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Research Paper

Chemotherapy and Stem Cell Transplantation Increase $p16^{INK4a}$ Expression, a Biomarker of T-cell Aging

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ABSTRACT

The expression of markers of cellular senescence increases exponentially in multiple tissues with aging. Age-related physiological changes may contribute to adverse outcomes in cancer survivors. To investigate the impact of high dose chemotherapy and stem cell transplantation on senescence markers in vivo, we collected blood and clinical data from a cohort of 63 patients undergoing hematopoietic cell transplantation. The expression of $p16^{INK4a}$, a well-established senescence marker, was determined in T-cells before and 6 months after transplant. RNA sequencing was performed on paired samples from 8 patients pre- and post-cancer therapy. In patients undergoing allogeneic transplant, higher pre-transplant $p16^{INK4a}$ expression was associated with a greater number of prior cycles of chemotherapy received ($p = 0.003$), prior autologous transplantation ($p = 0.01$) and prior exposure to alkylating agents ($p = 0.01$). Transplantation was associated with a marked increase in $p16^{INK4a}$ expression 6 months following transplantation. Patients receiving autologous transplant experienced a larger increase in $p16^{INK4a}$ expression (3.1-fold increase, $p = 0.002$) than allogeneic transplant recipients (1.9-fold increase, $p = 0.0004$). RNA sequencing of T-cells pre- and post- autologous transplant or cytotoxic chemotherapy demonstrated increased expression of transcripts associated with cellular senescence and physiological aging. Cytotoxic chemotherapy, especially alkylating agents, and stem cell transplantation strongly accelerate expression of a biomarker of molecular aging in T-cells.

1. Introduction

Hematopoietic stem cell transplantation (HSCT) is a potentially curative modality for high-risk hematologic diseases, but the procedure has profound and long-term effects on recipient hematologic and immune function. The long-term toxicity of HSCT may result from chemo-radiotherapy given at the time of transplantation (conditioning), from donor-host immune differences after allogeneic transplants or from accelerated stem cell exhaustion of transplanted stem cells (Hake et al., 2007). These late toxicities manifest as increased risk for infection, chronic graft-vs-host disease, bone marrow failure and acute leukemia.

Recent evidence has demonstrated that peripheral blood T-cells express markers of cellular senescence with physiological aging. The overall loss of physiological reserve that accompanies aging is associated with an accumulation of senescent cells (Sharpless and DePinho, 2007; Rodier and Campisi, 2011). Cellular senescence is triggered by activation of tumor suppressor mechanisms associated with cellular stressors, and results in expression of the $p16^{INK4a}$ tumor suppressor protein encoded by the *CDKN2a* locus, which has emerged as one of the more useful markers of senescence in vivo (Campisi, 2013; Sharpless and Sherr, 2015). Expression of $p16^{INK4a}$ in peripheral blood T lymphocytes increases exponentially with chronological age, doubling about every decade (Zindy et al., 1997; Krishnamurthy et al., 2004; Liu et al., 2009). Polymorphisms of senescence regulators have been associated with age-related conditions such as cancer, pulmonary fibrosis, glaucoma, atherosclerosis, and type II diabetes (Jeck et al., 2012; Siegel et al., 2012). Prior work has shown that several age-promoting stressors such as smoking, physical inactivity and chronic HIV infection accelerate the expression of $p16^{INK4a}$ and other markers of cellular senescence (Liu et al., 2009; Nelson et al., 2012). Importantly, we recently showed that cytotoxic chemotherapy, given in the adjuvant setting, markedly increases expression of senescence markers in the peripheral blood, consistent with ~15 years of chronological aging (Sanoff et al., 2014).

Increasingly, older individuals are considered for autologous or allogeneic transplantation. While age itself is usually not considered an absolute contraindication to transplantation, older individuals do have

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higher risks of acute transplant-related toxicities such as cardiac arrhythmias, diarrhea and mucositis (Wildes et al., 2014). Further, age-related comorbid illness is itself prognostic for outcomes in autologous and allogeneic transplant recipients, suggesting that functional, if not chronological, age of prospective transplant candidates is a potentially important variable for clinical decision-making. Lastly, survivors of transplants, regardless of age, are at risk for accelerated acquisition of several age-related syndromes such as endocrine dysfunction, cognitive impairment, cardiovascular morbidity, immune dysfunction, secondary neoplasms, and neuromuscular impairment (Fried et al., 2001).

In murine models, serial transplantation per se, in the absence of exposure to cytotoxic agents, is associated with accelerated aging of hematopoietic stem cells (HSC), manifesting as 'HSC exhaustion' (Harrison and Astle, 1982). Likewise, evidence suggests HSC exhaustion occurs in humans as well. HSC yields for autologous transplant from patients that have undergone significant prior chemotherapy are significantly depressed compared to yields from less heavily treated individuals (Clark and Brammer, 1998), and the transplantation of insufficient numbers of HSC is associated with long term graft failure (Perez-Simon et al., 1999). Additionally, transplantation is associated with an increased rate of telomere shortening, which has been associated with certain adverse outcomes in transplant recipients (Lee et al., 1999; Lewis et al., 2004; Akiyama et al., 2000; Pipes et al., 2006). Because individuals with hematologic malignancies have an increasing array of transplant approaches of varying intensity as well as non-transplant treatment approaches available to them, understanding the impact of treatment upon functional aging may have important implications for the care of both prospective transplant candidates as well as transplant survivors. Toward that end, we measured expression of $p16^{INK4a}$, a marker of molecular age that can be serially assessed, in HSC-derived T-cells before and after stem cell transplantation. Additionally, we performed whole transcriptomic RNA sequencing in a subset of paired samples to further examine the effects of chemotherapy or transplantation on T-cell function.

2. Materials and Methods

2.1. Patients

For the transplant patient population, participants were over the age of 18 and underwent either autologous or allogeneic stem cell transplantation for any hematologic malignancy between 2010 and 2013 at the University of North Carolina (UNC) Hospitals. Patient samples were obtained from two non-randomized, non-blinded observational cohorts: a study investigating symptom burden after transplantation, and a generic tissue procurement protocol. Studies were approved by the UNC Institutional Review Board (11–0600 and 13–1705), with study procedures confirming to standards indicated by the Declaration of Helsinki. Eligible patients were identified from the electronic medical records and approached by research personnel prior to scheduled transplantation for provision of signed informed consent. Patients undergoing concurrent radiation, chemotherapeutic, or investigational therapy other than transplant-related therapy were excluded. All patients received standard-of-care therapies and treatments as clinically needed. Medical history and treatment information were abstracted from the medical record. Samples were obtained in both cohorts from just before transplantation, and paired samples from 6 months post-transplantation were also obtained if available. Molecular analyses were performed by investigators blinded to patient data, and investigators collecting clinical information were blinded to laboratory results until data collection was complete. For the breast cancer patient population, T-cell RNA collected in the study Sanoff et al. (Sanoff et al., 2014) was used in the RNA sequencing analysis.

2.2. Assessment of $p16^{INK4a}$ expression

See Sanoff et al. (Sanoff et al., 2014) for details. In brief, $CD3^+$ T-cells were isolated from up to 10-ml of peripheral blood using anti-CD3 microbeads and an AutoMACS^{PRO} separator (Miltenyi Biotec, San Diego, CA). Purity of T cells was determined to be ~95% when isolated from fresh blood and ~50% when isolated from cryopreserved PBMCs in pilot experiments. T cell purity in clinical trial samples was monitored by measuring expression of the gamma subunit of the *CD3*. Total RNA was isolated using RNeasy Mini Kit (Qiagen) and cDNA were prepared using ImProm-II reverse transcriptase kit (Promega). Expression of $p16^{INK4a}$ was measured by TaqMan quantitative reverse-transcription polymerase chain reaction specific for $p16^{INK4a}$ and normalized to *YWHAZ* housekeeping gene (Mane et al., 2008; Dheda et al., 2004).

