ORIGINAL ARTICLE

Immunosenescent characteristics of T cells in young patients following haploidentical haematopoietic stem cell transplantation from parental donors

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Abstract

Objectives. Paediatric and adolescent patients in need of allogeneic haematopoietic stem cell transplantation (HSCT) generally receive stem cells from older, unrelated or parental donors when a sibling donor is not available. Despite encouraging clinical outcomes, it has been suggested that immune reconstitution accompanied by increased replicative stress and a large difference between donor and recipient age may worsen immunosenescence in paediatric recipients. **Methods.** In this study, paired samples were collected at the same time from donors and recipients of haploidentical haematopoietic stem cell transplantation (HaploSCT). We then conducted flow cytometry-based phenotypic and functional analyses and telomere length (TL) measurements of 21 paired T-cell sets from parental donors and children who received T-cell-replete HaploSCT with post-transplant cyclophosphamide (PTCy). **Results.** Senescent T cells, CD28$^-$ or CD57$^+$ cells, were significantly expanded in patients. Further, not only CD4$^+$CD28$^-$ T cells, but also CD4$^+$CD28$^+$ T cells showed reduced cytokine production capacity and impaired polyfunctionality compared with parental donors, whereas their TCR-mediated proliferation capacity was comparable. Of note, the TL in patient T cells was preserved, or even slightly longer, in senescent T cells compared with donor cells. Regression analysis showed that senescent features of CD4$^+$ and CD8$^+$ T cells in patients were influenced by donor age and the frequency of CD28$^-$ cells, respectively. **Conclusion.** Our data suggest that in paediatric HaploSCT, premature immunosenescent changes occur in T cells from parental donors, and therefore, long-term immune monitoring should be conducted.

INTRODUCTION

Haploidentical haematopoietic stem cell transplantation (HaploSCT) has been used worldwide over the past two decades in cases where a HLA-matched donor is not available.[1] The innovative development of post-transplant high-dose cyclophosphamide (PTCy) to prevent graft-versus-host disease (GVHD) has made HaploSCT simpler and safer to conduct by selective elimination of alloreactive T cells. Recently, HaploSCT with PTCy has shown promising clinical outcomes in both children and adults.[2-5]

The clinical outcome of haematopoietic stem cell transplantation (HSCT) is greatly impacted by the speed and success of immune cell reconstitution, which is achievable through marked proliferation of donor HSCs after infusion.[6-9] However, this process is unavoidably accompanied by replicative stress and accelerated telomere shortening in the immune cells of adult recipients.[10-12] Considering that replicative stress is a potent driver of cellular senescence[13-15], rapidly proliferating cell types, such as T cells, are particularly susceptible and this can result in dysfunctional immune responses and fundamentally affect the health and survival of individuals.[16] It has been established that senescent T cells have the ability to produce large quantities of proinflammatory cytokines and cytotoxic mediators and are closely associated with a variety of deleterious health-related outcomes.[17,18] Moreover, enhanced homeostatic T-cell proliferation to compensate for lymphopenia contributes to premature immune ageing, which is an important risk factor for many ageing-associated chronic inflammatory disorders.[19-21]

Paediatric and adolescent patients requiring HSCT generally receive stem cells from an older person when a matched sibling donor is not available. With regard to HaploSCT in children, parents are the most accessible donors and are usually 25–35 years older than the patients. However, it is not known whether this age difference causes adverse clinical effects or whether the immune cells originated from older donors are functionally well maintained after reconstitution. Specifically, the ageing status, or presence of immunosenescent features, of T cells in paediatric patients who receive HaploSCT from a parental donor is poorly characterised. Given that more paediatric patients undergoing HSCT have become long-term survivors, owing to improvements of clinical practices of transplantation and supportive care, long-term monitoring of their immunologic recovery and maintenance is now necessary.

In the present study, we investigate whether patients who received T-cell-replete HaploSCT with PTCy from parental donors have immunosenescent alterations or functional defects of their reconstituted T cells. We also sought to examine whether which parameters contribute to the immunosenescent features of patient cells.

RESULTS

Patient characteristics and clinical outcomes

Clinical characteristics of the patients are summarised in Table 1. Fourteen paediatric, adolescent and young adult patients with malignant disease and seven with nonmalignant disease were included with a median post-transplantation period of 16.9 months (range, 12.4–38.8). The median area under the curve of the total infused busulfan was 73 984 μg × h L⁻¹ (range, 67 302–76 887). All patients achieved engraftment. The median number of neutrophil and platelet engraftment days was 14 (13–21 days) and 26 (13–87 days), respectively. With the exception of two, all patients experienced cytomegalovirus reactivation, which was treated by ganciclovir induction therapy who had cytomegalovirus antigenemia levels ≥ 10 per 100 000 cells ($n = 4$) or half-dose preemptive therapy ($n = 15$).[22] No patients showed cytomegalovirus disease or post-transplantation lymphoproliferative disease. Acute GVHD above grade 2 occurred in five patients (23.8%), grade 2 in four patients and grade 3 in one patient. The signs and symptoms of acute GVHD in all patients were resolved by treatment with systemic methylprednisolone. Extensive, chronic GVHD developed in three patients. Cyclosporine and prednisolone were used in two patients for 7 and 12 months, respectively. For the remaining patient, mycophenolate mofetil plus prednisolone was used for 8 months because of post-transplant

thrombotic microangiopathy. Resolution of chronic GVHD was achieved in all patients. With a median follow-up of 16.9 months (range, 12.4–38.8) after transplantation, all patients survived without evidence of chronic GVHD or relapse.

Accumulation of CD28⁻ T cells and CD57⁺ T cells in peripheral blood of young HaploSCT recipients

Reconstituted T cells in HaploSCT recipients may be prone to premature ageing as a result of the transplantation of immunologically aged cells from parental donors, which then experience replicative stress in order to restore the immune system. To test this hypothesis, we first analysed phenotypic features using paired T cells from the patients and donors (Figure 1). The frequency of $CD3^+$ T cells was similar; however, the ratio of $CD4^+$ to $CD8^+$ T cells (1.37 ± 0.20) was significantly

decreased in patients compared with donors (2.98 ± 0.46) (Figure 1a, b).

One of the major age-related changes in T cells is the progressive reduction of naive T cells accompanied by an expansion of effector memory cells.[23-25] In humans, functional T-cell subsets are defined by expression of CD45RA and CCR7.[26] Analysis of the distribution of functional T-cell subsets (Figure 1c) revealed that transplant patients had a lower frequency of naive $CD8^+$ T cells and CM subsets in both the $CD4^+$ and $CD8^+$ populations compared with donors. Conversely, the frequency of EMRA (effector memory cell re-expressing CD45RA) of $CD8^+$ T cells tended to increase in recipients, suggesting that paediatric patients undergo age-related changes in the distribution of T-cell subsets, which are even more marked than that of parental donors, despite age differences of approximately 30 years. There were no significant differences in the other subsets between patients and donors.

Table 1. Patient characteristics

	N = 21
Median age, Years (range)	12.2 (0.9–28.1)
Sex, No. (%)	
Male	14 (66.7%)
Female	7 (33.3%)
Median BSA, m² (range)	1.21 (0.47–1.91)
Median body weight, kg (range)	35.6 (9.8–76.8)
Diagnosis, No. (%)	
Acute lymphoblastic leukaemia	5 (23.8%)
Acute myeloid leukaemia	5 (23.8%)
Mixed phenotype acute leukaemia	1 (4.8%)
Other malignancies[a]	3 (14.3%)
Other nonmalignant diseases[b]	7 (33.3%)
Disease status	
CR1	11 (52.4%)
CR2	3 (14.3%)
N/A	7 (33.3%)
Donor	
Mother	12 (57.1%)
Father	9 (42.9%)
CMV serology (donor/recipient), No. (%)	
Positive/Positive	19 (90.5%)
Positive/Negative	2 (9.5%)
Infused busulfan AUC, µg × h L⁻¹ (range)	73 939 (67 302–76 887)
Median infused TNC, ×10⁸ kg⁻¹ (range)	13.9 (2.9–18.5)
Median infused CD34⁺ cells, ×10⁶ kg⁻¹ (range)	7.3 (2.8–23.6)

