論文内容の要旨

論文題目

Prolonged survival of transplanted allogeneic hematopoietic stem cells

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Introduction

The stem cell niche is a specialized microenvironment where stem cells reside and receive appropriate support for maintaining self-renewal, survival, quiescence and multi-lineage differentiation capacity. The niche may also protect stem cells from environmental insults including cytotoxic chemotherapy.

Whether the stem cell niche can protect stem cells from pathogenic immunity is an underexplored question. It is hypothesized that the critical role of the stem cell in developing,

maintaining and repairing tissues provides a biologic imperative to dampen pathogenic immunity and prevent excessive inflammation or autoimmunity against stem cells. The testis, ovary, hair follicle, and placenta are all sites of residence for stem cells and are immune privileged (IP) sites. Peter Medawar first proposed the concept of IP sites, locations where multiple mechanisms could conspire to prevent immune attack and where even a foreign allograft can survive in immune competent hosts without immune suppressive therapy.

With advances in stem cell biology, somatic stem cells have been defined and their localization in vivo was characterized. Whether these niches all provide some measure of IP mechanisms remains unknown and we sought to address the question in one of the best defined somatic stem cell niches in the bone marrow (BM), a site where immune reactivity exists. We hypothesized that there might be immune suppressive microenvironments within the BM based on the following observations: 1) The BM is a common site for human tumor metastasis, 2) human BM contains higher numbers of CD4+CD25+FoxP3+ regulatory T cells (Tregs) than other secondary lymphoid organs, and 3) BM Tregs are more potent at suppressing T cell activation compared to peripheral blood Tregs. Here we tested the hypothesis that the hematopoietic stem/progenitor cell (HSC) niche located on the endosteal surface of the BM is an IP site, using intravital fluorescence microscopy to visualize the localization of Tregs and transplanted HSCs within the BM of live animals. Imaging a relatively small number of transplanted HSCs in the BM is made possible by the use of a custom-built microscope developed in our laboratory specifically for live animal imaging at high speed and high spatial resolution, as well as provides a means to analyze the BM microanatomy by 3D optical sectioning microscopy.

Result

IP mechanisms of other sites such as the testis or eye were confirmed by the prolonged survival of transplanted allogeneic or xenogeneic cells without immune suppression. To test if the endosteal HSC niche is an IP site that meets the above criteria, we first examined if allo-HSCs transplanted into non-irradiated recipient mice can survive without any immune suppressive therapy. Ckit+Sca1+Lin- (KSL) cells, which are enriched in HSCs from the BM of C57BL/6 (B6) mice, were labeled by a lipophilic membrane dye, DiD. Then 5 x 10^4 DiD-labeled KSLs were injected into non-irradiated allogeneic BALB/c mice or syngeneic B6 mice via the tail vein. Using in vivo microscopy, we imaged identical areas (~1650 μm x 2310 μm) of the

skull BM of the recipients on day 1 and day 30 following injection. The imaging depth was about 150 μm below the intact bone surface, allowing us to reach approximately 40% to 60% of the entire BM cavity. On day 1, the numbers of DiD B6 KSLs homing to the skull BM were comparable for the syngeneic and allogeneic recipients (52±5 in B6 recipients (n=4), 52±5 in BALB/c recipients (n=3)). In both groups, approximately 90 % of DiD KSL HSCs homed within 15 μm of the endosteal bone surface. Surprisingly, even on day 30, we found no significant difference in the numbers of surviving DiD B6 KSLs between the two groups (51±4 in B6 recipients (n=4), 49±5 in BALB/c recipients (n=4), and approximately 90 % of donor cells in both recipients are on the endosteal surface, indicating that allogeneic KSLs on the endosteal surface were not rejected even in immune-competent recipients. The majority of DiD B6 KSLs in both groups retained high levels of DiD signal, indicating that KSL HSCs underwent cell division only rarely and no B6 derived cells were observed in the blood by flow cytometry.

To explore the possibility that immune ignorance may be the cause of the prolonged survival of B6 KSLs in non-irradiated BALB/c mice, we first examined the major histocompatibility complex (MHC) class I antigen expression on KSL cells. Flow cytometry analysis revealed that KSL HSCs expressed higher levels of MHC class I molecule, H2-Kb than CD19+ B cells in the spleen. KSL HSCs therefore express immunogeneic molecules that allogeneic T cells should potentially recognize and thus kill these foreign cells. To address the possibility that transplantation of KSLs alone without differentiated cells may fail to evoke a potent allogeneic immune response, we examined if transplantation of DiD-labeled KSLs (5×10^4 / mouse) together with non-labeled B6 whole BM cells (5×10^7 / mouse) into non-irradiated BALB/c mice results in the rejection of donor KSLs on day 30. In vivo imaging of the skull BM showed that the number of DiD positive B6 cells surviving for 30 days were comparable in transplantation with or without whole BM cells. Approximately 90 % of the surviving DiD B6 cells were less than 15 μm away from the endosteal surface. Together, these results indicate that even in transplantation of whole BM cells, allogeneic KSLs with high expression of MHC class I can survive near the endosteal surface without immune suppression. The inability of the host to reject these cells is therefore not likely due to immune ignorance, but may reflect active immune suppression. The prolonged survival of allo-HSCs in non-irradiated recipients is consistent with Peter Medawar's observation of prolonged survival of transplanted allogeneic/xenogeneic grafts in other IP sites, supporting our hypothesis that the endosteal HSC niche also has IP mechanisms that will shield transplanted allo-HSCs from allogeneic immune response.

