Utilizing the Multiparameter Capability of the ZE5 Cell Analyzer to Monitor T-Cell Exhaustion Following Immunotherapy

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Abstract

The immune system has evolved sophisticated mechanisms to distinguish between self and nonself, and dangerous and nondangerous signals leading to appropriate and controlled immune responses against infection and cancer or tolerance to avoid attacking the host body. One pathway of immune suppression is T-cell exhaustion, which shuts down effector T cells. T cells are immune cells that help protect the body from infection and fight cancer. T-cell exhaustion is exploited by chronic viral infections and cancers to escape being targeted by the immune system. It results in a sustained expression of suppressive cell surface markers and effector function in these cells, meaning they are no longer able to function to remove the cancerous and infected cells. Recently, researchers have focused on using monoclonal antibody drugs to block these suppressive cell surface markers and reinvigorate the T cells, restoring their effector capability to clear viral infection and cancer cells from the body.

In this study, we use a 16-parameter panel to examine in-depth changes to the T-cell phenotype, exhaustion, and cellular subset identity markers before and after chronic stimulation and in combination with a monoclonal antibody drug treatment targeting the programmed cell death protein 1 receptor.

Introduction

Genetic alterations to cancer cells provide epitopes that would normally allow the host immune system to target and remove the damaged cell. However, adaption and mutation by the cell can allow it to circumvent the immune system and potentially form a tumor. T-cell activation and regulation is a complex balance between stimulatory and inhibitory signals mediated via the type of inflammatory cells present, antigen concentration, and the cytokine environment. The tumor environment is inherently immunosuppressive with impaired antigen presentation leading to insufficient T-cell stimulation, high concentrations of inhibitory cytokines, and mechanisms of evasion of immunosurveillance. This environment can lead to T-cell exhaustion. Exhausted T cells express high levels of inhibitory receptors, including programmed cell death protein 1 (PD1), cytotoxic T-lymphocyte antigen 4 (CTLA4), lymphocyte activation gene 3 (LAG3) protein, and T-cell immunoglobulin and mucin domain–containing 3 (TIM3) protein. Blocking these immune checkpoint molecules with monoclonal antibodies, thereby reactivating the T cells, has been used successfully to treat a variety of cancers including melanoma, non–small-cell lung cancer, lymphoma, and others as well as potentially treating chronic infections, such as human immunodeficiency virus (Lee et al. 2015, Messal et al. 2011).

The objective of this study was to design and test a multiparameter flow cytometry panel for the ZE5 Cell Analyzer that could be used in an in vitro immunotherapy model to interrogate a range of biologically relevant markers to T-cell activation and immunotherapy targets that are currently being investigated. In addition, we surveyed a panel of long noncoding RNAs (lncRNAs) for differential gene expression of inflammation-related targets using Bio-Rad's lncRNA workflow, which is a reverse transcription quantitative PCR (RT-qPCR) workflow optimized for long noncoding RNA discovery and validation.

Materials and Methods

Pan T cells, obtained as frozen samples from AllCells, LLC, were thawed and cultured overnight in RPMI + 10% FBS at 37°C, 5% CO_2. Following a viability cell count using the TC20 Automated Cell Counter (Bio-Rad Laboratories, Inc.), cells were either stained with the T-cell phenotyping antibodies in Table 1 for day 0 control or transferred to a 6-well plate for stimulation and drug treatment experiments.

T-cell stimulation — T cells at a concentration of 2–3 x 10^6 cells/ml were stimulated with Dynabeads Human T-Activator CD3/CD28 according to the manufacturer's instructions (Thermo Fisher Scientific Inc.) with or without nivolumab (Selleck Chemicals), a monoclonal antibody targeting PD1 (20 and 40 µg/ml). Medium was changed every 48 hours with fresh drug being added. T cells were stimulated for 4 and 13 days. At each medium change, cells were recounted and resuspended at 2–3 x10^6 cells/ml.

T-cell phenotyping — 5 hours before harvesting, cells were exposed to 1x Brefeldin A (Bio-Rad) to inhibit protein transportation. At 0, 4, and 13 days cultured pan T cells were washed in PBS and any CD3/CD28 beads were removed with a magnet. T cells were then treated with VivaFix 353/442 Cell Viability Assay (Bio-Rad), and stained with antibodies against cell surface molecules for 30 min in the presence of BD Fc Block (BD Biosciences). Cells were then stained for internal cytokines using the True-Nuclear Transcription Factor Buffer Set (BioLegend), and finally resuspended in BD Horizon Brilliant Stain Buffer (BD Biosciences). The panel was fully validated with single stains and fluorescence minus one (FMO) controls (Table 1). Compensation panels were developed with AbC Total Antibody Compensation Beads (Thermo Fisher Scientific). Flow cytometry measurements were carried out on a ZE5 Cell Analyzer (Bio-Rad) and analysis was performed using FlowJo Software 10.0 (FlowJo, LLC).

