Thymus Reconstitution in Young and Aged Mice Is Facilitated by In Vitro-Generated Progenitor T Cells

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The prolonged lag in T cell recovery seen in older patients undergoing hematopoietic stem cell transplant (HSCT), after chemo-/radiotherapy, can lead to immune dysfunction. As a result, recovering patients may experience a relapse in malignancies and opportunistic infections, leading to high mortality rates. The delay in T cell recovery is partly due to thymic involution, a natural collapse in the size and function of the thymus, as individuals age, and partly due to the damage sustained by the thymic stromal cells through exposure to chemo-/radiotherapy. There is a clear need for new strategies to accelerate intrathymic T cell reconstitution when treating aged patients to counter the effects of involution and cancer therapy regimens. Adoptive transfer of human progenitor T (proT) cells has been shown to accelerate T cell regeneration in radiation-treated young mice and to restore thymic architecture in immunodeficient mice. Here, we demonstrate that the adoptive transfer of in vitro-generated proT cells in aged mice (18-24 months) accelerated thymic reconstitution after treatment with chemotherapy and gamma irradiation compared to HSCT alone. We noted that aged mice appeared to have a more limited expansion of CD4-CD8- thymocytes and slower temporal kinetics in the development of donor proT cells into mature T cells, when compared to younger mice, despite following the same chemo/radiation regimen. This suggests a greater resilience of the young thymus compared to the aged thymus. Nevertheless, newly generated T cells from proT cell engrafted aged and young mice were readily present in the periphery accelerating the reappearance of new naïve T cells. Accelerated T cell recovery was also observed in both aged and young mice receiving both proT cells and HSCT. The strategy of transferring proT cells can potentially be used as an effective cellular therapy in aged patients to improve immune recovery and reduce the risk of opportunistic infections post-HSCT.

Keywords: hematopoietic stem cell (HSC) transplantation, progenitor T cells, T cell development, thymus, homing
INTRODUCTION

T cells play a key role in the adaptive immunity to protect individuals from infections and malignancies. Most of the events during T cell development occur within the thymus, which is a primary immune organ that normally lacks self-renewing T cell progenitors (1). Therefore, the thymus relies on the semicontinuous supply of thymus-seeding progenitors (TSPs) from the bone marrow (BM) (2). TSPs receive strong Notch signals in the thymus (3), which guides them through a series of regulated developmental steps, including CD4+ CD8+ double negative (DN), CD4+ CD8+ double positive (DP), and CD4+ or CD8+ single positive (SP) stages (4). TSPs commit to the T lineage during intrathymic differentiation and become mature T cells when receiving signals from the specialized thymic microenvironment (5).

Interventions that disrupt the generation of T cells can lead to immunodeficiencies, such as irradiation/chemotherapy required for the treatment of some cancers (6, 7). Clinically, patients with hematological malignancies require myeloablative chemotherapy and/or radiotherapy to eliminate leukemic cells followed by hematopoietic and stem cell transplant (HSCT) from compatible donors to reconstitute the depleted BM niche. Although most blood borne cells recover relatively quickly, T cells have a prolonged recovery period, leading to higher risks of opportunistic infections in aged patients post-HSCT (8). This is likely due to the disrupted process of lymphopoiesis as a result of irradiation and chemotherapy (e.g., cyclophosphamide), particularly due to damage to thymic epithelial cells (TECs) (9, 10).

The delay in T cell recovery, which can last for over a year, is exacerbated in elderly patients (11), largely as a result of thymic involution, the natural age-related atrophy of the T-lymphopoietic organ. This has been modeled by showing that TECs in aged mice showed a higher rate of apoptosis and lower proliferative capacity (12), which was concomitant with a decline in the expression of the TEC-specific master transcription factor, Forkhead box N1 (FOXN1) (13, 14). In addition, aged thymus has a deficiency in the enzyme catalase, leading to an accumulation of damaging reactive oxygen species in TECs (15–17). The aged thymus also has a disorganized thymic structure, with a thinning of cortical regions, and more adipocytes and fibroblasts, providing a suboptimal microenvironment for the development and survival of proT cells (18, 19). This is further exacerbated by the absence of thymocyte/TEC crosstalk following HSCT, which may include RANK stimulation, required for proper thymic maintenance (20–22), potentially adding to the decline of the aged thymus compared to young (23).