AUC, area under the curve; BSA, body surface area; CMV, cytomegalovirus; CR, complete remission; N/A, not applicable; No., number; TNC, total nuclear cells.
[a]One case of Ewing sarcoma, 1 of T lymphoblastic lymphoma, and 1 of Hodgkin disease are included.
[b]Three cases of adrenoleukodystrophy, 2 of Krabbe disease, 1 of autoimmune lymphoproliferative syndrome, and 1 of beta-thalassaemia are included.
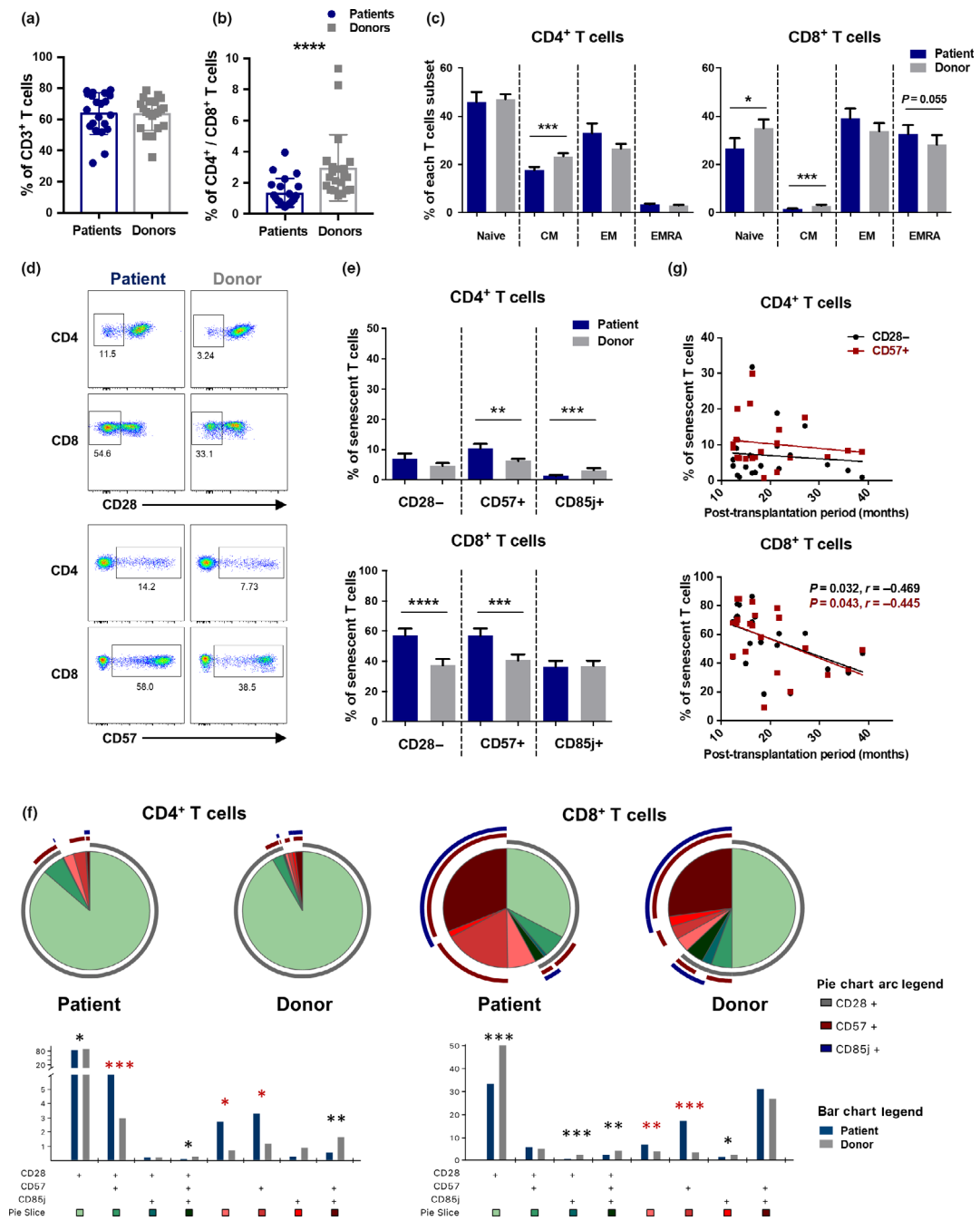
Figure 1. T cells in young HaploSCT recipients have phenotypic features typical of senescent T cells. Phenotypic characterisation of T cells in freshly isolated PBMCs from patients and parental donors (*n* = 21 pairs) by flow cytometry. **(a, b)** Frequency (%) of CD3$^+$ T cells **(a)** and ratio of CD4$^+$ to CD8$^+$ T cells **(b)** in patients and donors. **(c)** Distribution of functional T-cell subsets defined by the CD45RA and CCR7 expression on CD4$^+$ and CD8$^+$ T cells of patients and donors. Naive (CD45RA$^+$CCR7$^+$), central memory (CM: CD45RA$^-$CCR7$^+$), effector memory (EM: CD45RA$^-$CCR7$^-$) and CD45RA$^+$ effector memory (EMRA: CD45RA$^+$CCR7$^-$). **(d)** Representative FACS plot of CD28 and CD57 expression on CD4$^+$ and CD8$^+$ T cells from patients and donors. **(e)** Frequency (%) of CD28$^-$, CD57$^+$ and CD85j$^+$ cells in CD4$^+$ and CD8$^+$ T-cell populations of patients and donors. **(f)** Surface expression of senescence markers was assessed using Boolean combination gates in FlowJo software and analysed with the SPICE program. Pie charts demonstrate the relative contribution of each of eight possible subsets among total CD4$^+$ and CD8$^+$ T cells in patients and donors. Accompanying bar graphs depict the average frequency of each subset among the CD4$^+$ or CD8$^+$ T cells shown in **e**. **(g)** Correlation analysis between post-transplantation period and frequency (%) of CD28$^-$ or CD57$^+$ T cells of patients. Each data point represents an individual subject. Bar graphs show the mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001, ****P< 0.0001 by two-tailed paired non-parametric. The *P*-value in **g** was obtained using the Spearman correlation analysis.

Loss of CD28 is a typical feature of senescent T cells, is caused by repeated cell division,[17,27-29] and progressively increases with age. Moreover, CD28⁻ T cells concomitantly acquire CD57 and CD85j, which are associated with modulation of their functional activity.[23,30-33] In Figure 1d, we show that the patients had higher frequencies of CD28⁻ and CD57⁺ subsets of both CD4⁺ and CD8⁺ T cells compared with parental donors. In contrast, the frequency of CD85j⁺ cells in patients was either similar or decreased compared to donors (Figure 1e). In healthy individuals, senescent CD28⁻ T cells mostly co-express CD57 and CD85j molecules[30]; however, this mutually exclusive expression pattern was not apparent in the T cells of the patients (Figure 1f). Rather, they exhibited increased CD28⁺CD57⁺ and CD28⁻CD57⁻ T cells, which are atypical subsets of healthy donors.[34] Of interest, senescent CD28⁻ or CD57⁺ subsets of CD8⁺, but not of CD4⁺, T cells significantly decreased with time after transplantation (Figure 1g). These findings show that transplant patients have a higher frequency of phenotypically senescent T cells than parental donors, despite the considerably younger age of the patients.

The cytokine production profile of accumulated CD28⁻ T cells in patients

There is a growing body of research on the functional defects of senescent CD28⁻ and CD57⁺ T cells in the elderly.[18,27,29,35,36] To investigate whether the increased population of CD28⁻ T cells in the patients in this study exhibit functional defects, we first examined cytokine production and cytotoxicity of T cells. Cytokine production was partially impaired in CD4⁺, but not CD8⁺, T cells of patients compared with donors (Figure 2a–c). The frequency of PMA/ionomycin-induced IFN-γ, TNF-α and MIP-1β-producing CD4⁺ T cells was significantly lower in HaploSCT recipients. A similar trend was observed in CD4⁺ T cells stimulated with anti-CD3.

Considering that of CD4⁺ T cells, CD28⁻ cells are the main producers of IFN-γ, we further analysed cytokine production by assessing CD28⁺ and CD28⁻ T cells to explore whether impaired cytokine production is attributable to senescence in CD28⁻ T cells (Figure 2d, f). As expected, CD28⁻ T cells produced more substantial amounts of IFN-γ and TNF-α upon stimulation than did CD28⁺ T cells, and the frequency of IFN-γ and TNF-α-producing cells was diminished in CD4⁺CD28⁻

T cells of patients compared with donors (Figure 2e). However, the frequencies of IFN-γ and TNF-α-producing cells were significantly reduced in CD4⁺CD28⁺ T cells of patients as well; however, there was only a slight reduction in production of these cytokines by CD8⁺ T cells. Although the frequency of CD8⁺CD28⁻ T cells diminished gradually in transplant patients during the post-transplantation period (Figure 1g), the frequency of cytokine-producing cells among CD28⁻ cells was not altered (Figure 2g). These results indicate that in these patients not only accumulated CD4⁺CD28⁻ T cells but also CD4⁺CD28⁺ T cells have a reduced capacity for cytokine production. In contrast, the ability to produce these cytokines is relatively well maintained in CD8⁺ T cells.

Diminished polyfunctional T-cell responses in young HaploSCT recipients

To further investigate the impaired cytokine production by CD4⁺ T cells in these patients, polyfunctional analysis was performed using the dataset in Figure 2. Polyfunctional T cells producing multiple immune mediators, such as cytokines, provide a more effective immune response to a pathogen.[37,38] Figure 3a shows a polyfunctionality profile of CD4⁺ T cells in response to TCR stimulation based on their CD28 expression. The bar graphs represent the average frequency of the listed mediator combination (Figure 3b). These data revealed that the frequency of polyfunctional T cells was reduced in the patients and was more marked in CD4⁺ T cells than in CD8⁺ T cells and in CD28⁺ T cells than in CD28⁻ T cells (Figure 3b). Taken together, these findings demonstrate both quantitative and qualitative impairment in the ability of patient CD4⁺ T cells to produce major effector cytokines compared with donor cells.

No difference between patients and donors in the capacity of cells to proliferate

