Predicting Cytotoxic T cell Age from Multivariate Analysis of Static and Dynamic Biomarkers

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\textbf{Running title:} Predicting Cytotoxic T cell Age
Abbreviations:

7-AAD: 7-Aminoactinomycin D;

BCA: bicinchoninic acid;

CTL: cytotoxic T cell;

CV: coefficient of variation;

IRB: institutional review board;

PC: principal component;

PD: population doubling;

PDMS: poly (dimethylsiloxane);

PE: polyethylene;

PLSR: partial least square regression;

R-PE: R-Phycoerythrin;

SHB: staggered herringbone mixers;

TCR: T cell receptor
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Summary

Adoptive T cell transfer therapy relies upon *in vitro* expansion of autologous cytotoxic T cells that are capable of tumor recognition. The success of this cell-based therapy depends on the specificity and responsiveness of the T cell clones before transfer. During *ex-vivo* expansion, CD8+ T cells present signs of replicative senescence and loss of function. The transfer of non-responsive senescent T cells is a major bottleneck for the success of adoptive T cell transfer therapy. Quantitative methods for assessing cellular age and responsiveness will facilitate the development of appropriate cell expansion and selection protocols. Although several biomarkers of lymphocyte senescence have been identified, these proteins in isolation are not sufficient to determine the age-dependent responsiveness of T cells. We have developed a multivariate model capable of extracting combinations of markers that are the most informative to predict cellular age. To acquire signaling information with high temporal resolution, we designed a microfluidic chip enabling parallel lysis and fixation of stimulated cell samples on-chip. The acquisition of 25 static biomarkers and 48 dynamic signaling measurements at different days in culture, integrating single-cell and population based information, allowed the multivariate regression model to accurately predict CD8+ T cell age. From surface marker expression and early phosphorylation events following T cell receptor stimulation, the model successfully predicts days in culture and number of population doublings with $R^2 = 0.91$ and 0.98, respectively. Furthermore, we found that impairment of early signaling events following T cell receptor stimulation due to long term culture allows prediction of co-stimulatory molecules CD28 and CD27 expression levels and the number of population divisions in culture from a limited subset of signaling proteins. The multivariate
analysis highlights the information content of both averaged biomarker values and heterogeneity metrics for prediction of cellular age within a T cell population.
Introduction

Immune cell-based therapies hold promise in cancer therapy by harnessing the body's natural defense mechanisms against tumors, while leaving healthy cells unharmed (1, 2). Among those therapies, adoptive transfer of T cells has resulted in encouraging clinical trials for treating metastatic melanoma as well as non-Hodgkin's lymphoma, chronic lymphocytic leukemia and neuroblastoma (3-5). Although cancer cells are less immunogenic than pathogens, the adaptive immune system is able to recognize and eliminate tumor cells. Adoptive therapy with cytotoxic CD8+ T cells (CTLs) relies on the isolation of functional and tumor specific T cells and large in vitro clonal expansion. Once transferred back in the cancer patient, CTLs need to retain tumor specificity and proliferate further in vivo to establish an effective in vivo response and tumor shrinkage. In vivo persistence is a critical factor for elimination of residual or recurring malignant cells. The encouraging results of adoptive transfer therapy could be improved by enhancing the quality of transferred T cells. Cells derived from aged cancer patients have a skewed immune repertoire towards cells that underwent extensive clonal expansion against persistent antigens, resulting in few tumor-specific CTLs (6-8). Once isolated the tumor infiltrating cells go through a prolonged ex vivo culture process. T cells, as other somatic cells, have a finite clonal lifespan. Extensive ex vivo proliferation and clonal expansion result in T cell differentiation and ultimately replicative senescence (9). To obtain sufficient number of cells before transfer, tumor specific CTLs are activated and undergo several rounds of divisions, resulting in the progressive shortening of telomeres. Chronic antigenic stress and critically short telomere length lead CTLs to enter a state of senescence characterized by functional changes. Although extensively cultured CTLs retain antigen specificity for the tumor (10), they present
striking alterations in function and gene and protein expressions (8), e.g. they are in an irreversible cell cycle arrest, resistant to apoptosis, with short telomeres and unable to respond to antigenic cues or IL-2 stimulation. Once transferred in the cancer patient, these replicative senescent cells will not be able to eliminate tumor cells and further proliferate, thereby hindering the efficacy of these therapies (11, 12).

To ensure success of adoptive transfer therapy, it is desirable to evaluate T cell clones before transfer based upon their specificity or functionality, regardless of diverse in vitro priming, selection, or expansion methods. Similarly, evaluation of T cell clones in elderly population, or “immune signature”, can enable the identification of immune risk profiles correlated with increased risk of immune dysfunction and increased mortality (13). Phenotypic markers, such as the loss of expression of co-stimulatory markers CD27 (14) and CD28 (15), have been associated with senescent CD8+ cell populations; however, individual biomarkers are not sufficient to accurately measure the fraction of senescent non-responsive cells. Previous proteomics and gene array studies suggest that clusters of parameters would be more appropriate to quantify age-related alterations (16-19), and yet to date, prior proteomic and microarray studies have not attempted to determine the most informative metrics of cellular senescence for the purposes of generating predictive models of T cell function. To enable quantification of the “age” of T cells as they expand in culture through combinations of biomarkers, we applied a partial least square regression (PLSR) modeling analysis from data obtained under conditions consistent with in vitro expansion prior to adoptive transfer in patients. The multivariate model developed in the present study assesses the quality of T cell function through the use of phenotypic markers and protein signaling dynamics.
dynamics were acquired with a microfluidic device that samples rapid phosphorylation events by simultaneous lysing and fixing of stimulated cells. This technology takes advantage of the uniformity and controllability in sample handling and treatment to reduce error associated with biochemical assays; at the same time, it requires a small number of cells and performs the assay in a high-throughput and parallel fashion (20-22). Lysates provide population-averaged measurements compatible with downstream proteomic techniques, while fixed cells analyzed by flow cytometry reveal subpopulations and phenotypic variations within genetically identical cells. The dataset containing signaling dynamics, cellular morphology, and surface expression levels acquired under the uniform, precise conditions of the microfluidics chip allowed the model to determine relative contributions of each metric to the phenotype of replicative senescence despite large donor-to-donor variability. The multivariate analysis highlights the importance of both averaged values and heterogeneity in the cell population for prediction of the replicative senescence within a T cell population.

