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Harnessing bone marrow resident regulatory T cells to improve allogeneic stem cell transplant outcomes

Laetitia Le Texier[1] · Katie E. Lineburg[1] · Kelli P. A. MacDonald[1]

Abstract Regulatory T cells (Treg) are a suppressive T cell population which play a crucial role in the establishment of tolerance after stem cell transplantation (SCT) by controlling the effector T cell responses that drive acute and chronic GVHD. The BM compartment is enriched in a highly suppressive, activated/memory autophagy-dependent Treg population, which contributes to the HSC engraftment and the control of GVHD. G-CSF administration releases Treg from the BM through disruption of the CXCR4/SDF-1 axis and further improves Treg survival following SCT through the induction of autophagy. However, AMD3100 is more efficacious in mobilizing these Treg highlighting the potential for optimized mobilization regimes to produce more tolerogenic grafts. Notably, the disruption of adhesive interaction between integrins and their ligands contributes to HSC mobilization and may be relevant for BM Treg. Importantly, the Tregs in the BM niche contribute to maintenance of the HSC niche and appear required for optimal control of GVHD post-transplant. Although poorly studied, the BM Treg appear phenotypically and functionally unique to Treg in the periphery. Understanding the requirements for maintaining the enrichment, function and survival of BM Treg needs to be further investigated to improve therapeutic strategies and promote tolerance after SCT.

Keywords GVHD · Bone marrow · Treg · Mobilization

✉ Kelli P. A. MacDonald
    kelli.macdonald@qimr.edu.au

[1] The Antigen Presentation and Immunoregulation Laboratory, QIMR Berghofer Medical Research Institute, 300 Herston Road, Herston, Brisbane, QLD 4006, Australia

Introduction

Allogeneic haematopoietic stem cell transplantation (SCT) is the preferred therapy for the majority of malignancies of bone marrow origin. Following transplantation, donor haematopoietic stem cells (HSC) are relied upon to successfully reconstitute the recipient's haematopoietic and immune systems. The curative property of SCT lies in the graft versus leukaemia (GVL) effect which is required for ablation of residual cancer burden. This process is absolutely dependent on donor T cells contained within the graft; however, these T cells are also the primary mediators of graft versus host disease (GVHD), a life-threatening complication of SCT. Establishing transplant protocols which maximize GVL but minimize GVHD for optimal transplant outcomes has been a long-term goal in the field.

