vascular imaging, contrast-enhanced ultrasound (CEUS) is able to enhance the lumen, improving delineation of blood vessel wall (45). In addition, microbubbles allow detection of adventitial neovessels (46), which is a potential marker for atherosclerotic plaque instability (47). Recently, CEUS has been proposed as a potentially useful imaging modality in assessing disease activity in LVV. In early reports by Giordana et al. (48) and Magnoni et al. (49), carotid CEUS was used to diagnose TAK and monitor response to treatment. The authors initially observed circumferential wall thickening in the common carotid artery with multiple vasa vasorum. After treatment, carotid CEUS was repeated and showed progressive reduction in vessel wall and vasa vasorum enhancement, suggesting decrease of inflammatory activity in the carotid artery. CEUS improved definition of borders of the vascular lesion and demonstrated the presence of large amount of contrast, visualized by moving bright spots, on the adventitial side of vascular lesions (**Figures 2A,B**). The latter phenomenon was interpreted as a signal coming from neovessels. More recently, Schinkel et al. (50) confirmed these results in a pilot study involving seven patients, of which five were affected by TAK and two by GCA. They showed that CEUS significantly improved image quality as compared to standard color Doppler ultrasound and allowed detection of vascularization in carotid vessel wall. CEUS can be employed for molecular imaging (51, 52); unlike freely circulating microbubbles used for vascular opacification, targeted microbubbles are designed to adhere to the endothelium through specific interactions. The adhesion is then detected as increase in echogenicity, which persists after circulating bubbles have been washed away in the site where molecular target is localized. Examples of targets successfully visualized in preclinical experimental models with molecular targeted CEUS include leukocyte adhesion molecules including ICAM-1, VCAM-1, and P-selectin. In the future, this technology will provide the possibility of directly visualizing pathophysiologic events, including inflammatory changes, occurring in the patient's blood vessels.

The fact that proliferation of vasa vasorum and intimal neovascularization may play a role in atherosclerosis was clearly shown by postmortem studies by Barger et al. (53), who showed that atherosclerotic segments of coronary arteries present a rich vascular network extending from the adventitia to the intima. Atherosclerotic plaque neovascularization may contribute to the progression of a fibrotic, stable lesion to an unstable lesion at high risk of rupture (47, 54). Visualization of intra-plaque neovascularization may therefore provide a way to identify high risk, vulnerable plaques. Detection of blood vessel wall enhancement by CEUS was shown to correlate well with histological evidence of plaque neovascularization, as defined by presence of CD31 positive cells (46, 55). This technique may thus have a relevant future role in risk assessment of atherosclerotic plaques also in patients with immune-mediated disorders.

TRANSESOPHAGEAL ECHOCARDIOGRAPHY

Transesophageal echocardiography (TEE) is a semi-invasive ultrasound imaging technique that allows high-quality evaluation of the heart and the aortic root. The exam is carried out using an ultrasonographic transducer mounted on the tip of a modified steerable gastroscope inserted in the esophagus (56). This

Table 3 | Summarizes key studies concerned with imaging of inflammation in blood vessels in LVV, and their main results.

Technique	Study	Number of patients	Results	Reference
Color Doppler ultrasound	Ball et al., The British Journal of Surgery, 2010	998	Meta-analysis of 17 studies showing a sensitivity of 69% and a specificity of 89% for the halo sign in temporal artery	(32)
	Arida et al., BMC Muscoloskeletal Disorders, 2010	504	Meta-analysis of 8 studies showing a sensitivity of 68% and a specificity of 91% for the halo sign in temporal artery	(31)
	Maeda et al., Ultrasound Med Mol, 1991	23	"Macaroni" sign detected carotid artery involvement in 19 out of 23 patients with TAK	(37)
	Habib et al., Clin Rheumatol, 2012	32	Halo sign decreases after a mean of 21 days from beginning of therapy	(39)
Contrast-enhanced ultrasound	Schinkel et al., European Heart Journal Cardiovascular Imaging, 2013	7	Ultrasonographic contrast allowed better delineation of carotid arteries lesions. It also allowed vessel wall neovascularization in five out of seven patients (TAK or GCA)	(50)
Transesophageal ultrasound	Bezerra Lira-Filho et al., Journal of the American Society of Echocardiography, 2006	14	71% of thoracic aorta segments were found to be thickened, and 37% dilated in the 14 TAK patients studied by transesophageal echocardiography	(60)
	Espinola-Zavaleta et al., Echocardiography (Mount Kisco, NY). 2005	15	In the studied TAK patient cohort, 67% of patients had aortic regurgitation, 60% mitral or tricuspid regurgitation and 33% reduced coronary reserve measured with contrast enhancement	(61)
CT angiography	Khandelwal et al., European Journal of Radiology, 2011	15	CT angiography showed variable thickening of aorta and main branches in patients with active TAK	(65)
	Prieto-Gonzalèz et al., Annals of the Rheumatic Diseases, 2012	40	CT angiography was able to detect large-vessel involvement in 67% of patients with GCA. The proportion was higher for treatment naïve patients (77% vs 29%)	(73)
	Kang et al., Radiology, 2014	111	53% of patients had coronary artery involvement, while only 29% were symptomatic for heart disease	(72)
PET using FDG	Besson et al., European Journal of Nuclear Medicine and Molecular Imaging, 2011	101	Meta-analysis of six studies on patients with GCA, showing a sensitivity of 80% and a specificity of 89% for FDG-PET	(81)
	Blockmans et al., Arthritis and Rheumatism, 2006	35	Vascular FDG uptake was shown in 83% of 35 patients with GCA. It decreased after 3 months of effective therapy, but no further decrease was documented at 6 months follow-up	(88)
	Fuchs et al., European Journal of Nuclear Medicine and Molecular Imaging, 2012	30	PET was shown to increase diagnostic accuracy for LVV from 54 to 71%	(87)

(Continued)

Table 3 | Continued

Technique	Study	Number of patients	Results	Reference
PET with PK11195	Pugliese et al., Journal of the American College of Cardiology, 2010	15	PET/CT allowed visualization of tracer uptake in the vessels of all the six patients with active disease, but in none of the controls	(96)
Magnetic Resonance Imaging	Mavrogeni, J Am Coll Cardiol, 2004	13	Agreement between bright-blood MRI angiography and coronary X-ray angiography in identifying coronary aneurysms in KD	(106)
	Comarmond, Am J Cardiol, 2014	27	Myocardial ischemia detected by LGE at CMR was > 5 × greater in patients with TA compared to matched controls	(133)
	Li, J Comput Assist Tomogr, 2011	42	Whole body MRI; wall thickness and post-contrast signal higher in TAK patients with active disease	(134)
	Koenigkam-Santos, J Clin Rheumatol, 2011	28	GCA/polymyalgia rheumatica; CE-MRA identified extracranial involvement with good interobserver agreement	(135)
	Mavrogeni, Inflamm Allergy Drug Targets, 2013	28	CMR in Churg–Strauss syndrome showed cardiac involvement, with worse prognosis in presence of diffuse sub-endocardial fibrosis	(136)

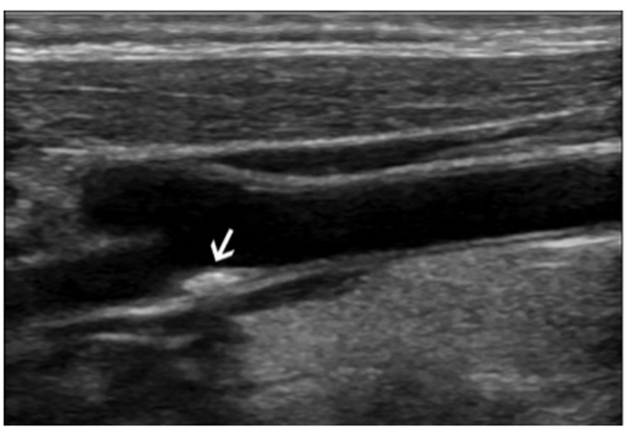

FIGURE 1 | Ultrasound imaging of the right carotid artery bifurcation of a 44-year-old woman with a 10 years history of systemic lupus erythematosus: the arrows show an atherosclerotic plaque extending toward the internal carotid artery.

technique allows correct identification of eventual thoracic aorta and coronary arteries involvement in LVV. Although several reports of utilization of TEE in the evaluation of LVV, especially in the setting of perioperative evaluation (57–59), few clinical studies have been performed using this technique. Bezerra Lira-Filho and colleagues described the most common lesions in 14 patients affected by TAK compared with age-matched controls: aortas of the patients were found to be thicker, more dilated, and stiffer as compared with controls (60). In another study, Espinola-Zavaleta et al. assessed coronary reserve in 15 patients with TAK using contrast-enhanced TEE: 33% of the patients were found to have reduced coronary reserve, while aortic and mitral valve regurgitation was found in 67% and 60%, respectively (61). CEUS can also improve the TEE (62).