**Experimental procedures**

*Cell isolation and expansion*

Following institutional review board (IRB) approval, CD8+ T cells were obtained from blood donors using standard isolation procedures. Briefly, 40 mL of fresh blood was collected in EDTA coated tubes from four healthy donors (21-35 years old) under written informed consent. Peripheral blood mononuclear cells were isolated by density centrifugation using Lymphoprep (VWR), and CD8+ T cells further purified using the Dynabeads® Untouched™ Human CD8 T Cells isolation kit (Invitrogen) (>92 % purity as checked by flow cytometry). The cells were expanded
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in RPMI 1640 medium with L-glutamine (Sigma-Aldrich) with 10 mM HEPES, 1 mM sodium pyruvate, and 1X MEM nonessential amino acids, and 100 units.mL⁻¹ penicillin/streptomycin (Cellgro) and 10% certified heat-inactivated fetal bovine serum (Sigma-Aldrich). The culture medium was supplemented with 50 U/mL of recombinant IL-2 (Sigma-Aldrich) and Dynabeads® Human T-Activator CD3/CD28 (Invitrogen) at 1:1 bead to cell ratio (kept constant for the entire culture period) for rapid cell expansion (23, 24). Cell cultures were checked daily and resuspended in fresh medium when needed. The number of population doublings (PDs) was calculated from the average cell count using the following equation:

\[
P D = \frac{\log_{10} n_{\text{post}} - \log_{10} n_{\text{init}}}{\log_{10} 2}
\]

where \( n_{\text{post}} \) represents the number of cells counted after expansion and \( n_{\text{init}} \) represents the number of cells initially seeded.

The following table presents the different time points at which 26·10⁶ cells from each donor cell culture were assayed for signaling, intracellular and surface marker expression. Sample times were chosen when the total number of cells in culture for each donor exceeded 32·10⁶ cells.

| Donor 1 | Day in culture when assayed | Corresponding PD |
|---------|----------------------------|-----------------|
|         | 4                          | 2.0             |
|         | 6                          | 4.1             |
|         | 8                          | 5.9             |
|         | 11                         | 8.6             |
|         | 13                         | 9.1             |

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| Donor 2 | 6  
|         | 8  
|         | 11 
|         | 13 |
|         | 3.8
|         | 6.5
|         | 9.5
|         | 10.5|
| Donor 3 | 4  
|         | 6  
|         | 8  
|         | 11 
|         | 13 |
|         | 2.5
|         | 4.1
|         | 7.3
|         | 9.7
|         | 10.9|
| Donor 4 | 6  
|         | 9  
|         | 13 
|         | 16 |
|         | 4
|         | 6.5
|         | 8.1
|         | 10.8|

Microfluidic device fabrication

The two-module device was fabricated using standard soft lithographic techniques (20, 25). Briefly, the modules were molded in poly (dimethylsiloxane) (PDMS) (Dow Corning Sylgard 184, Essex-Brownwell Inc.) from a two-layer SU-8 (Microchem Corp.) master. One layer of 70 μm-thick SU-8 2050 was spun onto 100 mm silicon wafer, prebaked, and exposed under UV light to define a negative image of the channel system in the resist, following the manufacturer’s instructions. After postbaking to crosslink the exposed resist, another layer of 40 μm thick SU-8 2020 was spun on top. This layer formed the staggered herringbone arrays (20). After the same prebake and expose process, the wafers were developed using propylene glycol monomethyl ether acetate (Doe & Ingalls, Inc.). The wafer surface was treated with vapor-phase tridecafluoro-1,1,2,2-tetrahydrooctyl-1-trichlorosilane (United Chemical Technologies, Inc.) for passivation. PDMS was cast on the SU-8 master and baked for 3 h at 70 °C to cross-link. The PDMS was then peeled off from the mold and individual devices were cut to size. Access holes were punched using stainless steel needles (McMaster Carr). The devices were plasma bonded...
to glass slides or PDMS. Medical grade polyethylene (PE) tubing (Scientific Commodities) of various lengths and inner diameters were used for fluidic connections.

**Device operation**

A syringe pump (Chemyx Fusion 200 series) controlled the flow to the four inlets at 44 \( \mu \text{L} \cdot \text{min}^{-1} \).

Before running an experiment, a solution of 2% BSA in PBS was flown through the device with plugged outlets to pressurize the device and remove any air bubbles. Then the outlets were opened to atmospheric pressure and PBS with 2% BSA was flowed through the device for an additional 15 min. Seven hours before the device operation, 20·10^6 cells were resuspended in fresh medium without IL-2 and anti-CD3/CD28 beads. During device operation, cells were delivered in 1.5 mL of a PBS + 7% w/v dextran solution to match the cell density and avoid cell settling (20). To further avoid cell loss in the syringe, a small cubic magnet with 2-mm edges was inserted with the cell suspension, and intermittently agitated during the experiment. The stimulus consisted of PBS supplemented with 2 \( \mu \text{g/mL} \) of anti-CD3, clone OKT3 (eBioscience), and 2 \( \mu \text{g/mL} \) of anti-CD28 (BD Bioscience). Ice-cold freshly-prepared lysis buffer as previously described (20) and a 10% formalin solution (Sigma-Aldrich) were delivered at the inlets of the second module. Cells, stimulus, lysis buffer and fixing solution were flowed for 9 min to allow steady state for the different time points to be reached before the 8 fixed samples and 8 lysates were collected in ice-cold 96-well plates covered with paraffin for 20 additional min.