lncRNA analysis — we customized the PrimePCR lncRNA Inflammation Panel (Bio-Rad) to include two additional targets: HULC and LINC01158 (and removed RP11-282O18 and BX322557.10). We used iScript Explore One-Step RT and PreAmp Kit (Bio-Rad) to remove genomic DNA, generate cDNA, and preamplify 96 targets and controls from each RNA sample in duplicate 50 µl reactions. Next, each sample (collected at the time points above) was treated in a one-step RT and preamplification (14 cycles) in a reaction mix consisting of 1x SsoAdvanced PreAmp Supermix, iScript Explore Reverse Transcriptase, iScript Explore Reaction Booster, Inflammation lncRNA PreAmp Pool, and PreAmp Assays for HULC and LINC01158 (all Bio-Rad). Each PreAmp reaction was diluted 1:10 in TE buffer, and 1 µl was used for each 10 µl qPCR reaction in 1x SsoAdvanced SYBR® Green Supermix (Bio-Rad). Four replicates were run for each target and sample. Two CFX384 Touch Real-Time PCR Detection Systems were run simultaneously using the CFX Automation System II in conjunction with CFX Maestro Software v1.1 (all Bio-Rad).

Table 1. Fluorophores used in flow cytometry panel.

Laser	Filter	Target	Fluoresent Tag	Isotype	Source
355	700LP	CD25	BUV737	IgG1κ	BD Biosciences
355	447/60	VivaFix 353/442	VivaFix 353/442	N/A	Bio-Rad
355	387/11	IFN-γ	BUV 395	IgG1	BD Biosciences
405	420/10	LAG3	Brilliant Violet 421	IgG1κ	BioLegend
405	525/50	CD28	Brilliant Violet 510	IgG1κ	BioLegend
405	615/24	TIM3	Brilliant Violet 605	IgG1κ	BioLegend
405	670/30	CD127	Brilliant Violet 650	IgG1κ	BioLegend
405	720/60	IL-2	Brilliant Violet 711	IgG2aκ	BioLegend
405	460/22	CD4	Pacific Blue	IgG1	Bio-Rad
488	750LP	TNF-α	PE-Cy7	IgG1κ	BioLegend
488	525/35	PD1	Alexa Fluor 488	IgG1	Bio-Rad
561	615/24	CCR7	PE–Dazzle 594	IgG2aκ	BioLegend
561	577/15	CTLA4	PE	IgG2a	Bio-Rad
561	720/60	CD3	PE-Cy5.5	IgG1	Bio-Rad
640	670/30	CD45RA	Alexa Fluor 647	IgG1	Bio-Rad
640	720/60	CD8	Alexa Fluor 700	IgG1	Bio-Rad

Results

Results are shown in Figures 1–4.