We next examined the ability of patient immune cells to proliferate upon TCR stimulation (Figure 4). Consistent with previous findings,[39,40] CD28⁻ cells showed significantly reduced proliferation compared with CD28⁺ T cells (Supplementary figure 1). However, there was no difference in the frequency of cells that underwent cell division between patients and donors (Figure 4a–c), indicating an intact proliferative capacity of accumulated CD28⁻ T cells in the patients.
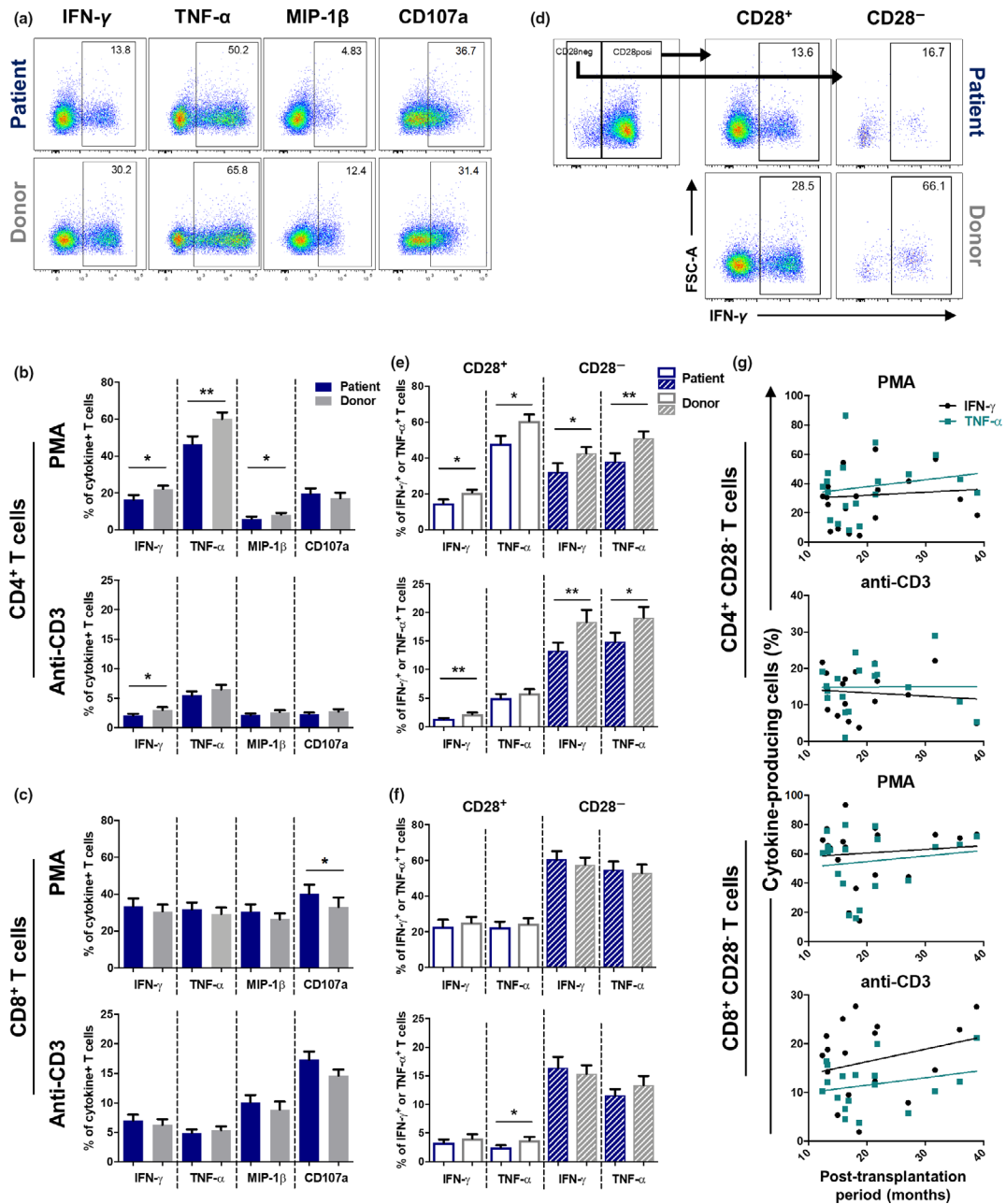
Figure 2. Cytokine production is reduced in CD4+ T cells of young HaploSCT recipients compared with parental donors. Frozen PBMCs of patients and donors were thawed and stimulated with anti-CD3 and anti-CD28 Abs for 5 days. PBMCs were re-stimulated for 4 h with PMA/ionomycin (n = 19 pairs) and plate-bound anti-CD3 Ab (n = 18 pairs), followed by intracellular cytokine staining (ICS). **(a)** Representative FACS plot of cytokine-producing CD4+ T cells upon stimulation with PMA/ionomycin in patients and donors. **(b, c)** Frequency (%) of cytokine-producing CD4+ **(b)** and CD8+ **(c)** T cells in patients and donors in response to PMA/ionomycin or anti-CD3 Abs. **(d)** Representative FACS plot of cytokine-producing cells in CD4+CD28+ or CD28− cells of patients and donors. **(e, f)** Frequency (%) of cytokine-producing CD28+ or CD28− cells in CD4+ **(e)** and CD8+ **(f)** populations of patients and donors. **(g)** Correlation analysis between post-transplantation period and frequency (%) of cytokine-producing cells in CD28− of CD4+ and CD8+ T cells of patients in response to the indicated stimuli. Bar graphs show the mean ± SEM. *P < 0.05 and **P < 0.01 by a two-tailed paired non-parametric t-test **(b, c, e, and f)**.

Telomere length is preserved in T cells of young HaploSCT recipients

Telomere attrition caused by extensive proliferation has been reported in T cells of HSCT recipients and may play a role in, or be a marker of, long-term outcomes after HSCT.[41] To analyse TL in different T-cell subsets, we utilised telomere sequence-specific Flow-FISH combined with cell surface staining (Figure 5 and Supplementary figure 2). Consistent with previous reports,[42,43] TL in senescent CD28^{−} T cells was found to be significantly decreased compared to naive T cells in both patients and donors (Figure 5a, b). Of

interest, the TL of CD28^{−} senescent T cells in patients was comparable, or even significantly longer, than that of donors (Figure 5c, d). This suggests that the TL of patient T cells is relatively well preserved during immune reconstitution. However, the TL of patient T cells predominantly correlated with that of donor T cells (Figure 5e).

CD28^{−} T cells of young HaploSCT recipients exhibit increased expression of γ-H2AX

It has been demonstrated that cellular senescence is associated with an increased DNA damage.[13] γ-H2AX is used as a DNA damage marker, and its
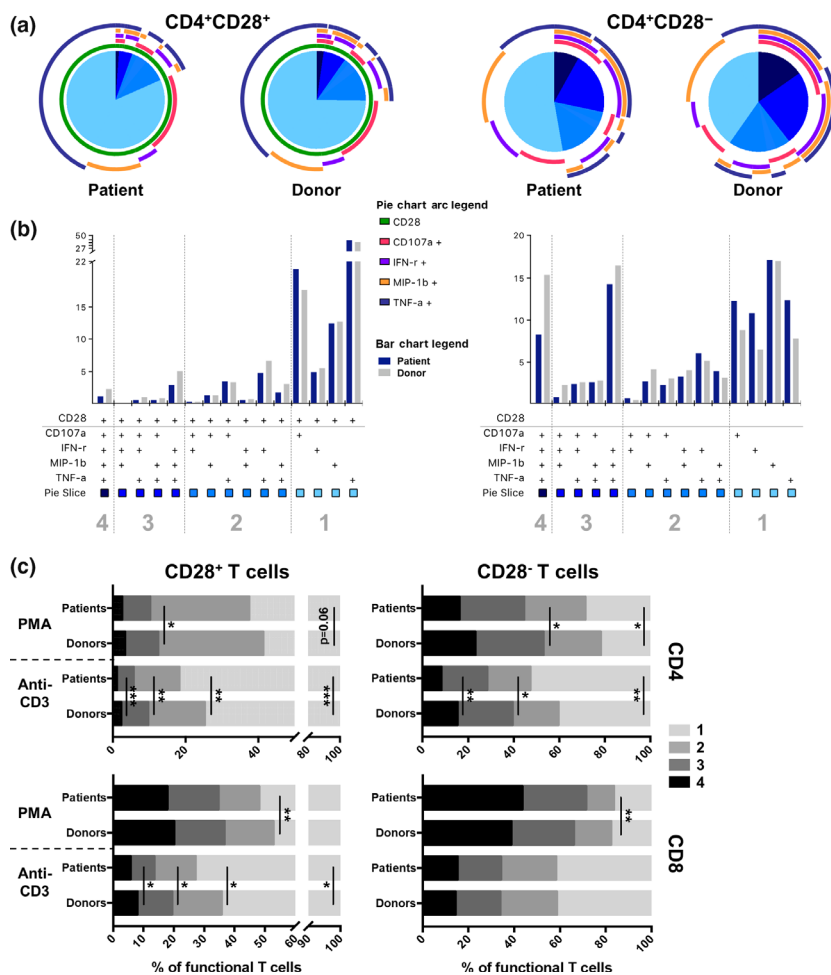
Figure 3. Polyfunctional T-cell responses are weakened in young HaploSCT recipients. The cytokine production profile was analysed using Boolean combination gates in FlowJo software and the SPICE program using ICS data from Figure 2. **(a)** Pie charts demonstrate the relative contribution of each subset to the total functional response, with each colour representing the number of cytokines or cytotoxic mediators simultaneously produced in the CD28^{+}CD4^{+} or CD28^{−}CD4^{+} T cells. **(b)** Bar graphs represent the average frequency (%) of all 15 functional mediator combinations among CD28^{−}CD4^{+} or CD28^{+}CD4^{+} T cells of patients and donors upon stimulation with anti-CD3 Abs (*n* = 18 pairs). **(c)** Frequency (%) of the relative contribution of subsets representing the number (1–4) of cytokines or cytotoxic mediators produced by the indicated T-cell subsets upon various stimulations. Bar graphs and stacked bar graphs show the mean (**b** and **c**, respectively). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$ by a two-tailed paired non-parametric *t*-test **(c)**.
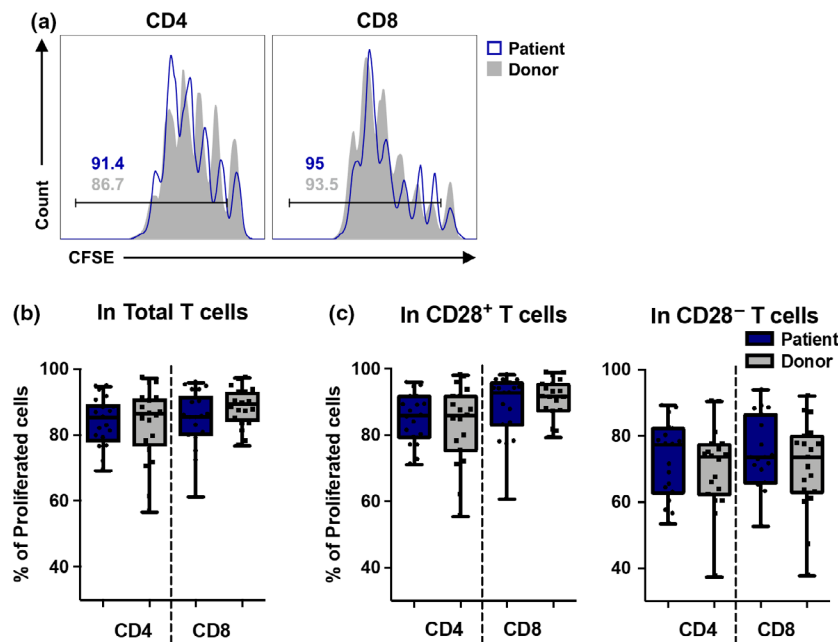
Figure 4. Accumulated CD28⁻ T cells from young HaploSCT recipients have intact proliferative capacity. Frozen PBMCs of patients and donors (*n* = 19 pairs) were thawed and labelled with CFSE followed by stimulation with anti-CD3/CD28 Abs for 5 days. The cells were stained with Abs against CD3, CD4, CD8 and CD28 followed by flow cytometric analysis. **(a)** Representative histogram plot of CFSE dilution assay in patients and donors. Numbers are the percentage of cells that underwent cell division. **(b, c)** Comparison of the proliferation capacity of the indicated T-cell populations between patients and donors. Box plots displaying medians, 25th and 75th percentiles as boxes, and minimum and maximum values as whiskers. Statistical analysis was performed by a two-tailed paired non-parametric *t*-test **(b, c)**.