2.3. RNA Sequencing

RNA was extracted and rRNA was removed using the Ribo-Zero kit. RNA libraries were prepared by using the Illumina TruSeq RNA Sample Preparation Kit v2 and then sequenced by Illumina HiSeq2000. Reads were subjected to quality control as previously described (Cancer Genome Atlas Research, 2012). RNA reads were aligned to human hg19 genome assembly using Mapsplice (Wang et al., 2010). Gene definitions were obtained from the UCSC known Gene table. Gene expression was estimated using RSEM (RNA-Seq by Expectation Maximization) (Li and Dewey, 2011). Genes differentially expressed due to treatment were identified by DESeq2 (Love et al., 2014) using a bivariate model to adjust for subject specific effects. The resulting statistics were subjected to gene set enrichment analysis by using the GSEA (Gene Set Enrichment Analysis) rank test (Subramanian et al., 2005). Expression estimates were normalized to a fixed upper quartile and log2 transformed prior to visualization.

2.4. Statistical Analyses

The sample size was determined by the availability of clinical specimens from the two study cohorts as described. Log_2-transformed $p16^{INK4a}$ expression values were standardized through conversion to *Z*-score to facilitate combining the two sample sets. Z-scores were calculated separately for the two transplant cohorts using the formula:

$$Z_i = (X_i - \mu)/\sigma$$

where μ is population mean, and σ is standard deviation.

For samples present in both cohorts, individual Z scores were averaged. Associations between $p16^{INK4a}$ expression and pre-transplant variables were performed using linear regression (for continuous variables) or one-way analysis of variance (for categorical variables). A paired *t*-test was used to compare $p16^{INK4a}$ expression before and after transplant. Data were analyzed by N. Mitin using JMP11 (SAS, Cary, NC) and A. Snavely using R. All tests of statistical significance were two-sided. *P* values of 0.05 or less were considered statistically significant.

3. Results

Two observational cohorts (Table 1) were combined for our analysis, and the baseline characteristics of the aggregated cohort are shown in Table 2. In order to compare samples analyzed from the two observational cohorts, we converted all $p16^{INK4a}$ expression values to a normalized *Z*-score as described in the methods. Using this conversion, we found excellent correlation among Z-scores for the 17 patients that had separate samples obtained in both cohorts (Table 3), suggesting the aggregation of the observational cohorts for analysis is valid. In the combined cohort there were 26 unique patients who underwent autologous transplantation and 37 who underwent allogeneic

Table 1
Summary of clinical samples used in this study.

	Number of samples
Symptom burden observational study[a]	
Baseline samples	28
Autologous transplant	8
Allogeneic transplant	20
6-month follow up samples	16
Autologous transplant	5
Allogeneic transplant	11
Tissue procurement observational study	
Baseline samples	35
Autologous transplant	18
Allogeneic transplant	17
6-month follow up samples	11
Autologous transplant	1
Allogeneic transplant	10

[a] 17 patients participating in the symptom burden study also had specimens stored in the tissue procurement facility and collected in the second cohort. In all, 21 samples were shared between the two cohorts- 17 baseline samples and four 6-month follow-up samples. These samples are not counted in the second cohort summary table above.

transplantation, for a total of 63 unique patients (Table 2). A majority of autologous transplant recipients had myeloma, and the rest had lymphoma as an underlying diagnosis. Most allogeneic transplant recipients had leukemia as their underlying diagnosis. Five allogeneic transplant recipients had previously undergone autologous transplantation.

Among autologous HSCT recipients, there were no baseline characteristics that were associated with pre-transplant $p16^{INK4a}$ expression. Among allogeneic HSCT recipients, however, a greater number of cycles of chemotherapy received before allogeneic transplant, history of prior autologous transplantation, and history of alkylating agent exposure were all significantly associated with higher pre-transplant $p16^{INK4a}$ expression ($p < 0.01$ for each, Table 4A and B). In accord with results in patients treated with chemotherapy for breast cancer (Sanoff et al., 2014), prior exposure to alkylating agents was associated with a 1.9-fold increase in $p16^{INK4a}$ expression (absolute value, log2 = 0.91). Moreover, there was a 2.3-fold increase in $p16^{INK4a}$ expression level (absolute value, log2 = 1.22) in the small number of allogeneic patients that had undergone prior autologous transplant. Previously, we have shown that a 2-fold increase in $p16^{INK4a}$ expression is equivalent to ~10 years of chronological aging (Sanoff et al., 2014). Therefore, these data suggest that extensive exposure to alkylating agents or autologous transplantation is equivalent in molecular terms to a chronological decade or more increase in the chronological age of Peripheral Blood T-Lymphocytes (PBTL).

Table 3
Comparison of $p16^{INK4a}$ expression levels in T cells isolated from fresh blood (symptom burden cohort (SB)) or frozen PBMCs (tissue procurement cohort (TP)). A Z score was calculated for samples in each cohort and the values of 20 samples shared between cohorts is shown ($r^2 = 0.6$).

	p16_SB	p16_TP
A101	1.83	0.34
A103	−1.34	−1.40
A108 6 months	−0.33	0.14
A108 BL	−0.74	−1.94
A109 6 months	0.44	0.68
A109 BL	−1.52	−1.04
A110	−1.59	−0.50
A202	−0.50	−0.96
A203	0.52	−0.05
A204	0.13	1.39
A208 6 months	1.55	1.90
A208 BL	−0.63	−1.17
B202 6 months	−0.39	0.65
B202 BL	−0.61	−0.54
B204	−1.32	−1.45
B205	0.06	−0.38
B206	0.94	2.05
B209	−0.63	−0.26
B211	1.61	1.27
B212	−0.11	−0.28

Next, we determined the effects of HSCT on molecular age of PBTL as measured by $p16^{INK4a}$ expression by comparing pre- and post-HSCT $p16^{INK4a}$ levels within an individual. As shown in Table 5, hematopoietic stem cell transplantation was associated with a significant increase in $p16^{INK4a}$ expression levels for both autologous and allogeneic transplant recipients. Allogeneic transplant recipients showed a pronounced increase in $p16^{INK4a}$ expression post-HSCT (1.93-fold, $p = 0.0004$). In the allogeneic setting, the PBTL pre-HSCT were derived from the host, whereas the PBTL post-HSCT were largely if not entirely donor-derived (given near 100% chimerism in patients post-engraftment). Since the hosts, who generally were transplanted for AML, had experienced prior chemotherapy and, in a small number of cases, auto-HSCT, we would expect that the pre-HSCT levels of $p16^{INK4a}$ would be considerably higher than the expression of $p16^{INK4a}$ in their healthy donors. Therefore, the measured change in $p16^{INK4a}$ from pre-HSCT to post-HSCT in allogeneic recipients likely underestimates the age-promoting effects on the graft of HSCT, given that the pre-HSCT levels were

Table 2
Baseline characteristics of hematopoietic stem cell transplantation (HSCT) patients in this study.