In the last two decades, there has been a significant shift in the stem cell sources used for SCT such that the majority of SCT is now undertaken using G-CSF mobilized blood (G-PBSC) products. The use of G-PBSC in place of steady-state bone marrow (BM) has been readily adopted in part due to the ease of harvest, but also due to favourable HSC numbers and the association with accelerated engraftment and decreased graft failure rates post-transplant [1]. Notably, while G-PBSC grafts contain 10–50-fold more T cells than standard BM grafts [2, 3], the incidence of acute GVHD (aGVHD) is not increased, and patients receiving either graft source have similar overall survival rates [1]. However, it is now clear that the use of G-PBSC grafts is associated with a significant increase in the incidence and severity of chronic GVHD (cGVHD) (Fig. 1) [1]. Thus, HSC mobilization with G-CSF exerts immunomodulatory effects on the graft that significantly impact on transplant outcomes [4–7]. Recent investigations
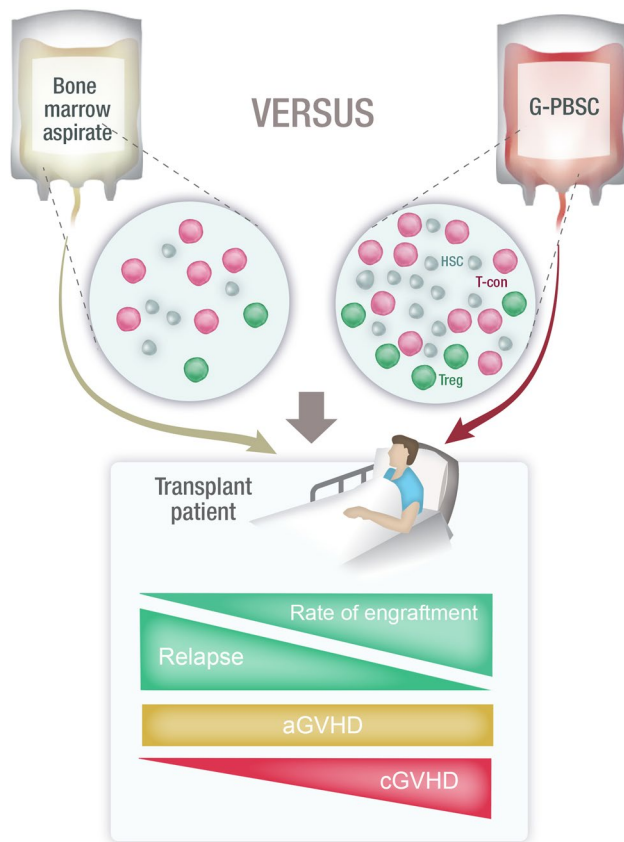
Fig. 1 BM versus G-PBMC: benefits and detriments. Compared to unmanipulated BM, G-PBSC grafts are enriched in HSC which promote an increased rate of engraftment post SCT. While G-PBSC is enriched in conventional T cells (Tcon) which contribute to the diminished risk of relapse, strikingly, the risk of development of aGVHD is unchanged. This effect can be attributed to the increased number of Treg in G-PBSC in addition to other tolerogenic effects of G-CSF on the immune system. Notably, however, the use of G-PBSC grafts is associated with a significant increase in the incidence and severity of cGVHD

into the immunomodulatory effects of G-CSF have identified G-CSF-mediated alterations in the number and function of $CD4^+FoxP3^+$ regulatory T cells (Treg) as an important mechanism contributing to the attenuation of aGVHD observed following SCT [8–10]. Treg represent a heterogeneous immunosuppressive T cell population that regulates immune responses and are critical for the induction and maintenance of tolerance after SCT [11]. Indeed the inverse correlation between Treg numbers contained within the donor graft and incidence of GVHD has been demonstrated both clinically and in preclinical models [10, 12–14]. In this review, we will discuss the mechanisms underlying the propensity of G-CSF administration to enhance Treg number and function within G-PBSC grafts, and strategies to promote their engraftment at this BM site to improve transplant outcomes.

Treg are critical for the control of alloreactive T cells after SCT

Donor T cells are central mediators of GVHD and aGVHD is absolutely dependent on the presence and function of donor T cells in the graft. Following SCT, tissue injury and inflammation characterized by proinflammatory cytokine release is initiated by the conditioning regimen [15, 16]. These cytokines, together with LPS released from damaged gut tissue, result in the activation of host antigen-presenting cells (APC). Activated host APC then prime naïve donor T cells and promote the differentiation and expansion of effector $CD4^+$ (Th) and $CD8^+$ (Tc) T cells which through cytokine production and cytolytic effector function mediate target tissue destruction [17]. During chronic GVHD both T cells contained in the graft and thymically derived auto- and alloreactive T cells contribute to pathology [18]. It is now clear that, in both acute and chronic forms, GVHD is characterized by an imbalance of T effector and Treg which results in uncontrolled T effector expansion and cytokine production. In this regard, both preclinical and clinical studies demonstrate that donor graft Treg number inversely correlates with aGVHD [10, 12, 13] and overall survival after SCT [19]. Moreover, a failure of Treg reconstitution after SCT contributes to both acute and chronic GVHD-associated pathology [17, 20–23]. As such, therapeutic strategies designed to increase the number of functional Treg within the graft and promote Treg survival following transplant represent areas of intense focus.