level is increased in CD28⁻CD57⁺ T cells.[44] We found that the patients had a higher level of γ-H2AX-expressing CD28⁻ senescent T cells compared with the donors (Figure 6). Lanna *et al.* recently reported that spontaneous activation of p38 in senescent CD4⁺ T cells can be driven by DNA damage signalling.[45,46] Therefore, we examined whether accumulated CD4⁺CD28⁻ T cells in patients exhibited phosphorylation of p38. The CD28⁻ T cells, but not the CD28⁺ T cells, showed a tendency to have increased levels of phosphorylated p38 compared with donors (Supplementary figure 3). Our data suggest that in patients CD28⁻ T cells exhibit signs of DNA damage, although their telomere length does not change.

Statistical analysis of the factors influencing senescent features of CD4⁺ and CD8⁺ T cells in patients

We sought to investigate which age-related parameters of donors associate with the senescent features of patient T cells. Thus, regression analysis of the measured parameters was performed in patients and donors. The features of immune ageing of CD4⁺ T cells in patients were primarily affected by the age of donors, whereas the CD8⁺ T cells of patients were more influenced by the frequency of CD8⁺CD28⁻ T cells in donors than their age (Figure 7a–f). Since the age difference between donors and recipients was relatively constant (31.33 ± 4.22 years) (Supplementary figure 4a), the age-related features associated with the age of donors may also be attributed to the age of patients. To test this, correlation analysis was conducted between the measured parameters of patients and their age (Figure 7g–i and Supplementary figure 4b). Overall, the measured parameters, which are related to immunosenescent changes, showed more robust correlation with patient age than with donor age, implicating that the young immune environment of patients may in part influence the senescent features of their T cells. Additionally, CMV reactivation status stratified by CMV antigenemia levels had no effect on the data presented in this study (Supplementary figure 5). These findings suggest that immunosenescent features of CD4⁺ and CD8⁺ T cells in young
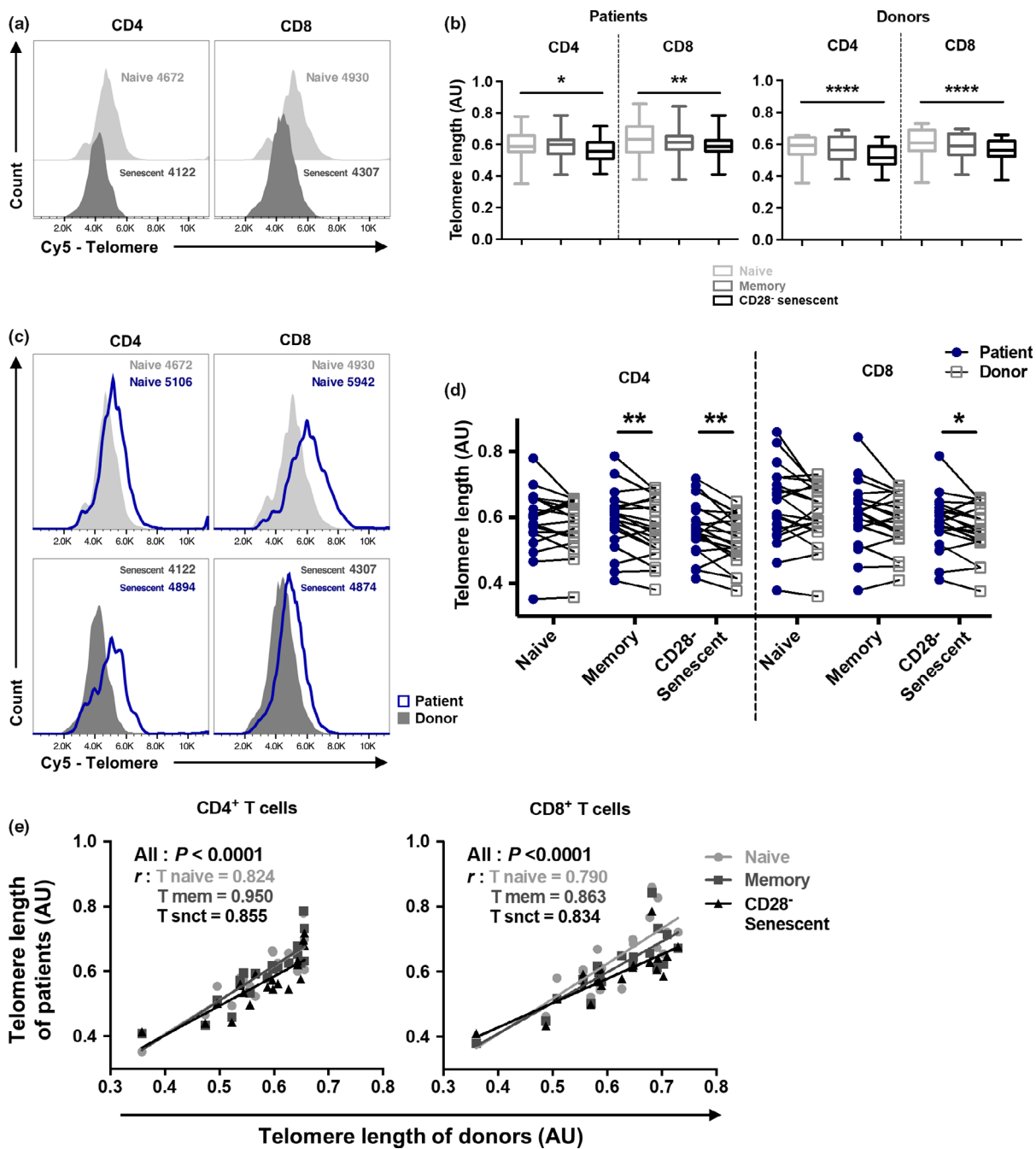
Figure 5. Telomere length is preserved in T cells of young HaploSCT recipients. Telomere length in different T-cell subsets was evaluated using telomere sequence-specific Flow-FISH combined with cell surface staining (*n* = 20 pairs). T-cell subsets are defined by the expression of CD45RA and CD28 (naive, CD45RA$^+$CD28$^+$; memory, CD45RA$^-$CD28$^+$; senescent, CD28$^-$) **(a)** Representative histogram plot of telomere length distribution for naive and senescent donor CD4$^+$ and CD8$^+$ T cells. Numbers are mean fluorescent intensity (MFI) of telomere-specific signal. **(b)** Telomere length (arbitrary unit: AU) of each functional subset in CD4$^+$ and CD8$^+$ T cells of patients and parental donors. **(c)** Representative histogram plot of telomere length distribution in naive and senescent CD28$^-$ CD4$^+$ and CD8$^+$ T cells from patients and donors. **(d)** Comparison of telomere length between patients and donors. Each data point represents an individual subject. **(e)** Telomere length of each functional CD4$^+$ or CD8$^+$ T-cell subset in donors positively correlates with that of counterpart subsets in recipients. Each data point represents an individual subject. Box plots displaying medians, 25th and 75th percentiles as boxes, and minimum and maximum values as whiskers. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$, ****$P < 0.0001$ by a two-tailed paired non-parametric *t*-test **(b–d)**. The *P*-value in **e** was obtained using the Spearman correlation analysis.
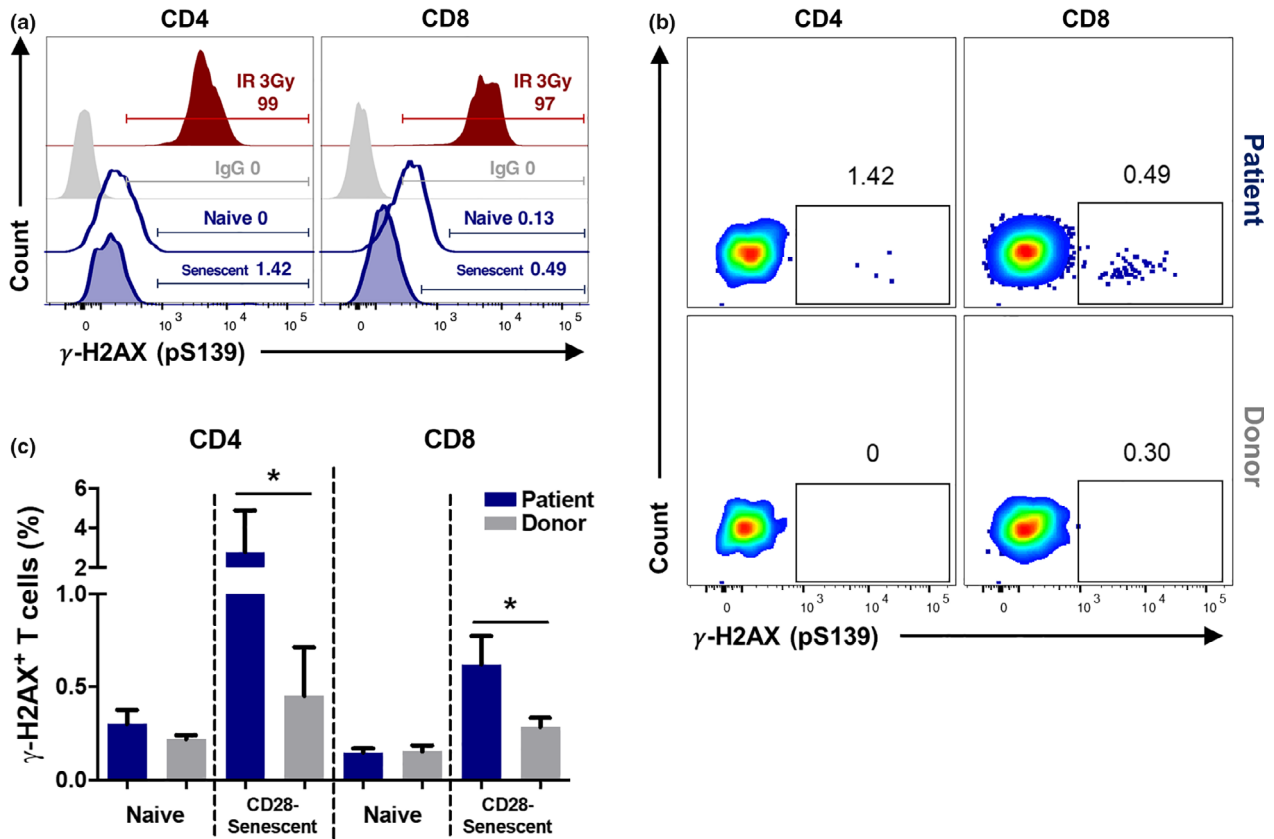
Figure 6. CD28⁻ T cells of young HaploSCT recipients have an increased frequency of γ-H2AX-expressing cells. γ-H2AX in different T-cell subsets was measured using phosphorylation-specific flow cytometric analysis (phosphoflow) with cell surface staining ($n = 11$ pairs). **(a)** Representative histogram plot of γ-H2AX distribution in naive and senescent CD28⁻ CD4⁺ and CD8⁺ T cells from patients. Numbers are the frequency of γ-H2AX-expressing cells. **(b)** Representative FACS plot of γ-H2AX⁺ senescent T cells of patients and donors. **(c)** Frequency of γ-H2AX⁺ T cells in senescent CD4⁺ or CD8⁺ T cells of patients and donors. Bar graphs show the mean ± SEM. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ by a two-tailed paired non-parametric *t*-test **(c)**.