Characteristic	Autologous HSCT	Allogeneic HSCT	Total
N	26	37	63
Age (mean (SD))	59.0 (8.6)	54.2 (11.4)	56.2 (10.5)
Gender			
Male	15 (58%)	24 (65%)	39 (62%)
Female	11 (42%)	13 (35%)	24 (38%)
Race			
Caucasian	24 (92%)	30 (81%)	54 (86%)
Malignancy			
Myeloma	14 (54%)	1 (3%)	15 (24%)
Lymphoma	10 (38%)	4 (11%)	14 (22%)
Acute leukemia	0	19 (51%)	19 (30%)
Prior treatments			
Chemotherapy exposure in months (mean (SD))	6.6 (5.2)	7.2 (10.2)	7.0 (8.5)
Number of chemotherapy regimens (mean (SD))	1.9 (1.2)	2.4 (1.7)	2.2 (1.5)
Number of chemotherapy cycles (mean (SD))	8.0 (5.2)	5.9 (5.0)	6.8 (5.1)
Anthracycline	13 (50%)	22 (59%)	35 (56%)
Nucleoside analogue	4 (15%)	22 (59%)	26 (41%)
Lenalidomide or thalidomide	14 (54%)	2 (5%)	16 (25%)
Bortezomib	12 (46%)	0	12 (19%)
Prior Autologous transplant	1 (4%)	4 (11%)	5 (8%)
Comorbidity score (HCT-CI) (mean (SD))	3.7 (2.8)	2.7 (2.0)	3.1 (2.4)

Table 4
Pre-transplant $p16^{INK4a}$ expression is associated with amount and type of pre-transplant chemotherapy and history of prior autologous transplant in univariate analysis. Linear regression analysis (A) and one-way analysis of variance (B) demonstrate association of $p16^{INK4a}$ with patient's characteristics and therapies.

A

	Autologous HSCT (N = 26)		Allogeneic HSCT (N = 37)	
Baseline characteristic	Estimate (log 2)	p-Value	Estimate (log 2)	p-Value
Age	−0.002	0.94	0.001	0.92
Comorbidity score (HCT-CI)	−0.081	0.18	−0.40	0.60
Number of cycles of chemotherapy	0.014	0.68	**0.086**	**0.003**

B

	Autologous HSCT (N = 26)		Allogeneic HSCT (N = 37)	
Baseline characteristic	Difference (log 2)	p-Value	Difference (log 2)	p-Value
Prior autologous transplant	NA	NA	**1.22**	**0.01**
Exposure to alkylator – incl prior auto	0.20	0.56	**0.91**	**0.01**
Exposure to alkylator – not incl prior auto	0.19	0.6	0.62	0.13
Exposure to anthracycline	0.28	0.41	−0.04	0.91
Exposure to nucleoside analogue	0.70	0.13	−0.23	0.45
Exposure to lenalidomide or thalidomide	−0.04	0.91	0.75	0.26
Exposure to bortezomib	0.05	0.88	NA	NA

elevated in the recipients from prior therapeutic exposure. In the allogeneic HSCT setting, it is possible that other unaccounted factors (e.g. post-transplant calcineurin-inhibitor exposure) may have also contributed to the observed effect.

In order to directly measure the effects of human HSCT per se on molecular age, we turned to an analysis of patients undergoing autologous HSCT. In accord with our finding that autologous HSCT prior to allogeneic transplant was associated with elevated $p16^{INK4a}$ in pooled patients (Table 4), we noted that within any given patient, autologous HSCT was associated with a marked increase in $p16^{INK4a}$ expression from pre-transplant to 6 months post-transplant (3.05-fold, p = 0.002). This finding suggests that the forced bone marrow repopulation associated with transplantation per se accelerates the molecular aging of PBTL, in the absence of significant chemotherapy exposure to the graft.

3.1. Relationship of Transplantation and Chemotherapy with Peripheral Blood T-cell Gene Expression

To further understand the long-term effects of DNA damaging agents and HSCT on PBTL, we performed whole transcriptome RNA sequencing before and after cancer therapy. In order to identify transcriptional changes that were not specific to a particular noxious insult, but instead were induced by the generic insult of forced bone marrow regeneration, we performed an analysis of samples pre- and post-autologous transplantation (3 pairs of samples) or pre- and post-cytotoxic chemotherapy (5 pairs of samples). In order to focus on durable changes to the T-cell transcriptome, PBTL were collected 6 months post-autologous HSCT or 6–12 months post-adjuvant chemotherapy with doxorubicin and cyclophosphamide for breast cancer (these samples are described in (Sanoff et al., 2014)). We performed whole transcriptome RNA sequencing of ribosome depleted total RNA on pre- and post-therapy T-cell samples, and compared expression profiles through pairwise supervised analysis. This approach identified a signature of ~500 coding transcripts whose expression was significantly (adjusted p-value < 0.05) and durably altered by these types of cancer therapy. If restricted to genes whose expression changed > 1.9-fold from baseline, we identified 134 coding transcripts that significantly increased in expression, and 47 transcripts that significantly decreased in expression (Supp. Tables 1, 2), which are presented as a representative subset in Fig. 1 and overall by hierarchical clustering in Fig. 2. These data demonstrate a potent and lasting effect on transcription of the T-cell organ after forced bone marrow regeneration.

In order to determine the effects of auto-transplant or chemotherapy on T-cell function, we performed several types of bioinformatic analysis on these RNA sequencing data. We used gene set enrichment analysis (GSEA) and supervised gene set analysis (SAM-GSA) to compare the genes altered by cancer therapy to ~8000 known gene sets in the Molecular Signatures database (Subramanian et al., 2005). These analyses identified a large number of published signatures with significant overlap with our gene list, of which the vast majority (>90%) were immunologic. Using an adjusted p-value of 0.05, 211 gene sets significantly overlapped with transcripts more highly expressed in the pre-therapy samples (Supp. Table 3), and 98 overlapped with transcripts that were more highly expressed in the post-therapy samples (Supp. Table 4). Hierarchical clustering of representative GSEA signatures are shown in Figs. 3–7. These GSEA results appear to be predominantly explained by changes in the composition of the CD3+ fraction of lymphocytes post-cancer therapy. For example, transcripts that were more highly expressed in memory CD4+ cells relative to naïve cells increased after cancer therapy (Fig. 3), and the same was true for transcripts that were more highly expressed in effector memory compared to central memory CD4+ cells (Fig. 4). Transcripts that were more highly expressed in regulatory T cells compared to conventional T cells also increased following cancer treatment (Fig. 5). As shifts from central memory and naïve cells to effector cells and Tregs are hallmark changes of peripheral T-cells with aging (Desai et al., 2010), these data provide further evidence that cancer therapy accelerates the changes in T-cell subsets that are associated with aging of the cellular immune system.

An analysis of specific genes altered by cancer therapy was also illuminating (selected transcripts are clustered in Fig. 1). For example, we observed a robust increase in transcripts associated generally with cellular senescence (e.g. *CDKN2a*, *IL8*, *HMGA2*, *CCL4*) (Narita et al., 2006; Acosta et al., 2008; Hammond and Sharpless, 2008; Cheng et al., 2015) or telomere shortening in T-cells (e.g. *KLRK1*, *NUAK1*, *DSP*, *PIF1*) (Humbert et al., 2010; Soriani et al., 2014; Textor et al., 2011; Lansdorp, 2007; Robin et al., 2014). Additionally, we noted changes that have been previously associated with T-cell aging and immunosenescence: decreased *CD28* expression (Effros et al., 1994) and increased expression of several NK markers (e.g. *CX3CR1*, *KLRK1*, *KLRA1*) (Goronzy and Weyand, 2005; Sciume et al., 2011; Bauer et al., 1999; Bull et al., 2000). Consistent with the GSEA results and known patterns of human T-cell aging, there was a relative depletion of

Table 5
High-dose chemotherapy increases $p16^{INK4a}$ expression. Changes in $p16^{INK4a}$ expression between baseline and follow-up in aggregated cohort in autologous-HSCT, allogeneic-HSCT or breast cancer patients.