CT AND CT ANGIOGRAPHY

Computed tomography is well suited to demonstrate pathological changes in large, deep blood vessels. While it is a widely available and reproducible technique, it involves the use of ionizing radiation and carries a risk connected to the use of iodinated contrast material although radiation dose to the patient has declined steadily in the past few years due to different technological advances (63). CT angiography is able to show alterations both in the wall and in the lumen of affected vessel in LVV (64). In early vasculitis, concentric mural thickening of the involved arteries is typically observed (65, 66). On pre-contrast scanning, the mural thickening has a higher attenuation as compared to the lumen, while in enhanced images it displays a double ring enhancement pattern, most evident in the venous phase (67). In particular, the wall shows a poorly enhanced inner ring and a more obviously enhanced outer ring; it has been proposed that the inner ring represents a swollen intima while the outer one represents active inflammation in the intima and in the media (67, 68). Mural enhancement usually resolves after successful treatment,

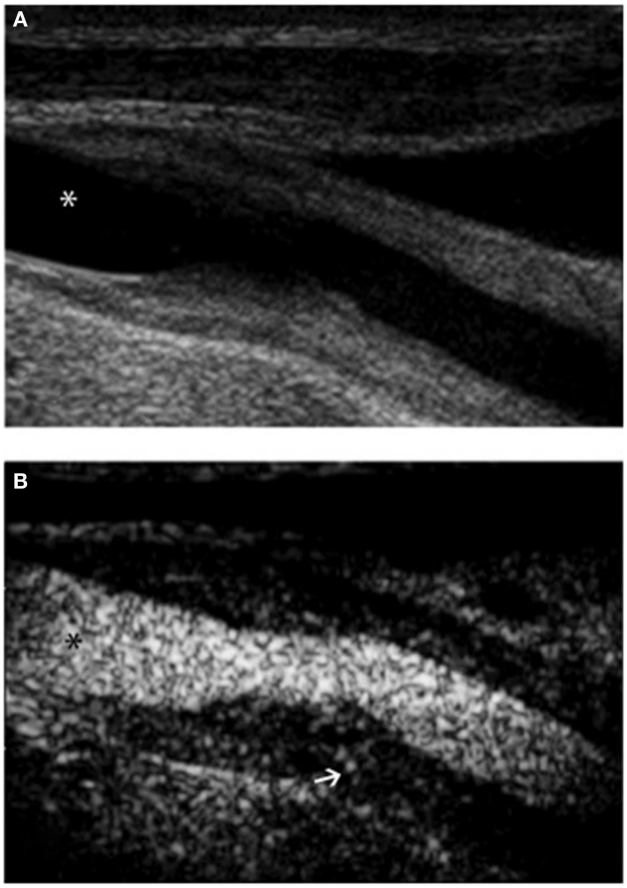

FIGURE 2 | Ultrasound examination of right proximal common carotid artery of a patient affected by Takayasu arteritis. B-mode ultrasound **(A)** shows long, smooth concentric thickening of the arterial wall. Contrast-enhanced ultrasound **(B)** using Optison (GE Healthcare, Little Chalfont, UK), a contrast media made up of human albumin microbubbles filled with perflutren, improves definition of the lesion border. Extensive enhancement can be seen within the vessel wall (arrow). In both panels, asterisk marks the vessel lumen.

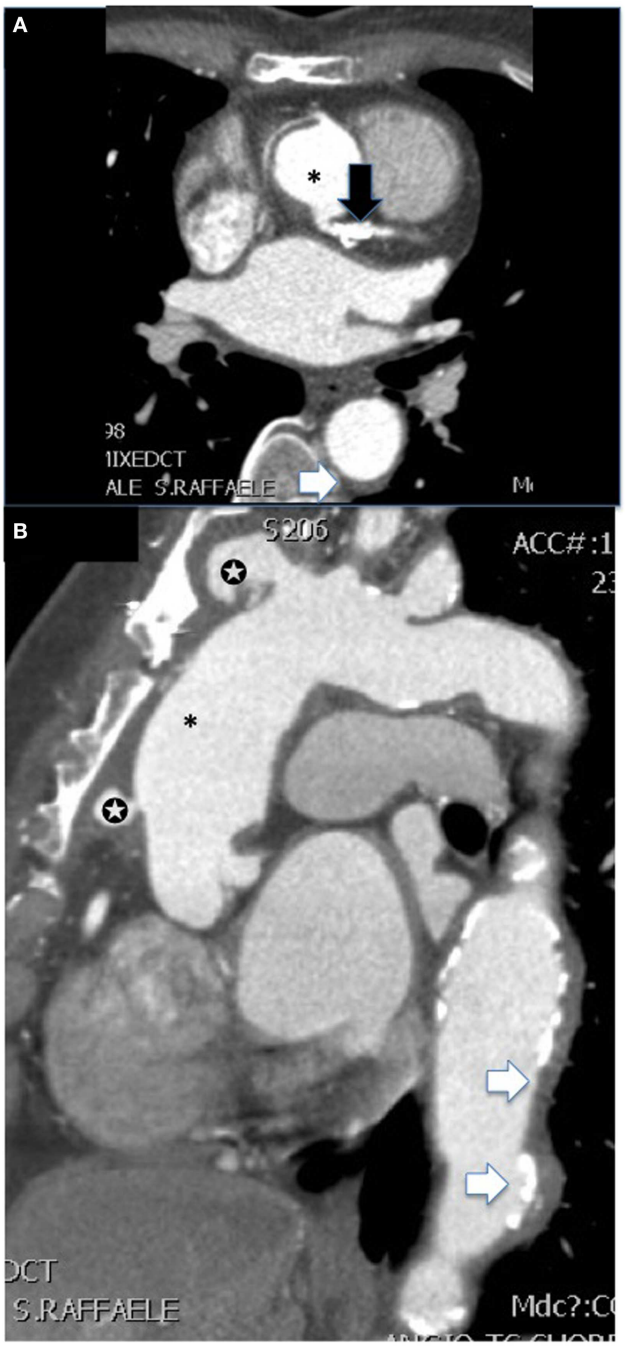

FIGURE 3 | CT angiography [(A) shows axial and (B) sagittal view] of the aorta of a female patient with advanced Takayasu arteritis who underwent previous surgical repair of an ascending aorta aneurysm (*). Several features of the disease are summarized in these images: post-surgical complications such as pseudoaneurysms at the level of proximal and distal anastomosis of the vascular graft with the native aorta (white stars), extensive concentric mural thickening with calcifications of the aorta (white arrows), and presence of a stent in the main steam with a calcific stenosis (black arrow).

although its improvement may lag behind clinical and laboratory improvement (69, 70). In advanced disease, CT angiography shows typical late stage complications such as aneurysms, vessel stenosis, or occlusion (66) (**Figure 3**). Compared with conventional angiography, CT was able to accurately assess stenotic lesions in brachiocephalic trunks, carotid arteries, and subclavian arteries in patients with TAK, with a sensitivity and specificity of 93% and 98%, respectively (71). In addition, coronary arteries assessment with CT was able to show coronary artery lesions in 53% of 111 TAK patients, while only 29% had cardiac symptoms (72). The most common lesions were ostial stenosis, non-ostial stenosis, and coronary aneurysms. In another study, CT was able to detect large-vessel involvement in 27 of 40 patients affected by GCA (73).

PET AND PET/CT

Imaging with PET offers unrivaled sensitivity and specificity for research into tissue perfusion, biochemical pathways, and pharmacological mechanisms *in vivo*. The success of PET is founded on the properties of positron emitters. Their short physical half-lives make it possible to administer a tracer dose high enough to obtain useful data, but such that the radiation burden to the patient is acceptably low. A tracer is a measurable substance used to mimic,

