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EDITED BY

Guido Moll,
Charité Universitätsmedizin Berlin,
Germany

REVIEWED BY

Steven O'Reilly,
STipe Therapeutics, Denmark
Rusan Ali Catar,
Charité Universitätsmedizin Berlin,
Germany

*CORRESPONDENCE

Maria Carolina Oliveira
mcarolor@usp.br

†These authors have contributed
equally to this work and share
first authorship

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Reconstitution of the immune system and clinical correlates after stem cell transplantation for systemic sclerosis

Marianna Y. Kawashima-Vasconcelos^{1,2†},
Maynara Santana-Gonçalves^{1,3†}, Djúlio C. Zanin-Silva^{1,4},
Kelen C. R. Malmegrim^{1,5} and Maria Carolina Oliveira^{1,6*}

¹Center for Cell-Based Therapy, Regional Hemotherapy Center of the Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, ²Internal Medicine Graduate Program, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, ³Oncology, Stem Cell and Cell-Therapy Graduate Program, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, ⁴Basic and Applied Immunology Graduate Program, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil, ⁵Department of Clinical, Toxicological and Bromatological Analysis, School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo, Ribeirão Preto, Brazil, ⁶Department of Internal Medicine, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, Brazil

Systemic sclerosis (SSc) is a chronic autoimmune disease that includes fibrosis, diffuse vasculopathy, inflammation, and autoimmunity. Autologous hematopoietic stem cell transplantation (auto-HSCT) is considered for patients with severe and progressive SSc. In recent decades, knowledge about patient management and clinical outcomes after auto-HSCT has significantly improved. Mechanistic studies have contributed to increasing the comprehension of how profound and long-lasting are the modifications to the immune system induced by transplantation. This review revisits the immune monitoring studies after auto-HSCT for SSc patients and how they relate to clinical outcomes. This understanding is essential to further improve clinical applications of auto-HSCT and enhance patient outcomes.

KEYWORDS

systemic sclerosis, hematopoietic stem cell transplantation, immune reconstitution, immune monitoring, immune tolerance, cellular therapy

Introduction

Autologous hematopoietic stem cell transplantation (auto-HSCT) is considered for patients with severe and progressive autoimmune diseases. In the past 30 years, much has improved in the field, especially concerning patient care. Developments in patient selection, choice of conditioning regimens, and intra-transplant patient management

have significantly decreased transplant-related toxicity and improved patient outcomes (1). Recently, auto-HSCT has been included in the recommendations for treating diseases such as systemic sclerosis and multiple sclerosis (2–4). For the remaining autoimmune diseases, transplants are mostly limited to clinical trials.

Auto-HSCT eliminates abnormal immune cells and reconstitutes a new, self-tolerant, long-lasting immunological system (1, 5). As a first phase of the procedure, autologous hematopoietic stem and progenitor cells are mobilized from the bone marrow to the peripheral blood, harvested by apheresis, and subsequently cryopreserved (1). Then, a conditioning regimen is administered to ablate the patient's autoreactive immune system using chemotherapy or radiation agents associated with lymphocyte-depleting strategies, either by antibodies or graft selection (6). The conditioning regimen may be myeloablative, such as total body irradiation, or less intense, such as high-dose cyclophosphamide (1, 7). Reduced-intensity regimens are safer and usually preferred by most transplant centers to treat autoimmune diseases. However, some authors debate that myeloablative regimens may promote better control of autoreactivity and therefore longer lasting disease control (8). Finally, the previously collected cells are reinfused intravenously to the patient and warrant reconstitution of the immune system. These cells may be administered unselected or undergo CD34⁺ selection, which is also debated. While unselected grafts may increase the risk of reinfusing autoreactive cells and perpetuating the autoimmune disorder, selected grafts delay immune reconstitution and may increase the risk of viral infections (9, 10). Following autologous cell infusion, neutrophil engraftment usually occurs within two weeks, which is very similar to the time for engraftment of neutrophils following autologous and allogeneic transplants for conventional hematological indications using peripheral blood as source for hematopoietic stem and progenitor cells (11–13). In most cases, the newly recovered immune system is self-tolerant and allows control of the disease activity without further immunosuppressant drugs.

Immune monitoring studies have shown profound and long-lasting changes in the immune system of patients with autoimmune diseases treated with auto-HSCT (14–20). **Table 1** summarizes the main clinical studies on multiple sclerosis, systemic lupus erythematosus, type 1 diabetes, idiopathic juvenile arthritis, and Crohn's disease that address how the autoimmune pathology is affected by auto-HSCT. Additional mechanisms, specific to auto-HSCT in SSc patients, will be discussed later in this review. Collectively, these studies show a modulation of the inflammatory and autoreactive profile, reactivation of thymic function, increased diversity of the T cell receptor (TCR) repertoire, and improvement of regulatory mechanisms after auto-HSCT (21–38). Importantly, these studies show that patients who reactivate the autoimmune disease after auto-HSCT have a different post-transplant

immune profile than those with sustained clinical remission. Multiple sclerosis patients who fail to respond to auto-HSCT present less diversity in the T cell receptor (TCR) repertoire early in the immune reconstitution process, when compared to patients that remain in remission (17). Similarly, SSc patients who reactivate the disease after auto-HSCT have lower regulatory T and B cell counts, less TCR repertoire diversity, and lower PD-1 expression on lymphocytes when compared to patients with sustained disease control (20, 39). Type 1 diabetes patients that remain insulin-free for longer periods after auto-HSCT have improvement of the immunoregulatory cell frequencies, not detected in patients with shorter insulin independency (31).

These evaluations are essential to understanding mechanisms and indicate possible pathways to be improved in the clinic. This review revisits the immune monitoring studies after auto-HSCT, specifically in SSc patients, and how they relate to clinical outcomes.

Clinical outcomes of systemic sclerosis patients after transplantation

Systemic sclerosis is a chronic autoimmune disease with complex pathogenesis that includes diffuse microvasculopathy, fibrosis, and inflammation (40). Skin fibrosis is the hallmark of the disease, but internal organ involvement, mainly heart and lungs, is frequent and usually associated with a poor prognosis (41). Conventional treatment is based mainly on immunosuppressive and vasodilator approaches, with antifibrotics paving the way more recently, and has modest benefit in controlling disease progression (42). Patients with severe and progressive disease benefit from auto-HSCT, and SSc is a growing indication in the field. According to international registries, the number of SSc patients that undergo auto-HSCT has nearly doubled in the past ten years (1), and patient outcomes have improved (43).