**Signaling measurements**

Total protein concentration of the lysates was determined with a BCA assay kit (Pierce). The analysis of phosphorylation dynamics was performed with a Bio-Plex 200 instrument (Bio-Rad) using commercially available Luminex bead assays. The quantification of proteins downstream
of TCR (Beadlyte 7-plex Human T cell Receptor Signaling Kit, Millipore) was completed according to manufacturers’ protocols. Results for all data are presented as the average of triplicates, normalized to values from GAPDH beads (Millipore), and further normalized to the maximum value for separate sets of proteins per donor. Phosphoprotein staining was performed as described in Krutzik et al. (26). Briefly, formalin-fixed stimulated cells eluted from the device were washed with PBS, permeabilized with ice-cold methanol and stored at -20°C. Cells were stained with pERK antibody pT202/pY204 (BD Bioscience) at a dilution of 1:100 (in 2% BSA in PBS). After 30 min of incubation at 4°C, cells were washed and resuspended in PBS with anti-mouse Alexa488 antibody (Invitrogen), incubated for 30 additional min at 4°C, washed and analyzed by flow cytometry.

**Flow Cytometry analysis**

Surface marker protein expression was determined by direct immunostaining. Stimulated CD8+ T-cells (5·10⁵ total) were removed from the beads and resuspended in 500 μL solution of PBS, 2% BSA, with the following antibodies: 10 μL of PE-labeled-αCD27, 10 μL PeCy5-labeled-αCD28, 10 μL FITC-labeled-αCD57 and 2.5 μL PeCy7-labeled-αCD45RO (BDBioscience). After 30 min of incubation on ice, cells were washed with PBS and analyzed by flow cytometry. To determine p16<sup>ink4</sup> and profilin-1 expression as well as cell cycle, stimulated CD8+ T-cells were removed from the beads, resuspended at 10⁷ cells·mL⁻¹ in ice cold methanol and stored at -20°C. After being washed in PBS, cells were resuspended at 10⁷ cells·mL⁻¹ in 50 μL of PBS, 2% BSA, and incubated for 30 min with αprofilin-1 antibody (Cell Signaling), washed in PBS, incubated with FITC-labeled αp16<sup>ink4</sup> antibody (BD Bioscience) and αrabbit R-PE antibody (Invitrogen) for an additional 30 min at room temperature. After the final wash step, cells were resuspended in
500 μL of PBS and 2.5 μL of 7-AAD to measure DNA content. After 30 min of incubation at room temperature, samples were analyzed by flow cytometry. A minimum of 10,000 cells per condition were analyzed on a BD LSR II flow cytometer. Flow cytometry data were analyzed with appropriate gating and compensation controls using the software FlowJo (TreeStar, Inc.). Cell cycle analysis was performed on the FlowJo cell cycle analysis platform using the Dean Jett Fox model.

**Partial Least Square Modeling**

Statistical modeling was performed using the SIMCA-P software (Umetrics). All signals were mean centered and unit variance scaled prior to analysis to allow all variables to be considered equally scaled in principal components (27). The data set was divided into two matrices: Y ∈ \( \mathbb{R}^{18 \times 2} \) consisting of measures of age in culture (dependent variable block), and X ∈ \( \mathbb{R}^{18 \times 140} \), denoting the measured protein phosphorylation signals as well as surface markers, cell morphology and intracellular proteins (independent variable block). Instantaneous derivatives of the signaling dynamics, corresponding to the slopes of the phosphorylation dynamics signals, were also added to the X block. Table S1 in the supplementary information recapitulates the variables assayed for each observation. PLSR can accommodate data sets that are not fully complete (i.e., yielding matrices that are not of full rank), providing the missing values are randomly distributed. The quality of a PLSR model can be summarized by two primary metrics: how well it is able to mathematically reproduce the data of the training set (given by the parameter \( R^2_Y \)) and how reliably it can predict the next experiment’s outcome (given by \( Q^2 \)). A good \( Q^2 \) is considered to be above 0.5.
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\[ R^2_Y = 1 - \frac{RSS}{SSY_{tot.corr}} \]  \hspace{1cm} (2)

\[ Q^2_Y = 1 - \frac{PRESS}{SSY_{tot.corr}} \]  \hspace{1cm} (3)

where RSS represents the residual sum of squares of predicted Y, SSY_{tot.corr} the total variation in the Y matrix after mean centering and scaling and PRESS, the predictive residual sum of squares defined as

\[ PRESS = \sum(y_{im} - \bar{y}_{im})^2 \]  \hspace{1cm} (4)

calculated by cross validation. The appropriate number of components is defined as the optimum trade-off between goodness of fit and predictive ability. For more detailed description on PLSR modeling we refer to a previous explanation (28).