A potential strategy to circumvent the paucity in T cell regeneration seen with HSCT is to adoptively transfer in vitro-generated progenitor T (proT) cells along with HSCT to facilitate thymic engraftment and accelerate T cell reconstitution (23–25). We define mouse proT cells as CD25+ DN cells (DN2 and DN3 stages) that can home to thymus, while possessing limited potential for non-T lineages outcomes. ProT cells have been shown to effectively reconstitute a host thymus and differentiate into all T cell subsets. Since donor proT cells undergo positive- and negative-selections in the host thymus, newly generated T cells are then restricted to host self-MHC, and tolerate to self-antigens, free from the risk of graft-vs-host-disease (26). Additionally, we have shown that through lymphocyte/TEC crosstalk, proT cells improve the thymic architecture of immunodeficient mice and enhance subsequent recruitment of bone marrow-derived progenitors (27). However, the kinetics of thymus recovery after combined therapy of proT cell and HSCT in aged mice remained unknown.

Here, we show that co-administration of in vitro-generated proT cells and HSCT can accelerate thymic reconstitution in aged and young mice after chemotherapy and radiotherapy, as compared to HSCT alone. Furthermore, we demonstrate that aged mice receiving proT cells have accelerated T cell recovery in the periphery, as compared to mice given HSCT alone. Notably, proT cells showed a similar ability to home to the thymuses of aged and young mice. However, aged recipient hosts showed a more limited expansion of donor thymocytes and slower kinetics of T cell development as compared to the young mice. Our preclinical results confirm that co-transferring proT cells with HSCT can potentially be used as an effective cellular therapy to enhance the immune recovery and lower the risk of opportunistic infections in aged patients post-HSCT.

METHODS

Mice

C57BL/6 (B6 CD45.2) and congenic B6.SJL-Ptprca Pepcb/BoyJ (B6 CD45.1) mice were purchased from The Jackson Laboratory (stock numbers 000664 and 002014, respectively). Young (8 to 12 weeks), and in-house aged (18–20 months) cohorts of mice were used. Green fluorescent protein (GFP+) hematopoietic cells were generated by breeding ROSA26-rTA transgenic mice (3) to Vav-iCre transgenic mice (4) to establish Vav-iCre-Rosa26rtTA mice on the B6 CD45.2 background. In hematopoietic cells, GFP was expressed upon Cre-dependent removal of a loxP-stop-loxP cassette within the ROSA26 locus. DsRed (B6.Cg-Tg(CAG-DsRed*MTST1)1Nagy/J) transgenic mice were purchased from The Jackson Laboratory (stock number 006051). All mice were maintained and bred at Sunnybrook Health Sciences Centre, and all animal procedures were approved by the Sunnybrook Health Sciences Centre Animal Care Committee.

Progenitor T Cell Co-Cultures With OP9-DL4-7FS Cells

Lineage-negative (Lin−) Sca-1+ Kit+ (LSK)/OP9-DL4 cell co-cultures were implemented, as previously described (5) with several modifications. Briefly, we cultured mouse-BM-derived LSK cells with the newly generated OP9-DL4-7FS cell line, transduced to express the Notch ligand Dll4 as well as human cytokines IL-7, FLT3-L, and SCF, as described (28). BM cells were collected from wild type B6 mice by dissecting and crushing the leg bones using sterile utensils in Hanks’ Balanced Salt Solution (HBSS). BM cells were then filtered through 40 μm filter to get a single cells suspension. CD117+ (Kit+) cells were enriched using anti-CD117-MicroBeads and LS column
Adoptive Transfer of Progenitor T Cells
B6 CD45.2 or CD45.1 congenic hosts were IP-injected with 150 µg/kg of Cyclophosphamide (Procytox (CTX), Baxter Corp.) 5 days and 3 days prior to exposure to 1.05 Gy total body irradiation using a Cs137 source gamma irradiator. 4-6 hours post-irradiation, all mice were intravenously injected with 10^6 B6 GFP+ BM-extracted cells from VaviCre-ROSA26rtTA mice that were T cells depleted (T-depleted Bone Marrow, TDBM). T cells in the BM VaviCre-ROSA26rtTA mice were depleted by anti-CD3-MicroBeads (Miltenyi Biotec). The "proT+TDBM" experimental group also received 5×10^5, 1×10^6, 2×10^6, or a different number of available thymic niches under steady state conditions, we adoptively transferred increasing numbers of in vitro-generated proT cells into non-irradiated mice in each age group (Figure 1). To this end, sorted mouse bone marrow (BM) Lineage- Sca-1+ Kit+ (LSK) cells were cocultured with OP9-DL4-7FS cells (28) for 10 days to generate CD25+ proT cells at the flow through (CD25- cells) was also collected for injection in some experiments.

