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The role of innate immune cells in systemic sclerosis in the context of autologous hematopoietic stem cell transplantation

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Summary

Systemic sclerosis (SSc) is a complex, heterogeneous autoimmune connective tissue disease. Autologous hematopoietic stem-cell transplantation (AHSCT) has emerged as a valuable treatment option for rapidly progressive diffuse cutaneous SSc (dcSSc) patients, and thus far is the only treatment that has been shown to have a long-term clinical benefit. AHSCT is thought to reintroduce immune homeostasis through elimination of pathogenic self-reactive immune cells and reconstitution of a new, tolerant immune system. However, the mechanism of action underlying this reset to tolerance remains largely unknown. In this study we review the immune mechanisms underlying AHSCT for SSc, with a focus on the role of the innate immune cells, including monocytes and natural killer (NK) cells, in restoring immune balance after AHSCT.

Keywords: autologous hematopoietic stem-cell transplantation, diffuse cutaneous systemic sclerosis, immunological tolerance, innate immunity

Introduction

Systemic sclerosis (SSc) is a complex, heterogeneous autoimmune connective tissue disease mainly characterized by vascular abnormalities, immune involvement and extensive fibrosis of the skin and internal organs. Although the exact pathogenesis of SSc remains to be elucidated, microvascular changes and endothelial cell dysfunction are thought to be the earliest events in the disease, followed by dysregulation of innate and adaptive immune responses, eventually leading to myofibroblast differentiation and increased deposition of extracellular matrix material [1].

Autologous hematopoietic stem-cell transplantation (AHSCT) is the only therapy with long-term clinical benefit in rapidly progressive dcSSc [2]. Until now, three randomized controlled trials comparing AHSCT to monthly intravenous cyclophosphamide pulse treatment in SSc have been published: ASSIST (American Scleroderma Stem cell *versus* Immune Suppression Trial) [3], ASTIS (Autologous Stem cell Transplantation International Scleroderma Trial) [4] and SCOT (Scleroderma Cyclophosphamide or Transplantation) trials [5]. AHSCT was shown to have a superior effect over cyclophosphamide, yielding significant improvements in survival, quality of life, functional capacity, modified Rodnan skin score (mRSS; skin thickening) and lung function. The main rationale for applying AHSCT to SSc as well as other autoimmune diseases (ADs) is to restore immune homeostasis. This is achieved by first applying an intensive immunoablative conditioning regimen, which eliminates the pathogenic self-reactive immune cells, followed by the reconstitution of a new immune system from reinfused hematopoietic precursors. However, the exact mechanisms underlying this 'immune reset' by AHSCT are not yet understood completely, leaving numerous unanswered questions regarding the molecular and immunological background of AHSCT. Immune monitoring studies after AHSCT aim to answer these questions and improve our understanding of how exactly immune tolerance after transplantation is established. In this review, we will summarize the current knowledge on the immune mechanisms involved in AHSCT for SSc and other ADs, with a focus on the innate immune system.

Adaptive immune system in SSc after AHSCT

The majority of the studies into the immune reconstitution and functioning after AHSCT in SSc so far have focused on the adaptive immune system, investigating its effects on the kinetics of reconstitution of different lymphocyte subsets (T and B cells), effects on the T cell receptor (TCR) repertoire and modulation of autoantibody levels.

During the first 3 months following AHSCT the immune system is in a lymphopenic state, marked by a reduction

of absolute numbers of circulating T cells (especially $CD4^+$ T cells) and B cells [6,7]. Thereafter, the numbers of B cells and $CD8^+$ T cells begin to rise and achieve reconstitution to baseline numbers after approximately 1 year [6,8], while the recovery of $CD4^+$ effector and memory T cells is slower and often remains incomplete (Fig. 1). Notably, a slower reconstitution of T cells has been observed in non-responder/relapsing patients, which can be due either to incomplete abrogation of pretransplant self-reactive T cells or a sustained activation and persistence of an underlying autoimmune mechanism in these patients [9]. Absolute numbers of T_{regs} increase after AHSCT compared to the numbers observed before AHSCT [10,11], and percentages of T_{regs} are higher in responders compared to non-responders [10]. These data suggest that generation of T_{regs} probably has a critical role in establishing immune tolerance after AHSCT.