Results

**Microfluidic chip design and operation**

Figure 1 illustrates the single-layer, modular microfluidic chip capable of precisely stimulating suspended cells and subsequently lysing and fixing the flowing cells in parallel. The two-module approach enables flexibility in the time points sampled, while keeping precise control over the stimulation time (Fig. 1a). In the first module, cells and soluble anti-CD3 and anti-CD28 antibody stimuli are mixed, and split into 8 different channels, corresponding to 8 different time points sampled (20) (Fig. 1b). Rapid mixing is achieved by chaotic mixing using the staggered herringbone mixers (SHM) in the channels (Fig. 1d) (20, 29). We have previously showed that eight cycles of herringbones are sufficient to mix the solutes completely (20), but we observe that this configuration leads to a predictable cell focusing pattern in specific outlets, insensitive
to the additional number of cycles of SHM used or the Reynolds number. To redistribute cells more evenly to facilitate the downstream cell and lysate collection, grooves with randomizer geometries and a large splitting area were added after 9 cycles of herringbones. A flow rate of 44 μL/min was chosen as it led to the most uniform cell distribution in the 8 outlets (Fig. 1b).

The second module receives the cells that have been stimulated for the desired time in the tubings. The length of the tubings was determined using the following formula:

\[ L_T = \frac{t \times Q}{\pi \times R_T^2} \]  

(5)

where \( L_T \) represents the length of a tubing, \( t \) represents time spent in the tubing, \( Q \) is the volumetric flow rate and \( R_T \) corresponds to the radius of a tubing. Time spent in the tubing is defined as

\[ t = t_{desired} - t_r \]  

(6)

where

\[ t_r = \frac{L \times H \times W}{Q} \]  

(7)

represents the time spent in the first module and in the pressure drop channels with rectangular cross sections (\( L, H \) and \( W \) are respectively the length, height and width of the channels) and \( t_{desired} \) is the total stimulation time desired.

The pressure drop channels ensure all cells are maintained at the same flow rate (Fig. 1c). The circular geometry of those channels minimizes shear forces that cells are subjected to. The pressure drop channels did not cause measurable inertial focusing or separation of cells. After stimulation, cells are split into two equal populations for lysis or fixation to quench the
reaction. Thus, one single experiment yields 8 lysates and 8 fixed cell populations for 8 different time points, from 0.5 minutes to 7 minutes. The modularity of the chip gives the possibility to sample at various time points but since we were interested in sampling early dynamics after TCR engagement, we chose to only measure protein activation within 7 minutes of stimulation. Each of the eight lysates was analyzed for six proteins; therefore each experiment resulted in 48 dynamic measurements.

Figure 2 compares the dynamics of ERK phosphorylation acquired by flow cytometry and Luminex bead-based assay for different donors and different times in culture. The trends of the signaling dynamics are conserved between both methods (Fig. 2a-b) with a Pearson’s correlation coefficient $R = 0.61$ (Fig. 2c). Changes in the sensitivity, and/or specificity of the antibodies used in both assays, as well as differential inactivation of phosphatases in the two sample acquisition methods (30) might explain differences observed in phosphorylation levels between those two assays. In addition, flow cytometry and bioplex analysis techniques have different dynamic range, indicated by the non-unity slope in the linear fit presented in Fig. 2c. Our data suggests that flow cytometry has a larger dynamic range than our Luminex bead based assay.

**Global characteristics of age-associated protein expression and activation changes in human CD8+ T cells**

Although aging of immune cells has been the focus of intense research, it is difficult to reconcile reports characterizing a variety of biomarkers over a range of culture conditions. By maintaining uniform expansion and sampling conditions, we obtained the data in figure 3 presenting trends
across multiple donors for limitations of population doublings, cell growth arrest, surface marker expression, and T cell activation dynamics. The final dataset consists of >2500 measurements generated from 4 donors.

**Human CD8+ T cells cultured with IL-2 and bead-based CD3/CD28 stimulation reach replicative senescence after 12 population doublings:** Primary CD8+ T cells, cultured with IL-2 and chronic bead-based anti-CD3, anti-CD28 stimulation, achieved 12.2 ± 0.9 population doublings, i.e. about 1,000-fold expansion, which is consistent with previous observations using this culture method (31, 32). After 12 population doublings, an increase in IL-2 stimulation or in the number of beads did not allow for further growth (Fig. S1). The cell population could be maintained in culture for several weeks with appropriate culture conditions (IL-2 stimulation and fresh media), suggesting the state of replicative senescence had been reached (33). At different stages in culture, cells were sampled in order to observe changes in cell morphology, cycle, phenotypical markers and signaling as they “aged” in culture (Fig. S1).

**Changes in cell morphology and cell growth arrest:** We observed a progressive decrease in cells in S/G2 phases as cells expand in culture, as others have reported (34). At the end of the proliferation phase, cells were found primarily in the G0/G1 phase of cell cycle (Fig. 4a). We also observed changes in cell size and shape as cells age through forward and side scatter detection by flow cytometry. Cell size progressively decreased with age and the variance in cellular shape increased (Fig. S2). Prior proteomic analysis of *in vitro* cultured CD4+ T cells identified profilin-1 as a potential biomarker for senescence (19); therefore this protein was included in our panel. However, no statistically significant changes in profilin-1 expression were observed in our CD8+
cell population as a function of days in culture (Fig. S2). Accumulation of the cell cycle checkpoint p16\textsuperscript{ink4} has been observed in senescent T cells (34, 35). However, in our study, the increase in p16\textsuperscript{ink4} expression during in vitro aging was found to be not significant (Fig. S2).