Flow Cytometric Analysis
Single cell suspensions of dissected thymus, spleen, and BM were prepared by mashing followed by filtering through 40 µm cell strainers (Thermo-Fisher). Single-cell suspensions were labelled with fluorescently-conjugated antibodies purchased from BioLegend as follows: CD45.1(A20)-Percp/Cy5.5, CD45.2(104)-APC/Cy7, CD4 (GK1.5)-Alexafluor 700, CD8(53,6,7)-PE/Cy7, CD44(IM7)-PE, CD25-APC, CD3(17A2)-PE/Cy7, CD11b(M1/70)-APC, CD19 (1D3)-PE, CD45(30F-11)-APC/Cy7. Flow cytometry was performed on LSR II (BD Biosciences). Dead cells were excluded by 4,6-diamidino-2-phenylindole (DAPI) uptake. Data were analyzed using FlowJo Version 10.8.1 software (TreeStar).

Statistical Analysis
Statistically significant difference between different adoptive transfer dosages of proT cells and between aged and young mice in their thymuses and spleens were analyzed using one-way or two-way ANOVA and were performed using Prism software. All data are represented as mean ± SEM in error bars, with asterisks representing statistical significance (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

RESULTS
Available Thymic Niches Are Similar in Young and Aged Mice
To determine whether the thymuses of aged and young mice have an intrinsically different capacity to recruit proT cells and/or a different number of available thymic niches under steady state conditions, we adoptively transferred increasing numbers of in vitro-generated proT cells into non-irradiated mice in each age group (Figure 1). To this end, sorted mouse bone marrow (BM) Lineage- Sca-1+ Kit+ (LSK) cells were cocultured with OP9-DL4-7FS cells (28) for 10 days to generate CD25+ proT cells at the

FIGURE 1 | Thymus engraftment by in vitro-generated progenitor T cells in unmanipulated young and old mice. (A) CD25+ DsRed+ donor proT cells per 10^7 host thymocytes (top) and splenocytes (bottom) isolated from host thymuses and spleens of non-irradiated aged and young mice. Each group of mice (n=4-5) were injected with either 1, 3, or 9 x10^6 proT cells. Error bars depict SEM; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 analyzed by two-way ANOVA. (B) Representative flow cytometric analysis of detected DsRed+ proT cells from the thymus of a host mouse (top). Aggregated percentages of DsRed+ proT cells in blood vessels out of total DsRed+ proT cells from host thymuses injected with either 1, 3, or 9 x10^6 proT cells (bottom). Hosts were intravenously injected with CD45-AF700 antibodies 3 minutes prior to sacrificing. Error bars depict SEM; difference between the aged and young mice groups was not significantly (ns) different as analyzed by two tailed unpaired student’s t-test.
DN2/DN3 stage of T cell development (Supplementary Figure 1A). Cocultures were subjected to magnetic-assisted cell sorting (MACS) to enrich for CD25+ and the CD25- subsets.

CD25+ proT cells (1, 3, or 9 x10⁶ CD25+ cells), in vitro-generated from LSK cells from DsRed mice, were injected intravenously (i.v.) into host mice, and the presence of donor cells within the thymus was assessed at 40 h after injection. To rule out proT cells outside the thymic parenchyma, a labelled anti-CD45 antibody was injected i.v. just prior to sacrificing the host mice. Flow cytometric analysis of thymuses from young mice revealed the presence of donor DsRed+ cells when injected with 1 x10⁶ proT cells, and the numbers of donor cells appeared to increase linearly when 3 and 9 x10⁶ donor proT cells were adoptively transferred (Figure 1A). However, the thymus of aged mice showed saturated niches with increasing numbers of injected proT cells. Of note, the frequency of proT cells present outside the thymic parenchyma, or perivascular space, was on average ≤5% in both aged and young mice (Figure 1B).