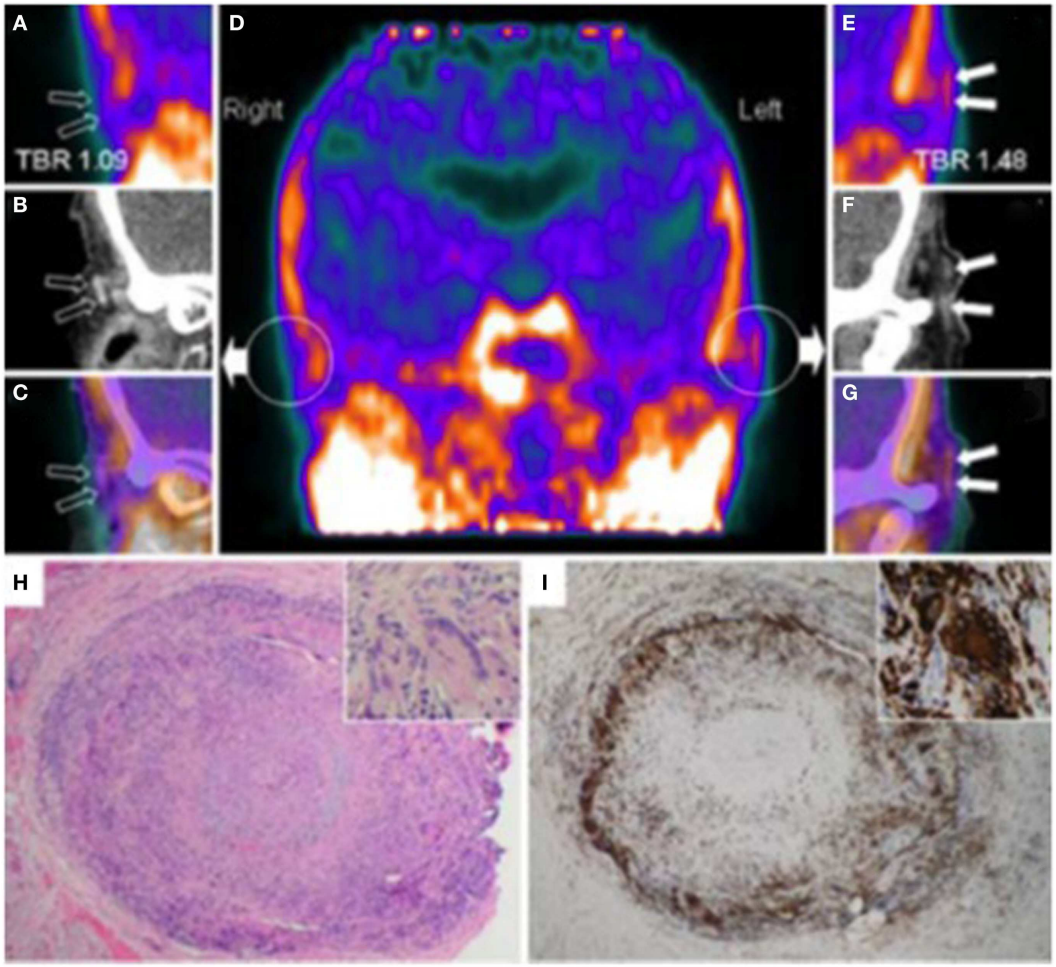

FIGURE 4 | Hybrid PET with PK11195 and CT angiography imaging of an 88-year-old woman presenting with left scalp tenderness, jaw claudication, and night sweats. The coronal reconstruction **(D)** and magnifications of PET **(A,E)**, and contrast-enhanced CT **(B,F)** and PET/CT fusion images **(C,G)** show focal PK11195 uptake in the left temporal artery above the zygomatic process (solid arrows) compared to the contralateral artery (open arrows). The arterial target-to-background ratio (TBR) was higher in the left than in the right temporal artery. On CT angiography, the left temporal artery lumen (1.8 mm diameter) was irregular with reduced contrast opacification compared with the contralateral (2.2 mm diameter). The H&E biopsy specimen **(H)** of the left temporal artery (4 × objective) shows transmural granulomatous infiltration (containing activated lymphocytes, macrophages, and multinucleated giant cells), secondary myofibroblast proliferation, and significant intimal thickening leading to luminal obliteration. Large part of the media and the internal elastic lamina are destroyed. The inset (H&E, × 100 objective) demonstrates a multinucleated giant cell. CD68 (macrophage marker, shown in panel I) staining brown with immunoperoxidase (×4 objective) shows dense macrophage infiltration with multinucleated cells (inset, ×100 objective). [adapted with permission from Springer]

follow or trace a chemical compound or process without disturbing the process under study. In the case of PET, this is made possible by: (1) the high sensitivity of PET imaging which enables the measurement of radiolabeled tracers administered in picomolar concentrations, which are sufficiently low so as not to disturb the processes under study; and (2) the ability of current PET scanners to perform rapid dynamic imaging and/or list mode acquisitions that provide good temporal resolution. Although it shows great sensitivity to even small amounts of probe mass, PET has a relatively poor spatial resolution (23), compared to CT and MRI. For this reason, coregistration of PET images with contrasted CT images has been developed, to provide good anatomical localization of functional data (74). Using this technique, Gaemperli et al. first reported detection of temporal artery inflammation using PET in a patient with GCA (**Figure 4**) (75).

PET AND PET CT USING FDG

[18F]-fluorodeoxyglucose (FDG) is a radiolabeled glucose analog, which competes with glucose for transport across the sarcolemma and phosphorylation by hexokinase and is avidly accumulated by metabolically active cells. Currently, PET imaging with FDG plays a major role in the management of cancer patients (76). Activated inflammatory cells also overexpress glucose transporters and extract increased amounts of glucose and hence FDG (77). In large-vessel vasculitides, FDG uptake in the vascular wall is increased. The uptake is commonly classified on a four-point

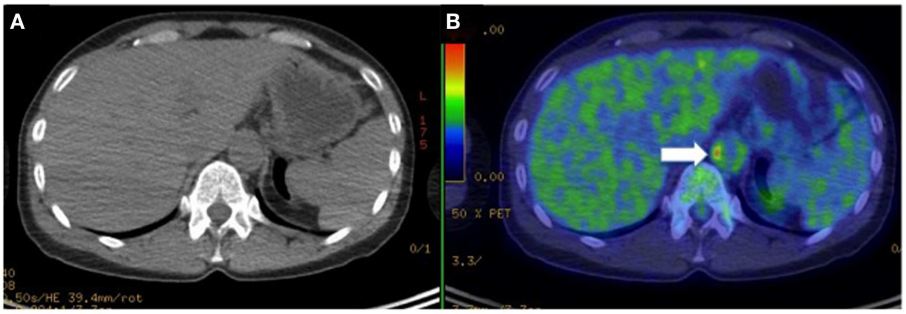

FIGURE 5 | Hybrid CT (A) with PET imaging (B) to identifies [^{18}F]-fluorodeoxyglucose (FDG) accumulation in the vascular wall of abdominal aorta (arrow) in a female patient with Takayasu arteritis.

visual grading system: no uptake (grade 0), less than liver uptake (grade 1), uptake similar to that of the liver (grade 2), and uptake higher than the liver (grade 3) (78). Grades 2 and 3 appear to be specific for vasculitis (79). Alternatively, the semi-quantitative aorta to liver ratio proposed by Hautzel et al. provides an excellent global performance for the diagnosis of GCA, with a sensitivity of 89% and a specificity of 96%, together as robustness as an observer-independent method (80). According to a recent meta-analysis, FDG-PET has a sensitivity of 80% and a specificity of 89% in the diagnosis of GCA, using ACR criteria as reference standard (81). FDG-PET was also shown to be useful in early diagnosis of TAK, showing a sensitivity ranging from 65 to 100% and high specificity (82, 83). Combination of PET with CT imaging was shown to increase diagnostic yield for LVV, allowing for better visualization of tracer accumulation in the vascular wall (84, 85) (see **Figure 5** as an example of female patients with inflammation localized at the abdominal aortic wall identified by accumulation of FDG) and visualization of small diameter arteries such as temporal artery (86). Diagnostic accuracy of FDG-PET dramatically decreases after initiation of appropriate therapy (87), providing a valuable tool for disease monitoring (88, 89). Furthermore, PET was shown to reliably predict GCA complications, e.g., aortic dilation (90), but it was unable to identify patient at higher risk of relapse (88). A recent study formally assessed the impact of FDG-PET on the diagnosis of LVV (87). A panel of international experts was asked to diagnose and manage patients with suspect LVV either with or without having access to PET results: PET was shown to significantly increase diagnostic accuracy from 54 to 71%.

PET/CT USING [^{11}C]-PK11195

[*N*-methyl-^{11}C]-(R)-1-(2-chlorophenyl)-*N*-(1-methylpropil)-3-isoquinoline carboxamide, also known as [^{11}C]-PK11195, is a radiolabeled ligand that specifically binds to the translocator protein (TSPO, 18 kDa), formerly known as peripheral benzodiazepine receptor (91). TSPO has been shown to be present at high density in circulating human phagocyte populations, in particular in monocytes and neutrophils (92). Stimulated human monocytes further increase TSPO expression, indicating that its overexpression associates with activation of phagocytes (93). Over the last two decades, [^{11}C]-PK11195 has been extensively used in a number of neuroinflammatory and neurodegenerative conditions due to its high uptake in activated microglia and low uptake in neurons (94). Subsequent reports have demonstrated [^{11}C]-PK11195 uptake in synovial macrophages of patients affected by rheumatoid arthritis (95). Pugliese et al. first applied [^{11}C]-PK11195 in LVV imaging in a small, proof-of-principle study involving 15 patients, predominantly affected by TAK and GCA (96). In this study, all five patients with clinically active vasculitis had markedly increased vascular uptake of the tracer as compared with patients with quiescent disease. Standardized uptake values for [^{11}C]-PK11195 correlated well with quantitative total intra-plaque volumes of distribution, calculated from dynamic tissue kinetic modeling using a one-tissue compartment model (97). In one patient, PET/CT images were obtained after a 20-week course of oral corticosteroids: vascular uptake of [^{11}C]-PK11195 was markedly reduced, and the reduction was paralleled by clinical improvement and decrease in biochemical markers of inflammation (96). The estimated effective dose for CT was 6.0 ± 0.5 mSv and 2.1 ± 0.2 mSv for PET scan and the mean total effective dose was 8.1 ± 0.6 mSv (96). These values are comparable to those of a cardiac FDG scan (98). Finally, [^{11}C]-PK11195 uptake was found to correlate with presence of inflammatory cells in atherosclerotic plaque specimens obtained by carotid artery endarterectomy (99). In the same study, carotid uptake of the radiotracer was higher in patients who had suffered from ischemic stroke, suggesting that this technique may be useful in detecting unstable high-risk plaque (24, 99). Despite these promising results, widespread use of [^{11}C]-PK11195 may be limited by its short physical half-life (20 min), which mandates an on-site cyclotron facility. New ^{18}F-labeled TSPO ligands, now undergoing preclinical investigation, may overcome some of these barriers (100).