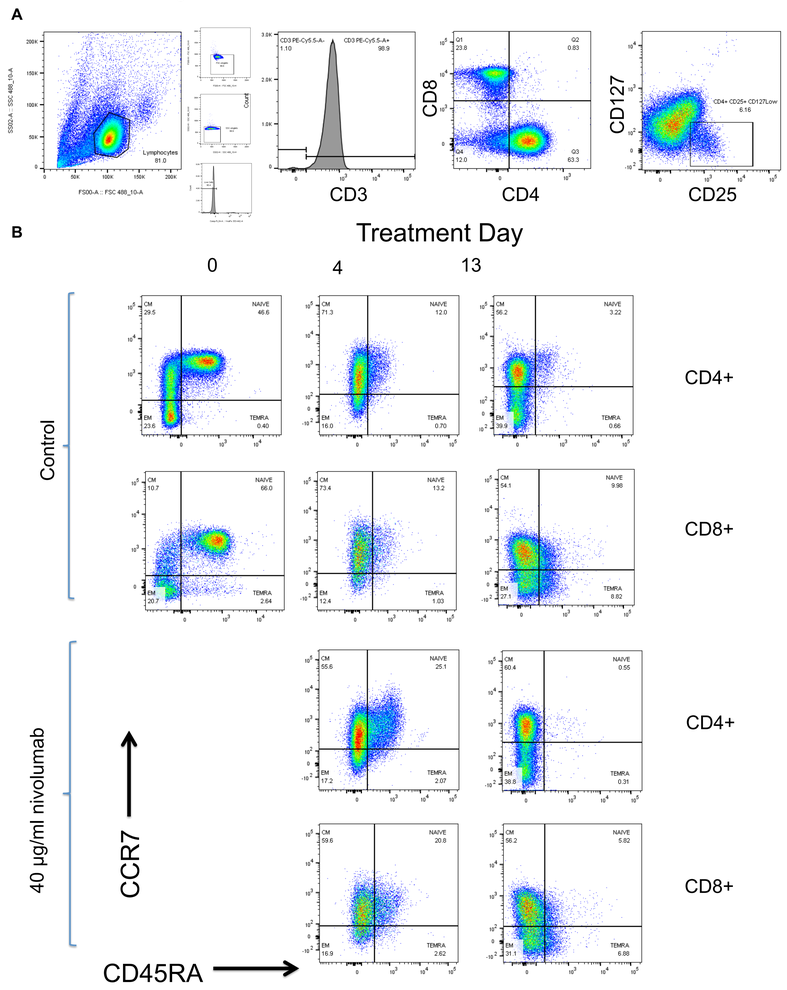

Fig. 1. Gating strategy for interrogating T-cell subsets. A, CD4/CD8 discrimination as well as identifying CD4+ CD25^{high}/CD127^{low} are shown; **B**, variation in naïve, central memory (CM), and effector memory (EM) T-cell subsets following stimulation with CD3/CD28 activation beads at days 4 and 13 with and without 40 µg/ml nivolumab.

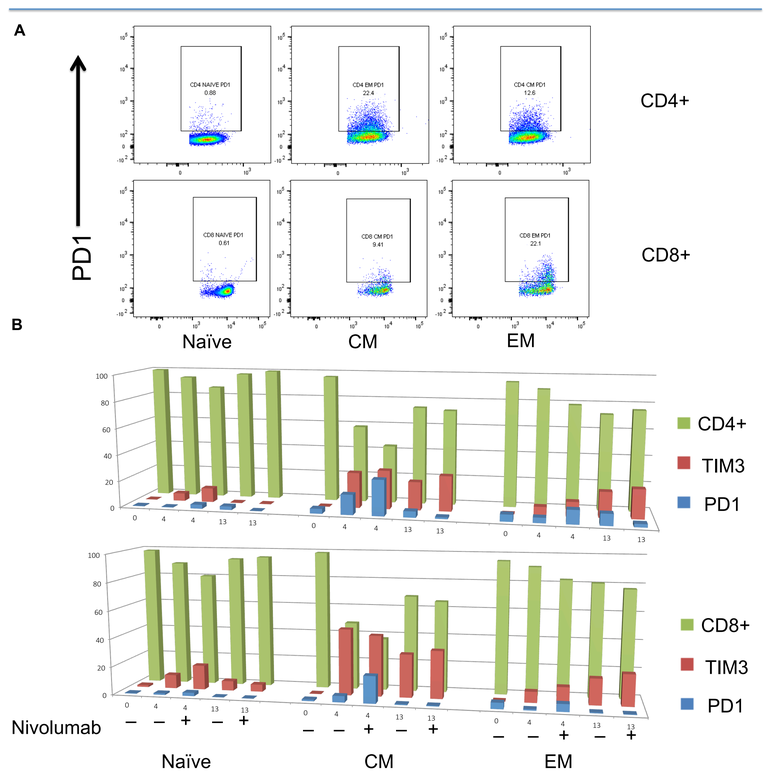

Fig. 2. Determination of exhaustion marker expression. PD1 expression is altered in the T-cell subsets. **A**, changes in expression of PD1 and TIM3 were observed over time in T-cell subsets following continued activation; **B**, these changes were altered following treatment with nivolumab. We observed no changes to CTLA4 or LAG3 expression at these time points. We saw no effect on CD28 expression following treatment with nivolumab.