Over the years, knowledge about patient selection and intra-transplant management has significantly reduced transplant-related mortality to the current rate of 3 to 5%. Nevertheless, SSc is still an autoimmune disease with high transplant-related mortality, primarily due to baseline organ involvement, mainly the heart (44). Recent strategies to decrease cardiac toxicity associated with auto-HSCT include extensive cardiac evaluations before enrolling for transplant and conditioning regimens with lower doses of cyclophosphamide, a drug known to potentially damage the heart (43, 45). Further studies will show if such interventions impact long-term patient outcomes.

Since the early 2000s, phase I and II studies have shown the potential of auto-HSCT to reverse skin involvement and at least stabilize interstitial lung disease in patients with SSc

TABLE 1 Main clinical studies on autologous HSCT for autoimmune diseases (systemic sclerosis excluded) addressing mechanisms.

Diseases	References	Study design	Clinical evidence	Immune mechanism
Multiple Sclerosis	Muraro et al. (2005) (21)	7 patients 2-year follow-up	Long-lasting clinical remission	Reactivation of thymic function (RTEs) Renewal of the TCR repertoire
	Darlington et al. (2013) (22)	14 patients 2-year follow-up	Long-lasting clinical remission Abrogation of new disease activity	Reactivation of thymic function (RTEs, TRECs) Diminished capacity for Th17 responses Transient increase in FOXP3 ⁺ T cells
	Abrahamsson et al. (2013) (23)	12 patients 2-year follow-up	EDSS improvement	Increase in FOXP3 ⁺ cells and CD56 ^{high} natural killer cells Depletion of pro-inflammatory CD8 ⁺ cells subsets
	Muraro et al. (2014) (24)	24 patients 1-year follow-up	Disease control (remission/relapse)	New repertoire of CD4 ⁺ cells and clonal expansion CD8 ⁺ cells
	de Paula Souza et al. (2015) (25)	16 patients 2-year follow-up	EDSS improvement	Normalization of gene expression in CD8 ⁺ and CD4 ⁺ T cells
	Arruda et al. (2015) (26)	24 patients 2-year follow-up	EDSS improvement	Increase in FOXP3 ⁺ cells and expression of CTLA-4 and GITR on CD4 ⁺ CD25 ^{high} T cells Modulation of immunoregulatory genes Homeostatic proliferation
	Cull et al. (2017) (27)	13 patients 2-year follow-up	EDSS stabilization 69% progression-free survival at 3 years	Reactivation of thymic function Decrease in T-regulatory cells Transient decrease in Th17 cells
Type 1 Diabetes	Li et al. (2012) (28)	13 patients 31 to 54-month follow-up	Reduced doses of insulin Reduced levels of glycosylated hemoglobin	Reduced levels of serum autoantibodies Reduced levels of IL-1, IL-17 and TNF- α Recovery of lymphocyte subsets
	de Oliveira et al. (2012) (29)	14 patients 1-year follow-up	Insulin-free remission GAD65 levels	Modulation of pro-apoptotic genes
	Zhang et al. (2012) (30)	9 patients 12-month follow-up	Insulin-free remission	Recovery of lymphocyte subsets Modulation of T cell-related genes
	Malmegrim et al. (2017) (31)	21 patients 72-month follow-up	Long-term insulin-free remission Increase in C-peptide levels	Thymic reactivation (TRECs) Increased TCR diversity Decreased effector-memory CD4 ⁺ T cells Expansion of immunoregulatory T cells Decreased frequencies of islet-specific autoreactive CTLs
	Ye et al. (2017) (32)	18 patients 12-month follow-up	Decrease in anti-GAD levels Increase in C-peptide levels Reduced doses of insulin	Reduced Th1 and Th17 cell frequencies Changes in cytokine patterns Modulation of regulatory genes
Systemic Lupus Erythematosus	Alexander et al. (2008) (33)	7 patients 96-month follow-up	Clinical remission Decreased ANA titers	Thymic reactivation Increased TCR diversity Regeneration of FoxP3 ⁺ T cells Recovery of CD19 B cell subsets
	Zhang et al. (2009) (34)	15 patients 8-year follow-up	Clinical remission	Sustained elevation of FoxP3 ⁺ T cells
Juvenile Idiopathic Arthritis	de Klee et al. (2006) (35)	12 patients 2-year follow-up	–	Restoration CD4 ⁺ CD25 ^{high} T cell Reprogramming of autoreactive T cells
	Brinkman et al (2007) (36)	22 patients 80 months follow-up	Clinical remission	Recovery of lymphocyte subsets
	Wu et al. (2014) (37)	5 patients 3-year follow-up	Clinical remission	TCR diversity
Crohn's Disease	Corraliza et al. (2015) (38)	18 patients One-year follow-up	50% endoscopic drug-free remission	Expansion of naive B cells in the blood and intestinal mucosa. Intestinal T cell depletion correlating with mucosal healing (endoscopic remission)

RTE, recent thymic emigrants; TCR, T cell receptor repertoire; TREC, T-cell receptor excision circles; EDSS, Expanded Disability Status Scale; FOXP3, forkhead box P3; GITR, Glucocorticoid-induced TNFR related protein; GAD65, glutamic acid decarboxylase; CTL, Cytotoxic T lymphocytes; ANA, antinuclear antibodies.

(46–52). There is a significant reduction of the modified Rodnan's skin score (mRSS), used to clinically quantify the extent and severity of cutaneous involvement, mainly in the first year after auto-HSCT. The pulmonary function also ceases to decline after the procedure, indicating stabilization of lung

disease, and some studies were even able to show improvement of forced vital capacity measurements (52–54). Moreover, patients increase their quality of life and functional capacity measured by the six-minute walk test after auto-HSCT, independence and well-being indicators (55, 56). Three

randomized-controlled trials plus a non-randomized comparative study have proven the superiority of auto-HSCT over standard treatment with intravenous cyclophosphamide pulses in improving overall survival, progression-free survival, and quality of life (53, 57–59). These critical studies show that, unlike conventional treatment, auto-HSCT can change the course of the disease.

However, although patient outcomes have improved after auto-HSCT, a few questions remain unanswered. SSc-reactivation over the 5 to 7 years that follow auto-HSCT is estimated at approximately 20% of patients (44, 52, 57, 58, 60). We cannot predict or early detect patients that will reactivate the disease after auto-HSCT. Additional immune reconstitution studies are essential to answer these and other questions (Table 2).