MAGNETIC RESONANCE IMAGING

Magnetic resonance imaging is increasingly recognized as a valuable tool in the work-up of patients affected by large- and medium-sized arteries vasculitis (101). MRI has the advantage of avoiding radiations and nephrotoxic contrast medium, while allowing a high-resolution characterization of both vessel wall and lumen. Moreover, systemic vasculitis is increasingly being linked to the occurrence of heart failure secondary to myocardial damage and cardiac magnetic resonance (CMR) has proven to be a refined diagnostic procedure to evaluate both cardiac function and myocardial

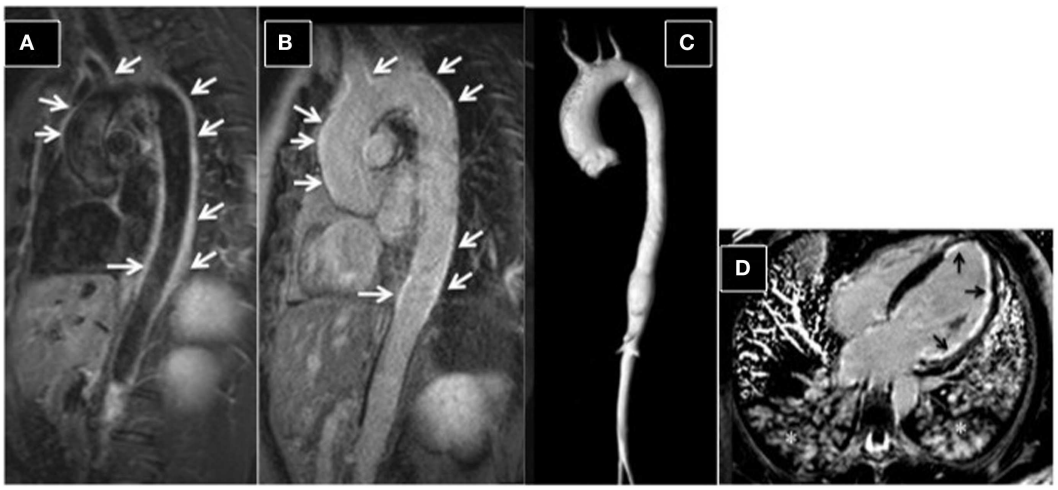

FIGURE 6 | Aortitis in Behcet's disease. **(A)** STIR T2 dark-blood imaging showing extensive edema of the aortic wall (arrows). Post-contrast late-enhancement imaging **(B)** shows extensive enhancement of the aortic wall (arrows). A typical Takayasu aortitis presenting with cardiac arrest and acute myocardial infarction in a 16-year-old girl. **(C)** 3D-MRA reconstructed image, showing dilation of the ascending aorta and narrowing of the descending aorta. **(D)** Post-contrast four-chamber view, showing extensive sub-endocardial and focally nearly transmural myocardial late enhancement (ischemic late-enhancement pattern) of the apical and lateral left ventricular walls. Asterisks highlight the presence of pulmonary edema; the patient was intubated during the scan.

tissue characterization (102). Avoidance of radiations and tissue characterization capability render MRI an ideal tool not only in defining diagnosis and disease extension but also in evaluating response to treatment and in follow-up. One of the strengths of MRI is the capability of tissue characterization even in basal conditions, before the administration of contrast, so a typical scan includes a pre- and a post-contrast phase; in patients in whom a cardiac examination is also performed, these two steps pertain also to heart evaluation.

PRE-CONTRAST MRI TECHNIQUES

1. Dark-blood morphologic images (HASTE; T1 or T2 turbo spin-echo images), acquired in the axial, sagittal, and coronal planes); in these images blood appears black, while the vessel wall is clearly depicted and its characteristics, such as thickness and regularity can be easily ascertained. Short-tau inversion-recovery images (STIR images) are dark-blood T2-weighted images in which the signal of fat is saturated and that allow the identification of tissue edema (103). This sequence is particularly useful to identify vessel wall active inflammation with edema in the acute phase and to monitor response to therapy and disease activity during follow-up (**Figure 6A**).
2. Bright-blood morphologic images (single-shot true-FISP images) in which blood appears bright and that allow the evaluation of vessel wall characteristics.
3. Non-contrast angiography. Time of flight angiography (TOF) is the most frequently non-contrast angiography used, especially to study intracranial and peripheral vessels. This technique can be exploited in patients in whom contrast administration is contraindicated (104). The presence of slow flow or vessel tortuosity can impair image quality and represent a limitation of this technique. Bright-blood whole-heart, self-navigated MRI allowing free-breathing is a technique steadily being improved to acquire 3D images of the aorta and coronary arteries without the need of contrast (104, 105). Techniques allowing the evaluation of coronary arteries are particularly useful when these vessels are involved. Mavrogeni et al. demonstrated complete agreement between bright-blood MRI angiography and coronary X-ray angiography in identifying coronary aneurysms in patients affected by KD (106). The avoidance of X-ray exposure is particularly advantageous in this young population and renders MRI the ideal tool for follow-up. Moreover, adding a complete cardiac evaluation with CMR allows a comprehensive evaluation of both the coronary tree and heart muscle (107).

POST-CONTRAST MRI TECHNIQUES

Three-dimensional (3D) contrast-enhanced magnetic resonance angiography (CE-MRA) is the corner stone for vessel lumen evaluation with high-spatial resolution (**Figure 6C**). At higher strength fields (3 T), an improvement in spatial resolution can be obtained with lower contrast dosage (104). Post-contrast acquisitions include T1-weighted images (e.g., VIBE) targeted at the evaluation of vessel wall, allowing the identification of thickening and thrombus stratification and late-enhancement images, obtained with the inversion-recovery technique, showing contrast accumulation (the so-called "late enhancement") in pathologic vessel walls (**Figure 6B**).

CARDIAC EVALUATION WITH CMR

Systemic vasculitidies can affect the heart either directly, through vasculitis-related myocarditis or indirectly, through myocardial ischemia secondary to coronary artery involvement (102, 108). CMR allows the evaluation of cardiac volumes and function through cine images, the assessment of valvular function with

phase-contrast images and the detection of myocardial edema, secondary to inflammation or ischemia through STIR T2-images. Post-contrast images obtained with the inversion-recovery technique highlight pathologic areas of contrast accumulation in the myocardium, identifying scar (**Figure 6D**). CMR is the ideal tool also to follow-up patients with cardiac involvement, to monitor response to therapy and disease activity.

IMAGING AND BIOMARKERS

The measurement of acute phase reactant, i.e., C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR), is currently recommended for LVV patients follow-up (12) and undoubtedly provides a useful aid for many patients. However, it is established that vascular inflammation and disease progression can occur in the face of normal levels of CRP or ESR, or both (109, 110). In addition, critical analysis of the utility of CRP and ESR, alongside other proposed biomarkers of disease activity in TAK, including soluble adhesion molecules and von Willeband factor, showed that they do not reliably identify active disease (111). The need for more effective markers has prompted the evaluation of several different new molecules, among which Pentraxin 3 (PTX3) deserves a special mention. PTX3 is a member of the pentraxin's superfamily and is induced by different cell types, including endothelial cells, smooth muscle cells, and mesangial cells upon inflammatory stimulation (112). Dagna et al. first reported that plasma levels of PTX3 were higher in patients with active TAK as compared with patients with inactive disease, and that TAK patients had higher PTX3 plasma concentration than healthy controls and subjects suffering from acute infections (113). Subsequent reports showed high sensitivity and specificity, 82 and 77%, respectively, of PTX3 for the detection of active disease (114, 115), suggesting that it may be of help in early detection of disease recurrence. Monitoring of LVVs by means of biomarkers, however, currently appears to be suboptimal and not fully reliable.

To the best of our knowledge, no systematic study has been performed to assess a correlation between serum level of biomarkers and imaging findings of inflammation. However, many studies report a lack of association between serum levels of CRP and ESR and imaging evidence of active inflammation during follow-up. In particular, a general trend toward persistence of low-grade positive imaging after normalization of acute phase reactants after initiation of treatment is shown for ultrasound (34, 116), FDG-PET (88) and MRI (117). Furthermore, a study performed with PK11195-PET showed that this imaging modality was more effective in identifying clinically active LVV than evaluation of ESR and CRP (96). While none of these findings appears to be conclusive, it is arguable that imaging may be more effective than currently available routine serological biomarkers in the assessment of LVV activity, and may provide an effective means of identifying subclinical active disease.