HaploSCT recipients are differentially influenced by various donor and patient factors.

DISCUSSION

HaploSCT is a curative treatment for paediatric and adolescent patients with high-risk haematologic malignancies or primary immunodeficiency diseases when HLA-matched sibling or unrelated donors are not available, or transplantation is urgently needed. Although accumulating evidence suggests that HSCs derived from older donors can result in ageing-related immune dysfunction in the recipient,[47-51] few studies have described the senescent features of these reconstituted immune cells. To our knowledge, this is the first paired comparison study of the immunosenescent characteristics of parent and child immune cells after HaploSCT.

Here, we found that patients had a marked increase in senescent CD28⁻ and CD57⁺ T cells and their T cells showed a reduced ability to produce cytokines and impaired polyfunctionality upon PMA or TCR stimulation compared with parental donors. However, the TCR-mediated proliferation capacity of patient T cells was intact and TL was preserved or longer in senescent CD28⁻ T cells compared with parental donors. Moreover, phosphorylation of H2AX (called γ-H2AX), a sensitive biomarker for DNA damage, was higher in senescent T cells of patients than in those of parental donors. Regression analysis revealed that senescent features of CD4⁺ and CD8⁺ T cells in patients are influenced by the age and CD28⁻ T-cell frequency of parental donors, respectively.

Immunosenescence, defined as age-related dysfunction of the immune system, contributes to deterioration of immune responses and
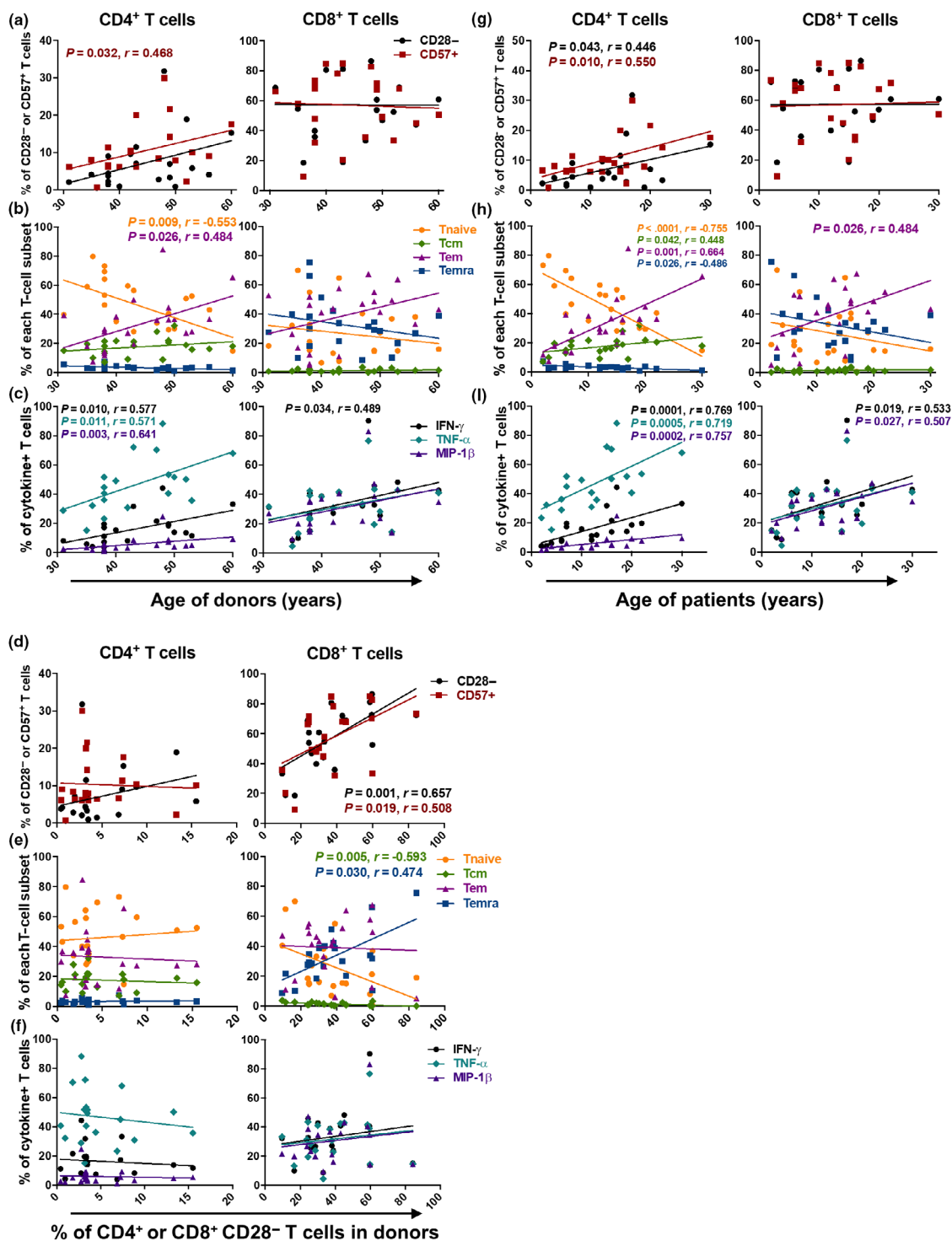
Figure 7. Analysis of the factors influencing senescent features of CD4^{+} and CD8^{+} T cells in patients. A regression analysis of the measured parameters between patients and donors was performed. Frequency of senescent cells, functional T-cell subsets (*n* = 21 pairs) and cytokine-producing cells (*n* = 19) was used. **(a–c)** Correlation between age of donors and frequency of CD28^{−} or CD57^{+} cells **(a)**, functional T-cell subsets **(b)** and cytokine-producing cells in response to PMA/ionomycin **(c)** of patients. **(d–f)** Correlation between frequency of CD28^{−} T cells in donors and frequency of CD28^{−} or CD57^{+} cells **(d)**, functional T-cell subsets **(e)** and cytokine-producing cells **(f)** of patients. **(g–i)** Correlation between patient age and frequency of CD28^{−} or CD57^{+} cells **(g)**, functional T-cell subsets **(h)** and cytokine-producing cells **(i)** of patients. *P*-values were obtained using the Spearman correlation analysis.

fundamentally impacts health and survival in the elderly.[17] Loss of CD28 is considered a hallmark of senescent T cells, and CD28$^-$ T cells gradually expand in the periphery with advancing age.[17,27-29] Our data clearly show that the frequency of senescent CD28$^-$ or CD57$^+$ T cells is higher in patients than in parental donors (Figure 1d, e). Although, because of ethical issues, only a limited number of studies have examined CD28$^-$ T cells in healthy children, both CD4$^+$CD28$^-$ (7.0 ± 1.63%) and CD8$^+$CD28$^-$ T cells (57.2 ± 4.37%) appear to be markedly increased in patients in our cohort compared with previous studies where approximately 2% of CD4$^+$ T cells were found to be CD28$^-$ and 15% of CD8$^+$ T cells were CD28$^-$ in healthy children with a median age of 9 years.[52,53] Of note, the frequency of CD28$^-$ T cells in our patients is comparable to that of approximately 60-year-old healthy individuals,[23,28] suggesting premature ageing in our patient group. A growing body of evidence has revealed that CD4$^+$CD28$^-$ T cells are significantly expanded in an age-inappropriate manner in patients with many chronic inflammatory diseases, such as autoimmune disorders and atherosclerosis, and play a role in the pathogenesis of these diseases.[18,27] Moreover, recent studies have demonstrated that CD8$^+$CD57$^+$ T cells have an adverse effect on many cardiovascular events including arterial stiffness, acute myocardial infarction and diabetes.[31,54,55] Considering that HSCT might favor the emergence of established risk factors, such as hypertension, diabetes, dyslipidemia[56] and expanded CD28$^-$ and CD57$^+$ T cells, long-term monitoring of these cells in patients is imperative.