Surprisingly, the thymus of aged mice showed a similar capacity in recruiting donor proT cells, with the thymus of young and aged mice failing to show a significantly different number of receptive niches. Similar to the thymus, host spleens showed a dose-dependent appearance of donor DsRed+ cells. However, the number of donor cells present in spleens was over 40-fold higher than what was detected in the thymus. This suggested that at steady state the thymus has a highly restricted entry and/or limited number of niches for proT cells.

Thymus Engraftment by In Vitro Generated ProT Cells

The use of non-irradiated host mice established that the thymus of both young and aged mice showed a similar but low number of receptive niches. We then assessed whether the use of clinically relevant conditioning regimens, including chemo/radiotherapy, would impact the effectiveness of proT cell engraftment in aged and young mice. To this end, young host mice were treated with cyclophosphamide (CTX, 150 µg/kg) 5 and 3 days prior to lethal irradiation (1.05 Gy) (Figure 2A). To verify that in vitro-generated proT cells and no other cocultured-derived cells would home to the thymus, as described before (24, 25) and shown above, we compared the homing and engraftment ability of LSK/OP9-DL4/FSC coculture-generated CD25+ DN proT cells to that of the remaining CD25- DN cells (Supplementary Figure 1A). In addition to the culture-derived cells, young mice (8-12 wks old) were co-injected with 1 x10⁶ GFP+ T cell-depleted BM (TDBM) cells. The thymuses of host mice were analyzed by flow cytometry 8 days (D8) after injection, and we noted a significantly greater number of donor CD25+ proT cells homing to and developing within the thymus, constituting ~50% of total thymocytes, including CD4+ CD8+ DP cells, in contrast to CD25- DN cells, which failed to engraft (Figure 2B). In this regard, mice injected with CD25- DN cells showed only host-derived cells in the thymus (Figure 2B). Remarkably, on D8, some of the CD25+ proT-derived thymocytes still exhibited CD25 expression, suggesting long-lasting self-renewal of proT cells within the thymus (Figure 2B, left panel). Nevertheless, mice injected with CD25+ DN cells showed a minor fraction (~0.5%) of donor cells within the thymus that progressed towards the DP stage of differentiation, which we attributed to the fact that the MACS-enriched CD25+ DN population contained about 8% CD25+ proT cells (Supplementary Figure 1A, middle panel). In contrast to the poor thymic engraftment, CD25- DN cells were readily detected in the spleen of host mice, comprising the 28% of splenocytes, with the vast majority expressing CD11b (Figure 2C and Supplementary Figure 1B). As expected, the other major contributor to CD11b+ myeloid cells in the spleen are derived from the GFP+ TDBM donor graft. Remarkably, the contribution of culture-derived donor cells to the BM was minimal for both CD25+ proT and CD25- DN cells, while as expected a strong contribution by GFP+ TDBM donor cells was observed (Figure 2D). Taken together, our findings further validate the use of in vitro-generated CD25+ proT cells as an effective thymus seeding cell in the context of HSCT.

Thymus Engraftment by Increasing Numbers of ProT Cells in Aged Mice

To determine the number of proT cells required to saturate thymic engraftment in aged mice, in the context of combined chemo/radiation conditioning and HSCT, we adoptively transferred 0, 0.5, 1, 2 or 4 x10⁶ congeneric in vitro-generated proT cells along with 1 x10⁶ GFP+ TDBM cells into aged mice, as illustrated in Figure 2A. On D14, we used flow cytometry to determine the contribution of host, GFP+ TDBM and proT cells to thymus cellularity (Figure 3). All aged mice receiving proT cells showed the presence of donor-derived DP cells by D14. Mice injected with only TDBM (0 proT) did not show the presence of DPs, either host or TDBM derived, rather contained host CD4 or CD8 SP T cells and very few, if any, TDBM derived cells, which were nearly all at the DN stage. There was a positive correlation between engraftment efficiency and the number of proT cells injected (Figure 3B), with the provision of 4 x10⁶ cells resulting in nearly 100% of thymic cellularity corresponding to proT-derived cells. This suggested that all the possible niches were likely occupied by the injection of 4 x10⁶ proT cells, competing out host and GFP+ TDBM donor cells to exclusively participate in early thymic engraftment.

