Utilizing the Multiparameter Capability of the ZE5 Cell Analyzer to Monitor T Cell Exhaustion and Effects of Immunotherapy

Chris Brampton, Angie Green, Kristin Lind, Laura Moriarty, Elizabeth Dreskin
Bio-Rad Laboratories, Inc., 2000 Alfred Nobel Dr., Hercules, CA 94547

Flow Cytometry

Bulletin 7125

Abstract

T cell exhaustion is a hyporesponsive state of T cells characterized by sustained expression of suppressive cell surface markers, decreased effector cytokines, and impaired cytotoxicity. Within the microenvironment of tumor cells or during chronic viral infections, T cells become exhausted, resulting in ineffective tumor and pathogen targeting by the immune system. High programmed cell death protein 1 (PD-1) expression is a major driver of T cell exhaustion and can be targeted by several drugs, which can reverse exhaustion and restore T cells' capabilities to clear viral infection and cancer cells from the body. This strategy of reawakening the immune system to fight disease is a growing field with new drugs and targets continuously being developed. The efficient monitoring of T cell surface markers, cytokines, and expression patterns is critical to the study of further immunotherapies. When working with limited patient samples, the ability to test multiple parameters simultaneously is highly valuable. Here, we successfully monitor in-depth changes to the T cell phenotype, exhaustion, and cellular subset identity markers using a 16-parameter panel developed for use with a 5-laser ZE5 Cell Analyzer. Using an in vitro model, we examined T cells before and after chronic stimulation, and in combination with a PD-1 receptor inhibitor, nivolumab.

Introduction

One of the hallmarks of cancer is its ability to evade the immune system. Although cancer cells do indeed have neoantigens, created through genetic aberrations, the tumor microenvironment is inherently immunosuppressive. Impaired antigen presentation, high concentrations of inhibitory cytokines, and mechanisms of evasion from immunosurveillance contribute to immune suppression and the uncontrolled growth of cancer cells. This environment pushes T cells into a state of exhaustion, in which the cells are functionally silenced. Exhausted T cells express high levels of inhibitory receptors (Messal et al. 2011), such as programmed cell death protein 1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), lymphocyte activation gene 3 protein (LAG-3), and T cell immunoglobulin and mucin-domain containing-3 (TIM-3). Blocking these receptors using targeted monoclonal antibodies has been demonstrated to successfully reactivate exhausted T cells (Lee et al. 2015) and has been used to treat a variety of cancers, including melanoma, non-small cell lung cancer, and lymphoma.

Ongoing and future studies of T cell activation using potential immunotherapies rely on the ability to detect the relevant markers that define T cell states. In this study, we developed and tested a 16-parameter flow cytometry panel for the ZE5 Cell Analyzer applicable to the interrogation of a range of biologically relevant markers of T cell activation and immunotherapy targets currently being investigated. In lieu of clinical samples, we also developed an in vitro model of T cell exhaustion by activating T cells with beads coated with anti-CD3 and anti-CD28 antibodies (CD3/CD28 activation beads). Nonactivated and exhausted cells were treated with nivolumab, a PD-1 inhibitor, resulting in significant changes in T cell markers detected with the multiparameter panel. Cells were stained and analyzed simultaneously for all 16 targets using a ZE5 Cell Analyzer configured with five lasers (355, 405, 488, 561, 640 nm), 16 fluorescence detectors, and forward and side scatter.

BIO-RAD

Materials and Methods

Cell Handling

Pan T cells obtained as frozen samples from AllCells, LLC (Alameda, CA) 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), cells were either stained with T cell phenotyping antibodies (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 per ml were stimulated with CD3/CD28 human T-Activator Dynabeads according to manufacturer's instructions (Thermo Fisher Scientific) with or without nivolumab (SelleckChem), a monoclonal antibody targeting PD-1 (20 and 40 µg/ml). Media was changed every 48 hours and fresh drug was added. T cells were stimulated for 4 and 13 days. At each media change cells were recounted and resuspended at 2–3 x 10^6 cells/ml.