Treatment type	Pre-treatment log2p16	Post-treatment log2p16	Change in $p16^{INK4a}$ expression	P-value
Autologous HSCT (mean (95% CI)); N = 6	−0.88 (−1.44, −0.32)	0.73 (0.20, 1.27)	3.05	0.002
Allogeneic HSCT (mean (95% CI)); N = 21	−0.13 (−0.53, 0.27)	0.82 (0.53, 1.11)	1.93	0.0004
Adjuvant breast cancer (mean (95% CI); N = 24	−0.48 (−0.12, −0.83)	0.48 (0.22, 0.74)	1.93	0.0001

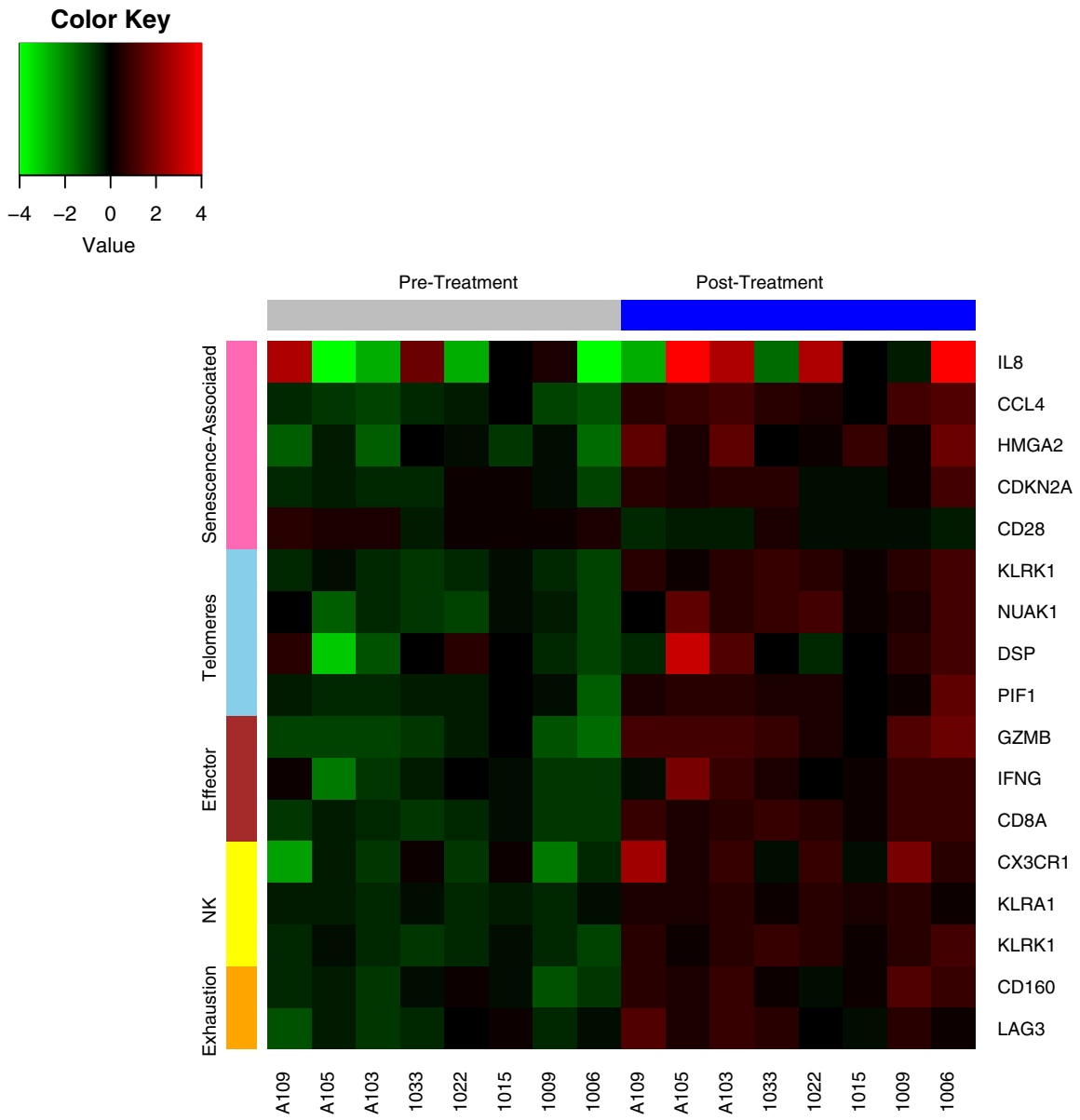

Fig. 1. A hierarchical clustering of representative transcripts whose expression significantly changes with cancer therapy (3 pair of auto-HSCT samples or 5 pair of chemotherapy-treated samples) in peripheral blood T-cells (CD3+). Pre-treatment samples are on the left (grey bar above the heatmap) and post-treatment samples are on the right (blue bar). Genes of interest are ordered along the y-axis as described in the Results section, with color-coding indicated to the left of the heatmap (purple = senescence associated; light blue = telomere shortening; brown = effector; yellow = NK; orange = exhaustion). Sample numbers are shown below the heatmap, with an "A" prior to sample numbers indicating patients undergoing auto-HSCT. Samples without an "A" indicate patients treated with chemotherapy and not transplantation. Samples are ordered along the x-axis.

transcripts associated with central memory cells with an enrichment of transcripts associated with effector CD8 + T-cells (e.g. decreased *CCR7* and *IL7Rα*, with increased *GZMB*, *CD8a* and NK markers (Fig. 1)). In accord with these processes, we observed that cancer therapy induced the expression of transcripts that are more highly expressed in T-cells from old versus young donors (Fig. 6). The effects on transcripts associated with T-cell exhaustion were less consistent: a few well-recognized markers of exhaustion significantly increased post-bone marrow insult (e.g. CD160 and LAG3, Fig. 1), whereas published signatures of T-cell exhaustion demonstrated a mixed pattern of expression in GSEA analysis (Fig. 7, see also Tables 8 and 9). These gene-specific data provide evidence beyond changes to $p16^{INK4a}$ (Table 2) that generic types of hematopoietic injury (e.g. forced bone marrow repopulation, cytotoxic chemotherapy) induces durable transcriptional changes characteristic of T-cell aging and immunosenescence, suggesting these insults accelerate T-cell molecular aging.

4. Discussion

In this work, we show that that DNA damaging chemotherapy and stem cell transplantation potently induce the transcriptional changes characteristic of aging in PBTL. This includes both changes associated with an altered composition of the T-cell organ with aging (e.g. decreased naïve and central memory cells versus increased effector memory cells and Tregs) as well as changes associated with telomere shortening and cellular senescence. While the notion that DNA damaging agents induce cellular senescence in vivo is non-controversial, a remarkable aspect of this work is the finding that autologous HSCT markedly induces the transcriptional changes of PBTL aging. For example, auto-HSCT increases the expression of $p16^{INK4a}$, a highly dynamic marker of senescence, to a degree comparable ~30 years of chronological age. A similar increase in $p16^{INK4a}$ expression with auto-HSCT has been reported in myeloma patients (Rosko et al., 2015). As the autograft