Immunological outcomes of systemic sclerosis patients after auto-HSCT

Reconstitution of innate immune cells in SSc after auto-HSCT

The innate immune system has a critical role in SSc pathogenesis (74, 75). Neutrophils from SSc patients exhibit distinct phenotypic and functional changes, such as deficiencies in cell migration, phagocytosis, and chemokine receptor expression (76). Monocytes are also in disbalance in dcSSc (diffuse cutaneous systemic sclerosis) since high numbers of circulating CD16⁺ monocytes have been detected and correlated with the severity of skin fibrosis (77). These cells further display the potential to differentiate into myfibroblasts, the primary cell type responsible for depositing extracellular matrix components (ECM) and fibrosis (78). Likewise, there are increased numbers of circulating natural killer (NK) cells with activated phenotype in dcSSc, and they produce high levels of IL-6 under stimulation (79). Differently, plasmacytoid dendritic cells (pDC) are reduced in the blood of SSc patients but accumulate in the skin and lungs, correlating with inflammation, leukocyte migration, and wound repair protein levels (80).

Most immune monitoring studies of auto-HSCT in autoimmune diseases have focused on the reconstitution of adaptive immune cells (81, 82). Nevertheless, a clear understanding of the reconstitution of all immune cells may be essential to predicting transplantation outcomes and improving patient care (83). Innate immune cells recover earlier after auto-HSCT than those from the adaptive system (61), indicating that they contribute to the regeneration of the adaptive immune system in SSc patients after auto-HSCT (Figure 1) (83).

Neutrophils are the first innate cell type to reconstitute, generally within the first 14 days after auto-HSCT (81, 84). Whole blood transcriptome from SSc patients show a significant decrease in neutrophil gene expression signatures after auto-HSCT compared to baseline, which also correlates with lung function improvements. However, it remains unknown if the transcriptional changes affect cell phenotype and function (68). Indeed, knowledge about the reconstitution of neutrophils and other granulocytes after auto-HSCT in SSc patients remains to be explored.

Reconstitution of NK cells also occurs early following auto-HSCT (Figure 1) (61, 81). CD56⁺ NK cells emerge within the first 30 days after the transplantation procedure (81). Cytotoxic NK cells transcript signature increases in dcSSc patients after auto-HSCT and correlates with a decline in skin fibrosis measured by mRSS (68). These results suggest that the transcriptomic signatures of the innate immune cells are normalized following transplantation and indicate a possible immunomodulatory role for the innate immune system early after auto-HSCT (68). Nonetheless, viral infections during transplantation may influence the function of the reconstituted NK cells. A case report of a SSc patient showed that cytomegalovirus (CMV) pneumonia after auto-HSCT was partially due to a hyperreactive NK cell response (85).

Monocytes may also have an essential function in controlling the immune response in the new post-transplantation microenvironment. CD14⁺ monocytes from blood samples of transplanted patients with hematological/immunological diseases, collected within the first six months after auto-HSCT, suppress T cell proliferation *in vitro* compared to healthy subjects (86). In SSc patients, serum levels of chemokines and cytokines related to the alternatively activated M2 phenotype, such as CCL-18, IL-6, and MCP-1, were significantly diminished after auto-HSCT. At the same time, no changes occurred in patients treated with monthly intravenous cyclophosphamide infusions (68). In another cohort, SSc patients treated with auto-HSCT had reduced serum concentrations of platelet-derived growth factor (PDGF) (65), an important molecule involved in fibrosis, playing a central role in the expansion of ECM-producing cells (87).

Recently, van der Kroef et al. (88) showed that the number of circulating monocytes positively correlated with plasma concentrations of PDGF-BB in SSc patients. These results suggest that reductions in PDGF and other circulating profibrotic mediators after auto-HSCT could be related to functional changes in monocytes and macrophages. These results highlight a possible role of monocytes in regulating T cell responses after auto-HSCT in SSc patients (Figure 1). We encourage future immune monitoring studies to determine the role of monocyte subsets in SSc pathogenesis and immune regulation after auto-HSCT (83).

TABLE 2 Overview of studies that evaluated immune reconstitution and clinical outcomes in SSc patients treated with auto-HSCT.

References	Number of patients	Duration of follow-up (mo)	Biological samples	Clinical evaluation	Laboratory analyses	Clinical association
Storek et al. (2004) (61)	30	1, 3, 6, 12, and 24	PBMC	Infection rates	Antibody levels CDR3 spectratyping Immunophenotyping Thymic size	Infection rates
Farge et al. (2005) (14)	7	3, 6, 9 and 12	PBMC	Cardiac and renal function HAQ mRSS	CDR3 spectratyping Immunophenotyping TRECs assay	Response or a relapse of disease
Bohgaki et al. (2009) (62)	10	3, 6 and 12	PBMC Serum	Cardiac, pulmonary, renal function mRSS	Antibody levels Foxp3 mRNA levels Immunophenotyping sjTREC assay	Response or a relapse of disease
Fleming et al. (2008) (63)	7	Until 72	Skin biopsies	Capillary counts, mRSS, MHAQ	Immunohistochemistry and mRNA <i>in situ</i> hybridization	–
Tsukamoto et al. (2011) (64)	11	1, 3, 6, 12, 24 and 36	PBMC Serum	mRSS DLCO KI-6 SP-D	Antibody levels Cytokine levels Immunophenotyping	mRSS
Baraut et al. (2014) (18)	7	24	PBMC	mRSS	Immunophenotyping suppressive capacity assay	–
Michel et al. (2016) (65)	20	6, 12, 24, 36 and 48	Serum	mRSS	Cytokine levels	–
Farge et al. (2017) (66)	10	24, 36, 48, 60, and 72	PBMC Serum	FVC mRSS	Antibody levels Immunophenotyping TCR repertoire	Response or a relapse of disease
Arruda et al. (2018) (39)	31	6, 12, 24 and 36	PBMC Serum	mRSS C-reactive protein	Antibody levels Cytokine levels Immunophenotyping Quantification of sjTREC, β TREC and Cj and sjKREC TCR repertoire	Response or a relapse of disease
Arruda et al. (2018) (67)	25	6, 12, 24 and 36	PBMC Serum	mRSS Lung, Gastrointestinal and renal involvement C-reactive protein	Antibody levels Cytokines levels Immunophenotyping Quantification of telomere length	Response or a relapse of disease
Assassi et al. (2019) (68)	62	8 and 26	Whole blood Serum	FVC mRSS	Gene expression profiling Serum protein composite score	FVC mRSS
Gernert et al. (2019) (69)	6	1, 2, 3, 5-7, 12-16	PBMC	–	Immunophenotyping Cytokines measuring	–
Gernet et al. (2020) (70)	17	4-14	Whole blood	mRSS Lungs and heart fuction	Immunophenotyping	Infectious complications
Arruda et al. (2020) (71)	8	18 (mean)	PBMC	mRSS Lung function	TCR diversity Frequency of CMV-specific clonotypes	Responder/non-responders/relapse
Lima-Júnior et al. (2021) (20)	22	1, 2, 3, 6, and 12	PBMC Serum	mRSS Lungs, heart, kidney and gastrointestinal tract function	Antibody levels Cytokines levels Immunophenotyping Suppressive capacity assay Signaling pathways	Responder/non-responders/relapse
Santana-Gonçalves et al. (2022) (72)	27	0,6,12,18,24,30 and 36	Serum Skin biopsies	mRSS Lungs, heart, kidney, gastrointestinal tract function and vascular involvement.	Cytokines levels Immunostaining in skin biopsies	Severity disease
Zanin-Silva et al. (2022) (73)	39	0 and 12	Serum Skin biopsies	mRSS Lungs, heart, kidney and gastrointestinal tract function	Cytokines levels Immunostaining in skin biopsies	Severity disease