T Cell Phenotyping and Staining

Five hours before harvesting, cells were exposed to 1x Brefeldin A (BioLegend) to inhibit protein transportation. At 0, 4, and 13 days, cultured pan T cells were washed in PBS, cleared of any CD3/CD28 beads, which were removed with a magnet, treated with VivaFix 353/442 Viability Assay (Bio-Rad Laboratories), and stained with antibodies against cell surface molecules (Table 1) for 30 min in the presence of BD Fc Block (BD Pharmingen). 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 Pharmingen). The panel was fully validated with single stains and fluorescence minus one (FMO) controls. Compensation panels were developed with AbC compensation beads (Thermo Fisher Scientific). Flow cytometry measurement was carried out on a ZE5 Cell Analyzer (Bio-Rad Laboratories) and analysis was performed using FlowJo v10 Software (FlowJo, LLC).

Table 1. Fluorophores used in flow cytometry panel.

Laser	Filter	Target	Fluorescent Tag	Isotype	Clone	Source
355	700LP	CD25	BUV737	IgG1 κ	2A3	BD Biosciences
355	447/60	Live/Dead	VivaFix 353/442	N/A	N/A	Bio-Rad
355	387/11	IFN-γ	BUV 395	IgG1	B27	BD Biosciences
405	420/10	LAG-3	Brilliant Violet 421	IgG1 κ	11C3C65	Biolegend
405	525/50	CD28	Brilliant Violet 510	IgG1 κ	CD28.2	Biolegend
405	615/24	TIM-3	Brilliant Violet 605	IgG1 κ	F38-2E2	Biolegend
405	670/30	CD127	Brilliant Violet 650	IgG1 κ	A019D5	Biolegend
405	720/60	IL-2	Brilliant Violet 711	IgG2a κ	MQ1-17H12	Biolegend
405	460/22	CD4	Pacific Blue	IgG1	RPA-T4	Bio-Rad
488	750LP	TNF-α	PE-Cy7	IgG1 κ	MAb11	BioLegend
488	525/35	PD-1	Alexa Fluor 488	IgG1	MIH4	Bio-Rad
561	615/24	CCR7	PE-Dazzle 594	IgG2a κ	G043H7	BioLegend
561	577/15	CTLA-4	PE	IgG2a	BNI3	Bio-Rad
561	720/60	CD3	PE-Cy5.5	IgG1	UCHT1	Bio-Rad
640	670/30	CD45RA	Alexa Fluor 647	IgG1	F8-11-13	Bio-Rad
640	720/60	CD8	Alexa Fluor 700	IgG1	LT8	Bio-Rad

Results

Expected and Rare T Cell Populations Were Distinguished Using Our Multiparameter Staining Strategy and the ZE5 Cell Analyzer

Untreated pan T cells were first analyzed for expected T cell populations to verify working conditions of our in vitro model of T cell exhaustion. We show that the pan T cells used in our model are of high cell viability and have a high percentage of CD3+ lymphocytes, as expected (Figure 1A). Using distinct gating strategies, our multiparameter panel, and the ZE5 Cell Analyzer, we were also able to discriminate between CD4+ and CD8+ cell populations and identify the rare regulatory T cell populations (Tregs), defined as CD4+CD25^{high}/CD127^{low} in this model.

T Cell Subsets Were Discriminated and Quantified before and after T Cell Exhaustion

To induce T cell exhaustion, pan T cells were incubated with CD3/CD28 activation beads for either 4 or 13 days. Cells were expected to transition into an exhausted state after 4 days and complete exhaustion after 13 days. Receptors CCR7 and CD45RA were used as markers to determine T cell states (Figure 1B). We found that in both CD4+ and CD8+ cell populations, the percentage of naïve cells dropped about fourfold after 4 days of activation and decreased even further after 13 days. Furthermore, significant increases and shifts in the percentage of central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory cells re-expressing CD45RA (T_{EMRA}) were seen in both CD4+ and CD8+ cell populations following activation. These shifts in T cell subpopulations illustrate that treatment with CD3/CD28 beads did drive naïve T cells into less active phenotypes.

Nivolumab Treatment Slows T Cell Exhaustion

In clinical studies, the PD-1 inhibitor nivolumab has been shown to reactivate T cells from an exhausted state. Here, we treated pan T cells with nivolumab during incubation with CD3/CD28 activation beads to examine its effect on T cell exhaustion in our in vitro model. We found that treatment with nivolumab delayed CD4+ and CD8+ cells from transitioning from the naïve state after 4 days of incubation with the beads. However the effect was lost after 13 days (Figure 1B). This suggests that nivolumab treatment has an effect on T cell phenotypic transition in this model.