Changes in surface marker expression with time in culture: Phenotypic markers of differentiation or co-stimulation have been associated with immunosenescence (14, 36-38). We observe a drop in CD28 expression (Fig. S3), from 84 ± 4% of CD28+ cells at PD 2 to 21 ± 3% at PD 12, consistent with replicative senescence (39). We also observe a decrease in CD27 expression (Fig. 4b and Fig. S3). Although the number of cells expressing the marker remains around 95%, there is a continuous decrease in mean fluorescence intensity, indicating a decrease in the number of costimulatory molecules present on the surface of each cell. After an initial drop in the number of CD57+ cells early in culture, the level of cells expressing CD57 remained constant (Fig. S3). We did not observe significant changes in the memory phenotype surface marker CD45RO (Fig. S3).

Overall decrease in protein activation following T cell receptor stimulation: Along with changes in surface marker expression, it has been proposed that T cell function decline with age could be due to the development of defects in the transduction of mitogenic signals following T cell receptor stimulation (40). From the lysates yielded by the microfluidic chip, we quantified the levels of phosphorylated CD3, Lck, Zap70, LAT, ERK and CREB within 7 min after T cell receptor ligation using a high-throughput, multiplex bead-based assay (20). T cell receptor stimulation led to an increase in the levels of phosphorylated proteins, with the magnitude and kinetics of activation dependent on the protein, the donor and the age of the cell population (Fig. 4c and...
Fig. S4). In general, a global decrease in the magnitude of the peak activation levels was observed (Fig. 4c and Fig. S4). Along with those lysates, the microfluidic chip provides fixed cells that have encountered the same stimulation conditions and that can be used for further single cell studies.

ERK activation has been shown to be essential in mediating proliferation and telomerase activation (41, 42) and displays digital or analog activation patterns depending on the strength and the nature of the stimulus (43-45); thus we investigated this protein by single cell analysis. Analog activation appeared with anti CD3-CD28 stimulation on our CD8+ T cell population throughout the duration of culture (Fig. 2d). This analog activation is consistent with observations by Singh et al. who also relied on anti-CD3/CD28 as the means of T cell activation (44). As with the lysate dynamics, an overall decrease in signaling was also observed with time in culture. We also measured the heterogeneity in cellular ERK activation within the population, as determined by the coefficient of variation of the histograms for each time point. Higher coefficients of variations are observed very early and late with respect to culture time (Fig. S5). Heterogeneity in the composition of the initial cell population isolated (naïve vs. effector vs. memory) might result in this high CV observed early in culture.

**Extraction of biomarker combinations of cellular age in culture and prediction of cellular age and quality:** Several biomarkers, such as CD28 and CD27, present modest correlation with time spent in culture; however their rate of change is very donor-specific and therefore individual markers are not sufficient to distinguish non-senescent populations with early-senescent
populations. We therefore sought a combination of markers suitable for quantifying age of a cell population. Hierarchical clustering is a common method used in microarray analysis to extract clusters of genes or proteins having similar response to the same environmental factors (46). Applied to our signaling and flow cytometry data, hierarchical clustering of protein phosphorylation time courses suggested higher phosphorylation values in “young” cells (Fig. 3). However, the clusters are very donor-specific. Combinations of biomarkers consistent for all donors do not emerge. In addition, this cluster analysis technique, very useful to interpret large dataset and group “like” variables, cannot convey quantitative contributions of markers with predictive power of T cell “age” or quality. Therefore, we applied further analytical methods for extracting markers of aging and their relative contribution to aging that would be universally predictive across donors.

*A combination of dynamic signaling and expression metrics (Lck, ERK, CD28 and CD27) are the most informative markers in predicting cellular age*

To relate cell age to network activation and phenotypic markers, we constructed a PLSR model. This data-driven modeling approach allows for complexity reduction in multivariate protein expression and signaling data to identify the most informative variables, and has been previously used to predict cellular fate or cytokine production (47, 48). The 140x18 data matrix was parsed by defining the dynamic activation profiles of 6 proteins phosphorylated after T cell receptor ligation (time-course measurements and instantaneous derivatives), cell morphology, cell cycle, costimulatory and differentiation surface markers, and heterogeneity in the population for the flow cytometry data all as independent, predictor variables (i.e. X-block) in
our data matrix (Fig. 3 and Supplemental Table 1). The number of days spent in culture or number of population doublings were defined as variables that depend on the X-block (i.e. Y-block) for regression purposes. The principal components derived by the PLSR model contain linear combinations of the predictor variables optimized for maximum covariance with the dependent outcomes. From this initial optimization, a pruning step was implemented by removing variables with both low importance in the projection and high uncertainty as determined by jack-knifing. The resulting 71x18 data matrix was fitted with a 3-component model with $R^2_Y = 0.96$ (goodness of fit). The model captures variance in the data with a $Q^2 = 0.78$, a measure of the cumulative fraction of the total X-block variation that can be predicted by all components. Individual observation sets, defined as all measurements for a specific donor for a specific day in culture, were mapped onto the first two principal components via their scores to determine how observations from different experimental conditions influenced the overall age prediction. Samples from late days in culture segregate with positive PC1 loadings, suggesting that the first component can be coarsely defined as an “age” axis (Fig. 5a). This first component can effectively estimate cell age with a $R^2_Y = 0.667$, $Q^2 = 0.53$. The second component captures additional 23% of the variance in the data.