The BM is a niche for highly functional memory Treg (Figs. 2 and 3)

Studies in both mouse and man have demonstrated that within the BM, the CD4 T cell compartment contains a substantially increased proportion of Treg compared to that of other peripheral sites including PB, spleen and lymph nodes (LN). Thus, within the BM, Treg represent 20–60% of the CD4 T cell compartment [24, 25], in contrast to the 7–15% typically found in the spleen [26]. Although the majority of studies have focused on Treg form and function in the periphery (PB in humans and spleen and LN in mice), the limited number of studies which have investigated BM Treg suggest that in addition to their enrichment, Treg at this site exhibit features distinct from Treg in the periphery. In this regard, comparison of the subset composition of Treg in the BM, thymus and spleen of mice shows ~50% of BM Treg express the co-inhibitory molecule TIGIT in contrast to ~10 or 30% in thymus or spleen, respectively. Treg can be broadly subdivided into two subpopulations: thymic-derived Treg (tTreg) and Treg induced in the periphery (pTreg) from $FoxP3^{neg}$ conventional CD4 T cells in response to immunosuppressive

Fig. 2 BM is enriched in Treg. Within the BM, the CD4 T cell compartment contains a substantially increased proportion of Treg compared to that of other peripheral sites. Through their CXCR4 expression, Treg preferentially home to and are retained in the BM where stromal cells produce high levels of SDF-1. Through their expression of CXCR4, CD127 and TIGIT, BM Treg appear as a distinct Treg population compared to those in the periphery
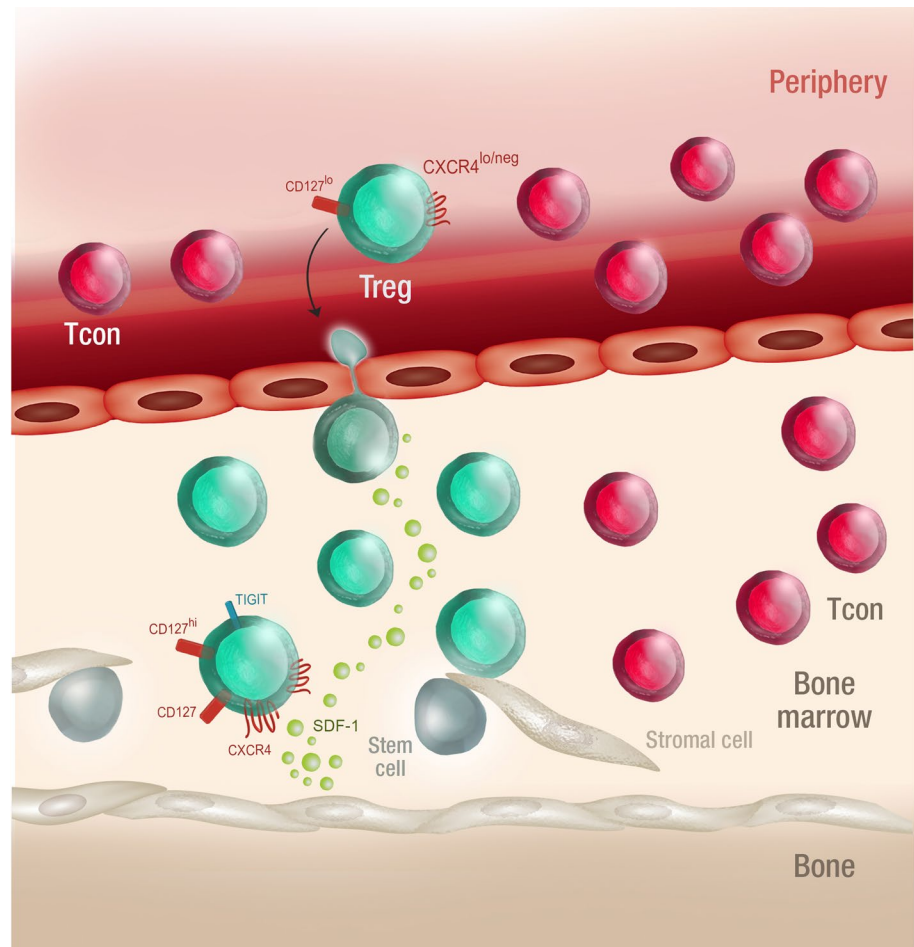
cytokines and antigen-specific activation. These Treg subsets are reported to have distinct but complementary roles in attenuating inflammation [27, 28]. To date, however, whether BM Treg represent pTreg or iTreg has not been elucidated. However, TIGIT expression marks a highly suppressive activated/memory Treg population which is required for the control of autoimmune disease and contributes to anti-tumour immune responses [29]. In further support of their activated/memory status, TIGIT$^+$ Treg express low levels of CD62L and high levels of CD44 and CXCR4 which is upregulated on Treg upon activation [24]. Indeed, the expression of CD44 on Treg is also strongly associated with superior suppressor function [30]. Thus, the BM appears to represent a niche for highly suppressive activated/memory Treg.