PBMC, peripheral blood mononuclear cells; HAQ, Health Assessment Questionnaire; SP-D, surface protein D (SP-D); MHAQ, Modified Health Assessment Questionnaire Disability Index; mo, months; mRSS, modified Rodnan Skin Score.

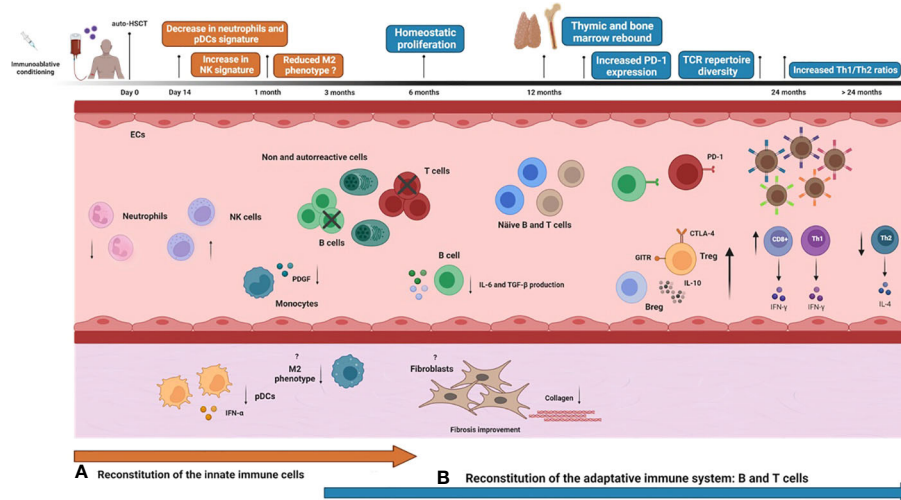


FIGURE 1

Immune reconstitution over time after auto-HSCT in systemic sclerosis patients. Systemic sclerosis (SSc) patients undergoing autologous hematopoietic stem cell transplantation (auto-HSCT) are treated with an immunoablative conditioning regimen consisting of high doses of chemotherapy/radiotherapy/immunotherapy agents, usually cyclophosphamide plus anti-thymocyte globulin. Then, previously collected autologous hematopoietic stem and progenitor cells (HSPCs) are thawed and administered to the patient intravenously. The graft may be CD34⁺ selected or non-manipulated, according to institutional protocols. After a period of bone marrow aplasia, there is hematological and immunological recovery, and the innate immune system recovers earlier than the adaptive system. Neutrophils are the first immune cell type to reconstitute, generally within the first 14 days after infusion of the HSPC graft. Neutrophil gene expression signatures significantly decrease after auto-HSCT. Other innate immune cells, such as monocytes, natural killer (NK) cells, and dendritic cells (DCs), achieve normal counts within the first month after transplantation. At this point, increased transcription signature of circulating NK cells is reported. In parallel, SSc skin biopsies post-auto-HSCT show significant reductions in mRNA expressions of plasmacytoid dendritic cells (pDC) and IFN- α responses. Alternatively-activated monocytes with an M2 phenotype significantly diminish post-transplantation. Plasma levels of platelet-derived growth factor (PDGF), an important molecule involved in fibrosis that positively correlates with the number of circulating monocytes in SSc, reduce significantly at six months after auto-HSCT. Improvement of skin fibrosis is evidenced within 6 months post-auto-HSCT, detected by decreasing modified Rodnan Score (mRSS) scores and reduced collagen deposition in the skin of SSc patients. Whether auto-HSCT affects the phenotype and function of fibroblasts from SSc patients is still unknown. B and T cells start to recover within the first six months after auto-HSCT. Early after transplantation, there is homeostatic proliferation, a process in which both cell subtypes expand in response to transplant-induced lymphopenia from residual cells that were not entirely depleted by the conditioning regimen or from cells that were re-infused with the graft. During the first-year post-transplantation, PD-1 expression is transiently increased on T and B cells as an important mechanism to control homeostatic activation. Circulating profibrotic IL-6 and TGF- β 1-producing B cell subsets transiently decrease at 6 and 12 months after auto-HSCT, possibly contributing to the amelioration of skin fibrosis. Thymic reactivation or rebound, usually detectable beyond the first-year post-transplantation, promotes the exportation of newly generated naive T-cells, including regulatory T cells, thereby increasing the peripheral TCR repertoire diversity. In parallel to the thymic rebound, there is also a parallel bone marrow rebound, marked by increased output of newly generated naive B cells. Functional and numeric recovery of Treg and Breg cells after auto-HSCT contributes to controlling autoreactivity and reestablishing self-tolerance by cell contact-dependent mechanisms, increased expression of GTR and CTLA-4, and increased production of IL-10. Finally, after transplantation, the Th1/Th2 ratio is rebalanced in SSc patients.

Considering the innate immune cells as the primary source of proinflammatory cytokines, investigations about how they contribute to T cell polarization and function in the context of auto-HSCT are essential. Auto-HSCT corrects the Th1/Th2 imbalance in SSc patients (64), but it is still unknown if the innate system contributes to this shift and which cell subtypes are involved (83).

Along with immune monitoring from blood samples, analyses of tissues affected by the disease, such as skin and lungs, may provide more insights into the local effects of auto-HSCT (83). Transplantation was able to reduce collagen deposition in the skin of SSc patients (73, 89), improved the microvascular morphology (63, 72, 90) and decreased lung infiltrates (91). In addition, the expression of IFN- α and

CD123⁺ (pDC markers) is associated with fibrosis in dcSSc (92). Skin biopsies of dcSSc patients showed significant reductions in IFN- α and CD123⁺ mRNA expression after auto-HSCT which inversely correlated with capillary numbers in the skin (63). These results demonstrate the positive effects of auto-HSCT on pDC phenotype and IFN- α response, with implications on microvasculature and fibrosis outcomes (Figure 1) (83).