In addition to their distinct phenotype, the impaired functions of senescent T cells, including hypoproliferative capacity and aberrant cytokine production, are well characterised.[29,39] Our data demonstrate that production of major cytokines, including IFN-γ and TNF-α, is reduced in patient CD4$^+$ T cells (Figure 2), whereas the overall proliferative capacity of these cells upon TCR stimulation is well maintained (Figure 4). Proliferation and effector function, especially cytokine production, are not rigidly linked in T cells; TCR-mediated proliferation requires a higher multiplicity of TCR-CD3 immunoreceptor tyrosine-based activation motifs than canonical TCR-induced signalling events that lead to cytokine secretion.[57,58] It has been suggested that age-related perturbations of the TCR signalling

pathway are closely linked to impaired functions in senescent T cells.[59,60] Thus, the functional impairment seen in patient T cells in this study may not be severe enough to influence proliferation in response to TCR stimulation. Of note, not only senescent CD28$^-$ CD4$^+$ T cells, but also non-senescent CD28$^+$ CD4$^+$ T cells showed qualitative (frequency) and quantitative (polyfunctionality) impairments of cytokine production (Figures 2 and 3). This may indicate that the reconstitution process imposes similar replicative stress on CD4$^+$ T cells regardless of their CD28 expression.

Unexpectedly, we observed a lack of obvious telomere attrition in patient T cells in this study (Figure 5c, d). Since successful immune cell reconstitution occurs through extensive proliferation,[6-9] we hypothesised that there would be shortened TL in T cells of patients compared with donors because of severe replicative stress during reconstitution. In fact, early studies reported telomere shortening in adult recipients of bone marrow transplantation (BMT) and its negative effect on clinical outcomes.[10,61] However, more recent studies suggested that telomere dynamics might be influenced by graft types (autologous vs. allogeneic), sources of cells (BMT vs. peripheral blood SCT (PBSCT)), and donor age.[8,41] Unlike most previous studies where telomere dynamics after HSCT were evaluated in BMT recipients,[10,61] Fibbe *et al*. reported that at 1 year after PBSCT, the TL in patient T cells was comparable to that of donors and suggested that this could be due to the presence of a higher number of HSCs in PBSCs.[62] This discrepancy may be explained by differences in the number of infused stem cells. In our study of PBSCT using a similar number of CD34$^+$ cells, the TL was relatively well preserved compared to that seen with BMT or PBSCT, which use a smaller number of infused HSCs.[7,62]

Because the T-cell-replete HaploSCT regimen contains mature T cells as well as HSCs, it is a growing interest on what extent infused T cells are involved in immune reconstitution. Recent studies on T-cell dynamics following HaploSCT illustrated that donor-derived naive T cells mainly differentiate into T memory stem cells (T$_{SCM}$) that play an important role for early T-cell reconstitution in patients. In the meantime, proliferating effector/memory T cells are preferentially depleted by PTCy administration.[63,64] Of importance, senescent CD28$^-$ T cells proliferate robustly to IL-15, which is a markedly increased homeostatic cytokine under

post-transplant lymphopenic milieu.[65,66] Therefore, it is likely that infused CD28⁻ T cells are efficiently depleted by PTCy administration. On the basis of the published data on T-cell dynamics and our data on TL of patients (Figure 5),[7,62] we presume that a majority of reconstituted CD28⁻ T cells in patients may be replenished from T_{SCM} or survived memory T cells that typically have a longer TL than donor-derived infused CD28⁻ T cells, whereas naive T-cell pool in patients is primarily reconstituted by thymus-dependent *de novo* generation from transplanted HSCs. In agreement with our data (Figure 5c, d), Sousa *et al.* showed that TL of reconstituted T cells in HaploSCT patients was preserved or slightly longer than relative parental donors although TL of patients was shortened compared with age-matched healthy donors.[7] Previous and our study suggest that telomere shortening, if any, in the T cells of HaploSCT recipients does not exceed age-dependent telomere shortening normally occurring in T cells of the parental donor.

Regression analysis revealed that donor age is the main factor associated with ageing of CD4⁺ T cells, whereas the frequency of CD28⁻ cells in CD8⁺ T-cell pool correlates with features of CD8⁺ T-cell ageing (Figure 7). The reconstitution dynamics of T cells differ between the CD4⁺ and CD8⁺ T-cell compartments although the underlying mechanism is unclear.[8] Recent studies suggest that CD4⁺ T-cell reconstitution is delayed compared with CD8⁺ T-cell reconstitution. This is likely due to greater reliance of CD4 T cells on *de novo* generation in the thymus.[67,68] Given the strong correlation between donor age and ageing of patient CD4⁺ T cells in our study, it seems likely that HaploSCT with younger parental donors would provide more favorable conditions for CD4⁺ T-cell reconstitution. However, future studies will be needed to determine whether this is the case.

Endogenous DNA damage leads to an increase of γ-H2AX in senescent T cells and enhanced autophosphorylation of p38 in senescent CD28⁻CD4⁺ T cells.[44-46] In this study, we found a significant increase in the expression of γ-H2AX, which indicates the presence of DNA double-strand breaks, in senescent CD4⁺ and CD8⁺ T cells of patients compared with donors (Figure 6). This increased DNA damage is likely caused by extensive proliferation or a reduction in the DNA repair capacity of the reconstituted T cells. Accumulation of γ-H2AX⁺ cells and downregulation of genes involved in DNA damage repair have been reported

in HSCs with advancing age,[69,70] and these changes directly contribute to functional defects of HSCs. Given their nature as precursors of blood cells, replicative stress is considered an important factor for increased DNA damage in HSCs.71 Thus, it is presumably because of excessive proliferation during reconstitution that there is an increase of γ-H2AX⁺ T cells in the patients in this study.

Our study does have limitations associated with the experimental design, including a relatively small number of paired samples and lack of a control population of patients receiving transplants from younger donors. Thus, we cannot exclude the possibility that one of the immunosuppressant treatments causes the ageing of these cells. Thus, further studies using well-designed larger cohorts will be needed to address these issues.

In conclusion, in paediatric HaploSCT recipients, T cells undergo premature immunosenescent changes and exhibit functional defects. Further, there is an increased level of DNA damage in patient CD4⁺ T cells compared to those of parental donors. Therefore, long-term, comprehensive immune monitoring of these patients is necessary.

METHODS

Study population and design

Twenty-one patients who underwent HSCT at Seoul National University Children's Hospital between February 2014 and January 2017 and the corresponding parental donors were enrolled. Patients, who received HaploSCT from parental donors, were alive at least 1 year after transplantation, and were free of primary disease and chronic GVHD without the use of any systemic immunosuppressant, were included. For T-cell analysis, peripheral blood samples were collected from patients and donors on the same day. Median initial sampling time from HaploSCT was 514 days (range, 377–1180 days), and 11 patient and donor pairs underwent additional sampling because the sample was insufficient to conduct some experiments. At the time of sampling, no patient had active infection or persistent viremia. This study was approved by the IRB of Seoul National University Hospital (H-1702-058-831), and all patients and donors provided written informed consent or assent prior to the study.

Transplantation protocol and supportive care

The conditioning regimen was composed of targeted busulfan with intensive pharmacokinetic monitoring, fludarabine (40 mg m⁻² once daily via IV from days −8 to

−4), and cyclophosphamide (14.5 mg kg^{-1} once daily via IV from days −3 to −2). The initial busulfan dose on day −8 was 120 mg m^{-2} for patients aged ≥ 1 year and 80 mg m^{-2} for patients aged < 1 year, administered once daily. The subsequent targeted dose of busulfan from days −7 to −5 was calculated by using therapeutic drug monitoring, targeting the total target area under the curve at 74 000–76 000 μg × h L^{-1}. The same regimen was used for malignant and nonmalignant diseases. For GVHD prophylaxis, PTCy (50 mg kg^{-1} once daily intravenously via IV on days 3 and 4), tacrolimus (from day 5) and mycophenolate mofetil (from days 5 to 35) were administered. Tacrolimus was generally used until 8 months after HSCT for malignant diseases and until 1 year after for nonmalignant diseases. Prophylactic treatments for veno-occlusive disease (VOD) and infections were performed according to our institutional guidelines for HSCT.[72] More detailed information is provided in our previous publication.[2] Donor chimerism was regularly measured through the molecular analysis of short tandem repeat regions, and all patients achieved complete donor chimerism. Neutrophil engraftment was defined as the first of 3 consecutive days on which the absolute neutrophil count was > 0.5 × 10^9 L^{-1}, and platelet recovery was defined as the day on which the platelet count was > 20 × 10^9 L^{-1}, without platelet transfusions in the prior 7 days.

Cell preparation and flow cytometric analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from blood by density gradient centrifugation (Biocoll Separating Solution; BIOCHROM, Cambridge, UK) and stained with at 4°C for 25 min with the antibodies (Abs) to CD4, CD8, CD28, CCR7 (four from BD Biosciences, Franklin Lakes, NJ), CD3, CD45RA (both from BioLegend, San Diego, CA), CD57 and CD85j (both from eBiosciences, San Diego, CA). All the information of Abs used in this study for flow cytometry analysis was listed in Supplementary table 1. Stained cells were acquired by Fortessa-X20 or LSRFortessa (BD Biosciences) and analysed using FlowJo software (Tree Star, Ashland, OR).