Next, we examined the relevance of Tregs to IP mechanisms in the HSC niche. Human BM contains a higher frequency of Tregs (24% of CD4 cells) than other secondary lymphoid organs

(12%, 14%, and 9% for lymph nodes, thymus, and peripheral blood, respectively). We confirmed that the mouse BM also harbors a high number of Tregs by FACS analysis of FoxP3-GFP KI mice in which FoxP3 Tregs specifically express GFP. The frequency of FoxP3+ in CD4 cells was 23% in the BM, 13% in the spleen, and 13% in lymph nodes. We hypothesized that Tregs abundant in the BM may provide the HSC niche with IP mechanisms.

The trafficking of FoxP3 Tregs to the BM critically depends on the stem cell chemo-attractant, SDF-1, which is expressed at high levels by osteoblasts on the endosteal surface. Based on this, we hypothesized that Tregs will accumulate on the endosteal surface and will render the HSC niche immune suppressive. The spatial localization of Tregs on the endosteal surface was examined in the BM of FoxP3-GFP KI mice by in vivo microscopy and confirmed in histological sections. We quantified the percentage of FoxP3-GFP Tregs that reside at the endosteal surface in 3D-stacks created from in vivo depth-sectioned images. Notably, 58% (203/350) of the total Tregs in the BM were found to be within 15 μm of the bone surface.

In addition to the endosteal niche, the BM sinusoidal vessel has also been suggested to be an HSC niche. Based on in vivo high-resolution 3D analysis, we reported recently that the endosteal surface is well vascularized and the osteoblastic niche is perivascular. Therefore, we examined the spatial distribution of Tregs relative to the BM vasculature. We visualized the BM sinusoidal vessels of FoxP3-GFP KI mice after intravenous injection of a vascular dye, Q-tracker, through the tail vein. As we expected, Tregs are located in the narrow space (approximately one to two cell diameters wide) between the endosteal surface and the sinusoidal vessels.

Next, we examined the spatial distribution of Tregs and HSCs on the endosteal surface. We measured the distance between Tregs and HSCs that home to the BM by acquiring 3D images 24 hours after intravenous injection of 5×10^4 DiD-labeled KSLs from B6 mice into FoxP3-GFP KI B6 mice. Remarkably, 89% (338/380) of DiD KSLs homed within 20 μm of Tregs on the endosteal surface. In contrast, when 5×10^4 Lin+ differentiated cells were injected into FoxP3-GFP KI mice, only 12% (42/330) of the Lin+ cells home within 20 μm of Tregs.

Because KSLs include many hematopoietic progenitor cells in addition to HSCs, we visualized the quiescent HSCs after administering the chemotherapy drug 5-fluorouracil (5-FU), which is known to selectively kill dividing cells. While most progenitor cells are dividing, many HSCs are quiescent. Therefore, the KSLs that survive after 5-FU therapy are considered to be quiescent HSCs. 48 hours after injection of DiD-labeled KSLs into FoxP3-GFP mice, we gave a lethal dose of 5-FU (250 mg/kg mice)[3]. 48 hours after the 5-FU treatment, we imaged the skull

BM of the FoxP3-GFP mice. As we expected, 82% (36/44) of the 5-FU resistant DID KSLs are within 20 μm away from the FoxP3-GFP Tregs, forming clusters with FoxP3-Tregs on the endosteal surface.

We next examined whether Tregs accumulate around allo-HSCs that survive in the BM niche without immune suppressive therapy. 30 days after intravenous injection of 10^5 DiD-labeled B6 KSLs into non-irradiated BALB/c FoxP3-GFP KI mice, we imaged the skull BM of the recipients. Notably, 74% (193/259) of DiD B6 KSLs were within 20 μm of Tregs on the endosteal surface. Imaging followed by intravenous injection of a vascular dye, Q-tracker, showed that B6 KSL HSCs are in close proximity to Tregs on the vascularised endosteal surface. Interestingly, time-lapse movie revealed that many FoxP3-GFP Tregs are actively moving around stationary allogeneic B6 KSL HSCs, suggesting a surveillance-like activity. This frequent co-localization of BM Tregs with allogeneic KSLs strongly supports our hypothesis that the accumulation of Tregs provides the HSC niche with IP mechanisms that enable allogeneic KSLs to escape rejection.