A remaining question is how the auto-HSCT rebalances the communication/interaction between innate immune cells, endothelial cells, and fibroblasts, which could explain the positive effects of transplantation in the fibrosis and vasculopathy pathological axes (83). SSc patients treated with auto-HSCT have improved microvascular morphology and

significantly reduced endothelial activation in the dermis (72). Auto-HSCT can also induce connective tissue remodeling and decrease inflammation markers in the skin, such as S100 calcium-binding protein A9 (S100A9) and NF- κ B (73). How these mechanisms interact is still to be determined.

Finally, other innate immune components involved in SSc pathogenesis, such as pattern recognition receptors (PRRs) signaling pathways, damage-associated molecular patterns (DAMPs), and innate lymphoid cells (75), should be investigated to provide scientific bases to understand the effects of auto-HSCT in SSc and potential biomarkers of response. Whether the recently emerged innate immune system initiates a permissive environment for the new and tolerant adaptive immune system after auto-HSCT regeneration remains elusive (83).

Reconstitution of T cells in SSc after auto-HSCT

T cells play a critical role in the pathogenesis of SSc (93–97). These cells infiltrate the skin before any evidence of cutaneous fibrosis, suggesting their participation in the initial events of the disease (93). T cell receptors from skin infiltrates show oligoclonal repertoires, evidencing failure of tolerance mechanisms (98). Overlapping TCR β repertoires in CD4⁺ and CD8⁺ T cells from peripheral blood of SSc patients are highly stable over time, indicating temporal persistence of a low diversity T cell repertoire (99). In this context, auto-HSCT ablates autoreactive clones of T and B cells, renews the immune system, and reestablishes immune tolerance (Table 3) (5, 6, 100).

For the past two decades, immune monitoring studies have investigated the role of T cells in auto-HSCT for SSc and how they influence patient clinical outcomes (14, 18, 39, 61, 62, 64, 66).

Homeostatic proliferation

After the immunosuppressive regimen of auto-HSCT, T lymphocytes can recover from two sources: expansion of residual T cells, known as homeostatic proliferation (HP), or thymopoiesis, generating new naive T cells (101). In the early post-transplantation periods, naive and memory T cells that survive the conditioning regimen or are infused with the graft expand in response to lymphopenia (101).

In SSc, CD8⁺ T cells recover earlier than CD4⁺ T cells, achieving normal levels at 1 to 3 months after auto-HSCT, regardless of the clinical outcomes after the procedure (14, 61, 62, 64). The rapid reconstitution of CD8⁺ T cells also occurs in other clinical scenarios of lymphopenia, such as sepsis, post-

chemotherapy, and auto-HSCT for other autoimmune diseases (Figure 1) (102–21).

Studies report divergent results about the reconstitution of naive CD4⁺ T cells. Farge and collaborators described that the absolute number of naive T cells remained lower than baseline levels during the first nine months after auto-HSCT and reconstituted faster in patients who reactivated the disease after transplantation than those who remained in remission. Memory CD4⁺ T cell reconstitution did not differ between groups (14). Another study observed a similar trend, with naive CD4⁺ T cell remaining low until six months post-transplant in patients who sustained disease remission while reconstituting earlier in patients who reactivated the disease after the procedure (62). Tsukamoto et al. demonstrated that in SSc patients transplanted with CD34⁺ selected grafts, naive CD4⁺ T cells remained lower than baseline until 36 months, while memory CD4⁺ T cells returned to baseline levels at 24 months after auto-HSCT (64). Homeostatic proliferation after auto-

TABLE 3 Immunophenotyping of peripheral blood T and B cell subsets in SSc patients undergoing auto-HSCT.

CELL SUBSET	PHENOTYPE	REFERENCES
Total CD3, CD4, CD8	CD3 ⁺ ; CD3 ⁺ CD4 ⁺ ; CD3 ⁺ CD8 ⁺	(14, 39, 62, 64, 66)
Recent thymic emigrants	CD3 ⁺ CD4 ⁺ CD45RA ⁺ CD31 ⁺	(39)
Naive T cells	CD4 ⁺ CD45RA ⁺	(14, 62, 64, 66)
Memory T cells	CD4 ⁺ (CD8) CD27 ⁺ CD45RO ⁻ CD4 ⁺ CD45RO ⁺	(39) (14, 64)
Central-memory T cells	CD4 ⁺ (CD8)	(39)
Effector-memory T cells	CD27 ⁺ CD45RO ⁺	(39)
Effector T cells	CD4 ⁺ (CD8)CD27 ⁻	(67)
Senescent T cells	CD45RO ⁺ CD4 ⁺ (CD8)CD27 ⁻ CD45RO ⁻ CD8 ⁺ CD28 ⁻ CD57 ⁺ CD8 ⁺ CD28 ⁺ FoxP3 ⁺ CD3 ⁺ CD4 ⁺ (CD8)PD1 ⁺	(67) (67) (67) (67)
Th1	CD3 ⁺ CD8 ⁺ INF γ ⁺	(62)
Th2	CD4 ⁺ INF γ ⁺	(64)
Tc1	CD3 ⁺ CD8 ⁺ IL-4 ⁺	(62)
Tc2	CD4 ⁺ IL-4 ⁺	(64)
Regulatory T cells	CD3 ⁺ CD8 ⁺ INF γ ⁺	(62)
Total CD19 cells	CD3 ⁺ CD8 ⁺ IL-4 ⁺	(62)
Transitional B cells	CD4 ⁺ CD25 ⁺	(62, 64)
Post-switched memory B cells	CD4 ⁺ Foxp3 ⁺ CD4 ⁺ CD25 ^{hi} Foxp3 ⁺	(62) (18, 39)
Pre-switched memory B cells	CD4 ⁺ CD25 ⁺ TGF- β ⁺ CD4 ⁺ CD25 ⁺ IL-10 ⁺	(18) (18)
Double-negative memory B cells	CD4 ⁺ CD25 ^{hi} CTLA-4 ⁺ CD4 ⁺ CD25 ^{hi} GITR ⁺	(39) (39)
Naive B cells	CD19 ⁺	(14, 20, 39, 66, 69, 70)
Transitional Bregs	CD38 ⁺ CD10 ⁺ IgD ⁺	(69, 70)
Memory Bregs	CD19 ⁺ CD27 ⁺ IgD ⁻ CD19 ⁺ CD27 ⁺ IgD ⁺ CD19 ⁺ CD27 ⁻ IgD ⁻ CD19 ⁺ CD27 ⁻ IgD ⁺ CD19 ⁺ CD24 ^{hi} CD38 ^{hi} CD19 ⁺ CD24 ^{hi} CD27 ⁺	(20, 69, 70) (20, 69, 70) (20, 69, 70) (20, 69, 70) (20, 39, 69, 70) (20) (20)

HSCT was also associated with transient telomere attrition and increased senescent CD8⁺CD28⁻CD57⁺ T cells (39). These cells have immunomodulatory properties and might have a role in controlling autoimmunity early post-transplant (103). These studies show that the delayed recovery of naive CD4⁺ T cells is associated with favorable clinical response in SSc treated with auto-HSCT (Figure 1).