Clusters of predictor variables that are highly correlated with outcome variables can be visualized by their proximities to one another on the weight plots in principal component space (Fig. 5b). Signaling information is mostly anti-correlated to age, consistent with the general understanding of altered signaling dynamics. An analysis of the weight of each variable highlights the importance of the proteins ERK, Lck and LAT for predicting cellular age (Supplemental Table 2-3). CD27 and CD28 expression, and number of cells in the G2/M cycle.
phase appear to be heavily negatively weighted in the first component. Somewhat surprisingly however, the heterogeneity of the cell population in cellular shape, the heterogeneity in CD57 expression, and the basal level in phosphorylated ERK emerged as being highly positively correlated with cellular age (Fig. 5c). The heterogeneity in ERK phosphorylation in stimulated cells emerges as important in the second component (Fig. 5d). P16\textsuperscript{ink4} and the percentage of cells in the G1 phase have the largest influence in the 3\textsuperscript{rd} component (not shown) and are positively correlated to age. This comprehensive model is capable of accurately predicting day in culture and number of divisions with regression coefficients (R\textsuperscript{2}Y) of 0.91 and 0.98 respectively (Fig. 5e-f). The robustness of the predictions was tested by iteratively omitting one donor set at a time and re-applying the algorithm on the remaining three donor sets. The regression is performed on the mean of four different predictions; errors in the predicted value generated in this manner (Fig. 5e-f) reflect variation in the ability of the model to be applied to new donor datasets that it has not been previously been trained on.

**Signaling information alone is sufficient for aging prediction:** While surface markers are commonly used as metrics of aging, to date a multivariate characterization of replicative senescence as a function of signaling dynamics has not been attempted. We sought to determine if a model containing only signaling information from lysates and flow cytometry enabled cell age prediction. The X-variance captured by this reduced model is lower than in the model previously described, with a Q\textsuperscript{2}=0.54; however this model is still able to accurately predict the number of days since isolation or the number of population doublings with a regression coefficient R\textsuperscript{2}Y of respectively 0.84 and 0.94 (Fig. 6). As in the previous model containing all types of data, the first component is loosely partitioned as a measure of age (Fig. 6).
6a). The instant derivatives of signaling at very early time points enabled by the fast sampling of the microfluidic device are important in this model (Supplemental Tables 4-5); the instant derivative of ERK phosphorylation after one minute of stimulation emerged as the most significant anti-correlated variable while the model also extracted the instant derivative of Zap70 phosphorylation at 1.5 min as a significant predictor. A simple multiple regression based on just ERK and Zap70 metrics enables age prediction with $R^2 = 0.65$. The most heavily weighted signaling variables have been sampled between 1 and 3.5 min after stimulation, pointing to the importance of very early signaling dynamics (Fig. 6b and Supplemental Table 4). An analogous modeling exercise was performed with only surface marker data (Fig S6). CD27 and CD28 expression, as well as CD57 coefficient of variation emerged as the most informative metrics. The PLSR prediction of days in culture was improved in the signaling model ($R^2_Y = 0.84$ vs. 0.78) but slightly less accurate in predicting population doubling ($R^2_Y = 0.95$ vs. 0.98).

**Signaling information can predict co-stimulatory molecule expression:** CD27 co-stimulation with TCR enhances cell expansion and promotes cell survival (49). The co-stimulatory molecule CD28 is involved in T cell receptor signaling amplification by PI3K activation and downstream calcium mobilization (50) as well as indirect ERK activation via Lck (51, 52). Therefore, the loss of CD28 and CD27 with cellular age would be expected to be directly correlated to altered T cell receptor downstream signaling. To test these relationships, we built a model that predicts phosphorylation levels from surface protein expression markers. This model behaved poorly ($R^2_Y=0.27; Q^2=0.1$). The expression of CD27 and CD28 is not sufficient to accurately predict any of the signaling variables. In contrast, a model based on signaling time courses and regressed against costimulatory surface marker expression is able to successfully predict both CD28 and
CD27 surface expression with a correlation coefficient ranging from 0.75 to 0.91 (Fig. 7c-e). CD27 mean fluorescence intensity is highly correlated to the instant derivative of ERK phosphorylation after one minute of stimulation and anti-correlated to the slope of deactivation of CREB after 2.5 min of stimulation. Lck phosphorylation is strongly correlated to CD28 expression and the rate of decay in LAT phosphorylation after 3.5 min of stimulation is strongly anti-correlated to CD28 expression. Globally, CD28 expression clusters with early dynamics (0.5-3.5 minutes) of ERK, Lck and LAT (Fig. 7b).