CONCLUSIVE CLINICAL REMARKS

Clinical evaluation still plays a major role in the diagnosis of LVV, also considering the fact that classification criteria have not yet been revised in response to the increased sensitivity of non-invasive imaging techniques (5). The likelihood of early diagnosis, which would be desirable in order to prevent irreversible vascular damage, is not facilitated by the protean manifestation of the diseases, and by the lack of constitutional symptoms in up to 50% of patients (118, 119). The index of suspicion should be held high for patients with unexplained acute phase response, hypertension, or symptoms of ischemia, and should trigger the request for non-invasive imaging (2, 5, 12). Although an early report questioned its utility in the evaluation of LVV (120), imaging of superficial vessels by color Doppler ultrasound was subsequently shown to have a good sensitivity and specificity for the diagnosis of these diseases (31, 32). Changes in ultrasonographic findings have been reported after initiation of an effective therapy (38–40, 121): alongside low economical and biological cost, this makes color Doppler ultrasound a suitable technique for patients' follow-up. CEUS may further enhance diagnostic power and role in the follow-up of the disease, but evidence is still lacking to warrant its use in clinical practice. For those patients in whom ultrasound examination is negative, PET scanning with FDG is the logical second-line approach. This technique has been shown to greatly increase diagnostic accuracy for LVV (87), and may be particularly useful for those patients with atypical presentation, in which LVV is only one of the several possibilities (122, 123). Although FDG uptake decreases with the initiation of an effective therapy, it is not unusual to detect persistent low-grade uptake in the involved arteries of patients who have normal acute phase reactants (124), which may be due to the persistent inflammatory infiltrates that can be detected in surgical specimens from patients with quiescent disease (110). This fact currently limits the utility of PET in patients' follow-up. These shortcomings have led investigators to explore new ligands for detection of vascular inflammation, such as [^{11}C]-PK11195, which has not been extensively validated yet, but for which promising preliminary result exist (96). MRI imaging can represent an alternative second-line examination, allowing complete evaluation of the aorta and its branches, but appropriate visualization of blood vessel lumen and wall necessitates of dedicated protocols not widely available (125, 126). In addition, MRI allows thorough evaluation of the heart, which is frequently involved, albeit sometimes silently, in LVV. The lack of ionizing radiation makes it suitable for repetition in patients follow-up. CT imaging has similar indications to MRI for what concerns blood vessels examination, and is more widely available. It may have a role in the evaluation of coronary arteries involvement in these patients. However, it involves the use of ionizing radiations and nephrotoxic iodinated contrast, which questions its safety for repeated exams in the follow-up of the patients. Current guidelines, while supporting the use of non-invasive imaging in the diagnosis and management of LVV (12), do not give systematic indications its use, deferring the selection of the approach to the practicing physician preferences and local expertize. The use of ultrasonography is well established in the diagnosis of GCA: while a positive ultrasound finding does not replace temporal artery biopsy for the diagnosis, it may provide the definite diagnosis for biopsy negative patients with a strong clinical suspicion for cephalic vessel GCA (127). Ultrasound may thus be the first imaging approach in such patients. FDG-PET, especially if combined with anatomical CT imaging, appears to be particularly appropriate subsequently, as it allows to detect lesions throughout the entire arterial tree (128). PET

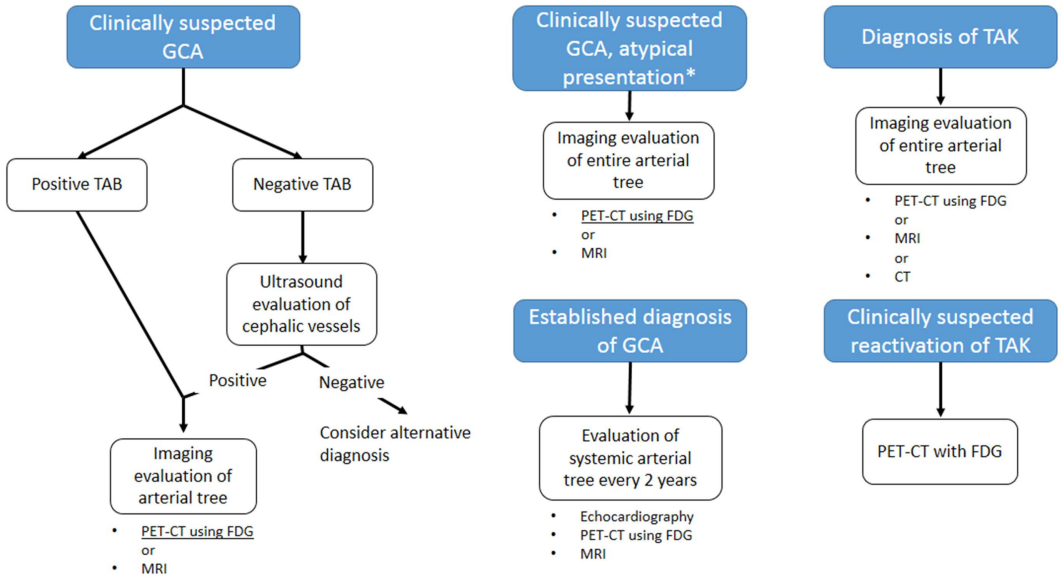

FIGURE 7 | Proposed guidelines-based algorithm for imaging evaluation of patients with large-vessel vasculitis. GCA: giant cell arteritis; TAK: Takayasu arteritis; TAB: temporal artery biopsy. *Atypical presentations for GCA include main large-vessel involvement or fever of unknown origin. MRI is a suitable approach only for the former presentation. Currently, no "gold standard" imaging is defined for TAK.

imaging may be particularly appropriate for patients affected by GCA (127) in whom large-vessel involvement and systemic symptoms, e.g., limb claudication, predominate, and in patients with atypical presentation, i.e., fever of unknown origin (122). MRI may have similar indications, although less established. In the subsequent management of the patient, imaging of the arterial tree is recommended every 2 years in order to monitor for lesion progression: echocardiogram for early detection of proximal aortic aneurysm, PET or MRI may be included (127, 129). For what concerns TAK, guideline indications are even less precise. A thorough assessment of the arterial tree is currently recommended upon diagnosis, and MRI, PET, and CT are well suited for this purpose (128), while none of these techniques appear to provide a "gold standard" (130). The use of ultrasound is not well established in this disease (131), but it may provide the tool for non-invasive assessment of cephalic vessel, especially carotid artery. The detection of the "halo sign" may provide information on disease activity. One of the major challenges in therapeutic management of TAK is in fact recurrence: even with effective corticosteroid therapy, up to 72% of the patients experience multiple recurrences within 6 months (119). While no routine imaging follow-up is currently recommended for early detection of recurrence, FDG-PET may be the imaging technique of choice upon clinical suspicion due to its strong correlation with disease activity (132). Diagrams in **Figure 7** summarize the suggested imaging approach.

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State of the art in the treatment of systemic vasculitides

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Anti-neutrophil cytoplasm antibodies (ANCA) are associated with small vessel vasculitides (AASV) affecting the lungs and kidneys. Structured clinical assessment using the Birmingham Vasculitis Activity Score and Vasculitis Damage Index should form the basis of a treatment plan and be used to document progress, including relapse. Severe disease with organ or life threatening manifestations needs cyclophosphamide or rituximab, plus high dose glucocorticoids, followed by lower dose steroid plus azathioprine, or methotrexate. Additional plasmapheresis is effective for very severe disease, reducing dialysis dependence from 60 to 40% in the first year, but with no effect on mortality or long-term renal function, probably due to established renal damage. In milder forms of ANCA-associated vasculitis, methotrexate, leflunomide, or mycophenolate mofetil are effective. Mortality depends on initial severity: 25% in patients with renal failure or severe lung hemorrhage; 6% for generalized non-life threatening AASV but rising to 30–40% at 5 years. Mortality from GPA is four times higher than the background population. Early deaths are due to active vasculitis and infection. Subsequent deaths are more often due to cardiovascular events, infection, and cancer. We need to improve the long-term outcome, by controlling disease activity but also preventing damage and drug toxicity. By contrast, in large vessel vasculitis where mortality is much less but morbidity potentially greater, such as giant cell arteritis (GCA) and Takayasu arteritis, therapeutic options are limited. High dose glucocorticoid results in significant toxicity in over 80%. Advances in understanding the biology of the vasculitides are improving therapies. Novel, mechanism based therapies such as rituximab in AASV, mepolizumab in eosinophilic granulomatosis with polyangiitis, and tocilizumab in GCA, but the lack of reliable biomarkers remains a challenge to progress in these chronic relapsing diseases.