Increased PD-1 expression

The lymphopenia that follows the intense immunosuppression regimen of auto-HSCT leads to homeostatic proliferation and may exacerbate the expansion of residual autoreactive T cells, perpetuating the autoimmune disease (104). The expression of the co-inhibitory programmed death-1 (PD-1) receptor is an essential mechanism for controlling homeostatic activation in the first months after auto-HSCT and keeping autoreactive T cell clones under control (105). In SSc patients, PD-1 expression transiently increases on CD4⁺ and CD8⁺ T cells during the first-year post-transplantation. In addition, CD4⁺ and CD8⁺ T cells from patients with better clinical outcomes have higher PD1 expression than patients who reactivate the disease after auto-HSCT, indicating that this is an important immune regulatory mechanism in the early stages after transplantation (67). (Figure 1). Once thymic rebound establishes itself, newly-generated naive and regulatory T cells are responsible for maintaining long-lasting self-tolerance (106).

Thymic reactivation

Thymic involution is a physiological process that includes atrophy, loss of cells, and structural changes of the organ, mainly associated with age (107). In immune monitoring studies after transplant, TCR rearrangement excision circles (TRECs) are used to assess thymic function. TRECs are small circles of DNA formed during the rearrangement of T cell receptors that do not duplicate during mitosis (108). TREC counts in the peripheral blood reflect new T cells exported by the thymus (109, 110).

In auto-HSCT, thymic rebound is defined by functional reactivation and volumetric enlargement of the thymus after the immunosuppression regimen and re-infusion of autologous hematopoietic stem and progenitor cells (111). The thymic reactivation generally occurs after one to two years post-transplantation, depending on the patient's age, and has been associated with favorable clinical response of autoimmune disease patients (21, 31, 31, 39). Storek et al. (61) showed an increase in thymic size detected by computed tomography at three and 12 months and increased TREC frequencies between one- and two-years post-transplantation in patients with multiple sclerosis and systemic sclerosis (Figure 1).

Farge et al. showed that in patients that reactivated SSc after auto-HSCT, TREC values transiently increased from 6 to 8 months after transplantation, decreasing thereafter (14). These results corroborate those from Bohgaki et al. demonstrating significantly reduced sjTREC levels at three months after auto-HSCT in patients with good outcomes compared with those that reactivated the disease (62). Arruda et al. reported a positive correlation between sjTREC levels and frequencies of early naive T cells, named recent thymic emigrants (RTE) with the phenotype CD3⁺CD4⁺CD45RA⁺CD31⁺ in the peripheral blood of SSc patients, an alternative and complementary method to quantify thymic function. The RTE number also correlated with regulatory T cell (Treg) counts and better clinical outcomes after auto-HSCT (39).

In summary, the thymic rebound is one of the main immunological mechanisms of auto-HSCT. Efficient production of new naive T cell cells generates a diverse TCR repertoire that has been associated with the control of the autoimmune disease after auto-HSCT (Figure 1).

TCR repertoire diversity

The generation of naive T cells after auto-HSCT -induced thymic reactivation directly affects TCR repertoire diversity (19). Farge et al. reported a disturbed T cell repertoire in SSc patients at baseline compared with age-matched controls, which did not change in the one-year follow-up after auto-HSCT (14). In a more extended follow-up study of six years, these authors showed late recovery of a polyclonal profile of the TCR repertoire similar to healthy individuals (66). However, there was no correlation between TCR diversity and clinical response to transplantation.

Arruda et al. demonstrated that SSc patients that reactivated the disease after auto-HSCT sustained a skewed TCR repertoire, indicating persistent autoreactivity one to two years after transplantation (39). In contrast, patients that remained in remission after transplantation presented a polyclonal TCR repertoire, similar to healthy individuals. In addition, patients that reactivated the disease after auto-HSCT presented increased frequencies of CMV-specific clones and a reduction of TCR diversity after the procedure. Oppositely, patients with good outcomes after transplantation showed an increase in TCR clonotypes specific to CMV, EBV, Influenza, and Dengue virus (71).

The renewal of the TCR repertoire following auto-HSCT has been proposed as a potential biomarker of therapeutic response (17). Patients with favorable clinical outcomes after auto-HSCT present low overlap of TCR clonotypes, reflecting the successful replacement of skewed and autoreactive T cell clones by a more polyclonal T cell repertoire (17).

Increase of Th1/Th2 ratios

The Th1/Th2 imbalance contributes to the pathogenesis of several organ-specific and systemic autoimmune diseases (112). The Th2 response, characterized by the production of profibrotic cytokines such as IL-4, IL-6, IL-5, and IL-13, has a critical relevance in the pathogenesis of SSc (113–115). These cytokines stimulate collagen synthesis by fibroblasts and are chronically elevated in the serum of SSc patients. In contrast, the anti-fibrotic Th1 cytokine IFN- γ is reduced in SSc patients (116–119).

Frequencies of IFN- γ -producing CD8⁺ T cells increase at 12 months after transplantation, regardless of the clinical outcomes after auto-HSCT (62). Tsukamoto et al. showed that the ratio of IFN- γ /CD4⁺ to IL-4/CD4⁺ increased one month after auto-HSCT, reaching a plateau at six months that was maintained for at least 36 months. However, despite the Th1/Th2 ratio increase, there were no correlations between changes in the Th profile and modified Rodnan skin score (mRSS) (64). There are few studies evaluating the reconstitution of T cell subsets after auto-HSCT. Therefore, more studies are warranted to understand how T cell subsets contribute to the different clinical outcomes in SSc patients after auto-HSCT.

Regulatory T cell recovery

Regulatory T cells (Tregs) expressing the transcription factor forkhead box P3 (FOXP3) represent 5–10% of the peripheral CD4⁺ T cells in humans and are crucial for the maintenance of self-tolerance and immune homeostasis (120, 121). SSc patients present decreased frequencies and reduced suppression capacity of circulating Tregs compared to healthy individuals (122–124).