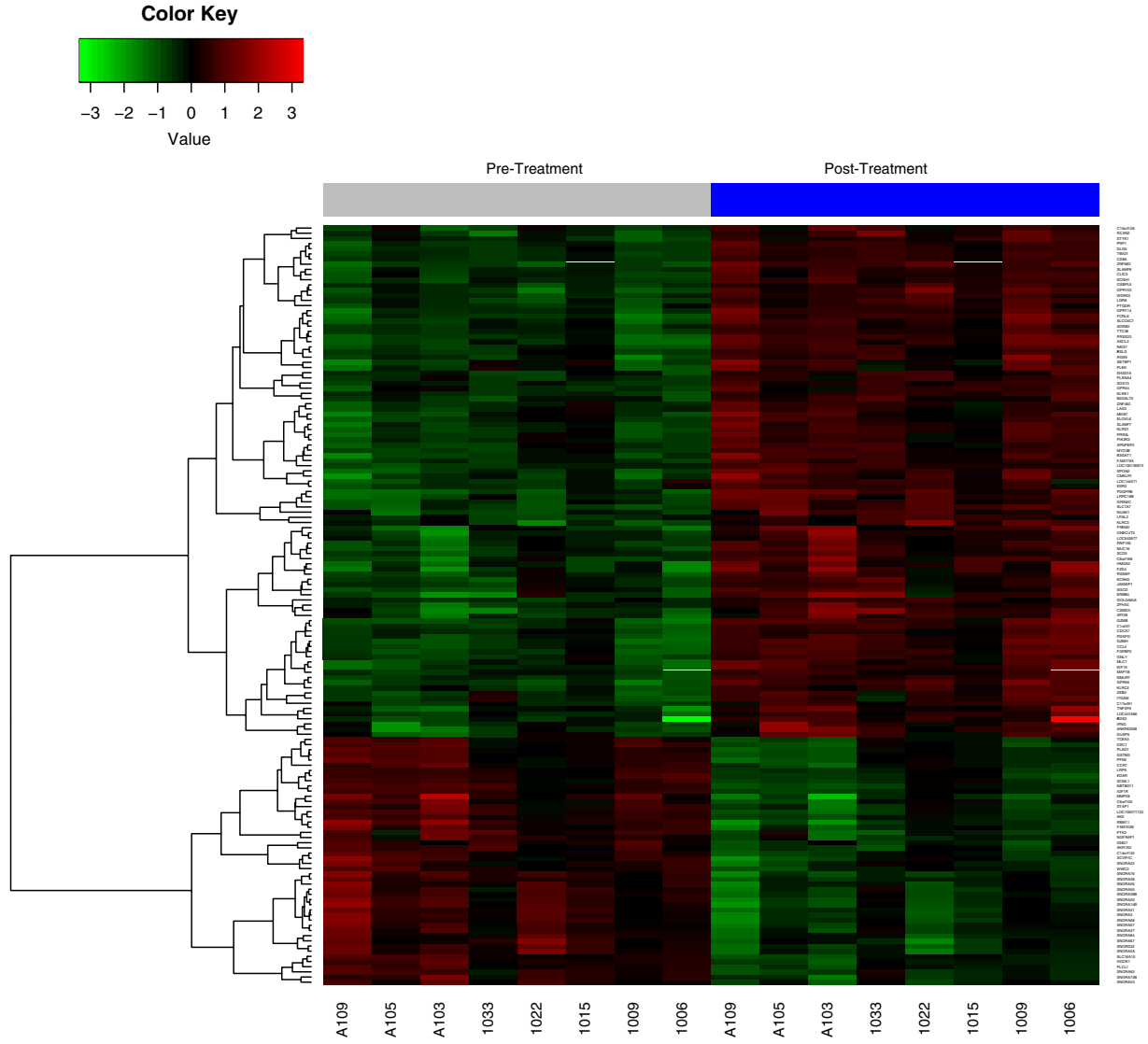

Fig. 2. A hierarchical clustering of all transcripts whose expression significantly changes with cancer therapy (3 pair of auto-HSCT samples or 5 pair of chemotherapy-treated samples) in peripheral blood T-cells (CD3+). Pre-treatment samples are on the left (grey bar above the heatmap) and post-treatment samples are on the right (blue bar). Sample numbers are shown below the heatmap, with an "A" prior to sample numbers indicating patients undergoing auto-HSCT. Samples without an "A" indicate patients treated with chemotherapy and not transplantation. Samples are ordered along the x-axis as in Fig. 1.

is not exposed to DNA damaging chemotherapy, this observation suggests that the forced proliferation that accompanies BM re-engraftment, even in the absence of exogenous clastogens, may be age-promoting in humans.

In accord with prior work (Sanoff et al., 2014), we observed relatively increased expression of PBTL $p16^{INK4a}$ in pre-treatment samples from patients undergoing allogeneic, but not autologous stem cell transplant. This observation likely reflects differences in prior therapy between these two patient groups: with allogeneic HSCT patients having received high doses of alkylating agents and anthracyclines for acute leukemia, whereas autologous patients received largely non-cytotoxic anti-myeloma therapies such as bortezomib and lenalidomide. This observation suggests that cytotoxic chemotherapy may be more age-promoting than non-cytotoxic drugs used for myeloma, and is consonant with the practice of avoiding alkylating agents in patients with myeloma because such drugs impair subsequent stem cell collection (Clark and Brammer, 1998).

It is perhaps surprising that autologous HSCT induces the strongest effect on PBTL molecular age, as measured by $p16^{INK4a}$ expression, of any noxious stimulus to date tested, including cytotoxic chemotherapy (Sanoff et al., 2014), chronic HIV infection (Nelson et al., 2012), tobacco use (Liu et al., 2009) or physical inactivity (Liu et al., 2009). We believe the most likely explanation for this finding is that the forced BM regeneration that accompanies re-engraftment induces intrinsic HSC aging. Serial transplant readily 'exhausts' HSC in murine models (Harrison, 1979), leading to a 'myeloid skewing' characterized by a marked decline in the per HSC capacity to produce naïve T-cells (Janzen et al., 2006), an observation that is in accord with our RNAseq analysis (Fig. 3). Furthermore, though pre-treatment samples from patients undergoing allogeneic HSCT had higher PBTL $p16^{INK4a}$ expression than those from patients undergoing autologous HSCT, it is possible that pre-transplant therapy had some age-promoting effect upon PBTLs in autologous HSCT recipients. Thus, in combination with forced BM regeneration, PBTLs of autologous HSCT recipients were "aged" twice due to therapy and then transplant, possibly helping to explain the larger effect on PBTL $p16^{INK4a}$ expression from autologous in comparison with allogeneic HSCT.

Beyond an effect on HSC, however, it is possible that damage to other tissues contributes to this effect. For example, the thymus is damaged by pre-transplant conditioning with high-dose chemotherapy, and it is