To examine whether Tregs play a critical role in IP mechanisms of the HSC niche, we tested if Tregs depletion by anti-CD25 antibody therapy resulted in the rejection of B6 KSL HSCs transplanted into non-irradiated BALB/c mice without any immune suppression. After intravenous transplantation of DiD-labeled B6 KSL HSCs (5×10^4 / mouse) into BALB/c mice, we gave anti-CD25 antibody therapy on day 0 and day 2. In vivo imaging of the skull BM of the recipients on day 11 showed that there are few FoxP3-GFP Tregs in the skull BMs of anti-CD25 antibody treated recipients, and that Treg depletion by anti-CD25 antibody therapy led to 90 % reduction in the number of surviving donor cells compared with control antibody treated recipients. This indicates that Tregs critically provide IP mechanisms in the HSC niche.

Finally, we explored the possible mechanism controlling the strength of IP mechanisms of the HSC niche. Osteoblasts lining on the endosteal surface are critical cell constituents of the HSC niche, providing HSCs with signals to regulate HSC function/number and to protect HSCs from environmental insults such as toxic substances. We hypothesized that PTH stimulation of osteoblasts, known to increase HSC number, will control IP mechanisms through increasing Treg frequency, based on the finding that PTH stimulation of osteoblasts increases expression level of SDF-1, critical chemokine ligand for Treg homing to the BM. To this end, by using flow cytometry, we analyzed BM Treg frequency in OB-PPR mice whose osteoblasts express constitutively activated PTH receptors and KSL frequency is 2-fold compared with control mice. Unexpectedly, flow cytometry analysis of Treg frequency in OB-PPR mice with intracellular

stating showed that the frequency of FoxP3+ Tregs in total CD4 T cells in the BM is significantly lower in OB-PPR mice than that in control mice (22.2 % vs 55 %, p=0.0015), while the frequency in the spleen and lymph nodes are comparable between in OB-PPR mice and in control mice. The frequency of Tregs in whole bone marrow cells is comparable between OB-PPR mice and wild mice. This indicates that PTH stimulation of osteoblasts negatively regulates BM Treg frequency. Decreased Treg frequency in Col-PPR mice is contrary to our expectation and somewhat surprising because even many other osteoblast secreting molecules reported to be elevated in response to PTH stimulation also have the potential to expand Treg population. PTH stimulation was shown to increase osteoblast secretion of TGF-β, cytokines critical for Treg survival and proliferation in vivo as well as for the maintenance of Treg immune suppressive property. RANKL, expressed by osteoblasts following PTH activation, was also shown to expand Treg population in the skin. This contradictory result could be explained by the following reasons; 1) Since the continuous PTH stimulation of PTH/PTHrP receptors was often reported to have the opposite effect on downstream signaling compared to the pulsatile PTH stimulation [16], osteoblasts from Col-PPR mice whose osteoblasts express constitutively activated PTH receptors could secret lower amount of TGF-β and RANKL compared to control osteoblasts. 2) Decreased Treg frequency in PPR mice may be due to increased secretion of IL-6 from osteoblasts, which is known to impede the conversion of naïve T cells into FoxP3 Tregs in combination with TF-betta. 3) Abnormally expanded HSCs in Col-PPR mice may compete for the endosteal niche occupancy with Tregs, leading to decreased Treg frequency. Future investigation is warranted.

Discussion

It has been known for decades that some stem cell niches such as the testis and ovary are IP sites that protect stem cells from pathogenic immunity and prevent excessive inflammation and autoimmunity. We show here that anatomic sites where immune activity is otherwise known to occur, may have discreet sites of IP contributing to a somatic stem cell niche. Allogeneic KSL HSCs, immunogenic cells that highly express MHC class I molecules, survived on the endosteal surface for 30 days without immune suppressive therapy with the same survival frequency compared to transplanted sygeneic KSL HSCs. This finding is analogous to Peter Medawar's observation of prolonged survival of transplanted allogeneic/xenogeneic grafts that defined IP sites. Moreover, FoxP3 Tregs enriched in the BM accumulated on the vascularised endosteal surface, and frequently formed clusters with transplanted HSCs where Tregs are actively moving suggesting a surveillance-like activity. Treg depletion by anti-CD25 antibody resulted in a

significant reduction of B6 KSLs survival in BALB/c mice. Together, the data indicate that Treg accumulation provides the HSC niche with IP mechanisms, shielding allo-HSCs from host immunity. We additionally showed that BM Treg frequency in OB-PPR mice is lower than that in control mice, indicating that PTH stimulation of osteoblasts negatively regulates BM Treg frequency, possibly as well as the strength of entire IP mechanisms.