Bohgaki et al. showed that CD4⁺CD25⁺ T cells increased at 12 months in SSc patients treated with unselected CD34⁺ grafts, regardless of clinical outcomes (62). FOXP3 gene expression levels did not correlate with the therapeutic response or graft selection. Another immune monitoring study of autologous transplants for SSc using selected grafts showed a severely delayed reconstitution of Tregs (64). The frequencies of CD4⁺CD25⁺ and CD4⁺FOXP3⁺ T cells remained lower than baseline even at 36 months post-transplantation (Figure 1) (64).

Baraut et al. evaluated the suppressive capacity of Tregs by co-culture with autologous effector T cells (18). The suppressive function of CD4⁺CD25^{high}CD127^{low} Tregs was restored 24 months after auto-HSCT, along with increased numbers of IL-10-producing CD4⁺CD25⁺ Tregs. On the other hand, CD4⁺CD25⁺TGF- β T cell counts remained significantly decreased before and after auto-HSCT (18). These findings indicate an improvement of the suppressive capacity of Tregs by cell contact-dependent mechanisms and the production of the inhibitory cytokine IL-10.

Patients with good clinical response to auto-HSCT present higher CD4⁺CD25^{high}FOXP3⁺ Treg percentages after the procedure than those that reactivate the disease (39). Tregs from responsive patients also show increased expression of GITR and CTLA-4 molecules compared to the patients that reactivate the disease after auto-HSCT (39).

Although the results diverge across different studies, the reported findings indicate that auto-HSCT induces a functional recovery of Treg with increased IL-10-production, improved cell contact-dependent suppression, and higher expression of GITR and CTLA-4. Effective functional Treg reconstitution has been related to favorable clinical outcomes of SSc and is currently considered another pivotal mechanism of auto-HSCT.

Changes in serum cytokines

Several T cell-related cytokines are implicated in SSc pathogenesis and associated with fibrosis and vascular damage (18, 114, 125, 126). Auto-HSCT changes serum levels of inflammatory and pro-fibrotic cytokines by regenerating the immune system (39, 64, 65, 72, 73). High serum levels of tumor necrosis factor-alpha (TNF- α), IL-6, and soluble interleukin 2 receptor (sIL-2R) are found at baseline in the SSc patients, with a significant decrease after auto-HSCT (64). Serum levels of IL-2, IL-8, and TGF- β also transiently decrease after auto-HSCT. Still, changes in levels of the profibrotic cytokine TGF- β are not associated with the improvement of the skin score (mRSS) (55). Other cytokines, such as IL-6, IL-10, and IFN- γ , are increased at baseline and do not decrease for at least 48 months after transplantation (65).

Expression of tumor necrosis factor receptor (TNFR) was found higher on dermal T lymphocytes from SSc patients than in healthy controls. Activated peripheral blood lymphocytes also secreted more IL-6, soluble IL-6 receptor and IL-13, and induced higher type 1 collagen expression in fibroblasts compared to lymphocytes from healthy controls. In one subject that had been treated with auto-HSCT, expression of TNFR and IL-6 decreased in the dermis at the six-month time point after the procedure. These results indicate a therapeutic potential of auto-HSCT in improving the inflammation-fibrosis axis in SSc (127).

Recently, Zanin-Silva et al. observed alterations in connective tissue and fibrosis-related molecules after auto-HSCT (63). One-year post-transplant, SSc patients exhibited significant decreases in serum concentrations of platelet-derived growth factor (PDGF)-AA, PDGF-BB, tissue inhibitor of metalloproteinases (TIMP)-1 and S100A9. On the other hand, serum levels of collagen I alpha 1 (COL1A1) increased after transplantation, indicating collagen degradation (73).

Assassi et al. investigated molecular changes in the peripheral blood cell transcriptome in 62 SSc patients treated with auto-HSCT. At 26-months post-transplantation, the IFN

transcript score decreased significantly, indicating a long-lasting effect. In contrast, patients treated with conventional cyclophosphamide did not present significant changes in the molecular signatures (68).

Santana-Gonçalves et al. evaluated serum levels of markers of inflammation, angiogenesis, and endothelial activation before and until 36 months post-transplant (72). IL-6, von Willebrand factor (vWF), CXC Motif Chemokine Ligand 8 (CXCL8), Endothelin-1, epidermal growth factor (EGF), VEGFA, Pentraxin-3, Intercellular Adhesion Molecule 1 (ICAM-1), E-selectin, P-selectin, Thrombomodulin and IL-18 levels were significantly higher at baseline in SSc patients when compared to healthy controls, except for ICAM-1. After auto-HSCT, all biomarkers remained stable at high levels until 36 months of follow-up, indicating persistence of the vascular injury (72).

The transplant-induced changes in serum cytokine levels associated with inflammation and fibrosis indicate an at least partial systemic modulatory effect of this therapeutic approach. However, alterations in cytokine profiles are only partially responsible for the clinical outcomes of SSc patients treated with auto-HSCT.

Reconstitution of the B cell compartment in SSc after auto-HSCT

B cells have been widely investigated in SSc pathogenesis, especially since B cell-targeting therapies have become available. Autoreactive B cells produce autoantibodies against nuclear autoantigens, such as anti-topoisomerase I (anti-Scl-70), anti-centromere, and anti-RNA polymerase III, which are associated with different disease phenotypes (42, 128). Autoantibodies directed against endothelial cells and fibroblasts are also found in SSc patients, suggesting a contribution of B cells to tissue fibrosis and vasculopathy (129, 130).

Target organs, such as the lungs and the skin, show B cell infiltrates, underscoring the importance of these cells in disease pathogenesis (131–133). In the skin, B cell infiltrates are implicated in the early stages of the disease, preceding the establishment of fibrosis (134). This is important since B cells from SSc patients can induce the production of IL-6, TGF- β , and collagen by fibroblasts (135).

Moreover, B cells with a hyperactivated phenotype have been identified in the peripheral blood of SSc patients, with overexpression of the CD19 surface signaling molecule and correlation with autoantibody production, indicating that CD19 regulation may be functionally linked with autoantibody production in SSc (136). Meanwhile, regulatory B cells (Breg) are decreased in numbers and functionality, with deficient IL-10 production (137) and inversely correlating with disease activity (138). Additionally, Breg subpopulations from SSc patients fail to suppress CD4⁺ T cells (139).

In the context of auto-HSCT, pre-existing autoreactive B cells are depleted by the immunoablative regimen (17, 21, 140). Different studies with SSc patients have assessed the kinetics of the B cell reconstitution after auto-HSCT (Figure 1) (1, 14, 20, 39, 66, 69, 70, 141). Memory B cell counts increase early after auto-HSCT due to homeostatic proliferation but significantly diminish after that, while numbers of naive B cells newly produced by the bone marrow increase (14, 39). Indeed, CD19⁺IgD⁺CD27⁻ naive B cell frequencies increase early after auto-HSCT compared to baseline (20). In parallel to the thymic rebound, there is also a comparable “bone marrow rebound”, with increased output of newly generated naive B cells, which is not observed in SSc patients treated with conventional therapies (39).