CFSE dilution assay and intracellular cytokine staining

Frozen PBMCs were thawed and immediately incubated in RPMI 1640 medium supplemented with 10% FBS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 2 mM L-glutamine (henceforth, complete RPMI 1640) for 12 h at 37°C and 5% CO$_2$. To analyse proliferative capacity of T cells, cells were labelled with carboxyfluorescein succinimidyl ester (CFSE; Invitrogen, Carlsbad, CA) and stimulated for 5 days on the 96-well flat-bottom plate coated with 1 μg mL^{-1} of anti-CD3 and anti-CD28 Abs (OKT3 and CD28.2, respectively; eBiosciences). For intracellular cytokine staining (ICS), cultured cells were re-stimulated for 4 h with PMA and ionomycin (both from Sigma-Aldrich, St. Louis, MO) or plate-bound anti-CD3 Ab in the presence of Brefeldin A (Cell Signaling Technology, Danvers, MA) and anti-CD107a Ab. Stimulated cells were stained with Abs to CD3, CD4, CD8 and CD28, followed by fixation and permeabilisation (BD Cytofix/Cytoperm Solution Kit; BD Biosciences). Fixed cells were stained with Abs to IFN-γ, TNF-α and MIP-1β. Stained cells were acquired by Fortessa-X20 or LSRFortessa and analysed using FlowJo software and SPICE program (ver. 5.0, NIH, Bethesda, MD).

Measurement of telomere length

Telomere length was analysed in PBMCs by flow cytometry-based fluorescence *in situ* hybridisation (Flow-FISH) combined with cell surface staining as previously described.[73,74] In brief, thawed PBMCs (5 × 10^6 cells 100 μL^{-1}) were stained at 4°C for 25 min with Abs to CD3, CD28, CD4, CD8 and CD45RA. After washing with staining buffer, cells were stained with Cy3-conjugated streptavidin (BioLegend). Samples were washed and re-suspended in 200 μL of PBS (-), followed by fixation with 200 μL BS^3 solution (final 4 mM, Thermo Scientific) for 30 min on ice. After incubation, 8 μL of 1 M Tris–HCl (pH 8.0, final 20 mM) was added into samples for quenching. Cells were washed twice and mixed with 1.2 × 10^6 mouse splenocyte as an internal control, followed by re-suspension with 300 μL of hybridisation buffer supplemented with 70% deionised formamide (Invitrogen), 10 mM Tris, and 1% BSA with or without 20 nM of the telomere-specific PNA TelC-Cy5 probe (Panagene, Daejeon, Korea). After heat denaturation of DNA for 10 min at 80°C using a Thermomixer (Eppendorf, Hamburg, Germany), samples were rapidly chilled on ice for 2 min and left to hybridise for 2 h at room temperature in the dark. Calibration beads (Rainbow particle; BioLegend) were acquired alongside samples at each experiment to correct daily fluctuations in the intensity and alignment of the laser.

Phosphoflow of p38 and γ-H2AX

Freshly isolated PBMCs were incubated in complete RPMI 1640 medium for 2 h at 37°C with 5% CO$_2$. Cells were stained at 4°C for 25 min with Abs to CD4, CD8, CD28, and CD57. Samples were fixed with 2% paraformaldehyde at 37°C for 10 min, followed by permeabilisation with 1 mL ice-cold 70% MtOH on ice for 30 min. After washing steps, cells were stained with Abs to CD3, CD45RA, phosphor-p38 or γ-H2AX at 4°C for 30 min in the dark. The stained cells were washed in staining buffer and immediately acquired by Fortessa-X20.

Statistical analysis

Two-tailed Wilcoxon matched-pairs signed rank tests, Mann–Whitney *U*-tests or Spearman correlation analysis were utilised to analyse data using Prism 7 software (GraphPad Software Inc.). *P*-values of <0.05 were considered statistically significant.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

GHL and KTH participated in the design of the study; performed all the experiments, data collection and analysis; and drafted the manuscript. JYC participated in the design of the study, data collection and analysis. HYS conceived the study and participated in its design and coordination. WWL and HJK participated in its design and coordination; performed data analysis and writing of the manuscript; and has full access to all the data in this study and provided financial support. All authors have read and approved the final manuscript.

REFERENCES

1. Passweg JR, Baldomero H, Bader P *et al*. Is the use of unrelated donor transplantation leveling off in Europe? The 2016 European Society for Blood and Marrow Transplant activity survey report. *Bone Marrow Transplant* 2018; **53**: 1139–1148.

2. Hong KT, Kang HJ, Choi JY *et al*. Favorable outcome of post-transplantation cyclophosphamide haploidentical peripheral blood stem cell transplantation with targeted busulfan-based myeloablative conditioning using intensive pharmacokinetic monitoring in pediatric patients. *Biol Blood Marrow Transplant* 2018; **24**: 2239–2244.

3. Sugita J, Kagaya Y, Miyamoto T *et al*. Myeloablative and reduced-intensity conditioning in HLA-haploidentical peripheral blood stem cell transplantation using post cyclophosphamide. *Bone Marrow Transplant* 2019; **54**: 432–441.

4. Mariotti J, Devillier R, Bramanti S *et al*. T cell-replete haploidentical transplantation with post-transplantation cyclophosphamide for Hodgkin lymphoma relapsed after autologous transplantation: reduced incidence of relapse and of chronic graft-versus-host disease compared with HLA-identical related donors. *Biol Blood Marrow Transplant* 2018; **24**: 627–632.

5. Martinez C, Gayoso J, Canals C *et al*. Post-transplantation cyclophosphamide-based haploidentical transplantation as alternative to matched sibling or unrelated donor transplantation for Hodgkin lymphoma: a registry study of the lymphoma working party of the European Society for Blood and Marrow Transplantation. *J Clin Oncol* 2017; **35**: 3425–3432.

6. de Koning C, Plantinga M, Besseling P, Boelens JJ, Nierkens S. Immune reconstitution after allogeneic hematopoietic cell transplantation in children. *Biol Blood Marrow Transplant* 2016; **22**: 195–206.

7. Azevedo RI, Soares MV, Albuquerque AS *et al*. Long-term immune reconstitution of naive and memory t cell pools after haploidentical hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2013; **19**: 703–712.

8. Chang YJ, Zhao XY, Huang XJ. Immune reconstitution after haploidentical hematopoietic stem cell transplantation. *Biol Blood Marrow Transplant* 2014; **20**: 440–449.

9. Chang YJ, Zhao XY, Huo MR *et al*. Clinical impact of absolute lymphocyte count on day 30 after unmanipulated haploidentical blood and marrow transplantation for pediatric patients with hematological malignancies. *Am J Hematol* 2011; **86**: 227–230.

10. Wynn RF, Cross MA, Hatton C *et al*. Accelerated telomere shortening in young recipients of allogeneic bone-marrow transplants. *Lancet* 1998; **351**: 178–181.

11. Gadalla SM, Wang T, Haagenson M *et al*. Association between donor leukocyte telomere length and survival after unrelated allogeneic hematopoietic cell transplantation for severe aplastic anemia. *JAMA* 2015; **313**: 594–602.

12. Helby J, Petersen SL, Kornblit B *et al*. Mononuclear cell telomere attrition is associated with overall survival after non-myeloablative allogeneic hematopoietic cell transplantation for hematologic malignances. *Biol Blood Marrow Transplant* 2019; **25**: 496–504.

13. d'Adda di Fagagna F. Living on a break: cellular senescence as a DNA-damage response. *Nat Rev Cancer* 2008; **8**: 512–522.

14. Ben-Porath I, Weinberg RA. The signals and pathways activating cellular senescence. *Int J Biochem Cell Biol* 2005; **37**: 961–976.

15. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. *Cell* 2013; **153**: 1194–1217.

16. Goronzy JJ, Weyand CM. Successful and maladaptive T cell aging. *Immunity* 2017; **46**: 364–378.

17. Yu HT, Park S, Shin EC, Lee WW. T cell senescence and cardiovascular diseases. *Clin Exp Med* 2016; **16**: 257–263.

18. Broux B, Markovic-Plese S, Stinissen P, Hellings N. Pathogenic features of CD4$^+$CD28$^-$ T cells in immune disorders. *Trends Mol Med* 2012; **18**: 446–453.

19. Goronzy JJ, Weyand CM. Immune aging and autoimmunity. *Cell Mol Life Sci* 2012; **69**: 1615–1623.

20. Muller M, Wandel S, Colebunders R *et al*. Immune reconstitution inflammatory syndrome in patients starting antiretroviral therapy for HIV infection: a systematic review and meta-analysis. *Lancet Infect Dis* 2010; **10**: 251–261.

21. King C, Ilic A, Koelsch K, Sarvetnick N. Homeostatic expansion of T cells during immune insufficiency generates autoimmunity. *Cell* 2004; **117**: 265–277.

22. Ju HY, Kang HJ, Hong CR *et al*. Half-dose ganciclovir preemptive treatment of cytomegalovirus infection after pediatric allogeneic hematopoietic stem cell transplantation. *Transpl Infect Dis.* 2016; **18**: 396–404.

23. Czesnikiewicz-Guzik M, Lee WW, Cui D *et al*. T cell subset-specific susceptibility to aging. *Clin Immunol* 2008; **127**: 107–118.

24. Akbar AN, Fletcher JM. Memory T cell homeostasis and senescence during aging. *Curr Opin Immunol* 2005; **17**: 480–485.

25. Naylor K, Li G, Vallejo AN *et al*. The influence of age on T cell generation and TCR diversity. *J Immunol* 2005; **174**: 7446–7452.

26. Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature* 1999; **401**: 708–712.

27. Lee GH, Lee WW. Unusual CD4+CD28- T cells and their pathogenic role in chronic inflammatory disorders. *Immune Netw* 2016; **16**: 322–329.

28. Fagnoni FF, Vescovini R, Mazzola M *et al*. Expansion of cytotoxic CD8+CD28- T cells in healthy ageing people, including centenarians. *Immunology* 1996; **88**: 501–507.

29. Weng NP, Akbar AN, Goronzy J. CD28- T cells: their role in the age-associated decline of immune function. *Trends Immunol* 2009; **30**: 306–312.

30. Gustafson CE, Qi Q, Hutter-Saunders J *et al*. Immune checkpoint function of CD85j in CD8 T cell differentiation and aging. *Front Immunol* 2017; **8**: 692.

31. Yu HT, Youn JC, Kim JH *et al*. Arterial stiffness is associated with cytomegalovirus-specific senescent CD8+ T cells. *J Am Heart Assoc* 2017; **6**: e006535.

32. Pita-Lopez ML, Gayoso I, DelaRosa O *et al*. Effect of ageing on CMV-specific CD8 T cells from CMV seropositive healthy donors. *Immun Ageing* 2009; **6**: 11.

33. Onyema OO, Njemini R, Forti LN *et al*. Aging-associated subpopulations of human CD8+ T-lymphocytes identified by their CD28 and CD57 phenotypes. *Arch Gerontol Geriatr* 2015; **61**: 494–502.

34. Brenchley JM, Karandikar NJ, Betts MR *et al*. Expression of CD57 defines replicative senescence and antigen-induced apoptotic death of CD8+ T cells. *Blood* 2003; **101**: 2711–2720.

35. Focosi D, Bestagno M, Burrone O, Petrini M. CD57+ T lymphocytes and functional immune deficiency. *J Leukoc Biol* 2010; **87**: 107–116.

36. Strioga M, Pasukoniene V, Characiejus D. CD8+CD28- and CD8+CD57+ T cells and their role in health and disease. *Immunology* 2011; **134**: 17–32.

37. Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* 2008; **8**: 247–258.

38. Kannanganat S, Ibegbu C, Chennareddi L, Robinson HL, Amara RR. Multiple-cytokine-producing antiviral CD4 T cells are functionally superior to single-cytokine-producing cells. *J Virol* 2007; **81**: 8468–8476.

39. Azuma M, Phillips JH, Lanier LL. CD28- T lymphocytes. Antigenic and functional properties. *J Immunol* 1993; **150**: 1147–1159.

40. Chiu WK, Fann M, Weng NP. Generation and growth of CD28null CD8+ memory T cells mediated by IL-15 and its induced cytokines. *J Immunol* 2006; **177**: 7802–7810.

41. Gadalla SM, Savage SA. Telomere biology in hematopoiesis and stem cell transplantation. *Blood Rev* 2011; **25**: 261–269.

42. Batliwalla FM, Rufer N, Lansdorp PM, Gregersen PK. Oligoclonal expansions in the CD8+CD28- T cells largely explain the shorter telomeres detected in this subset: analysis by flow FISH. *Hum Immunol* 2000; **61**: 951–958.

43. Rufer N, Zippelius A, Batard P *et al*. Ex vivo characterization of human CD8+ T subsets with distinct replicative history and partial effector functions. *Blood* 2003; **102**: 1779–1787.

44. Mondal AM, Horikawa I, Pine SR *et al*. p53 isoforms regulate aging- and tumor-associated replicative senescence in T lymphocytes. *J Clin Invest* 2013; **123**: 5247–5257.

45. Lanna A, Henson SM, Escors D, Akbar AN. The kinase p38 activated by the metabolic regulator AMPK and scaffold TAB1 drives the senescence of human T cells. *Nat Immunol* 2014; **15**: 965–972.

46. Lanna A, Gomes DC, Muller-Durovic B *et al*. A sestrin-dependent Erk-Jnk-p38 MAPK activation complex inhibits immunity during aging. *Nat Immunol* 2017; **18**: 354–363.

47. Sudo K, Ema H, Morita Y, Nakauchi H. Age-associated characteristics of murine hematopoietic stem cells. *J Exp Med* 2000; **192**: 1273–1280.

48. Kollman C, Howe CW, Anasetti C *et al*. Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age. *Blood* 2001; **98**: 2043–2051.

49. Liang Y, Van Zant G, Szilvassy SJ. Effects of aging on the homing and engraftment of murine hemato-poietic stem and progenitor cells. *Blood* 2005; **106**: 1479–1487.

50. Dykstra B, Olthof S, Schreuder J, Ritsema M, de Haan G. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med* 2011; **208**: 2691–2703.

51. Geiger H, de Haan G, Florian MC. The ageing haematopoietic stem cell compartment. *Nat Rev Immunol* 2013; **13**: 376–389.

52. George RP, Mehta AK, Perez SD *et al*. Premature T cell senescence in pediatric CKD. *J Am Soc Nephrol* 2017; **28**: 359–367.

53. Knipp S, Feyen O, Ndagijimana J, Niehues T. *Ex vivo* apoptosis, CD95 and CD28 expression in T cells of children with juvenile idiopathic arthritis. *Rheumatol Int* 2003; **23**: 112–115.

54. Tae YuH, Youn JC, Lee J *et al*. Characterization of CD8+CD57+ T cells in patients with acute myocardial infarction. *Cell Mol Immunol* 2015; **12**: 466–473.

55. Lee YH, Kim SR, Han DH *et al*. Senescent T cells predict the development of hyperglycemia in humans. *Diabetes* 2019; **68**: 156–162.

56. Tichelli A, Bucher C, Rovo A *et al*. Premature cardiovascular disease after allogeneic hematopoietic stem-cell transplantation. *Blood* 2007; **110**: 3463–3471.

57. Laouar Y, Crispe IN. Functional flexibility in T cells: independent regulation of CD4+ T cell proliferation and effector function *in vivo*. *Immunity* 2000; **13**: 291–301.

58. Guy CS, Vignali KM, Temirov J *et al*. Distinct TCR signaling pathways drive proliferation and cytokine production in T cells. *Nat Immunol* 2013; **14**: 262–270.

59. Goronzy JJ, Li G, Yu M, Weyand CM. Signaling pathways in aged T cells – a reflection of T cell differentiation, cell senescence and host environment. *Semin Immunol* 2012; **24**: 365–372.

60. Fulop T Jr, Larbi A, Dupuis G, Pawelec G. Ageing, autoimmunity and arthritis: Perturbations of TCR signal transduction pathways with ageing – a biochemical paradigm for the ageing immune system. *Arthritis Res Ther* 2003; **5**: 290–302.

61. Akiyama M, Asai O, Kuraishi Y *et al*. Shortening of telomeres in recipients of both autologous and allogeneic hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2000; **25**: 441–447.

62. Roelofs H, de Pauw ES, Zwinderman AH *et al*. Homeostasis of telomere length rather than telomere shortening after allogeneic peripheral blood stem cell transplantation. *Blood* 2003; **101**: 358–362.

63. Roberto A, Castagna L, Zanon V *et al*. Role of naive-derived T memory stem cells in T-cell reconstitution following allogeneic transplantation. *Blood* 2015; **125**: 2855–2864.

64. Cieri N, Oliveira G, Greco R *et al*. Generation of human memory stem T cells after haploidentical T-replete hematopoietic stem cell transplantation. *Blood* 2015; **125**: 2865–2874.

65. Russo A, Oliveira G, Berglund S *et al*. NK cell recovery after haploidentical HSCT with posttransplant cyclophosphamide: dynamics and clinical implications. *Blood* 2018; **131**: 247–262.

66. Alonso-Arias R, Moro-Garcia MA, Vidal-Castineira JR *et al*. IL-15 preferentially enhances functional properties and antigen-specific responses of $CD4^+CD28^{null}$ compared to $CD4^+CD28^+$ T cells. *Aging Cell* 2011; **10**: 844–852.

67. Adkins B, Hamilton K. Developmental ages of the thymic epithelium and of the T cell precursors together determine the proportions of peripheral $CD4^+$ cells. *J Immunol* 1994; **153**: 5359–5365.

68. Al-Shami A, Spolski R, Kelly J *et al*. A role for thymic stromal lymphopoietin in $CD4^+$ T cell development. *J Exp Med* 2004; **200**: 159–168.

69. Rossi DJ, Bryder D, Seita J, Nussenzweig A, Hoeijmakers J, Weissman IL. Deficiencies in DNA damage repair limit the function of haematopoietic stem cells with age. *Nature* 2007; **447**: 725–729.

70. Rube CE, Fricke A, Widmann TA *et al*. Accumulation of DNA damage in hematopoietic stem and progenitor cells during human aging. *PLoS One* 2011; **6**: e17487.

71. Flach J, Bakker ST, Mohrin M *et al*. Replication stress is a potent driver of functional decline in ageing haematopoietic stem cells. *Nature* 2014; **512**: 198–202.

72. Lee JW, Kang HJ, Kim S *et al*. Favorable outcome of hematopoietic stem cell transplantation using a targeted once-daily intravenous busulfan-fludarabine-etoposide regimen in pediatric and infant acute lymphoblastic leukemia patients. *Biol Blood Marrow Transplant* 2015; **21**: 190–195.

73. Riddell NE, Griffiths SJ, Rivino L *et al*. Multifunctional cytomegalovirus (CMV)-specific $CD8^+$ T cells are not restricted by telomere-related senescence in young or old adults. *Immunology* 2015; **144**: 549–560.

74. Lee WW, Nam KH, Terao K, Yoshikawa Y. Age-related telomere length dynamics in peripheral blood mononuclear cells of healthy cynomolgus monkeys measured by Flow FISH. *Immunology* 2002; **105**: 458–465.

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Supplementary figures 1-5

Supplementary table 1