ProT Cells Accelerate Thymus Engraftment in Both Young and Aged Mice

Having established the number of proT cells required to fully engraft the thymus in aged mice, we then compared proT cell engraftment and differentiation in aged vs. young mice. We analyzed host mice, young and aged, in 2-week intervals following the administration of 4 x10⁶ proT cells and 1 x10⁶ GFP+ TDBM cells (Figure 4A). On D14, proT-derived cells constitute ~95% of thymocytes present in young mice and ~87% in aged mice (Figure 4B), with most cells (~90%) having reached the CD4/CD8 DP stage of differentiation in both young and aged thymuses. Of note, a major difference between young and aged host mice was the number of thymocytes after engraftment, with thymus of young mice having ~5-fold higher total cellularity.
than aged mice at D14 (Figure 4C). In contrast, mice receiving only TDBM cells showed a delayed thymic engraftment. Importantly, at D14, TDBM mice had ~4-fold fewer thymocytes when compared to their age-matched mice that had also received proT cells, confirming that proT cells significantly enhance thymic reconstitution in both young and aged mice.

In vitro generated proT cells may represent a finite source of thymic seeding cells, after entering the thymus and differentiating into later stages of T cell development. However, whether proT cells could undergo limited self-renewal after thymic entry was addressed by establishing whether proT-derived short lived DP cells, as well as SP cells, could be seen at later time points. We noted an increase in the proportion of proT-derived SPs in both young and aged thymus by day 28 (Figure 4A). However, the thymuses of young mice revealed the presence of a large percentage of proT-derived DP cells at D28. Of note, even as late as D42, proT-derived DP cells were still detected in the thymus of young mice, albeit coming from a much-reduced frequency of proT-derived cells.

By day 28, the next wave of TSPs derived from the GFP+ TDBM cells becomes apparent. We observed a significant decline in the percentage of total thymocytes that were derived from proT cells in young mice, from 96% by D14 to an average of 36% by D28 and only 7% by D42 (Figure 4B). This was concomitant with a shift towards an increase in GFP+ donor-derived cells within the thymus of young mice (Figure 4B). Additionally, total thymus cellularity in young mice, reached ~84 x10^6 cells by D42, which is similar to unmanipulated aged-matched mice (Figure 4C). Remarkably, we noted a dip in thymocyte cellularity at D28 in young mice receiving proT cells (Figure 4C), which though statistically not significant, it appears to correspond to the transition from proT-derived cells being the major contributor of thymic cellularity to GFP+...
donor-derived cells taking over, with over 90% derived from GFP+ donor cells (Figure 4B).

In aged mice, the frequency of proT-derived thymocytes also significantly declined in percent contribution over time, as these cells are replaced by donor GFP+ cells, but also by host cells as well; this latter occurrence was not as readily observed in young mice (Figure 4). On D28, both proT- and TDBM-injected aged mice host cells contributed to about 50% of the thymus cellularity, in contrast to less than 5% host contribution in young mice (Figure 4B). A similar trend was observed on D42, with the thymuses of aged mice showing a large fraction of host-derived cells irrespective of whether they received proT or TDBM GFP+ donor cells.

**Accelerated Peripheral T Cell Reconstitution in Aged ProT-Treated Mice**

We next examined whether the rapid thymic engraftment observed in proT-injected mice had an effect on the appearance of peripheral T cells in the spleen. Mice receiving chemo/radio conditioning treatment showed altered immune cell subset distribution in their spleens. While there is variation amongst mouse strains, as well as effects of ageing on the cellular composition of spleen in control unmanipulated mice (29, 30), we observed on average 62% B cells, 25% T cells, 7% myeloid cells and 6% other cells in C57BL/6 spleen. In contrast, on D14 following conditioning, myeloid cells made up the majority of the splenocytes in young or aged mice, whether given proT cells or GFP+ TDBM cells (Figure 5A). By D28, the proportion of B cells began to recover and approached normal levels, which were reached by D42, and by this time point the proportion of myeloid, B and T cells had returned to control levels.