A remaining and intriguing question of the auto-HSCT scenario is whether complete removal of memory B and T cells is required for full therapeutic efficacy of transplant. Incomplete immunoablation or even reinfusion of autoreactive and memory cells within the autologous stem cell graft may trigger disease reactivation (100, 142). Patients with autoimmune diseases, including SSc, have more circulating double-negative memory B cells, characterized by absent expression of CD27 and IgD (143, 144). This cell subpopulation transiently increases in the first month after auto-HSCT in SSc patients, followed by a sustained decrease in later time points (20). Combined with the expansion of switched and non-switched memory B cells after transplantation, the increase of double negative memory B cells at this stage could indicate the existence of residual B-cells, either non-depleted by the transplant conditioning regimen or re-infused within the graft (20). The transiently high expression of the negative co-stimulatory molecule PD-1 in B cells of SSc patients in these early periods after transplantation may have a role in preventing the exaggerated proliferation of autoreactive B cells (20). A similar mechanism of control is described in multiple sclerosis (MS) patients, correlating with good neurological outcomes after auto-HSCT (19).

Circulating CD19 and CD20 B cell counts are inversely associated with clinical response, suggesting that pathogenic B cell clones may preferentially expand in patients with less favorable outcomes (14, 66). Additionally, in patients with a good response to auto-HSCT, there is a sustained and positive slope of B cell reconstitution, which may reflect increased numbers of B cell subsets that promote disease control, especially those with a regulatory phenotype and function (66).

Timely reconstitution of regulatory T and B cells after transplantation is considered a key element in modulating the activation and proliferation of potent autoreactive cells after auto-HSCT (19, 39, 145). Multiple studies have shown that the T cell-mediated regulatory network improves in SSc and other autoimmune diseases following auto-HSCT (17, 21, 146). However, recent studies have also shown an increased

frequency of B cells with regulatory and anti-inflammatory phenotypes in SSc patients after auto-HSCT (20, 139). IL-10 release by peripheral B cells of SSc patients post-transplantation significantly raises compared to baseline (141).

Additionally, transitional Bregs (tBregs, CD19⁺CD24^{high}CD38^{high}) and memory Bregs (mBregs, CD19⁺CD24^{high}CD27⁺) are IL-10-producing cells (147–149) that increase after auto-HSCT (20). Patients with disease remission after auto-HSCT present higher frequencies of tBregs than those who reactivate SSc, both at baseline and 360 days post-transplant. Moreover, tBregs also recover their ability to suppress the production of Th1 cytokines by CD4⁺ T cells after auto-HSCT (20).

Previous studies have shown that phosphorylation of ERK1/2 and p38 MAPK contributes to IL-10 production (150, 151) and that SSc Bregs have impaired p38 MAPK phosphorylation pathways (135). Lima-Júnior and collaborators demonstrated that Bregs increased the phosphorylation of ERK1/2 and p38 MAPK proteins after auto-HSCT (20). Thus, an increase in ERK1/2 and p38 MAPK phosphorylation may be one of the mechanisms responsible for improving Breg suppressive function in SSc treated with auto-HSCT (20). Circulating profibrotic subsets of IL-6 and TGF- β 1-producing B cells also decreased after auto-HSCT, possibly contributing to the observed amelioration of skin fibrosis (20). Future studies should investigate a possible relationship between the decreased numbers of these B cell subsets and tissue fibrosis.

We suggest that the profound alterations in the B cell compartment in SSc patients induced by auto-HSCT cannot be achieved with conventional B cell-depleting therapies. Rituximab, a monoclonal antibody against CD20 molecule, has been used to selectively deplete B-cells in many autoimmune diseases, with transient and variable responses (152–154). Treatment of rheumatoid arthritis (RA) with rituximab promotes B-cell depletion and is associated with clinical response (155). However, half of the rheumatoid arthritis patients eventually lose responsiveness over time, requiring additional rituximab infusions (154). Three-quarters of these patients recover disease control after consecutive treatment cycles with rituximab, indicating that loss of response is reversible and that patients may still improve (154). However, for type 1 diabetes (T1D) patients, rituximab was ineffective in resetting defective B cell tolerance checkpoints (153).

SSc patients treated with rituximab showed benefits in the skin (155–157) and lung involvements (158). In parallel, treatment with belimumab, another monoclonal antibody that leads to B cell apoptosis and decreases autoantibody production (159) resulted in significant improvement of mRSS in SSc patients with early disease and alterations in the expression of the profibrotic gene pathways in B cells. These studies indicate that although the B cell-depleting therapies are effective in the control activity of specific autoimmune diseases, they do not

promote profound or long-lasting modifications in the patient's immune system. Therefore, auto-HSCT may be a better therapeutic alternative than conventional B cell-directed therapies for promoting long-lasting improvements in the B-cell compartment.

Conclusions and perspectives

In conclusion, auto-HSCT involves multiple immune mechanisms that collectively improve SSc patient clinical outcomes. Reconstituted neutrophils, monocytes, natural killers, and dendritic cells may exert an important role in producing signals that contribute to the new regulatory microenvironment promoted by auto-HSCT. However, the literature lacks studies regarding these cells and how the innate and adaptative immune systems interact after auto-HSCT. Future investigations of the innate immune cell subsets, including their phenotype, numbers, and functionality, may increase the understanding of SSc pathogenesis, perhaps even beyond the transplantation scenario.

In the context of auto-HSCT for autoimmune diseases, including SSc, the roles of homeostatic proliferation, thymic and bone-marrow rebound, T cell repertoire diversity, and increase of T and B cell-mediated regulation are currently well-described mechanisms. Nonetheless, more vigorous efforts are needed to better correlate the immunological mechanisms of auto-HSCT with patient clinical outcomes. Further cellular function assessments and comprehensive molecular analyses may identify immune signatures associated with disease remission or reactivation after auto-HSCT. Collaborative approaches to evaluate the immune profile of more significant numbers of transplanted SSc patients worldwide may provide helpful answers. We convene the academic community to pursue these research gaps and further improve clinical transplant protocols, allowing more SSc patients to benefit from this therapeutic approach.

Author contributions

MK-V, MS-G, and DZ-S wrote the manuscript and performed the literature review. MS-G and DZ-S created the images. MS-G, KM, and MO critically revised the final version. All authors contributed to the article and approved the submitted version.

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