Keywords: vasculitis, cyclophosphamide, rituximab, ANCA, glucocorticoid, plasmapheresis, methotrexate, azathioprine

INTRODUCTION

The systemic vasculitides are a complex set of overlapping conditions whose natural history has been significantly modified by current therapies but continue to challenge patients and clinicians. We expect survival in over 90% (compared to over 90% mortality untreated) in the first year; about 70% with small vessel vasculitis survive up to 5 years, giving a mortality ratio of 2.6 (95% CI 2.2–3.1) compared to background (1, 2).

In large vessel vasculitis, mortality is low (3) but morbidity is high. In giant cell arteritis (GCA), visual loss occurs in up to 35% (4). In Takayasu arteritis, ischemic claudication of limbs and great vessels can require surgical reconstruction (5).

Current therapies minimize systemic and local inflammation and can preserve organ function. Immunosuppressive agents are combined with supportive management, which includes: compensating for organ dysfunction (e.g., treating hypertension or providing dialysis); dealing with or preventing comorbidity, which might arise from treatment (e.g., infection, steroid related osteoporosis, or cataract); worsening of pre-existing comorbidity (e.g., worsening of ischemic heart disease or obesity); or development of new comorbidity.

We need to ensure that we identify what we are actually treating so that we tailor the choice of treatment at the right dose and at right time for each individual.

WHAT ARE WE TREATING?

Making an accurate diagnosis of the type of vasculitis is an important part of treatment choices. **Figure 1** illustrates a typical plan of management for patients with vasculitis. There are no diagnostic criteria for the vasculitides; Chapel Hill Consensus Conference definitions are widely applied (6). Classification criteria for vasculitis are currently problematic (7) and research is underway to improve them (8). However, the diagnostic label is not enough. The patient's status should include assessment of disease severity and the context in which the disease occurs in individuals. **Table 1** outlines the immunosuppressive therapies used to manage vasculitis.

The range of diseases encompassed includes small, medium, and large vessel vasculitis; small and medium vessel diseases are grouped together because the standard treatment approaches are very similar; however, they are starting to diversify as we develop more targeted agents.

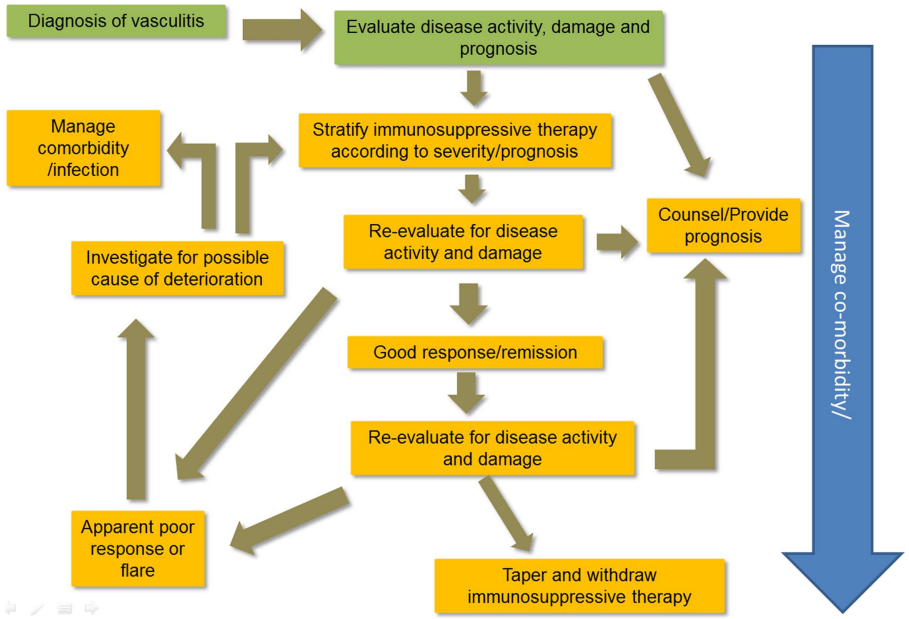

FIGURE 1 | Managing systemic vasculitis.

For patients with a virus associated vasculitis, treatment of the virus is a prerequisite to controlling disease. Polyarteritis nodosa (PAN) related to hepatitis B (HBV-PAN), a typical form of PAN, is characterized by the absence of glomerulonephritis and the absence of anti-neutrophil cytoplasm antibodies (ANCA); relapses are rare, and never occur once viral replication has stopped and seroconversion has occurred (18). Eradication of hepatitis B is part of the management for HBV-PAN (18).Combining an anti-viral drug with plasmapheresis facilitates seroconversion and prevents the development of long-term hepatic complications of HBV. The incidence of HBV-PAN has decreased 10-fold as a result of improved blood safety and vaccination campaigns (19). In a study of 80 patients with HBV-PAN given anti-viral therapy plus immunosuppression, 5% relapsed and 30% died compared with 14.3% relapses and 48.6% deaths among 35 patients treated with immunosuppression alone. Patients who seroconverted achieved complete remission and did not relapse.

Unfortunately, the eradication of hepatitis C has been more problematic; patients with cryoglobulinaemic vasculitis may continue to require ongoing anti-viral therapy. Combination anti-viral therapy is more effective, as shown in a study of cryoglobulinaemic vasculitis; 69% of 23 cases treated with a combination of pegylated interferon alpha, ribavirin, and a protease inhibitor had achieved undetectable viral loads and a good clinical response in the majority including complete remission in 57% (20).

In small vessel vasculitis associated with ANCA, these antibodies are intricately involved in the pathogenesis (21). The role of conventional immunosuppressive agents remains important. Cyclophosphamide is the gold standard for multi-system small vessel vasculitis (22, 23); for less aggressive forms of disease, there is a potential role for leflunomide (24), methotrexate (25) or in one small series, high dose intravenous azathioprine (13). Whilst small open label studies of tumor necrosis factor (TNF) inhibition have suggested benefit (26) in disease control and improvement in abnormal endothelial dysfunction, a large randomized placebo controlled trial of etanercept, a TNF receptor protein, has shown no benefit in patients with GPA; in fact these patients had an increased risk of malignancy, which may in part have related to the inclusion of patients previously exposed to large doses of cyclophosphamide (27). Direct targeting of B cell production of antibody is an effective therapy for many but not all patients (28–30).

For patients with large vessel vasculitis such as GCA or Takayasu arteritis, the primary treatment is glucocorticoids, but as we identify disease mechanisms, we should be able to use targeted therapies, avoiding the use of high doses of steroids, which result in very significant toxicity in over 80% (31).

MECHANISM SPECIFIC VS. GLOBAL IMMUNOSUPPRESSION

Immunosuppressive therapy results in global effects on the immune system, which can be both good and bad. Glucocorticoids produce a rapid improvement in all types of vasculitis by genomic effects on the cytosolic and more rapid non-genomic effects on the membrane bound glucocorticoid receptor (32), but these effects are short lived in small vessel vasculitis. By contrast, most patients experience significant steroid toxicity (over 80% for GCA) and this relates to the total steroid load (31). It is important to tailor the dose of steroids, often used together with an immunosuppressive agent (see **Table 1**), to minimize the harm, while still controlling disease.

Specific targeting of inflammatory immune mechanism in vasculitis is increasingly practical as we identify the molecular pathways that are primarily responsible for the disease. The role of complement in ANCA-associated vasculitis (33) has led to the

Table 1 | Immunosuppressive therapies used to treat systemic vasculitis.

Drug	Phase of therapy	Dose	Indication/comments	Common adverse effects
NON-BIOLOGICAL IMMUNOSUPPRESSIVE THERAPIES USED TO TREAT SYSTEMIC VASCULITIS				
Glucocorticoids	Induction and maintenance	Varies but usually required at high initial dose (0.75–1 mg/kg/day) tapering after 4 weeks with good disease control Subsequent reduction of steroids is more rapid in the first 4–6 months (e.g., 5–15 mg per day reduction every 1–2 weeks), then much slower (e.g., 1 mg every 1–2 months) for large vessel vasculitis; in small and medium vessel vasculitis, because the patient is usually also managed with another immunosuppressive agent, glucocorticoid reduction protocols can be more aggressive Pulse high dose intravenous methylprednisolone (500–1000 mg) may be indicated for organ or life threatening manifestations, but the evidence base for its use is poor	For GCA and Takayasu arteritis, this may be the only immunosuppression given. For most other forms of systemic vasculitis, additional immunosuppressive agents are mandatory. Increasingly, we recognize the adverse effects of glucocorticoid therapy and the aim is to minimize their use In small and medium vessel multi-system disease such as GPA, MPA, EGPA, and PAN, glucocorticoid therapy remains essential to the management, except for Kawasaki disease where it is rarely used	Weight gain Hyperglycemia Mood swings Easy bruising Infection risk Cataracts Hypertension Osteoporosis Cushing's syndrome
Cyclophosphamide	Induction	Usually given intravenously as high dose intermittent pulses of 15 mg/kg/dose on 6–10 occasions, 2–3 weeks apart. Oral pulse therapy is feasible and delivers higher level of active metabolites (due to first pass metabolism in liver to active compound) Continuous daily oral cyclophosphamide is also effective but the cumulative dose is much higher after 6 months compared to pulse therapy	Most forms of small vessel ANCA vasculitis, some patients with PAN and some with large vessel vasculitis require cyclophosphamide Rituximab is increasingly used as an alternative for patients with ANCA vasculitis who have failed cyclophosphamide or in whom cyclophosphamide is contraindicated	Cytopenias Nausea and vomiting Diarrhea Hair loss Teratogenesis (avoid in pregnancy) Hemorrhagic cystitis Long-term risk of infertility and malignancy (especially bladder carcinoma) relate to cumulative dose life-time exposure especially above 35 g
Plasmapheresis	Induction	Additional to conventional immunosuppression. No standard volume of exchange. A typical regimen would be to use between 7 and 10 exchanges (4 l each) in first 10 days of induction therapy (9). It is not clear which method of plasmapheresis (centrifugation or filtration) is superior	Evidence from one large randomized controlled trial suggests that additional plasmapheresis is renal sparing (10), but a follow up study of the same patient group suggested that the benefit did not last (11), suggesting that plasmapheresis may not be effective if used in patients with established kidney scarring A smaller study of 32 patients with GPA with 5-year follow up showed that plasmapheresis plus cyclophosphamide and glucocorticoids followed by ciclosporin maintenance therapy was effective in patients with a creatinine of >250 μmol/l at baseline (12)	Increased risk of sepsis especially if combined with cyclophosphamide Potential risk of transmission of viral infection if using infected blood products