The prolonged survival of allo-HSCs without immune suppression is surprising and appears contrary to clinical experience that indicates strong immune suppressive therapy is required to prevent rejection in allogeneic BM transplantation. We propose that our data may not be in conflict with that well-defined experience since we are observing events at the level of the HSC in non-irradiated hosts (in which the HSCs rarely proliferate), while the clinical experience is related to the daughter cells of the HSC that provide the manifestations of stem cell engraftment. Since we have previously shown that more mature hematopoietic progenitor populations are located further from osteoblastic cells and the endosteal surface, it may be that rejection is accompanied by cells exiting the sanctuary provided by Tregs in the stem cell niche. In the relative absence of proliferation, the small number of surviving HSCs we observed in our 3D imaging experiment may not be detected by conventional flow cytometry and histology. Indeed, after injecting the same number of KSLs (5×10^4 cells) into non-irradiated syngeneic recipients, we were unable to detect these cells by flow cytometry even on day 1 (data not shown).

The BM sinusoidal vessels lack the "blood-tissue barrier" that is believed to play an important role in maintaining IP mechanisms in other IP sites by limiting the free transport of soluble factors and cytotoxic T cells. However, the fact that the hair follicle stem cell niche also lacks the blood-tissue barriers indicates that this property is not a requirement for all IP sites. Moreover, in the HSC niche, other mechanisms can work in concert with Tregs to help maintain local IP. For example, the HSC niche is thought to be hypoxic, which exerts immune suppressive effects in tumor microenvironments. In addition, BM stromal cells have strong immune suppressive effects both in vitro and in vivo. These additional IP mechanisms can explain why even after Treg depletion by anti-CD25 antibody treatment, a small fraction (~10%) of allo-HSCs survived for 30 days on the endosteal surface.

Tregs accumulate in the endosteal HSC niche and may provide the HSC niche with IP mechanisms, enabling transplanted allo-HSCs to escape from allogeneic rejection. IP mechanisms of the HSC niche will shield endogenous HSCs from autoimmunity or excessive

inflammation, and will help even malignant cells derived from HSCs to escape from the host immunity. This work raises the possibility of niches in other tissues serving as IP sites.

Method Summary

Mouse All mice were housed according to IACUC guidelines and used for experiment when 8–14-weeks old. Wild-type C57BL/6 mice were HSC donors when recipients were C57BL/6 mice, BALB/c mice, and FoxP3-GFP mice (C57BL/6 or BALB/c background). Col2.3-GFP mice whose mature osteoblasts express GFP under the promoter of Col2.3 were adoptively transferred with CD4+CD25+ T cells isolated from C57BL/6 mice. OB-PPR mice whose osteoblasts express constitutively activated parathyroid hormone/parathyroid hormone related protein receptors were used to analyze Treg frequency in Spleens, lymph nodes, and bone marrow cells by flow cytometry.

In vivo imaging Mice were anaesthetized and prepared for in vivo imaging. The mouse was held in a heated tube mounted on a precision 3 axis motorized stage (Suter MP385). All mice were imaged with a custom-built confocal two-photon hybrid microscope specifically designed for live animal imaging. Using the crossing of the central vein and coronal sutures as landmarks, we imaged identical areas of the skull (~1650 μm x 2310 μm) encompassing most of the parasagittal BM cavities. We acquired 3D stacks consisting of 31 optical sections with 5 μm z spacing, which provide image volumes 150 μm in depth, reaching approximately 1/3 to 1/2 into the BM cavity. After in vivo imaging, the scalp was re-closed using 3 M Vetbond veterinary glue and post-operative care was provided.

Fluorescence cell labelling Before intravenous injection into the recipients, Sca1+Ckit+Lin- HSCs, Lin+ differentiated cells, and CD4+CD25+ Tregs were fluorescently labelled by incubation with the dialkylcarbocyanine membrane dyes, "DiD" (invitrogen). Cells in culture media were incubated with 10 μm dye for 30 minutes at 37°C. Cells were then centrifuged and the pellet washed in PBS x 3 prior to injection in animals.

Flow cytometry analysis Whole bone marrow cells, spleen cells, and lymph node cells were isolated and stained with anti-CD4, CD25, NK1.1 antibodies (eBioscience). Intracellular staining of FoxP3 was performed using FoxP3 intracellular staining kit (eBioscience) according to manufacture's protocol. Stained cells were analyzed and sorted by FACS Aria.

Data analysis Images were assigned RGB colors and merged using Image J software and Adobe Photoshop, and HSC-microenvironment distance measures were obtained using Image J and Microsoft Excel. A two-tailed type 2 t-test was applied to all data. P values < 0.05 were considered statistically significant.