Focusing on T cells, we noted that the frequency of T cells remain low at D14, both in young and aged mice (Figure 5B). By D28, a clear recovery in the percentage of T cells was observed in young mice that were given either proT or TDBM cells, with an increase in T cells to ~14% and ~10%, respectively. Aged mice given proT cells showed an equal recovery, with an increase in T cells to ~8%; whereas, aged mice given TDBM cells failed to recover the proportion of T cells, and remained at less than 2% (Figure 5B). This suggests that aged mice given TDBM remain T cell deficient for a significantly longer period when compared to proT-injected aged mice. Nevertheless, by D42 we observed the recovery of T cells in TDBM-injected aged mice.

In terms of the contribution of host, GFP+ TDBM or proT-derived cells to splenic T cell population, we found that for proT-injected mice, whether young or aged, the majority of T cells present on D28 were derived from proT cells (Figure 5C). This trend continued up to D42, even as the frequency of GFP+ TDBM- and host-derived T cells increased, but remained a minority. For young and aged mice given TDBM, there was a trend towards an increased contribution of GFP+ donor cells over host cells at all time points. Taken together, these findings support the notion that aged mice given proT cells exhibit an earlier recovery of their T cell compartment.

**DISCUSSION**

One of the clinical shortcomings of HSCT is the extended period of time needed for the T cell compartment to reemerge, leading to increased susceptibility to infections and relapse. This is exacerbated in elderly patients (≥60 years old) that comprise the majority of individuals undergoing HSCT for leukemia (6, 31, 32). Adoptive transfer of proT cells into patients has been proposed as a viable future option to combat the prolonged paucity of T cells, since preclinical studies showed that proT cells, generated in culture from HSPCs, can engraft the thymus and accelerate recovery of functional T cells (24, 25). In this study, we addressed whether proT cells can function in a similar manner when given to aged mice, as a preclinical model for elderly patients receiving HSCT. To this end, we also included the administration of cyclophosphamide, a chemotherapy reagent, to better simulate conditioning regimens. Additionally, we expand the pool of proT cells to include all CD3+CD4-CD8- (DN) CD25+ cells, based on the observations that both DN2 and DN3 can engraft the thymus (24).
One of the issues with performing irradiation as the sole approach in mouse models, is that it does not reflect the reality of most clinical modalities, where multiple regimens of chemotherapeutic and radiotherapeutic treatments may be combined. There is also the issue of early auto-reconstitution of host thymocytes by the endogenous radioresistant DN2 subset, which reduces the availability of niches for thymic seeding cells (23). In our model and clinically, as radioresistant DN cells expand and differentiate to repopulate the thymus affected by chemotherapy, these cells are exposed to irradiation, leading to a reduction in the pool of radioresistant DN cells, and potentially less competition for incoming adoptively transferred proT cells. This notion is reflected in the reduction in the requirement of the number of proT cells, from 6-10 x 10^6 (23, 25, 33) to 4 x 10^6, to reach saturation of thymic niches, and with a concomitant increase in the average proportion of proT-derived thymocytes to ~90% within two weeks after adoptive transfer.

Consistent with previous results (12), engraftment of proT cells into the thymus of aged mice appeared to be very similar in efficiency as that seen in young mice, when comparing the number of incoming proT cells within 40 hours after adoptive transfer in absence of conditioning. We extended our analysis to young and aged mice receiving chemo/radio-conditioning and noted by day 14, the total cellularity of the thymus in young mice was 5-fold greater than that obtained in aged mice. This difference in cellularity is likely due to thymic involution, the natural age-related atrophy of the thymus, limiting the niches available for expansion (34). Our results confirmed the
likelyhood of limiting or poor-quality niches within the thymus of aged mice, affecting the proliferation of donor-derived thymocytes (12, 35).

In particular, we noted that, in the thymus of young mice, short-lived immature DP cells were still being generated from proT cell grafts even after one month since their adoptive transfer. Strikingly, this capacity of proT cells to give rise to DPs for such an extended period after transplant was not seen in the thymus of aged mice. These findings point to a major difference between the thymic microenvironment of young and aged mice, such that proT-derived cells were able to give rise to short-lived DPs for a much longer period of time within the young thymus than when seeding the thymus of aged mice, suggesting that their ability to undergo self-renewal was severely limited within the older thymic microenvironment. Understanding what these deficiencies are within the aged thymic niche will provide important insights as to how to improve T-lymphopoiesis in the elderly.