(Continued)

Table 1 | Continued

Drug	Phase of therapy	Dose	Indication/comments	Common adverse effects
			Avoid plasmapheresis shortly after administration of other IV therapies (otherwise they are removed)	
Methotrexate	Induction or maintenance	15–25 mg/week oral or sc	Can be used as effective induction therapy for non-organ or non-life threatening ANCA vasculitis. It provides some additional benefit in control of GCA. Avoid use in significant renal impairment	Nausea Diarrhea Mouth ulcers Hair loss Cytopenia Liver dysfunction
Leflunomide	Induction or maintenance	10–40 mg/day	This drug is used for inflammatory arthritis but has shown benefit in patients with localized GPA	Nausea Diarrhea Mouth ulcers Hair loss Cytopenia Liver dysfunction Hypertension
Mycophenolate mofetil	Induction or maintenance	2–3 g per day	Less effective than azathioprine as a maintenance agent, nevertheless this drug has a place in management of ANCA vasculitis. As an induction agent it appears to be as effective as cyclophosphamide	Nausea Diarrhea Mouth ulcers Hair loss Cytopenia Liver dysfunction
Co-trimoxazole	Induction or maintenance	960 mg twice a day or 960 mg 3× per week if used in combination with methotrexate	This simple antibiotic has immunomodulatory effects in patients with mild GPA and has been shown to improve upper airways disease, usually in combination with steroids. At the reduced dose it can be used as prophylaxis against pneumocystis jirovecii in patients receiving other immunosuppressive agents	Beware allergy to sulfonamide Nausea Diarrhea Cytopenia (avoid full dose if combined with methotrexate)
Azathioprine	Induction Maintenance	Usually given as 2 mg/kg/day for maintenance but there is one report of using high dose intravenous pulse therapy with 1200 mg per month for 6 months in very resistant disease (13)	This is a common maintenance agent, following successful induction therapy with either cyclophosphamide or rituximab	Nausea Diarrhea Mouth ulcers Hair loss Cytopenia Liver dysfunction Non-melanoma skin tumors (advise sun protection)
Ciclosporin	Maintenance	2–4 mg/kg/day in two divided doses	Less commonly used than other agents, largely due to its nephrotoxicity	Nausea Diarrhea Gingival hyperplasia Increased facial hair Cytopenia Renal dysfunction Hypertension

(Continued)

Table 1 | Continued

Drug	Phase of therapy	Dose	Indication/comments	Common adverse effects
Gusperimus	Relapse	0.5 mg/kg/day until neutropenia develops or for up to 21 days repeated every month for up to 6 months	Unlicensed in Europe, this immunomodulator therapy has been effective in relapsing GPA (14) Reversible and predictable neutropenia Potential risk of sepsis	Well tolerated but limited information because of very limited use
BIOLOGICAL IMMUNOSUPPRESSIVE THERAPIES USED TO TREAT SYSTEMIC VASCULITIS				
Intravenous immunoglobulin (IVIG)	Induction	2 g/kg single dose or divided over 5 days is typical therapy for Kawasaki disease (15). These doses are much higher than those used for immunodeficiency	Kawasaki disease is the main form of vasculitis responding to IVIG, in combination with high dose aspirin. ANCA vasculitis will respond temporarily, and this can be useful if patients are also septic, because it is an immunomodulating therapy. Check serum IgA to avoid allergic reactions in patients who are IgA deficient (because there is usually some IgA contamination). IVIG is prepared form pooled human serum, typically from thousands of donors. Viral screening of IVIG is now highly effective (previous IVIG therapy use has been associated with hepatitis C transmission)	Potential risk of transmission of viral infection if using infected blood products Allergic reaction in patients who are IgA deficient (due to expected levels of small amounts of IgA in the preparation) Headaches, flushing, fever, chills, fatigue, nausea, and diarrhea are transient reactions during infusions
Rituximab	Induction or maintenance	375 mg/m^2 every week for 4 weeks or 1 g $\times$2 14 days apart are typical induction regimens. Maintenance therapy (typically 1 g single infusion) can be given every 4–6 months afterward	Increasingly used in place of cyclophosphamide as induction therapy at initial presentation or during relapse for ANCA vasculitis	Infusion reactions neutropenia hypogammaglobulinemia Infections (including small risk of progressive multifocal leukoencephalopathy) Potential for viral reactivation (e.g., hepatitis B) Development of other autoimmune conditions
Tocilizumab	Relapse	4 mg–8/kg per month intravenously or 162 mg sc per fortnight if $<$100 kg or 162 mg sc every week if $\geq$100 kg	Limited evidence for effectiveness in large vessel vasculitis. A randomized controlled trial in GCA is currently underway (16)	Infection risk Potential masking of evidence of sepsis (by down regulating production of CRP) Increased lipid levels Neutropenia Liver dysfunction Infusion reactions are rare
Mepolizumab	Resistant disease	Two different regimens are being explored: 300 mg sc every 4 weeks 750 mg iv every 4 weeks	This interleukin five inhibitor is effective in hypereosinophilic states and the iv regimen has been shown to control resistant cases of EGPA (17). A randomized controlled trial using the sc regimen is underway (http://clinicaltrials.gov/show/NCT02020889)	Limited evidence available only to date No increase in toxicity compared to placebo (e.g., fatigue, nausea)

Patients are typically given intense induction therapy followed by maintenance. Most patients will require additional therapy to manage comorbidity and limit drug toxicity. Induction therapies can be repeated for relapse; however, it may be necessary to change the type of induction due to toxicity or poor initial response.

development of targeted therapy against complement 5A (C5a), which is currently being tested in clinical trials[1]. The involvement by ANCA itself has led to development of specific B cell ablation therapy using rituximab and now belimumab[2]. As newer understanding of disease mechanisms is revealed then more targets for therapy will be identified or at least we will have better recognition of how we are affecting the underlying pathways with existing therapies.

CAN WE INDUCE REMISSION?

The aim of managing the patients with vasculitis is to induce remission, which should be possible in the majority. However, this is a clinical remission not a cure and the majority of patients will relapse. We need to deliver treatment according to need without exposing patients to unnecessary risk whilst ensuring the maximum benefit. Conventional measurements of clinical remission are defined using disease activity scores, which are preferred to any current serological marker for small vessel and medium vessel vasculitis. In over 90% of patients with small vessel vasculitis, remission should be achieved by 6 months (22) using standard induction therapy. Further serial evaluation is important in order to detect and treat relapses.

By contrast, in large vessel vasculitis, the induction of remission is less easily documented. All the clinical symptoms and signs disappear rapidly with steroid treatment; by contrast, it is not so clear that we have adequately controlled disease at a sub-clinical level. Imaging is emerging as an effective technology to define disease activity. Unfortunately, it is expensive and can involve significant radiation exposure. The best imaging technique available is 18 fluorodeoxyglucose positron emission tomography with co-localized computerized tomography (FDG PET CT) to identify areas of abnormal glucose uptake. However, this involves exposure to an average of 14.4 mSv for females and 11.8 mSv for male patients (34) per scan. Nevertheless, it is important to quantify the presence of sub-clinical disease to find ways of preventing end stage ischemic complications or other vascular events such as thrombosis, dissection, and aneurysm. Non-invasive imaging protocols using magnetic resonance scans or ultrasound are being developed as ways of measuring change in disease state (35, 36).

HOW DO WE MEASURE REMISSION?

Serological measurements of disease activity are not reliable in systemic vasculitis (37). ANCA testing is very useful for diagnosis, but for subsequent follow up, levels can vary independently of future disease activity (38).