Discussion

The outcome of immune based therapies, such as adoptive T cell transfer therapy or engineered vaccines, is dependent on the quality of T cell clones used (11). Progress has been achieved towards improved T cell expansion methods in the past few years (53): new culture media as well as improved stimulation techniques have been developed (54-56), and cord blood as been harnessed as a source of non-senescent lymphocytes for tumor immunotherapy (57). To assess the quality of expanded cells, researchers/clinicians generally perform functional assays (e.g. cytokine production or cytotoxic T-lymphocyte (CTL) assays (58)) or examine surface marker expression, such as the extent of CD28 loss or the appearance of late differentiation markers such as CD57 (37). Univariate, static assays measuring expression of surface markers alone are not always accurate for prediction of cell functionality. While CD28 is considered as a biomarker of immunosenescence, its expression can be downregulated by tumor-necrosis factor (TNF) (59, 60) or upregulated by IL-12 in CD4+ cells (61). It was also shown that CD8+CD28- cells are able to proliferate (62) and therefore the loss of CD28 is not necessarily
associated with senescence. There is also contradictory evidence concerning loss of the co-stimulatory molecule CD28 and altered downstream signaling. Larbi et al. suggested that differential localization of CD28 in lipid rafts and not the actual number of receptor could explain disparities in response to stimulation for CD4+ cells (63). Hence, CD28 alone cannot be considered as a direct marker of senescence, and combinations of biomarkers need to be considered to quantitatively predict the level of senescence in a population. To measure T cell response, different assays exist to measure early (calcium influx, protein phosphorylation within a few minutes after stimulation), intermediate (degranulation or cytokine production) or late functions (proliferation and apoptosis) (64). Although cytolytic assays are very informative in assessing cell functionality, they rely on bulk population measurements which may mask deficiencies in subpopulations and usually require additional culture preparation of target cells (65). The analysis of signal transduction protein phosphorylation after T cell receptor stimulation can be used as a proxy for cell functionality, and offers insight on mechanistic details (41, 66). Flow cytometry can provide additional information regarding the heterogeneity of the early phosphorylation events in T cell signaling, while lysates provide multiplexed capabilities of many measurements at once. Although a combination of the two acquisition methods provided the optimal model, our results suggest that signaling information alone is comparable in prediction of cellular age and can also diagnose costimulatory surface marker expression. In contrast, the reverse is not true: while surface markers alone provide information on cellular differentiation state and can predict cell age, they cannot predict protein phosphorylation changes as a measure of cell functionality (data not shown).
Determining clusters of biomarkers and aging quantification is largely facilitated by the development of computational models, and several efforts to model and quantify cellular age in various cellular model systems have been published recently. Lawless et al. developed estimates of fraction of senescent cells in human and mouse fibroblasts using growth curves and candidate markers, such as p21 or DNA damage foci loci in paraffin-embedded tissue sections (67). Tsygankov et al. created a stochastic model that links p16\(^{inkd}\) expression with aging (68). Proteomic analysis of elderly patient-derived T cells expanded \textit{in vitro} to senescence used statistical discriminant analysis to identify potential biomarkers (19). Each of these prior studies has relied on static information from the cells rather than functional dynamics of TCR activation. As there is no consensus for the best biomarkers of \textit{in vitro} aging for CD8+ T cells, we chose to utilize a multivariate approach, combining surface phenotype of CD8+ lymphocytes and intracellular signaling.

In this work, we developed a microfluidic tool and a statistical model to evaluate cell responsiveness and accurately predict cell “age” and quality respectively. It has been observed that T cells are able to respond to stimuli within seconds, such as TCR ligation initiating a burst of calcium (69). The design of the microfluidic device enabled sampling of the rapid protein phosphorylation dynamics in the first few minutes following stimulation with minimal standard error, and allowed us to obtain accurate measurements in a high-throughput manner. As shown previously, chaotic mixing and flow in narrow channels at low Reynolds number does not elicit adverse stress response (20). The ability to reproducibly sample stimulated cells with 30-90 second intervals enabled the use of instant derivatives as additional variables in the regression model; thus, the microfluidic device provided an additional benefit in enhancing the...
information content from the signaling data. Although others have reported derivatives of time courses to be less informative in PLSR analysis (27, 47, 48, 70, 71), these studies relied on signaling dynamics that extended for hours rather than minutes. Because the device can stimulate a small number of cells with high temporal resolution and subsequently lyse and fix them in parallel, we could seamlessly “stitch” together complementary measurements for our statistical analysis from populations of cells treated identically, not only from day to day, but also within a particular stimulation experiment. Because donor-to-donor variability is a confounding factor in deriving robust biomarkers of senescence, the technological platform minimizes the experimental data variance so that meaningful dimension reduction could be performed, as shown by the conserved trends of signaling of phosphorylated ERK (Fig. 2). Cheong et al. have developed a microfluidic device able to measure time courses of signaling responses to continuous or with wave form soluble stimuli with immunofluorescence on chip on adherent cells (22). In contrast to our design that performs off-chip biochemical and cellular analysis, their immunostaining was performed on chip. Their device provided single-cell data on fixed cells, similar to live cell imaging and could be used to discriminate single cell versus population signaling dynamics. As with flow cytometry or microscopy, however, one is limited by the number of proteins that can be measured simultaneously. With the microfluidic chip presented here, the simultaneous fixing and lysing of cell populations which have encountered the same environmental conditions allowed us to use multiplexed capabilities of lysates while being able to probe for the heterogeneity in ERK phosphorylation in aging populations with flow cytometry. This technique could also prove useful in future applications for distinguishing
signaling in different subcellular compartments, e.g. nuclear transcription factors, not directly accessible with detergent-based lysing.

We developed multiple partial least square regression models to explore properties of the data and be able to predict physiological age of ex vivo expanded cells. PLSR models have been applied previously to understand complex signaling networks involving multiple inputs and multiple outputs, without prior knowledge of the network structure (27, 47, 48, 70, 71). Prior application of this type of modeling approach has demonstrated that antigenic information content is encoded within downstream phosphorylation events in T cells (48). This data-driven modeling technique is particularly well suited to carry multivariate analysis and predictions in extensive datasets, and therefore could be applied to extract correlations between age, signaling, and surface phenotype expression. From the analysis, CD28 and CD27 expression emerged as key markers in determining cellular age and also correlated strongly to intracellular phosphoprotein dynamics. Interestingly, the model did not find any positive correlation between the effector memory surface markers CD57 and CD45RO and age in culture, contrary to a previous observation (37). This might be due to the culture conditions specific to each study. It has been reported that the anti-CD3 and anti-CD28 presentation as used here preferentially expand CD45RO- cells (55). CD57 is a marker associated with the end-stage T cell differentiation. Elevated expression of CD57 has been observed on tumor-specific T cells. It may be the consequence of persistent chronic antigen stimulation, resulting in the accumulation of cells capable of rapidly secreting cytokines but that lack proliferative capacity (37). IL-2 supplementation may have induced an increased loss of CD28 expression (72), blocking cell proliferation before CD57 upregulation. Thus, this model is limited in scope to the
culture conditions assayed yet provides a generalizable approach that could be used for other expansion methods.