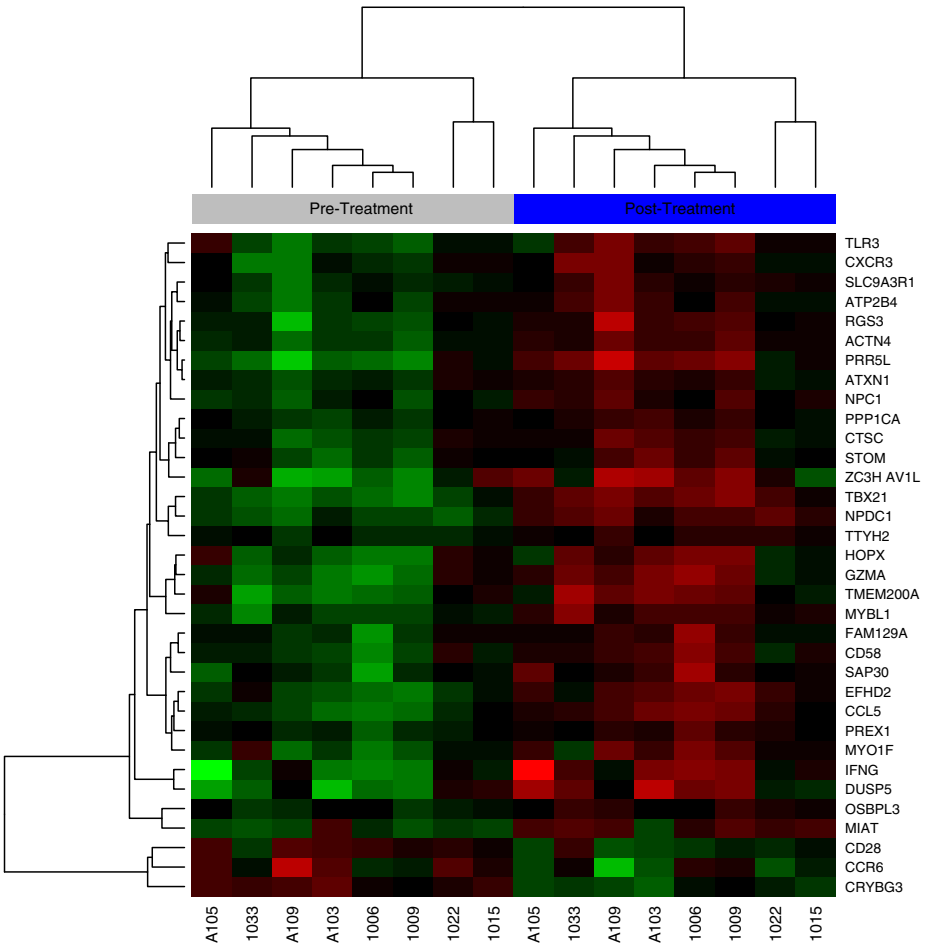

Fig. 3. A hierarchical clustering of transcripts from a Gene Set Enrichment Analysis Signature (GSE11057) with increased expression in memory CD4 + cells compared to naïve cells. Pre-treatment samples are on the left (grey bar above the heatmap) and post-treatment samples are on the right (blue bar). Sample numbers are shown below the heatmap, with an "A" prior to sample numbers indicating patients undergoing auto-HSCT. Samples without an "A" indicate patients treated with chemotherapy and not transplantation. Samples are ordered along the x-axis as in Fig. 1.

likely that graft-derived T-cells produced via a dysfunctional thymus exhibit accelerated aging (Min et al., 2005; Montecino-Rodriguez et al., 2013; Dorshkind et al., 2009; Linton and Dorshkind, 2004). Moreover, it is possible that not all host hematopoietic stem cells and self-renewing T-cells are destroyed by the conditioning regimen, and these surviving cells could exhibit accelerated aging as a result of exposure to conditioning. Since our analysis is performed on pooled PBTL, even a rare population of surviving cells that highly express $p16^{INK4a}$ could affect a post-transplant sample's results. While such effects could affect PBTL $p16^{INK4}$, it is unlikely that thymic damage or very rare surviving host T-cells would cause the wholesale transcriptional effects observed in the RNAseq analysis.

Our finding that post-transplant senescent T-cells were biased toward the expression of transcripts associated with CD8 + effector cells and NK cells is consistent with prior studies of T-cell aging and immunosenescence. (Perillo et al., 1993; Jaruga et al., 2000; Lemster et al., 2008). In aggregate, our work and other studies suggest that noxious stimuli such as DNA damage, telomere shortening and forced regeneration mimic the effects of chronologic age to augment the production of hypo-replicative T-cells with distinct immunophenotype (e.g. CD8 + CD28-) that express effector molecules such as Granzyme B and NK markers as well as markers of cellular senescence (e.g. $p16^{INK4a}$ and IL8). Such senescence-promoting stimuli have a more complex effect on markers of T-cell exhaustion, leading to increased expression of some exhaustion-associated markers and decreased expression of others. This finding is in accord with the notion that there are at least two different types of severely hyporeplicative states induced by excess T-cell proliferation—cellular senescence and exhaustion (Akbar and Henson, 2011)—with our work suggesting forced replication and DNA damaging agents induce the former, but not the latter, in humans.

This work has several implications. First, it is possible that an assessment of T-cell molecular age could be used to predict suitability for transplantation. In fact, prior studies have shown that older autologous HSCT recipients have a higher likelihood than younger recipients of failing to achieve complete peripheral blood count recovery at 1 year, particularly true in patients who have been exposed to prior cytotoxic chemotherapy (Woolthuis et al., 2014; Bhatia et al., 2005). Such a use would be similar to the suggestion that a renal allograft's molecular age, as measured by $p16^{INK4a}$ expression, is a stronger predictor of long-term graft function than donor age or telomere length (Koppelstaetter et al., 2008; McGlynn et al., 2009; Gingell-Littlejohn et al., 2013). Larger studies with longer follow-up would be needed to confirm the hypothesis that donor molecular age of HSC influences long-term hematopoietic graft function. Second, post-transplant T-cell senescence may have important implications with regard to T-cell function, with an increase in the frequency of senescent T-cells leading to greater long-term risk of infection or decreased response to vaccination (Targonski et al., 2007). Additionally, if increased molecular age of PBTL is the result of intrinsic HSC aging, this marker could also perhaps predict other late complications of HSCT such as bone marrow failure, MDS or secondary leukemia. Third, the finding that HSCT and DNA damaging agents promote the molecular aging of T-cells suggests such

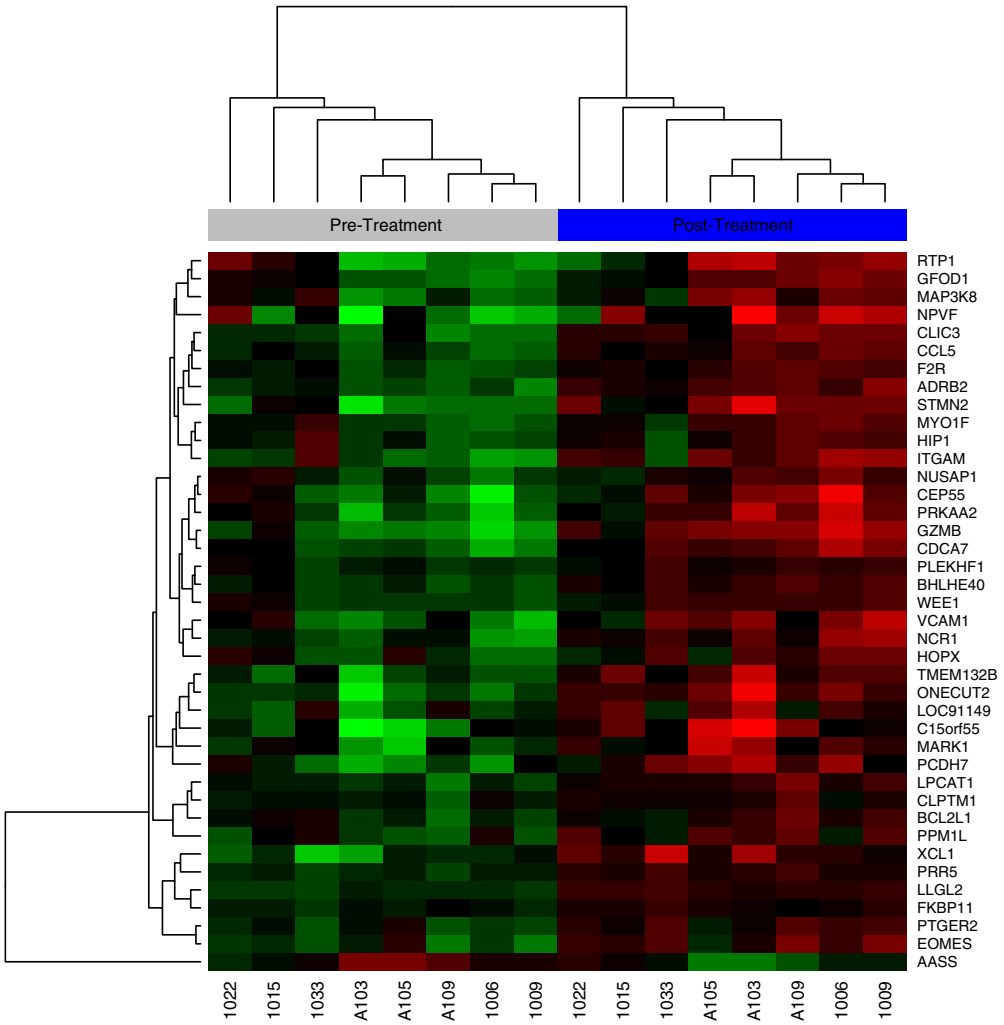

Fig. 4. A hierarchical clustering of transcripts from a Gene Set Enrichment Analysis Signature (GSE26928) with increased expression in effector memory compared to central memory CD4+ cells. Pre-treatment samples are on the left (grey bar above the heatmap) and post-treatment samples are on the right (blue bar). Sample numbers are shown below the heatmap, with an "A" prior to sample numbers indicating patients undergoing auto-HSCT. Samples without an "A" indicate patients treated with chemotherapy and not transplantation. Samples are ordered along the x-axis as in Fig. 1.