G-CSF mobilizes Treg into the periphery via disruption of the CXCR4/SDF-1 axis (Fig. 4)

Through their enhanced CXCR4 expression, activated Tregs preferentially home to and are retained in the BM where stromal cells produce high levels of SDF-1 [24, 31]. HSC are also known to be tethered in the BM via the CXCR4/SDF-1 interactions [32], and the dominant mechanism by which G-CSF mobilizes HSC from the BM is through the disruption of this chemo-attractive interaction [33]. As for HSC, studies in both mice and humans suggest that G-CSF administration increases the number of graft Treg through the disruption of this axis, which promotes the egress of Treg from BM into the periphery [24, 34–36]. Mechanistically this was confirmed by administration of the CXCR4 antagonist AMD3100, which resulted in the rapid mobilization of Treg into the PB. Notably, in both mice and primates, AMD3100 has been shown to mobilize Treg more efficiently than G-CSF [35, 37] highlighting the potential to further increase graft-associated Treg numbers by optimizing mobilization regimes. Indeed, a synergism between G-CSF and AMD3100 in HSC mobilization is established [38]; however, the combined effects of G-CSF and AMD3100 administration on Treg mobilization remain to be elucidated. Furthermore, in addition to the CXCR4/SDF-1 axis, several other adhesive interactions are known to contribute to the firm retention of HSC in the BM. In this regard, HSC expression of the integrins α4β1 (VLA4), α4β7, α9β1 and αLβ2 (LFA1) contributes to HSC retention through interactions with their cognate ligands VCAM-1,
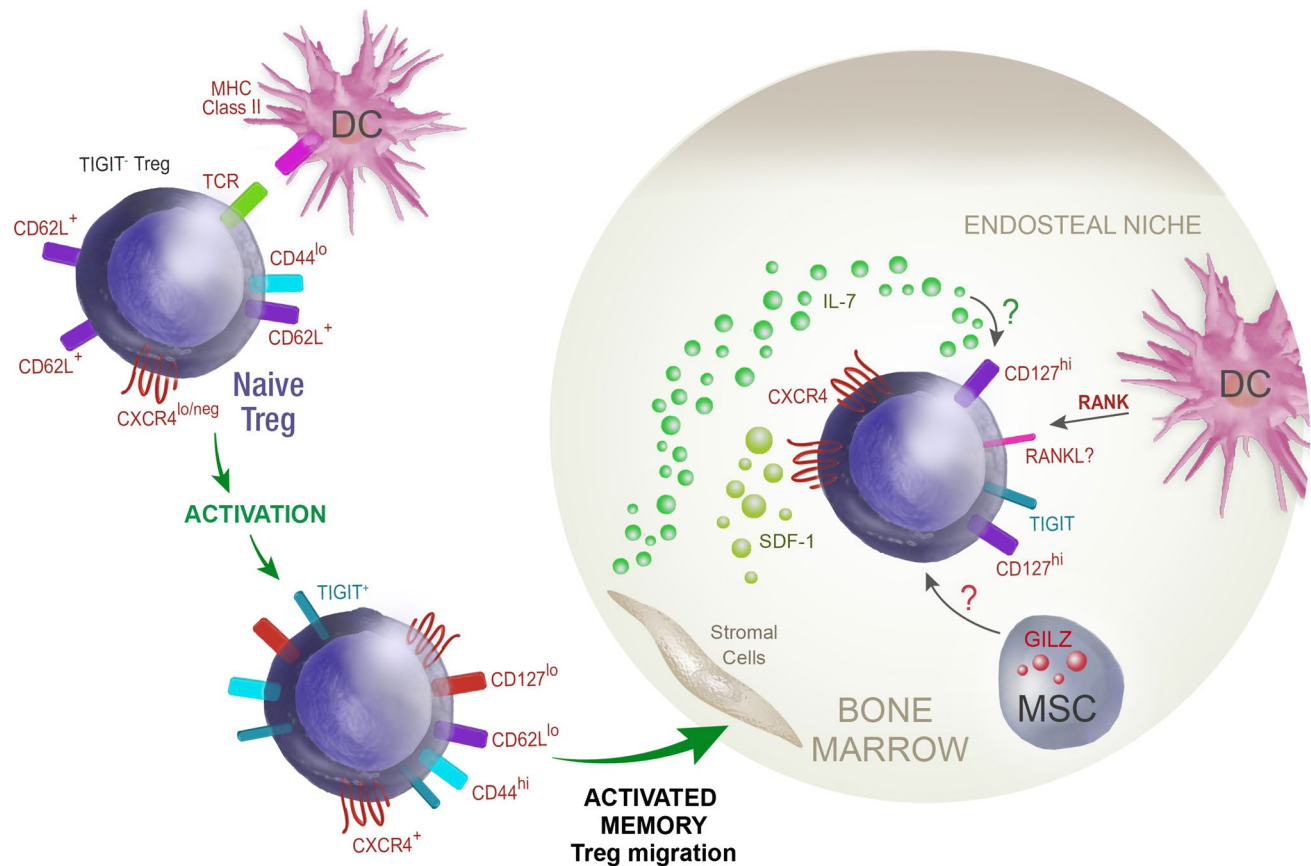
Fig. 3 The BM is a niche for memory Treg. In the periphery, APC (DC) MHC class II/TCR interactions promote naive CD62L$^+$ CD44^{lo} Treg activation and the upregulation of TIGIT and CXCR4 expression. Through the SDF-1/CXCR4 axis activated/memory TIGIT$^+$CXCR4$^+$CD44^{hi}CD62^{lo} Treg migrate to the BM endosteal niche. High-level CD127 (IL-7R) expression by BM Treg may contribute to Treg survival at this site, as IL-7 is enriched in the BM. DC and mesenchymal stem cells may also be involved in Treg maintenance in BM, putatively through RANK/RANKL signalling and GILZ production, respectively