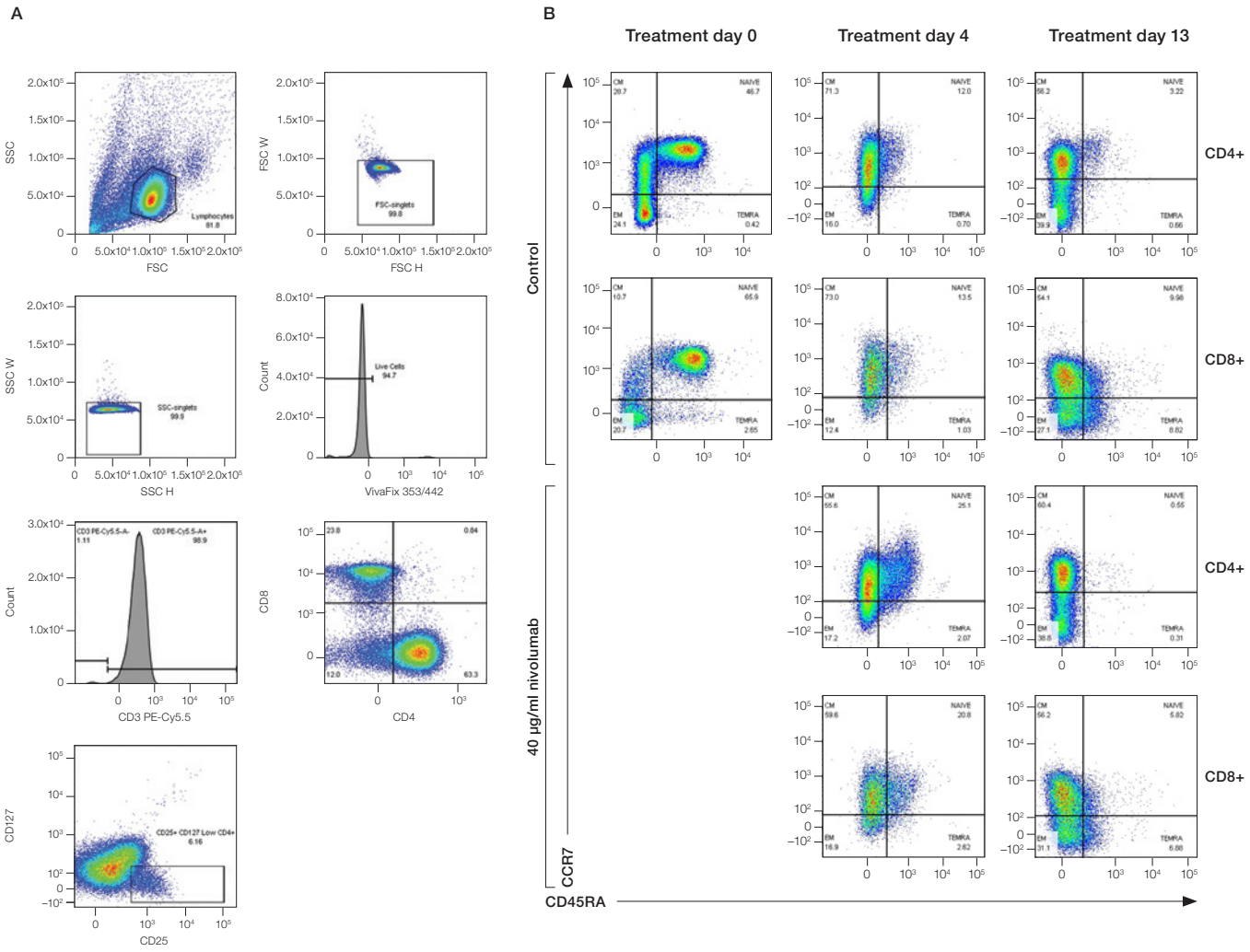

Fig 1. Gating strategy for interrogating T cell subsets. A, lymphocytes, single cells, viability, and CD3+ populations are identified. CD4+/CD8+ cells are discriminated and Tregs (CD4+ $CD25^{high}/CD127^{low}$) are identified. **B**, CCR7 and CD45RA markers are used to determine variation in T cell subsets, naïve (T_N), central memory (T_{CM}), effector memory (T_{EM}), and terminally differentiated effector memory cells re-expressing CD45RA (T_{EMRA}), following stimulation with CD3/CD28 activation beads at days 4 and 13 with or without 40 µg/ml nivolumab.