Despite the known age-associated decrease in thymic output (36, 37), we show here that the thymus of aged mice can export newly generated T cells to the periphery, as detected in the spleen by D28. Of importance, with respect to our modeling, we replicated the lag in the reemergence of peripheral T cells in aged mice receiving TDBM only, when compared to mice given both proT cells and TDBM cells, which showed an earlier appearance of donor-derived T cells in the periphery. We postulate that the aged mice with delayed T cell recovery would be more susceptible to infections, similar to what is seen in elderly patients receiving HSCT. While peripheral T cell reconstitution is accelerated by the provision of proT cells, it is not clear from our results whether earlier thymic engraftment by proT cells facilitated the subsequent wave of TSPs from the GFP+ TDBM donor graft, as we had postulated earlier (27). As such, for both the young and aged thymus, the initial delay in T cell cellularity seen at D14 when given only TDBM cells, is replaced by an equivalent or greater cellularity by D28 and beyond. This suggests that a delay in thymic crosstalk does not appear to significantly alter the recruitment capacity of the recovering thymus, despite the damage incurred by TECs due to the conditioning regimen.

Apart from lower cellularity, the thymuses of aged mice are quite distinct in their composition from their young counterparts, in that they showed a much larger contribution of host-derived cells. This may be due to the incomplete replacement of host-derived cells by GFP+ TDBM donor cells within the BM of aged mice, unlike their young counterparts. The ability of host cells to compete against the GFP+ graft and repopulate the BM conflicts with current literature showing many instances of aging cells being more susceptible to radiation exposure (38). One explanation could be that host cells in aged mice are being protected from the effects of radiation simply due to their higher body weight, as aged mice typically weighed twice as much as their young counterpart. While we set the CTX dose according to weight, a similar

![Figure 5](image-url)
increase in radiation dose according to weight may have led to irreparable cellular damage. Nevertheless, mouse weight and size, as well as other metabolic differences, may add to the complexity in comparing the effects of conditioning in aged and young mice. Future clinical applications of our findings will rely on the recent replacement of the xenogenic OP9-DL cells with a serum-free, cell-free system of plate-bound Dll4 or DL4-µbeads methods for the generation of human proT cells (39–41), which have increased the potential therapeutic use of proT cells. In addition, our findings strongly suggest that proTs could provide an immune boost to the elderly, the population that comprises the majority of patients undergoing HSCT. The next hurdles remaining before the therapeutic use of proT cells appear surmountable, though wide-ranging. Focusing on the preclinical side, it is paramount to demonstrate that proT-derived mature T cells can confer immunity against diseases in aged mice. Further, there is the need to address the standard practices of care in hospitals during HSCT and use them as a guide for our preclinical modelling. This includes details such as administration of both chemo- and radiation treatments, which we have done here, but also including anti-thymoglobulin (ATG) following HSCT (42) or the use of allogeneic grafts instead of congenic HSPCs. ATG treatment, for example, is given to curtail host vs graft rejection and other complications of HSCT, a practice that would likely counter proT cell therapy, as these cells would also be targeted by ATG. Thus, it requires further consideration before embarking on clinical trials.

In short, here we have improved upon the conditioning regimen and discovered that providing proT cells allows for the effective reconstitution of the aged mouse thymus with accelerated T cell regeneration. The favorable consequences afforded by rapid thymic reconstitution includes the appearance of mature T cells in secondary immune organs, providing a potential advantageous immune boost to aged recipients.

**ETHICS STATEMENT**

The animal study was reviewed and approved by Sunnybrook Health Sciences Centre Animal Care Committee.

**AUTHOR CONTRIBUTIONS**

MM, YRL and CL performed all the experiments. MM and YRL designed and analyzed the data, and wrote the manuscript. JCZ-P conceived the project, analyzed the data, and wrote and edited the manuscript. All authors contributed to the article and approved the submitted version.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2022.926773/full#supplementary-material

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