Our model emphasizes the importance of early signaling dynamics (1-3.5 min after TCR engagement) to explain and predict cellular age. Others have reported alterations in early signaling events in old mice (73) and in human T lymphocytes from elderly patients (74), furthering the common characteristics between ex vivo culture expansion and immunoosenescence. Three major proteins, Lck, LAT and ERK, were extracted from the model as having primary roles in accurately predicting cellular age. Lck is the first protein to be phosphorylated after TCR engagement, leading to phosphorylation of the adaptor protein LAT via Zap70, and downstream phosphorylation of ERK. Impaired redox regulation (75), reduced calcium release (73, 76) and altered membrane rafts composition (77) are possible explanations for the impaired activation of those proteins.

Previous studies have reported higher cell-to-cell variation with increasing age (78, 79); however the modeling results suggest that this trend is more nuanced and depends on the protein in question. We find that the heterogeneity in CD57 protein expression and cellular shape is correlated with cellular age. In contrast, no direct correlation between age and cell-to-cell variation of ERK phosphorylation was discovered (Fig. S5), and yet as part of a multivariate analysis this CV variable still possesses a high predictive power. This suggests robustness in ERK response to intracellular noise created with aging (80).

The multivariate PLSR model is able to assess the age and therefore the quality of a cell population for cells expanded using CD3-CD28 bead stimulation with IL-2 supplementation. Past
multivariate models associated signaling information with cellular fate (70), cytokine production (48), or drug response (81). These models enabled the creation of new hypotheses to test the validity of the model. In this application, aging is not a cell fate that we can modify and the biomarkers extracted are not correlated in a causal fashion to the age of the cells. This lack of a testable mechanism poses a limitation to the study; however, the model yields novel insight on the most informative markers of the array selected for sampling, and predicts cellular age from those specific markers. We also envision this model to be a possible diagnostic tool to quantify immune age of elderly individuals or individuals presenting accelerated immunosenescence, such as HIV patients.

In summary, the design of a novel microfluidic chip enabled the statistical analysis of T cell aging by minimizing error in the sample handling between days and across multiple donors. We took advantage of chaotic mixing geometries and a modular chip design to capture the early phosphorylation events associated with TCR ligation, which in turn proved to be highly informative in the partial least square regression prediction of population doubling and days spent in culture. Our findings point to a cell signaling-based assessment method that could quickly evaluate patient-derived cells for degree of population doubling. The general approach described here, combining fixation and lysing on-chip, can facilitate the integration of single-cell information with population-averaged techniques such as multiplexed immunoassays or mass spectrometry.

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Figure 1: Microfluidic Device for cell stimulation and simultaneous fixing or lyzing. a) Schematic of the two-module microfluidic chip. Module 1 receives the cells (1) and soluble stimuli (2), mixes these solutions and splits in 8 equals outlets. Module 2 is linked to module 1 by 8 tubings of different lengths and diameters and receives the cells in the circular pressure drop channels. Stimulated cells are mixed either with a fixing solution, here formalin (3) or lysis buffer (4). Lysates (5) and fixed cells (6) can be collected. b) Superposition of inverted bright field images showing cell distribution in the splitting area of module 1 at 44 µL/min. White dots are individual cells. c) Spiral shaped pressure-drop channel and splitting area for cell fixation or lysis (colors are from food coloring dye solutions that fill the channels). d) Mixing of cells (yellow) with lysis buffer (blue) or formalin (green).
Figure 2: Dynamics of ERK phosphorylation measured by flow cytometry on fixed samples and bead-based immunoassay on lysates. a-b) Representative traces of ERK phosphorylation for donor 2 at day 11 and 13. Flow cytometry and lysate data are normalized so that the mean value of the time course is equal to 100. c) Linear correlation of ERK phosphorylation between fixed and lysed samples. Data with residuals larger than expected in 90% of the observation were considered as outliers and removed from the regression. d) Representative flow cytometry time course of ERK phosphorylation (also displayed in 2a) showing analog activation.
Figure 3: Complete dataset. For each donor, dendrograms were generated with signaling measurements from lysates (left and middle), protein phosphorylation quantification and instant derivatives of those measurements. Flow cytometry dendrograms (right) contain ERK phosphorylation, cell cycle, cell morphology, surface marker, profilin-1 and p16^inkd expression. Each row of 3 dendograms corresponds to a different donor. In each dendogram, a row corresponds to a sampled time. Each column corresponds to a particular variable measured.
Figure 4: Cell cycle, surface marker expression and signaling trends over time in culture. a) Percent of cells in the G0/G1 phase determined by the Dean Jett Fox model. Restimulation with anti CD3, anti-CD28 was performed at passage 6 and 10. b) CD27 expression over time in culture (representative donor data). c) Lck and pErk activation profiles over time in culture. The green line corresponds to the mean of our 4 donors. The box plot represents the median, the 25th and 75th percentile as well as outliers.
Figure 5: Cellular age prediction with signaling measurements and phenotypic markers using a multivariate regression model. a) Loading plot of the observations in the reduced principal component space. b) Score plot of the predictor variables on the principal components space. Y-variables are highlighted in red box. c-d) Weight of the most important variables (weight>0.4) in the first (c) and second component (d). e-f) Prediction of day (e) and number of divisions in culture (f).
Figure 6: Cellular age prediction from signaling data only. a) Loading plot of the observations in the reduced principal component space. b) Score plot of the predictor variables on the principal components space. c-d) Prediction of day (c) and number of divisions in culture (d).
Figure 7: Surface marker prediction from signaling information during the aging process. a) Loading plot of the observations in the reduced principal component space. b) Score plot of the predictor variables on the principal components space. c-e) Prediction of the percentage of CD28+ cells (c), and CD27 (d) and CD28 (e) mean expression.