T Cell Exhaustion and Treatments with Nivolumab Alter the Ratio of Cell Surface Receptors on T Cell Subsets

The percentage of untreated cells displaying the PD-1 surface protein was determined for T cell subsets (naïve T_N, central memory T_{CM}, and effector memory T_{EM}) of both CD4+ and CD8+ cells (Figure 2A). The population of cells positive for PD-1 significantly increased in the less active states of T_{CM} and T_{EM} compared to the more active naïve state. This result is in line with the premise that PD-1 expression is associated with senescent T cells in vivo. We then determined the percentage of CD4+ and CD8+ T cell subsets expressing PD-1, TIM-3, CTLA-4, LAG-3, and CD28, cell surface proteins associated with T cell exhaustion and checkpoint inhibition, after 0, 4, and 13 days of activation with CD3/CD28 beads and with or without 40 µg/ml nivolumab treatment. We found in this model that PD-1 and TIM-3 expressions differ among the CD4+ and CD8+ T cell subsets over time following continued activation (Figure 2B). In addition, when compared to controls we measured increased PD-1 and TIM-3 expression at day 4 following treatment with nivolumab. By day 13, PD-1 expression had decreased from that measured at day 4. Also, there was less PD-1 expression in the nivolumab-treated cells compared to control samples. However, TIM-3 remained significantly elevated throughout the course of the study following stimulation with further increases following nivolumab treatment. Although we detected changes to CTLA-4 and LAG-3 over the course of the experiment in line with expectations for stimulated T cells, we measured no significant changes to these markers or to the CD28 surface protein expression between drug-treated and control T cells (Figure 2B).

T Cell Subsets Have Varied Expression of Cell Surface Proteins

For a comprehensive view of the changes in cell surface proteins, we further interrogated the individual memory T cell subsets for expression of the PD-1 and TIM-3 checkpoint inhibitors with and without nivolumab treatment. Prior to stimulation, PD-1 is expressed in the T_{CM} and T_{EM} subsets in both CD4+ and CD8+ cells. This balance is shifted at day 4 to a predominantly T_{CM} expression, before returning to an even T_{EM}, T_{CM} split at day 13 (Figure 3A). A different expression pattern was measured for TIM-3. In CD4+ and CD8+ cells prior to stimulation the majority of expression of TIM-3 is through the TN subset. TCM is responsible for most of the TIM-3 expression at day 4 in both CD4+ and CD8+. TIM-3 expression remains high through day 13 and is more weighted toward T_{CM} expression in CD8+ cells whereas the split between T_{CM} and T_{EM} in CD4+ cells is more even (Figure 3B). Overall, the addition of nivolumab did not alter the overall expression localization of either PD-1 or TIM-3 in the T cell subsets but did alter the level of expression of these markers. TIM-3 expression in CD4+ T cells and PD-1 expression in both CD4+ and CD8+ T cells treated with nivolumab was significantly higher than in control cells (Figure 3B). This result indicates potential co-signaling interactions between these two checkpoint inhibitors. Interestingly, combinatorial approaches concurrently targeting both PD-1 and TIM-3 are now being explored as a potential therapy in some circumstances.

Nivolumab Treatment Alters Expression of Cytokines in Activated T Cells

Analysis of the cytokine expression profile for CD4+ and CD8+ cells revealed that nivolumab treatment alters IL-2 expression in activated T cells. More specifically, treatment with nivolumab resulted in an increase in cells expressing IL-2, compared to control, for both CD4+ and CD8+ T cells at 4 and 13 days after activation (Figure 4A). TNF-α expression increased 4 days after stimulation, but nivolumab treatment had no effect on TNF-α expression at day 13. We also observed no effect from nivolumab treatment on IFN-γ expression at any of our three time points.

Breakdown of cytokine expression into individual T cell subsets revealed a similar story to that of the checkpoint inhibitors. Like the checkpoint inhibitor expression, the cytokine expression was mainly found in the CD4+ T_{CM} subset at 0, 4, and 13 days. Naïve CD8+ T cells had the highest expression of cytokines prior to stimulation, but again, the majority of expression was shifted to T_{CM} at 4 and 13 days. Treatment with nivolumab did not alter which of the T cell memory subsets expressed the cytokines (Figure 4B).