Apart from the reduction of the absolute numbers of T and B cells, AHSCT also affects the diversity of T and B cell repertoires, which forms another important aspect in immune system regeneration. TCR-Vβ repertoires reportedly display a skewed and oligoclonal expanded profile in dcSSc patients [6,9,10,12]. After AHSCT, TCR diversities (estimated by the Chao1 diversity index) increase significantly following thymic rebound compared to baseline and percentages of polyclonally expanded TCRs are raised, reflecting the reconstitution of a new immune system [6,9,10]. Differences in the clonality of the TCR repertoire have also been observed between responders and non-responders, with non-responders having a less diverse repertoire [6,10].

B cell reconstitution in SSc after AHSCT has also been studied recently in more detail, showing a shift from memory B cells to naive B cells [8,10,13]. Additionally, these B cells show an enhanced production of IL-10, an immune regulatory cytokine, after AHSCT. The fraction of B_{regs} was also increased post-AHSCT, and responders showed significantly higher levels of B_{regs} than non-responders [10,11]. Serum concentrations of the dcSSc-specific anti-topoisomerase I-antibodies (topo-I/Scl70) have also been shown to drop progressively after AHSCT [9,13,14]. Correlations between anti-Scl70 titers and clinical parameters have been reported, but these observations were not consistent throughout different studies.

Innate immune system in SSc after AHSCT

Although the innate immune system plays a pivotal role in SSc pathogenesis [15], the majority of the immune monitoring studies after AHSCT have focused on the adaptive immune response, as outlined above. Because the innate immune response recovers much faster than the adaptive immune system after AHSCT [16], and the formation of adaptive responses are largely dependent on co-stimulatory signals provided by the innate immune system, a clearer understanding of innate immune reconstitution after AHSCT can help to further improve transplant outcomes and patient care.

In order to gain more insight into how AHSCT affects the gene expression profile of circulating immune cells,