agents may compromise the post-transplant ability to respond to therapies intended to activate exhausted T-cells (e.g. anti-PD1 antibodies). Finally, the significant increase in T-cell senescence after HSCT further raises questions about the potential long term risks related to accelerated aging in recipients of two transplants (e.g. tandem autologous transplantation). For diseases in which credible non-transplant treatment approaches exist, pre-transplant "molecular age" and subsequent risk for further transplant-related accelerated aging could impact clinical decision-making.

We acknowledge limitations to our work. First, we did not have data to demonstrate a correlation between the observed changes in biomarker expression with T cell function. However, inasmuch as $p16^{INK4a}$ expression is arguably one of the best in vivo markers of cellular senescence and is directly associated with age-related deterioration (Baker et al., 2016), the signal of increased senescence early after transplantation is clear and concerning, even if a clearer explanation of this phenomena requires further study. Second, other aspects of post-transplant immune reconstitution also influence the relative contribution of CD8 and CD4 T cells to the CD3 population in the early post-transplant period. Third, the relative contribution of clinical factors to PBTL $p16^{INK4a}$ expression, including graft versus host disease, infection, and use of immunosuppressive agents, could not be discerned in this analysis. Fourth, we did not have sequential longitudinal post-transplant samples available to study changes in $p16^{INK4a}$ expression over time. Fifth, donor samples were limited. We were unable in this study to determine the source of the T cells that were undergoing changes in biomarker expression following allogeneic transplantation; a clear idea of which cells were donor-derived and which were recipient-derived would help to discern the relative effects of chemotherapy vs transplant upon $p16^{INK4a}$ expression.

In summary, we have shown that HSCT and cancer therapy strongly increase the expression in T cells of $p16^{INK4a}$, a well-known biomarker of cellular senescence. Further, this likely occurs via an effect on hematopoietic stem cells. $p16^{INK4a}$ expression was markedly increased following transplantation, and in association with amount and certain types of chemotherapy. This observation may have implications for the management of patients with cancer as well as cancer survivors, in order to limit pro-aging effects of treatment and to protect against the development of frailty and other aging-related syndromes. Additional studies to investigate the relationships between T-cell aging and adverse outcomes following cancer therapy are warranted.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ebiom.2016.08.029.

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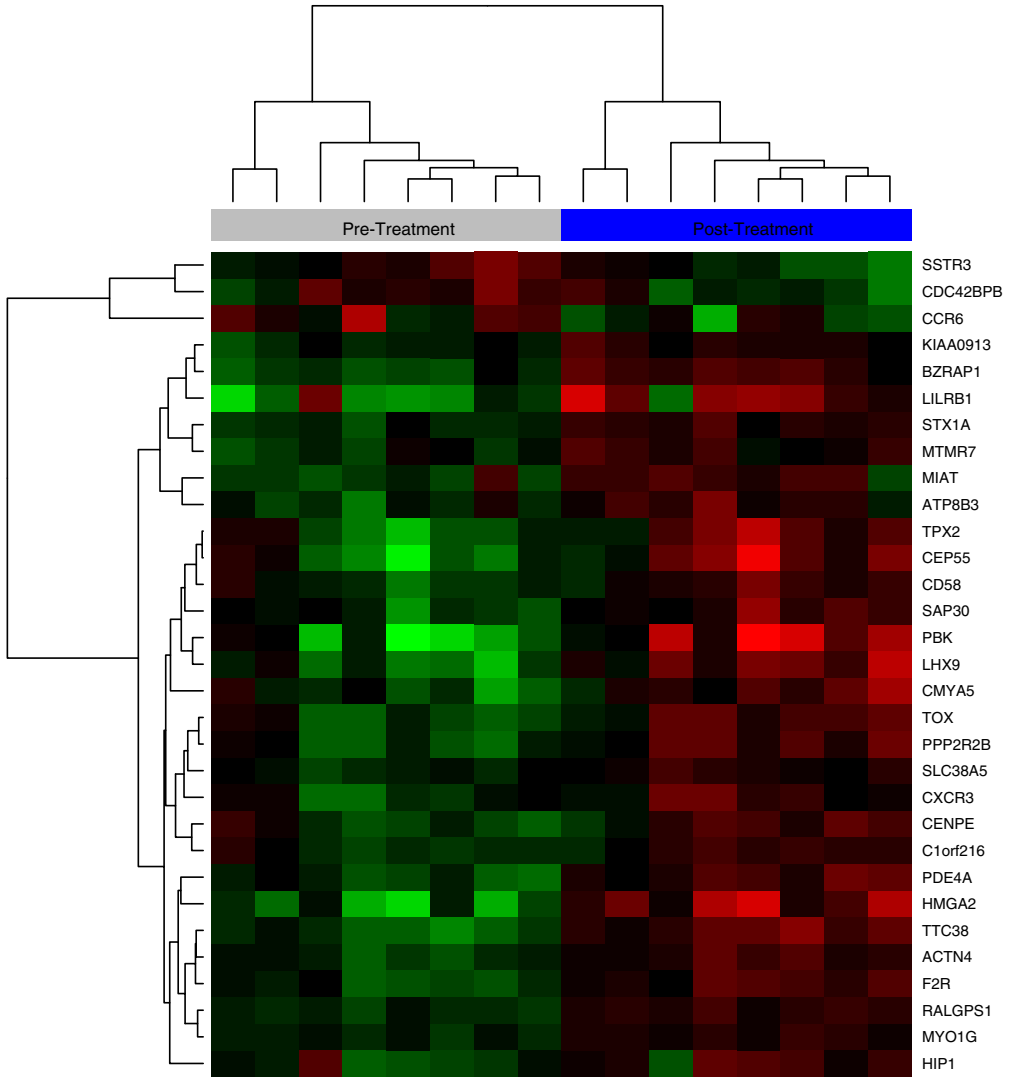

Fig. 5. A hierarchical clustering of transcripts from a Gene Set Enrichment Analysis Signature (GSE22045) with increased expression in regulatory T cells compared to conventional T cells. Pre-treatment samples are on the left (grey bar above the heatmap) and post-treatment samples are on the right (blue bar). Samples are ordered along the x-axis as in Fig. 1.

Conflict of Interest Statement

N.M. and N.E.S. are founders and hold equity in HealthSpan Diagnostics, which is developing clinical grade assays for molecular aging related to this work. K.J. and N.E.S. are inventors on an issued patent related to this work.

Author Contributions

W.A.W., J.K., N.M., C.T., J.S.P, T.C.S, J.S.S, and N.E.S. designed the research, performed research, and analyzed data. A.C.S. contributed to the statistical analysis. W.A.W. and N.E.S. wrote the paper. All authors contributed to the critical review and editing of the manuscript.