fibronectin, osteopontin and ICAM-1 in the BM microenvironment. Blocking these interactions using antibodies or small molecule inhibitors has proven effective at mobilizing HSC and in some cases, to enhance G-CSF-mediated HSC mobilization [39–46]. While the integrin expression profile of Treg residing in the BM remains largely undefined, it is important to note that integrins expressed by Treg contribute to both their function [47–50] and tissue tropism [48, 51–53]. Clearly, further investigations into the mechanisms by which Treg are recruited and retained within the BM are warranted and will be required to allow mobilization strategies optimal for harnessing Treg in SCT grafts.

Memory Treg are autophagy-dependant and G-CSF elicits autophagy in Treg

TIGIT$^+$ and TIGIT^{neg} Treg exhibit distinct survival requirements. In this regard, in TIGIT^{neg} Treg the Bcl2 anti-apoptotic pathway is more active, whereas TIGIT$^+$ Treg are critically reliant on autophagy for their survival [35]. Autophagy is a highly conserved process used to degrade and recycle damaged or unused intracellular components and is considered an adaptive response to stress which promotes survival [54]. Indeed disarming autophagy in the FoxP3$^+$ Treg specifically, results in a profound loss of activated/memory Treg in vivo [55], particularly within the BM [35], consistent with their enrichment at this site. Functionally, the loss of these Treg resulted in dysregulated effector T cell activation and expansion, and the development of multiorgan inflammation. Of note, mRNA transcriptome analysis of Treg isolated from splenic grafts from G-CSF-treated donor mice demonstrates an upregulation of genes associated with Treg survival, including genes associated with the autophagic pathway [34]. Furthermore, in vitro exposure to G-CSF has been shown to rapidly upregulate the autophagic pathway in Treg [35]. Thus, G-CSF mobilization results in grafts containing
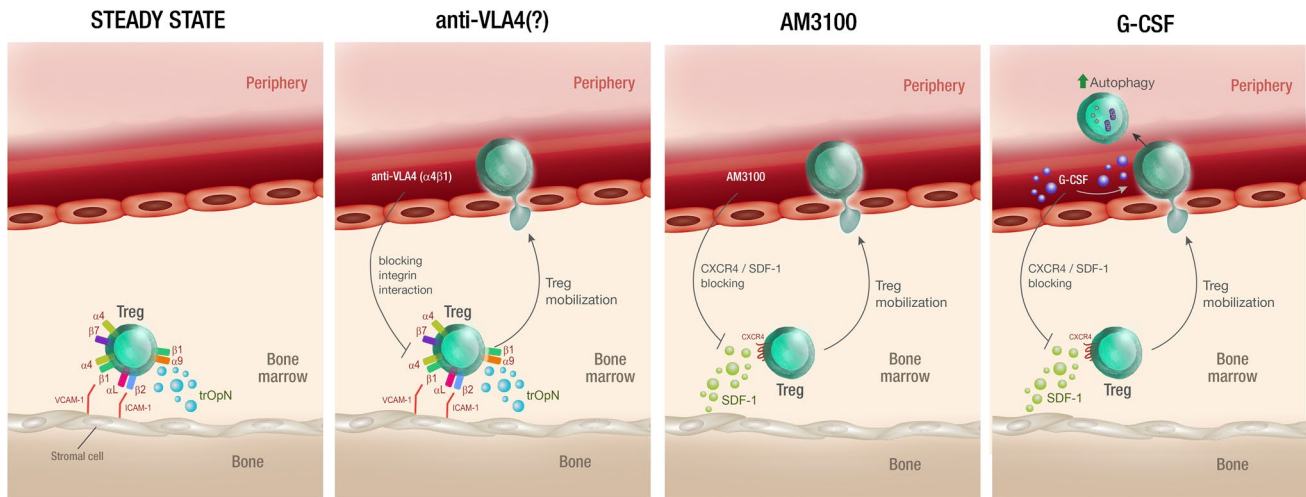
MOBILIZATION

Fig. 4 Treg mobilization via CXCR4/SDF-1 disruption or blocking of integrin interaction. As observed for HSC in the BM, integrin expression on BM Treg like α4β7, α4β1 α9β1 or αLβ2 may contribute to their retention in the BM via the interaction with their ligands, VCAM-1, thrombin-cleaved osteopontin (trOpn) or ICAM-1 expressed on stromal cells. Targeting these interactions using small molecules or blocking antibodies such as anti-VLA4 represents a putative strategy to enhance BM Treg mobilization. Through their expression of CXCR4, Treg are also maintained in BM via the interaction of SDF-1 produced by the stromal cells. CXCR4/SDF-1 disruption using AMD3100, a CXCR4 antagonist or G-CSF which decreases SDF-1 level in BM, induces BM Treg migration into the periphery. G-CSF also induces autophagy in Treg which promotes their survival after transplant

increased number of Treg poised for survival. In support of this notion, in a murine aGVHD model, Treg reconstitution was shown to be significantly improved in mice receiving allografts from G-CSF pre-treated donors [34]. Thus, G-CSF exerts additional beneficial effects on Treg beyond their mobilization, suggesting strategies to optimize Treg mobilization should be built around combination strategies where G-CSF is retained. In this regard, that AMD3100 was shown to mobilize Treg with increased efficacy than G-CSF leads to the speculation that a combined strategy of G-CSF plus AMD3100 mobilization would generate more tolerogenic grafts due to Treg content. However, in a recent preclinical study, grafts mobilized with combined G-CSF plus AMD3100 in fact elicited increased GVHD severity, although the mechanism was unclear [56].