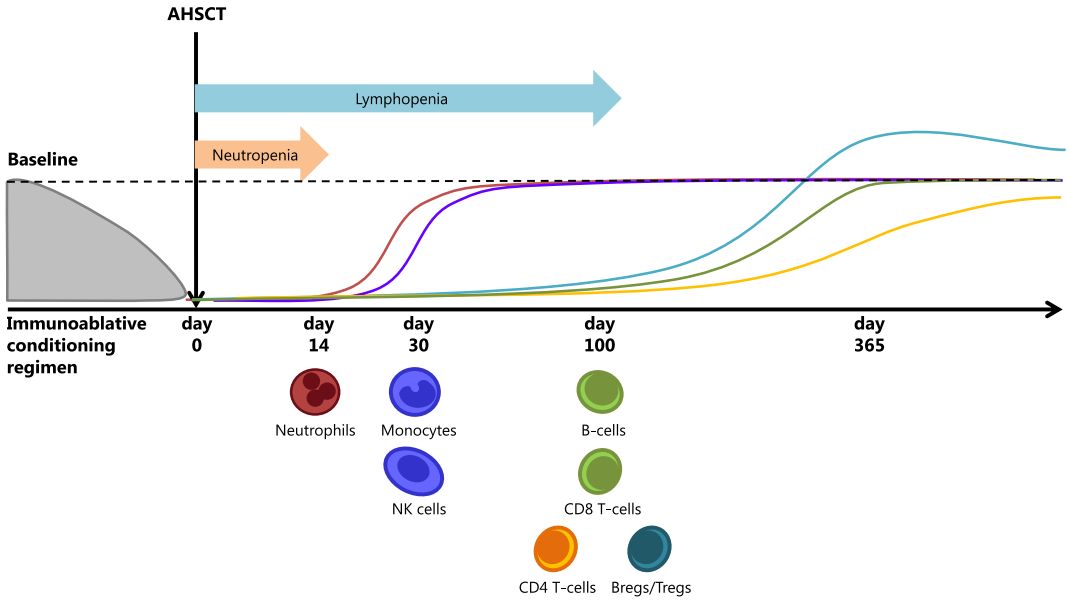

Fig. 1. Schematic overview of the kinetics of immune reconstitution after autologous hematopoietic stem-cell transplantation (AHSCT). Neutrophils are this first cells to reconstitute after approximately 14 days, followed by $CD14^+$ monocytes and natural killer (NK) cells. Lymphocytes reconstitution begins approximately 3 months post-AHSCT. Total $CD8^+$ T and B cell numbers achieve reconstitution to baseline in around 1 year after AHSCT, while $CD4^+$ T cell [non-regulatory T cell (T_{reg})] reconstitution is slower and remains lower than baseline. The numbers of T_{regs} and regulatory B cells (B_{regs}) increase post-AHSCT compared to baseline.

transcriptomic and protein profiling was performed on whole blood samples obtained from study participants receiving AHSCT in the SCOT trial [17]. First, the gene expression profile of peripheral blood mononuclear cells (PBMCs) from baseline samples of SSc patients were compared to healthy donors, and the presence of an interferon (IFN) signature was confirmed. Additionally, neutrophil and cytotoxic/NK cell signatures were described [17]. Interestingly, 26 months after AHSCT the IFN and neutrophil signatures were decreased, while the cytotoxic/NK cell signature was increased. Analysis of samples from patients treated with cyclophosphamide did not show this shift in gene expression. Moreover, the decline in the IFN and neutrophil gene signatures correlated with an increased improvement in lung function [forced vital capacity (FVC%)], while the increase in the cytotoxic/NK cell signature was significantly associated with a decrease in mRSS score [17]. These results indicate that dysregulated signatures of innate immune cells including neutrophils and NK cells are normalized after AHSCT, and associated with more effective clinical outcomes. Interestingly, neutrophils and NK cells are one of the first immune cell subsets to reconstitute after AHSCT [16,18], and the evidence that their transcriptomic signatures are normalized following AHSCT provides a potential role for these cells in early immunomodulating events after AHSCT.

Cytokines and chemokines produced by innate immune cells are fundamental for polarizing $CD4^+$ T cells into T helper type 1 (Th1) and Th2 responses [19]. Evidence for a shift to a predominant Th2 response has been observed in SSc and has been associated with clinical outcomes [20]. This shift in Th1/Th2 balance appeared reversible after AHSCT [13]. Thus far it is largely unclear how this predominant reconstitution of Th1 cells is induced and maintained in dcSSc patients after AHSCT. The previously observed increase in levels of profibrotic and Th2 cytokines [transforming growth factor (TGF)-β and IL-2, sIL-2R IL-8, respectively] in sera of SSc patients was shown to be partly reversed after AHSCT [13,21], which could possibly contribute to the shift in Th balance. Further studies on the balance between Th1/Th2 cytokines after AHSCT may provide more insight into the mechanisms behind the T cell polarizing shift. Additionally, as innate immune cells form a major source of proinflammatory cytokines, it would be interesting to investigate further if the shift from Th2 to Th1 after AHSCT is indeed mediated by innate immune cells and, if so, which subtype. Apart from circulating cytokines/chemokines, tissue levels of these molecules should provide more insight into the local effect of AHSCT on immune cells; for example, in skin or in lung. AHSCT has been shown to reduce collagen deposition in SSc skin [22], has a positive effect on the microvasculature [23,24] and decreases the extent of lung involvement [25]. Fleming *et al.* performed mRNA *in-situ* hybridization and immunohistochemistry on skin samples of dcSSc patients at baseline and after AHSCT [22]. Using these techniques, it was shown that IFN-α mRNA expression, $CD123^+$ (the marker for plasmacytoid dendritic cells) and signal transducer and activator of transcription 1 (STAT)-1 (an interferon-inducible transcription factor) expression were decreased in SSc skin biopsies after transplantation, and this was associated with an increase in capillary numbers in skin. As IFN-α has been identified as an inhibitor of angiogenesis [26], a decrease of IFN-α levels in the skin could contribute to the improvements in microvasculature observed after AHSCT. Furthermore, IFN-α expression and $CD123^+$ plasmacytoid dendritic cells are interesting in the context of fibrosis, as they have been shown to be directly involved in the establishment and maintenance of skin fibrosis in bleomycin mouse models of SSc [27]. In this context, it would also be interesting to investigate chemokine (C-X-C motif) ligand (CXCL4) in the skin of SSc patients after AHSCT, as plasma concentrations of CXCL4 (a chemokine secreted by plasmacytoid dendritic cells) strongly correlate with SSc disease activity and progression [28], and have been directly linked to fibrosis in SSc [27]. More functional studies that focus on the interplay between endothelial cells, fibroblasts, dendritic cells and other (innate) immune cells in SSc skin after AHSCT are essential to help understand the effect of AHSCT on fibrosis and angiogenesis.

