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

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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. Patients receiving autologous transplant experienced a larger increase in expression of $p16^{INK4a}$ expression (3.1-fold increase, p = 0.002) than allogeneic transplant recipients (1.9-fold increase, p = 0.05). 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 = 0.004). 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.

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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 chemoradiotherapy given at the time of transplantation (conditioning), from donor-host immune differences after allogeneic transplant 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-versus-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).

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
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 p16INK4a, 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, chemotherapy, 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\textsuperscript{INK4a} expression

See Sanoff et al. (Sanoff et al., 2014) for details. In brief, CD3\textsuperscript{+} T-cells were isolated from up to 10-ml of peripheral blood using anti-CD3 microbeads and an AutoMACS\textsuperscript{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\textsuperscript{INK4a} was measured by TaqMan quantitative reverse-transcription polymerase chain reaction specific for p16\textsuperscript{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 TrueSeq 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 log\textsc{2} 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\textsc{2} transformed p16\textsuperscript{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 \( \mu \) is population mean, and \( \sigma \) is standard deviation.

For samples present in both cohorts, individual Z scores were averaged. Associations between p16\textsuperscript{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\textsuperscript{INK4a} expression before and after transplant. Data were analyzed by N. Mitin using JMP11 (SAS, Cary, NC) and A. Snavey 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\textsuperscript{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...
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 p16INK4a expression. Among allogeneic HSCT recipients, however, a greater number of cycles of chemotherapy received before allogeneic transplantation, history of prior autologous transplantation, and history of alkylation agent exposure were all significantly associated with higher pre-transplant p16INK4a 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 alkylation agents was associated with a 1.9-fold increase in p16INK4a expression (absolute value, log2 = 0.91). Moreover, there was a 2.3-fold increase in p16INK4a expression level (absolute value, log2 = 1.22) in the small number of allogeneic patients that had undergone prior autologous transplantation. Previously, we have shown that a 2-fold increase in p16INK4a expression is equivalent to ~10 years of chronological aging (Sanoff et al., 2014). Therefore, these data suggest that extensive exposure to alkylation 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).

Next, we determined the effects of HSCT on molecular age of PBTL as measured by p16INK4a expression by comparing pre- and post-HSCT p16INK4a levels within an individual. As shown in Table 5, hematopoietic stem cell transplantation was associated with a significant increase in p16INK4a expression levels for both autologous and allogeneic transplant recipients. Allogeneic transplant recipients showed a pronounced increase in p16INK4a 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 of a chimeric origin (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 p16INK4a would be considerably higher than the expression of p16INK4a in their healthy donors. Therefore, the measured change in p16INK4a 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 1
Summary of clinical samples used in this study.

| Characteristic                        | Number of samples |
|---------------------------------------|-------------------|
| Symptom burden observational study    |                   |
| 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.

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)      | 7.9 (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 (3.0)       | 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-Cl) (mean (SD)) | 3.7 (2.8)      | 2.7 (2.0)       | 3.1 (2.4) |

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

In order to directly measure the effects of human HSCT per se on mo-
lecular age, we turned to an analysis of patients undergoing autologous
HSCT. In accord with our finding that autologous HSCT prior to allogene-
ic transplant was associated with elevated p16INK4a in pooled patients
(Table 4), we noted that within any given patient, autologous HSCT
was associated with a marked increase in p16INK4a expression from
pre-transplant to 6 months post-transplant (3.05-fold, \( p = 0.002 \)).
This finding suggests that the forced bone marrow repopulation associ-
ated 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 se-
quencing 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 re-
generation, we performed an analysis of samples pre- and post-autolo-
gous 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-autolo-
gous HSCT or 6–12 months post-adjuvant chemotherapy with doxor-
ubicin 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-ther-
apy 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 expres-
sion, 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 demon-
strate 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 Molecu-
lar Signatures database (Subramanian et al., 2005). These analyses
identified a large number of published signatures with significant over-
lap with our gene list, of which the vast majority (>90%) were immuno-
logic. Using an adjusted \( p \)-value of 0.05, 211 gene sets significantly over-
lapped 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 naive 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 naive 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 illu-
minating (selected transcripts are clustered in Fig. 1). For example, we
observed a robust increase in transcripts associated generally with cel-
lular 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, PIF1) (Humbert et al.,
2010; Soriani et al., 2014; Teixtor 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, KLRAR1) (Gotzmy 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 4

Pre-transplant p16INK4a expression is associated with amount and type of pre-transplant chemotherapy and history of prior autologous transplant in univariate analysis. Linear re-
gression analysis (A) and one-way analysis of variance (B) demonstrate association of
p16INK4a with patient’s characteristics and therapies.

| Baseline characteristic | Autologous HSCT (N = 26) | Allogeneic HSCT (N = 37) |
|--------------------------|--------------------------|--------------------------|
| Age                      | −0.002                   | 9.4                      |
| Comorbidity score (HCT-CI)| −0.081                   | 0.18                     |
| Number of cycles of chemotherapy | 0.014 | 0.68                      |

| Baseline characteristic | Autologous HSCT (N = 26) | Allogeneic HSCT (N = 37) |
|--------------------------|--------------------------|--------------------------|
| Prior autologous transplant | NA                      | NA                       |
| Exposure to  alkylation – incl prior auto | 0.20 | 0.56                      |
| Exposure to alkylation – not incl prior auto | 0.19 | 0.62                      |
| Exposure to anthracycline | 0.28 | 0.41                      |
| Exposure to nucleoside analogue | 0.70 | 0.13                      |
| Exposure to lenalidomide or thalidomide | −0.04 | 0.75                      |
| Exposure to bortezomib | 0.05 | 0.88                      |

### Table 5

High-dose chemotherapy increases p16INK4a expression. Changes in p16INK4a 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 p16INK4a 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 |
| Autologous HSCT (mean (95% CI)); N = 21 | −0.13 (−0.53, 0.27) | 0.82 (0.53, 1.11) | 1.93 | 0.0004 |
| Breast cancer (mean (95% CI)); N = 24 | −0.48 (−0.12, −0.83) | 0.48 (0.22, 0.74) | 1.93 | 0.0001 |

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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, CD8α 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 p16INK4a (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 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 p16INK4a, a highly dynamic marker of senescence, to a degree comparable ~30 years of chronological age. A similar increase in p16INK4a expression with auto-HSCT has been reported in myeloma patients (Rosko et al., 2015). As the autograft...
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 any noxious stimulus to date tested, including cytotoxic chemotherapy, and it is...
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 p16INK4a could affect a post-transplant sample’s results. While such effects could affect PBTL p16INK4a, 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 chronicologic 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. p16INK4a 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 p16INK4a 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
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.

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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. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
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