BM Treg contribute locally to the maintenance of the HSC niche and immune tolerance

Increasing evidence suggests that the Treg that reside within the BM are active and functionally relevant at this site. In elegant in vivo imaging studies, Fujisakie et al. demonstrated that Treg co-localize with HSC in the endosteal niche and are crucial for establishing an immune privileged environment [57]. In this study using non-irradiated recipients, transplanted purified syngeneic and allogeneic HSC were engrafted and were maintained in the niche in similar numbers. However, Treg depletion resulted in a dramatic loss of allogeneic donor HSC, which was associated with increased proinflammatory cytokine expression by BM resident CD4 and CD8 T cells. Mechanistically, IL-10 production by Treg was shown to be critical for Treg-mediated protection of allogenic HSC within the niche. In line with this, the infusion of Treg into patients prior to SCT improves lymphoid reconstitution and GVHD control after transplantation. Furthermore, as ascertained from studies of the FoxP3-deficient scurfy mice, BM Treg protect B lymphopoiesis integrity through the inhibition of cytokine production by effector T cells during inflammation [58]. Moreover, the BM is a recently recognized target of GVHD, which is characterized by donor T cell destruction of the BM HSC niche associated with delayed immune reconstitution and disrupted B cell lymphopoiesis [59, 60]. The observation that changes in the resident BM Treg population are associated with pathology further supports the importance of these cells in the regulation of disease. Studies report increased Treg in the BM of patients with metastatic prostate cancer compared to healthy donors. These BM Treg were functional, highly proliferative and found to suppress osteoclast differentiation, in turn contributing to the osteoblastic bone lesions observed in patients with prostate cancer. Several studies support this finding, most

notably Luo et al. demonstrated in vitro that Treg inhibit osteoclastogenesis and bone resorption through IL-10 and TGF-β secretion and that production of these cytokines was enhanced in the presence of estrogen [61]. In line with the suppressive role of Treg in BM, patients with acute myeloid leukaemia present with higher Treg numbers in the PBMC and in the BM. This is associated with poor prognosis and may contribute to the suppression of immune responses via IL-10 production in these patients [62]. In contrast to this, in the inflammatory setting observed after allogeneic SCT, GVHD is associated with a defect in Treg homeostasis and these findings lead to the question of the role of BM resident Treg in controlling GVHD locally. In this regard, the reconstitution of the BM Treg compartment following SCT appears important for the control of GVHD. Thus, in models of both acute and chronic GVHD, recipients of grafts from donor mice which harbour a Treg-specific autophagy deficiency resulted in reduced Treg engraftment, which was most striking in the BM. This reduction of BM Treg was associated with an expansion of CD8 effector T cells in the BM, exacerbated GVHD and reduced survival [35]. However, the impact of BM Treg deficiency on the HSC niche was not investigated. Thus, more than just a reservoir for Treg, the BM appears to be a site where Treg actively contribute to immune homeostasis and peripheral tolerance.

BM environment contributes to Treg maintenance (Fig. 3)