Implication of the innate immune system in AHSCT: evidence from other autoimmune diseases

Thus far, little is known about the effects on and the role of the innate immune system in SSc upon AHSCT. As AHSCT is also used to treat other (rheumatic) ADs, evaluating the role of the innate immune system in the context of AHSCT in these diseases might provide more clues about its potential role in SSc [29]. Suppression of T cell responses after AHSCT has largely been attributed to intrinsic T cell mechanisms [18]. However, it might be possible that other, non-T cell, populations are also able to exert inhibitory effects on T cells after AHSCT. In a cohort of patients transplanted for hematological/immunological diseases (allogenic and autologous HSCT), $CD14^+$ monocytes suppressed the proliferative capacity of T cells in the post-HSCT period [30]. Monocytes obtained from healthy donors did not have this inhibitory effect on T cells obtained from HSCT donors, indicating that HSCT might induce monocytes to become more suppressive. In another study, in a cohort of patients with Crohn's disease receiving AHSCT without $CD34^+$ selection, AHSCT resulted in a decrease in expression of TLR-4, TNF and IL-10 on circulating $CD14^+$ monocytes [31]. These studies suggest

that monocytes may have a role in controlling immune responses post-AHSCT. Controversially, Bonechi *et al.* showed that AHSCT does not modulate the TLR-4 signaling pathway in CD14$^+$ monocytes from multiple sclerosis (MS) patients [32]. Thus, the exact role of monocytes after AHSCT is still unclear. Interestingly, numbers of circulating monocytes are increased in PMBCs of SSc patients compared to healthy donors. In particular, the CD16$^+$ subpopulation is increased in dcSSc and also correlates with the severity of skin fibrosis evaluated by mRSS [33]. This CD16$^+$ monocyte subtype has especially been shown to display enhanced proinflammatory features on activation and exhibits properties for antigen presentation [34]. Furthermore, monocytes from SSc patients have the propensity to differentiate into myofibroblasts, which are the key effector cells in fibrosis in SSc [35]. Given the importance of monocytes in SSc and their potential to regulate T cell responses after AHSCT (Fig. 2), studying monocyte subsets after AHSCT in SSc could help to gain more insight into their role in SSc pathogenesis and immune regulation after AHSCT. Apart from monocytes, NK cells are also known to be able to regulate T cell responses. This regulation can be indirect, via the secretion of cytokines, or direct through their cytolytic activity [36]. Using a combination of mass cytometry (CyTOF) and multi-parameter flow cytometry, it was shown that NK cells were transiently increased after AHSCT in MS patients [37]. In a Canadian cohort of MS patients who received immunoablative chemotherapy followed by CD34$^+$ cells, this increase in NK cells was confirmed and was additionally found to be correlated with a decrease in Th17 cells [38]. Co-cultures of NK and Th17 cells from post-AHSCT patients were performed to further investigate the relationship between these two cell types and explore the regulatory potential of NK cells after AHSCT. Indeed, Th17 as well as Th1 cell activation was significantly lower in activated PBMC cultures that contained CD56$^+$ NK cells compared to NK cell-depleted cultures [38]. These results demonstrate that, at least in MS patients, NK cells can contribute to the regulation of T cell responses after AHSCT. As mentioned earlier, an increased cytotoxic/NK cell signature was also identified in PBMCs obtained from SSc patients after AHSCT, and is associated with a decrease in mRSS [16], suggesting that NK cells might also contribute to immunosuppression after AHSCT in SSc. The results described above suggest that monocytes and NK cells may contribute to generating a tolerant, immunosuppressive environment after AHSCT. It would be interesting to further explore the roles of these cells in SSc AHSCT, and also delineate how different monocyte/NK subpopulations influence this process.