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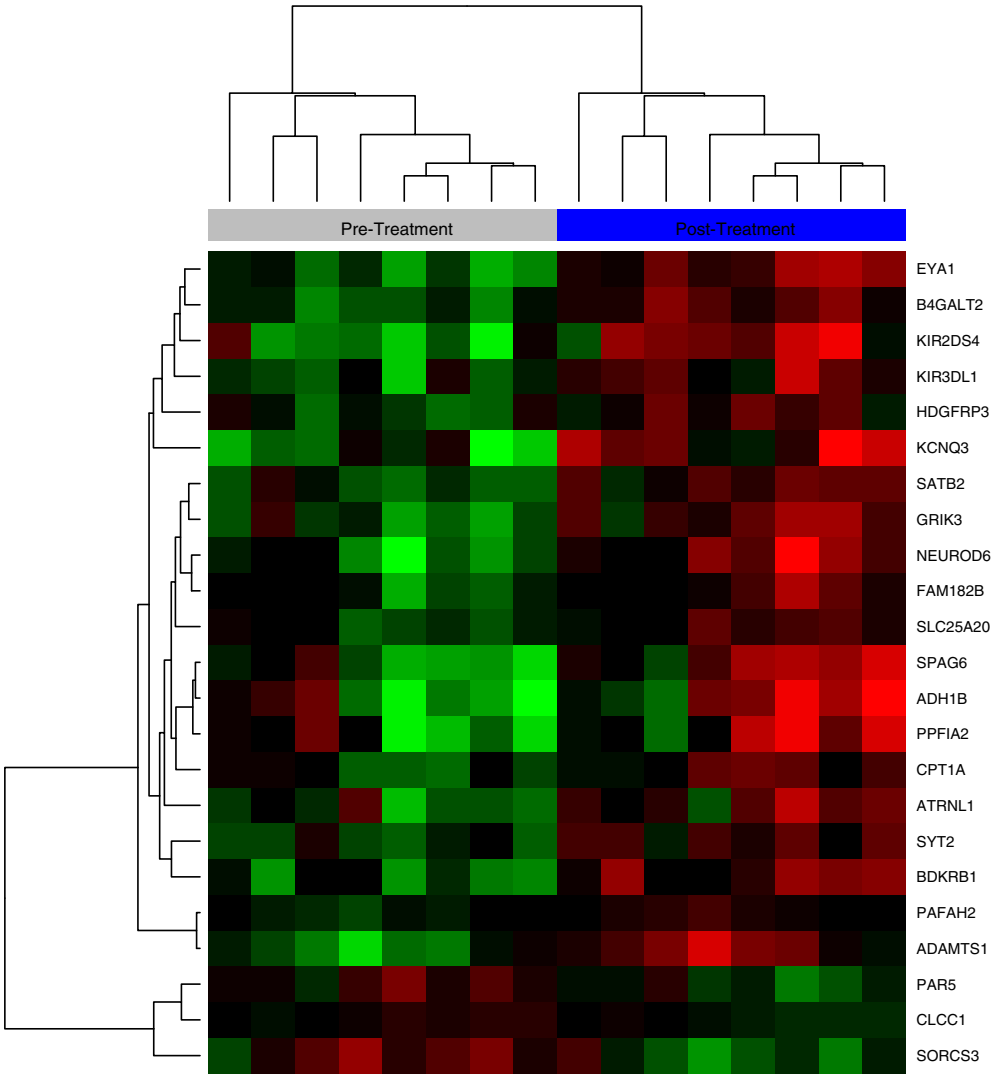

Fig. 6. A hierarchical clustering of transcripts from a Gene Set Enrichment Analysis Signature (GSE36476) with increased expression of transcripts that are more highly expressed in T cells from old versus young donors. Pre-treatment samples are on the left (grey bar above the heatmap) and post-treatment samples are on the right (blue bar). Samples are ordered along the x-axis as in Fig. 1.

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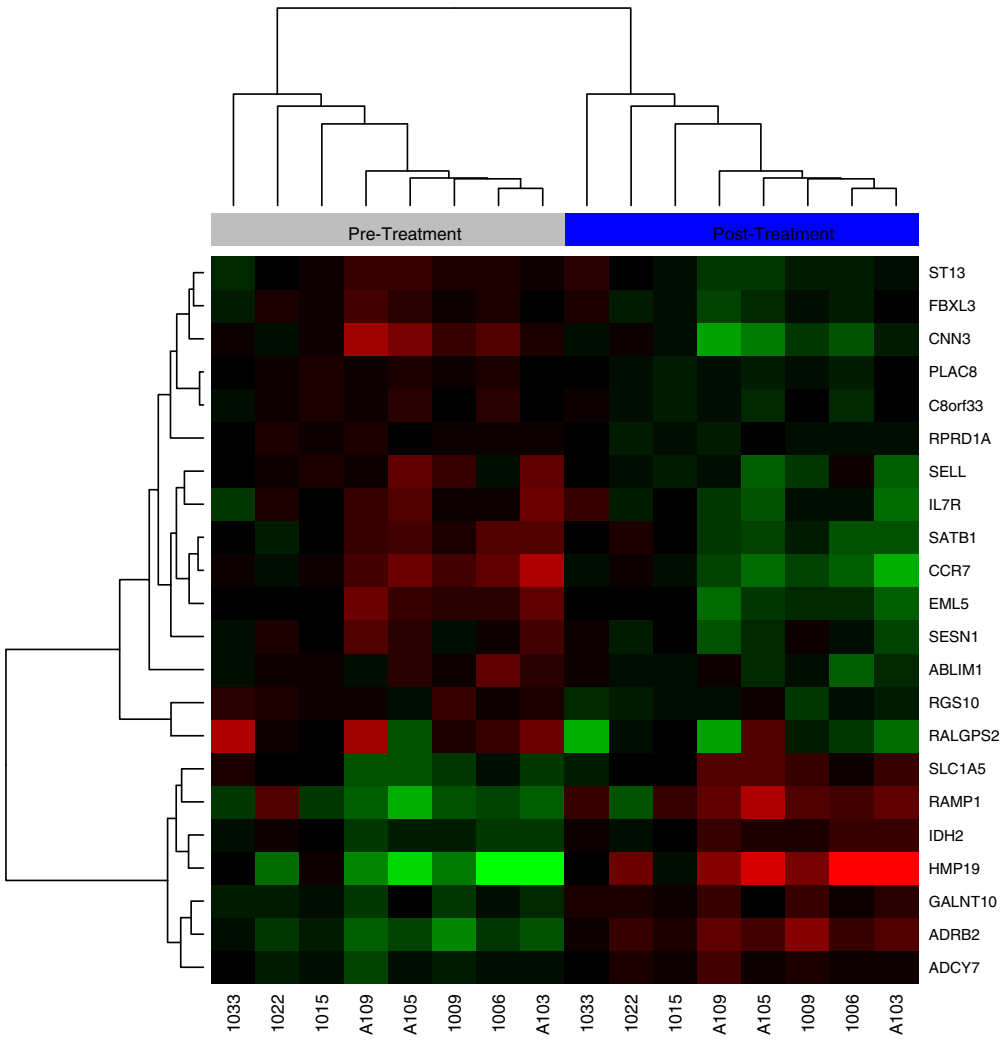

Fig. 7. A hierarchical clustering of transcripts from a Gene Set Enrichment Analysis Signature (GSE9650) with a mixed pattern of expression of signatures associated with naïve versus exhausted CD8+ cells. Pre-treatment samples are on the left (grey bar above the heatmap) and post-treatment samples are on the right (blue bar). Sample numbers are shown below the heatmap, with an "A" prior to sample numbers indicating patients undergoing auto-HSCT. Samples without an "A" indicate patients treated with chemotherapy and not transplantation. Samples are ordered along the x-axis as in Fig. 1.

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