The cytokine requirements for BM Treg also appear to differ from those in the periphery. While FoxP3 represents the definitive marker of Treg, the ability to functionally characterize Treg based on FoxP3 expression is limited by its intracellular localization, which requires fixation and permeabilization to visualize. To circumvent this, cell surface markers are routinely used to define and separate Treg from conventional T cells, thus the phenotype of high-level CD25 (IL-2Rα) expression in parallel with low-level CD127 (IL-7R) expression is widely used to identify Treg [63, 64]. IL-2 is widely recognized as being required for the proliferation, survival and function of Treg [65–68]. However, it is increasingly recognized that a significant proportion of $CD4^+FoxP3^+$ Treg is $CD25^{neg}$, and this is particularly true for BM Treg [69]. In line with this, $TIGIT^+$ Treg exhibit significantly diminished levels of CD25 as compared to the $TIGIT^{neg}$ Treg subset [35]. Furthermore, all Treg within the BM, irrespective of TIGIT expression, exhibit elevated levels of CD127, which has been reported to be upregulated on activated Treg [70]. As IL-7R signalling on Treg has also been shown to be required for the development, function and survival of Treg [71–73], together these findings suggest that BM Treg, in contrast to Treg in the periphery, may be more dependent on IL-7 than IL-2. Notably,

IL-7 is also critical for memory CD8 T cell maintenance [74, 75], their recruitment to and residency within the BM, and is required for their homeostatic proliferation [76, 77]. Within the BM, CD8 T cells are found in close proximity to stromal cells which produce large amounts of IL-7. Thus, common mechanisms may contribute to the maintenance of Treg and CD8 T cell populations in the BM, although overall, the influence of the BM environment on Treg homeostasis remains poorly defined. However, a study in prostate cancer demonstrated that RANK expressing BM resident dendritic cells promote the expansion of BM Treg in this pathogenic environment [25]. Moreover, the expression of the anti-inflammatory protein glucocorticoid-induced leucine zipper (GILZ) by BM mesenchymal stem cells (BMSC) was reported to contribute to Treg maintenance at this site [78]. GILZ, the expression of which is ubiquitously induced by glucocorticoids, has been reported to elicit immunosuppressive effects on T cells through the disruption of NFκB signalling and downstream proinflammatory cytokine production [79]. In transgenic mice in which GILZ overexpression is restricted to BMSC, the frequency of Treg was significantly increased within the BM in parallel with an increase in CD8 but not CD4 T cells. Mechanistically, BMSC overexpression of GILZ promoted increased IL-10, and diminished IL-6 and IL-12 production [78]. Thus, through their generation of a distinct cytokine milieu within the BM, BMSC and stromal cells appear to promote a Treg-supportive environment.

That failed Treg reconstitution following SCT has been demonstrated to be causative a lesion in GVHD has led the field to develop Treg restorative approaches. Based on the high-level expression of CD25 on peripheral Treg one approach currently being trialled in the clinic is the administration of low-dose IL-2 to preferentially expand Treg over T effector cells. Encouragingly, approximately 50% of patients show Treg expansion in the PB and improved disease status as long as therapy is continued [80, 81]. Whether low-dose IL-2 is effective in promoting the BM Treg compartment is unknown; however, given the perceived distinction between the cytokine requirements of Treg in the periphery and BM, one could speculate that IL-2 alone may not be optimal. Thus, deepening our understanding of the BM Treg requirements in both steady state and after SCT, should help direct further Treg restorative protocols.

To summarize, Treg are a suppressive T cell subset which play a crucial role in the establishment of tolerance after SCT. Recent findings highlight the enrichment of an activated/memory Treg population in the BM which are highly suppressive and functional at this site. Treg are attracted to, and maintained in, the BM through the CXCR4/SDF-1 axis, and the disruption of this pathway mobilizes Treg into the periphery. In contrast to its efficacy

in HSC mobilization, AMD3100 is superior to G-CSF in its ability to mobilize Treg. Given that HSC mobilization involves the disturbance of adhesive interactions beyond the CXCR4/SDF-1 axis, it will be important to determine if these interactions contribute to Treg retention in the BM, to allow the development of mobilizing regimes optimal for generating SCT grafts enriched in these cells. Importantly, strategies that promote autophagy to enhance Treg survival appear ideal. In addition to enhancing Treg numbers in the graft, collectively the literature suggests that promoting Treg engraftment in the BM after SCT will be beneficial for GVHD control. Clearly, further studies of the survival requirements of the BM Treg are required, and may lead to new therapeutic strategies to promote tolerance after SCT.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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