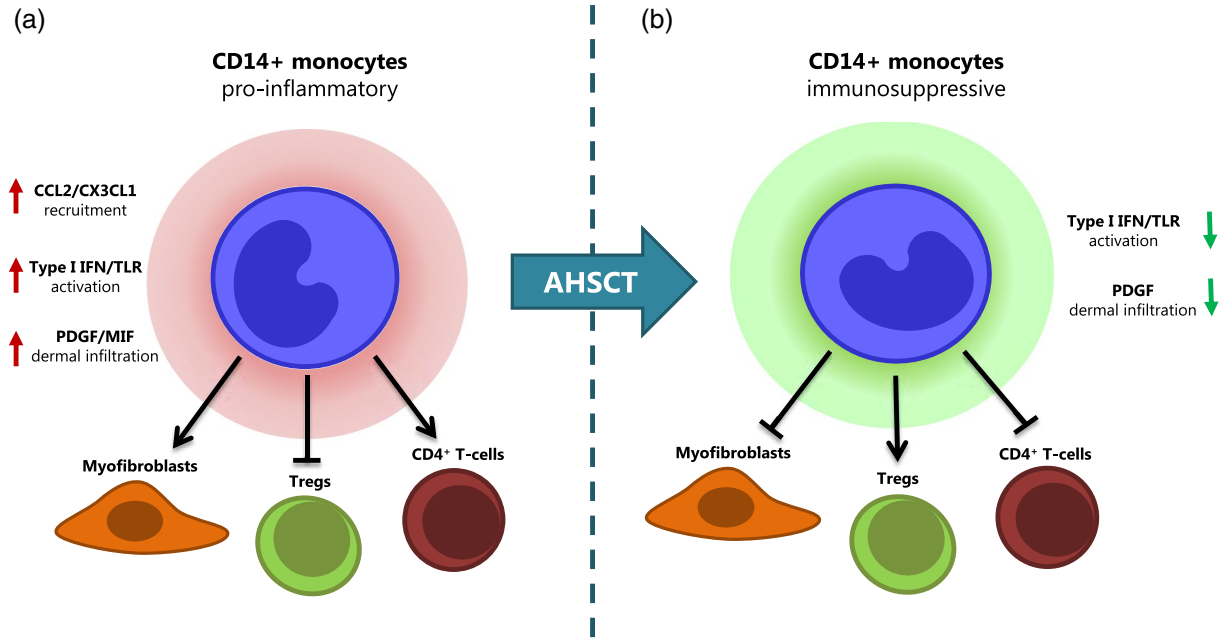

Fig. 2. Postulated role of monocytes in autologous hematopoietic stem-cell transplantation (AHSCT). (a) Monocytes are implicated in systemic sclerosis (SSc) pathology through different mechanisms. They are increased in circulation and skin of SSc patients, due to an increase in recruitment factors in these patients. Additionally, they display an inflammatory phenotype through increased Toll-like receptor (TLR) signaling and interferon (IFN) pathways. SSc monocytes have an enhanced potential to differentiate into myofibroblasts, and can contribute to the inhibition of T_{regs} while priming other autoreactive T cell responses. (b) After AHSCT, CD14$^+$ monocytes seem to display an immunosuppressive phenotype, evident by a decreased expression of TLR-4, tumor necrosis factor (TNF) and interleukin (IL)-10, and their ability to suppress the proliferative capacity of T cells in the post-HSCT period. Furthermore, we postulate that differentiation of monocytes into myofibroblasts is inhibited during AHSCT.