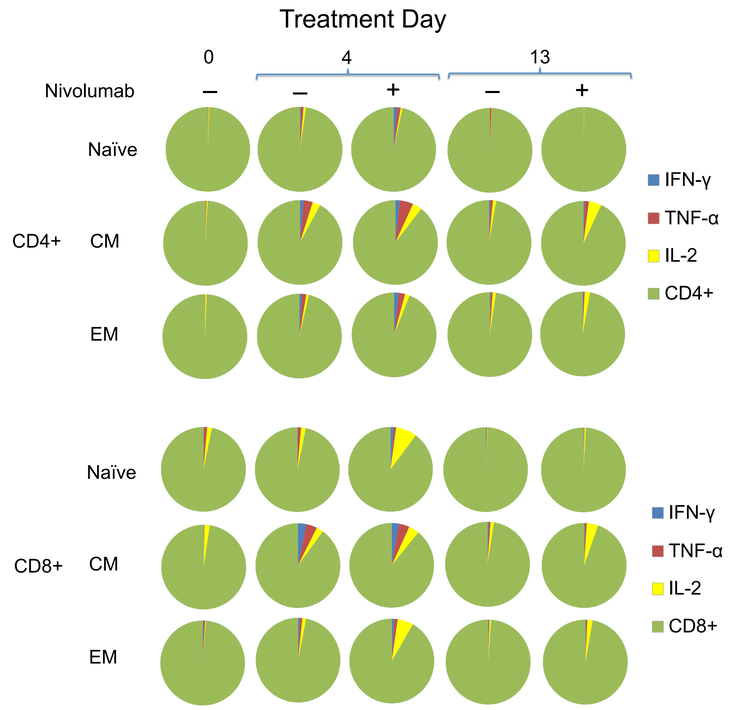

Fig. 3. Cytokine levels in T-cell subsets as frequency of CD4+ and CD8+ T cells. Expression of interferon gamma (IFN-γ), tumor necrosis factor alpha (TNF-α), and interleukin 2 (IL-2) at 0, 4, and 13 days after stimulation with CD3/CD28 activation beads in the presence or absence of 40 µg/ml nivolumab.

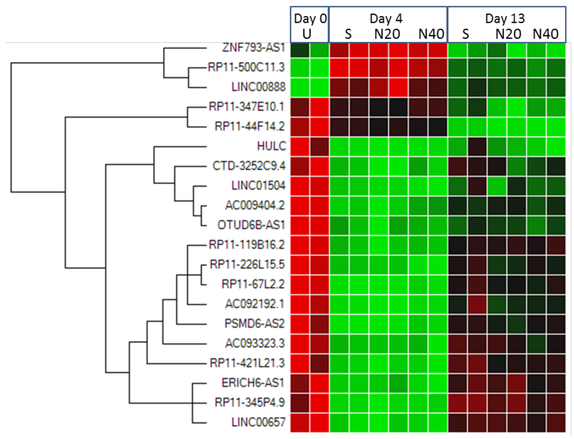

Fig. 4. Clustergram of lncRNAs expressed in unstimulated (day 0, U) and pan T-cell population following stimulation with CD3/CD28 activation beads (days 4 and 13). Eighty-six lncRNA targets were screened: 30 were not expressed, 18 were not expressed in unstimulated pan T cells, and 18 were expressed in all samples but were omitted from this analysis due to technical variation. The twenty targets shown here had *P* values less than 0.05 in stimulated samples (with and without drug) compared with unstimulated controls. No marked differences in lncRNA expression were observed in cells treated with either 20 ng or 40 ng nivolumab (N20 and N40) compared with the corresponding stimulated but untreated sample (S). However, several patterns were observed in comparing unstimulated pan T cells with the pan T cells stimulated for 4 and 13 days. The majority of the lncRNAs shown here were expressed at relatively high levels in unstimulated cells, downregulated at day 4, and then somewhat upregulated at day 13. Fewer lncRNAs followed the pattern of high expression in unstimulated cells, showing a drop at day 4 and a further drop at day 13. Another small group of targets was observed to be expressed at very low levels in unstimulated cells, with more highly expressed targets at day 4 and then lower levels at day 13.

Summary

- Stimulation of pan T cells with CD3/CD28 beads results in changes to naïve and memory T-cell population subsets that are affected by treatment with anti-PD1 mAb
- CD3/CD28 stimulation of pan T cells results in changes to checkpoint molecules PD1 and TIM3 but not CTLA4 and LAG3 at the time points measured. Nivolumab treatment resulted in an altered cytokine profile in both CD4 and CD8 central memory T cells
- Mutlicolor flow cytometry with the ZE5 Cell Analyzer allows a thorough analysis of multiple checkpoint inhibitors and activation markers with limited sample. Such detailed cell population discrimination is an essential tool for research investigating personalized cancer and chronic infection therapies
- Several patterns of lncRNA expression were observed in the entire (heterogeneous) population of pan T cells stimulated for 0, 4, and 13 days. Isolation of the specific populations of T cells identified here would allow investigation of these lncRNA targets in T-cell activation, clonal expansion, and exhaustion

References

Lee J et al. (2015). Reinvigorating exhausted T cells by blockade of the PD-1 pathway. For Immunopathol Dis Therap 6, 7–17.
Messal N et al. (2011). PD-L2 is expressed on activated human T cells and regulates their function. Mol Immunol 48 , 2,214–2,219.

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