The Birmingham Vasculitis Activity Score (BVAS) is the most effective validated tool to document disease activity; it can be used as to define remission, response to therapy and flare (37, 39, 40). The BVAS consists of a list of typical features of active systemic vasculitis related to each body system; each item is recorded as present only if it is judged to be due to active vasculitis. This is semi-subjective because items are derived from the patient history and physical examination and cannot always be confirmed with more objective testing. However, the BVAS is valid, reliable, and widely used in clinical trials in vasculitis to define the responsiveness to various agents including cyclophosphamide, methotrexate, mycophenolate, intravenous immunoglobulin, and rituximab. It is a valuable tool for the clinicians and strongly recommended as a routine part of disease management in small and medium vessel vasculitis (40, 41). Other versions of BVAS have been validated for use in individual forms of vasculitis, such as the BVAS/Wegener's granulomatosis, specifically for patients with GPA (42).

The Vasculitis Damage Index (VDI) is used to assess the outcome of vasculitis, by documenting the occurrence of damage as a result of having a diagnosis of vasculitis (43, 44). VDI is recommended as a cumulative measure to define the effectiveness of therapy (by limiting or preventing the accumulation of scarring). The VDI is strongly related to mortality. The presence of VDI levels of at least five points on a scale of 0–64 items (which occurs in about a third of patients (45) when measured 6 months from diagnosis) is associated with a much higher future mortality (approximately sixfold higher) than patients with less than five items of damage (46) 6 months from diagnosis.

WHAT DRUGS SHOULD WE USE?

Each patient's management should be based on their diagnosis and clinical state. Control of active vasculitis may be achieved using a range of therapies, depending on how rapidly and aggressively the treatment is required. The decision should be based on evidence, but interpreted for the individual to minimize harm, taking into account existing or likely co-morbidities. Some treatment protocols allow for this. For example, there are dose reductions for the dose of cyclophosphamide in older persons, those with renal impairment or with prior significant neutropenia (23).

The treatment protocol may need to be amended if unexpected changes occur in clinical status, either as a result of toxicity or if patients fail to respond to standard agents. Therapy should be withheld until inter-current infection is treated, or escalated in cases with poor initial response. Fundamental to these decisions is the regular careful clinical evaluation of patients to detect these changes. **Table 1** summarizes the immunosuppressive agents commonly used as well as describing potential future therapies under investigation.

IMMUNOSUPPRESSIVE THERAPIES

The main drug used is cyclophosphamide, cyclic nitrogen mustard, phosphamide ester, first used as a chemotherapeutic agent in the 1950s (47). Cyclophosphamide is a cytotoxic alkylating agent capable of killing B cells and T-cells. It is life-saving in patients with small and medium vessel vasculitis and is considered the drug of choice for multi organ disease (22).

However, the toxicity has been considerable, when used as continuous daily oral therapy for up to 2.7 years, providing over 100 g life-time exposure in some patients (48, 49), mainly due to its predicted cytotoxic effects on rapidly dividing normal cells. It can cause reversible nausea, vomiting, diarrhea, and hair loss; permanent infertility and malignancy occur with increasing cumulative doses, with an incidence of 5% at 10 years and 16% after 15 years (50). There is no absolute cut-off dose to avoid toxicity,

[1] http://clinicaltrials.gov/show/NCT01275287;
http://www.controlled-trials.com/ISRCTN53663626
[2] http://clinicaltrials.gov/show/NCT01663623

but the recent British Society for Rheumatology guidelines for management of ANCA-associated vasculitis recommend restricting total exposure to <25 g (41). Current cyclophosphamide protocols use a much lower cumulative dose and the bladder cancer incidence is not increased (51). However, in a study of male fertility risk in patients given cyclophosphamide for sarcoma (52), a total dose of >7.5 g/m^2 was associated with only a 10% chance of recovery of spermatogenesis compared to 70% for those given less than this dose. The use of short courses of high dose intravenous cyclophosphamide is likely to be safer than continuous daily oral therapy (53), chiefly due to the fact that the cumulative dose is typically 30–50% less.

Cyclophosphamide is effective in reducing the mortality in ANCA-associated vasculitis (22, 23). It can be given either as a pulse intravenous high dose therapy 15 mg/kg every 2–3 weeks on 6–10 occasions or as a continuous daily oral therapy at 2 mg/kg/day (54). The latter results in much higher cumulative dose of drug over 6 months period and there is evidence of equivalent of these two regimens; although the use of pulse cyclophosphamide is associated with a higher relapse rate (23). For less aggressive forms of vasculitis, there is a potential role for leflunomide (24), methotrexate (23, 25, 55) or in one small series, high dose intravenous azathioprine (13), and mycophenolate mofetil (56). These agents are usually less toxic but also less effective than cyclophosphamide.

For patients with large vessel vasculitis such as GCA or Takayasu arteritis, the primary treatment is to suppress systemic inflammation with glucocorticoid therapy in order to prevent significant vascular complications (such as loss of sight in GCA or aortic aneurysm formation or stenosis or occlusion of peripheral arteries in Takayasu arteritis. With better understanding of disease mechanisms, we might be able to use targeted therapies, perhaps even avoiding the use of steroids, which otherwise carry very significant risk of toxicity in over 80% (31).

There is some evidence for the effectiveness of TNF inhibition in small vessel vasculitis (26), but concerns about long-term toxicity (27).In Takayasu arteritis (35), studies show improvement but are limited to small numbers and there are no randomized controlled trials. This is partly due to the problem of not having adequate end points to demonstrate a potential treatment effect as well as due to the rarity of the condition (57). Direct targeting of B cells is an effective therapy for many patients; the response (64% in complete remission by 6 months) is similar to that achieved with cyclophosphamide (53% in complete remission by 6 months) for a group of 197 patients with new or relapsing ANCA-associated vasculitis; limited evidence suggests that in 102 patients with relapsing disease previously responding to cyclophosphamide, rituximab was more effective than cyclophosphamide (67 vs. 42% in complete remission respectively, $p = 0.01$) (28–30).

For any form of B cell depletion therapy, it is logical to assume that reconstitution of B cells after the end of treatment will lead to recurrence (58), although this is disputed, with at least one study demonstrating that disease relapse was independent of B cell numbers (59). Nevertheless, maintenance rituximab substantially reduces the risk of recurrence of disease from 73 to 12% after 2 years follow up in retrospective cohort data (60). Another retrospective observational study of 89 patients treated with rituximab for ANCA-associated vasculitis (60) suggests that there was additional protection against future relapse by using maintenance azathioprine, methotrexate, or mycophenolate mofetil compared to no additional immunosuppressive treatment [the hazard ratio for relapse was 0.53 (95% CI 0.29–0.97) if a maintenance drug was given].

DISCUSSION

The state of the art for therapy in vasculitis has improved, but remains unsatisfactory until we can completely control or cure the disease. We can prevent early mortality in multi-system vasculitis and have reduced the immediate effects of active vasculitis on organ function. However, our aim is to further improve the likelihood of survival and also the quality of life of those who survive, ensuring that we minimize disease activity and damage, drug toxicity, and impairment of quality of life.

With better understanding of the pathogenesis of vasculitis, we can target therapy against specific disease mechanisms. Examples of these are rituximab in ANCA-associated vasculitis; mepolizumab in eosinophilic granulomatosis with polyangiitis; complement 5a inhibition in ANCA vasculitis; and potentially Interleukin-6 (IL-6) inhibition with tocilizumab in large vessel vasculitis.

We are helped in our management of the disease by earlier diagnosis, so that treatment can be initiated before organ damage is established in most cases. Whilst the ANCA test is overused (61, 62), it has helped in earlier identification of patients with systemic vasculitis (63). Greater awareness of vasculitis as a cause of unexplained medical illness is leading to better case recognition. Imaging of arteries in large vessel vasculitis may become established as an early diagnostic test (64), which might change our current management approach.

Better management of comorbidity, particularly management of sepsis, control of hypertension, or management of renal failure have changed the outcome and potentially allowed more aggressive immunosuppression to be successful. However, in first 12 months after diagnosis of ANCA vasculitis, episodes of acute sepsis are now responsible for more deaths than vasculitis itself (49).

The role of glucocorticoids in vasculitis is being challenged. Whereas, previously they have been used at high doses for prolonged periods, we recognize their harm, coupled by the benefit from more specific therapy. We should see substantial reduction in toxicity in the coming decade, as we use lower doses or even steroid free regimens to control vasculitis.

Whilst the twentieth century has been dominated by use of the therapies for vasculitis designed to treat other conditions [e.g., cancer and rheumatoid arthritis (23, 65, 66)], drugs are now been designed specifically for vasculitis. We should see significant benefits for our patients, but we need to ensure that we measure their impact (for good and for harm). In the absence of reliable circulating biomarkers we need to use structured clinical assessment to document change in disease state in response to therapy. Development of effective, prognostic biomarkers for vasculitis would allow therapy to be targeted to disease mechanisms, tempered by safety assessments to prevent untoward harm.

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