Additionally, neutrophils should also be studied, as neutrophil gene expression signatures in PBMCs from dcSSc patients are decreased after AHSCT [17], and neutrophils are the first cells to reconstitute post-AHSCT [7].

Conclusions

Both adaptive and innate immune responses are modulated after AHSCT in SSc and contribute to the generation of a tolerant immune system. Although the adaptive immune system has been studied in more detail, the innate immune system also has a potential immunosuppressive/modulating role in regulating adaptive responses after AHSCT. In support of this theory, $CD14^+$ monocytes and NK cells have already been shown to be capable of regulating T cell responses after AHSCT [30,38]. Additionally, signatures implicating regulatory cytokines [13,21], NK cells and neutrophils [17] have been identified in SSc patients after AHSCT, and these signatures correlate with clinical parameters. Further investigation into the exact role of innate immune cells and their importance in regulating immune responses after AHSCT is needed. Better insights into these responses may help to further improve patient care and predict transplant outcomes more accurately, as well as helping to gain a better understanding of SSc pathogenesis.

Implications for future research

In order to further delineate the exact mechanisms underlying the induction of immune tolerance upon AHSCT, the interaction between innate and adaptive immune cells after AHSCT should be better established. One important question that remains unanswered is whether the innate immune system, which reconstitutes much faster than the adaptive immune system [7], creates a permissive environment for the regeneration of a tolerant adaptive immune system after AHSCT. In other words, is the reconstitution of innate immune cells such as monocytes, NK cells, neutrophils and dendritic cells after AHSCT a prerequisite for the formation of T_{regs}, B_{regs} and a more diverse TCR/BCR repertoire? In order to answer this fundamental question, innate immune cells should be studied in more detail, especially at early time-points after AHSCT. Furthermore, it would be interesting to investigate if innate immune reconstitution and priming of adaptive responses underlies the efficacy of AHSCT and whether this is different in responders *versus* non-responders.

Current immune-profiling studies after AHSCT focus on broad PBMC profiling. However, profiling of separate immune cell populations (for example, by performing RNA sequencing on isolated cell subsets, single-cell sequencing or CyTOF) could be more informative to delineate the precise contribution of these separate subsets to immune regulation after AHSCT. Additionally, apart from circulating immune cells, it is also crucial to further investigate the effects of AHSCT on tissue resident immune cells. In the context of SSc, it would be especially interesting to study macrophages, dendritic cells, neutrophils and myofibroblasts after AHSCT in skin as well as lung tissue to see how these cells are affected, and to determine if AHSCT also induces tolerance at these sites.

Additionally, more insights of immune cell reconstitution and modulation of immune cell function after AHSCT in other ADs than SSc may help to identify disease-specific patterns. Identifying commonalities as well as differences between immune tolerance induction after AHSCT in different ADs can help to solve questions in specific disease pathogenesis and aid patient classification.

Finally, many different conditioning regimens are used in clinical practice in the context of AHSCT in SSc. However, the optimal regimen has yet to be identified. Unfortunately, heterogeneity and the limited number of patients in the studies that have been performed in this field complicate comparison with regard to regimens and the impact on outcome and safety. Importantly, different conditioning regimens may affect (innate) immune cells in different ways, which can potentially impact efficacy of the strategy. More research is needed to enable comparison between different conditioning regimens (myeloablative *versus* non-myeloablative), as well as manipulation of the graft (with or without CD34 selection), in order to develop the optimal regimen that is both safe and effective.

Disclosures

The authors declare no conflicts of interest.

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