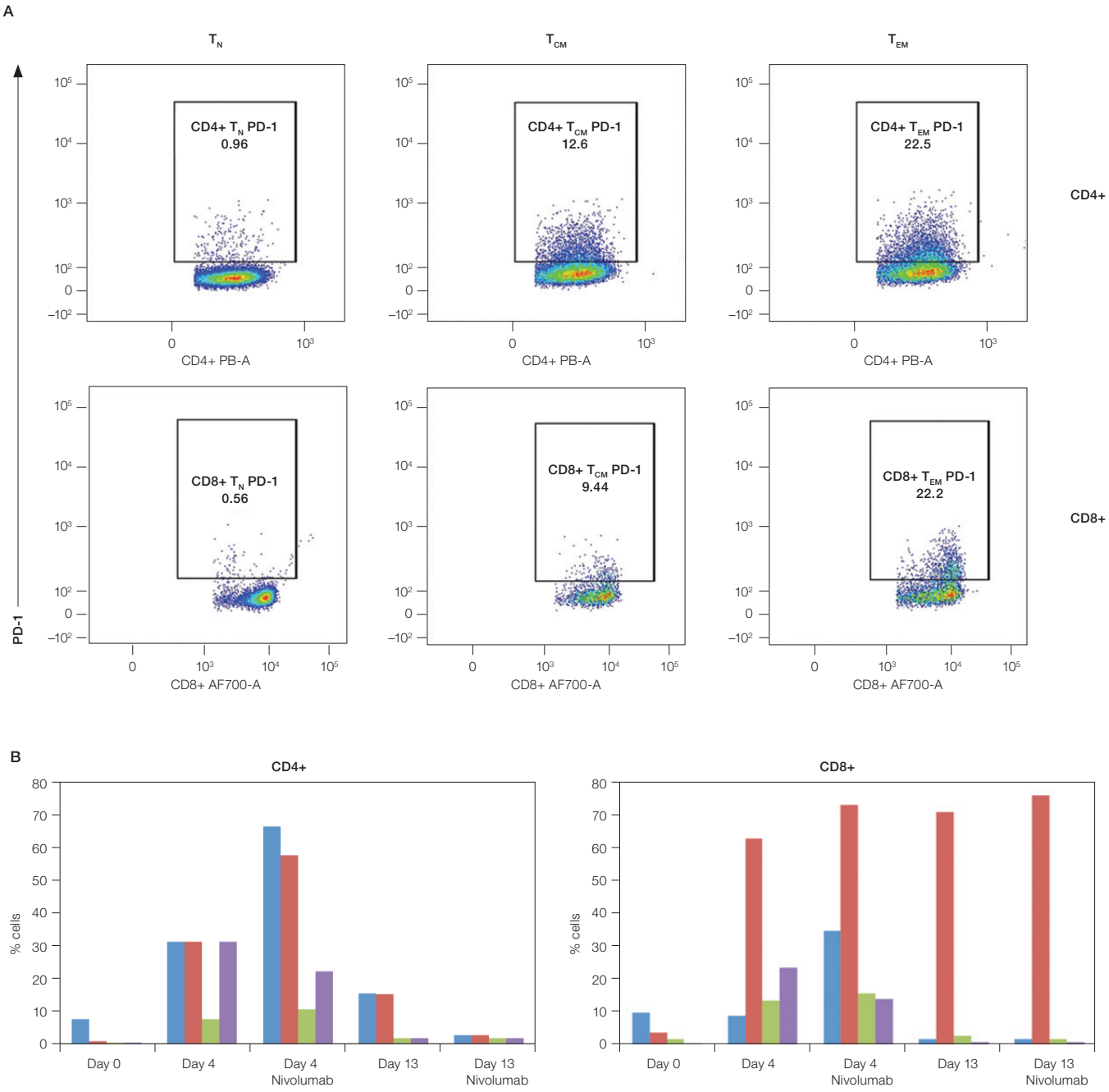

Fig. 2. Determination of exhaustion marker expression. The expression of checkpoint inhibitors PD-1, TIM-3, LAG-3, and CTLA-4 is altered over time in stimulated CD4+ and CD8+ T cells. **A**, PD-1 expression is determined by the type of memory T cell subset. **B**, the expression of TIM-3 and PD-1 is affected when stimulated T cells are treated with nivolumab. We saw no effect on CD28 expression following treatment with nivolumab. PD-1 (■); TIM-3 (■); LAG-3 (■); CTLA-4 (■).

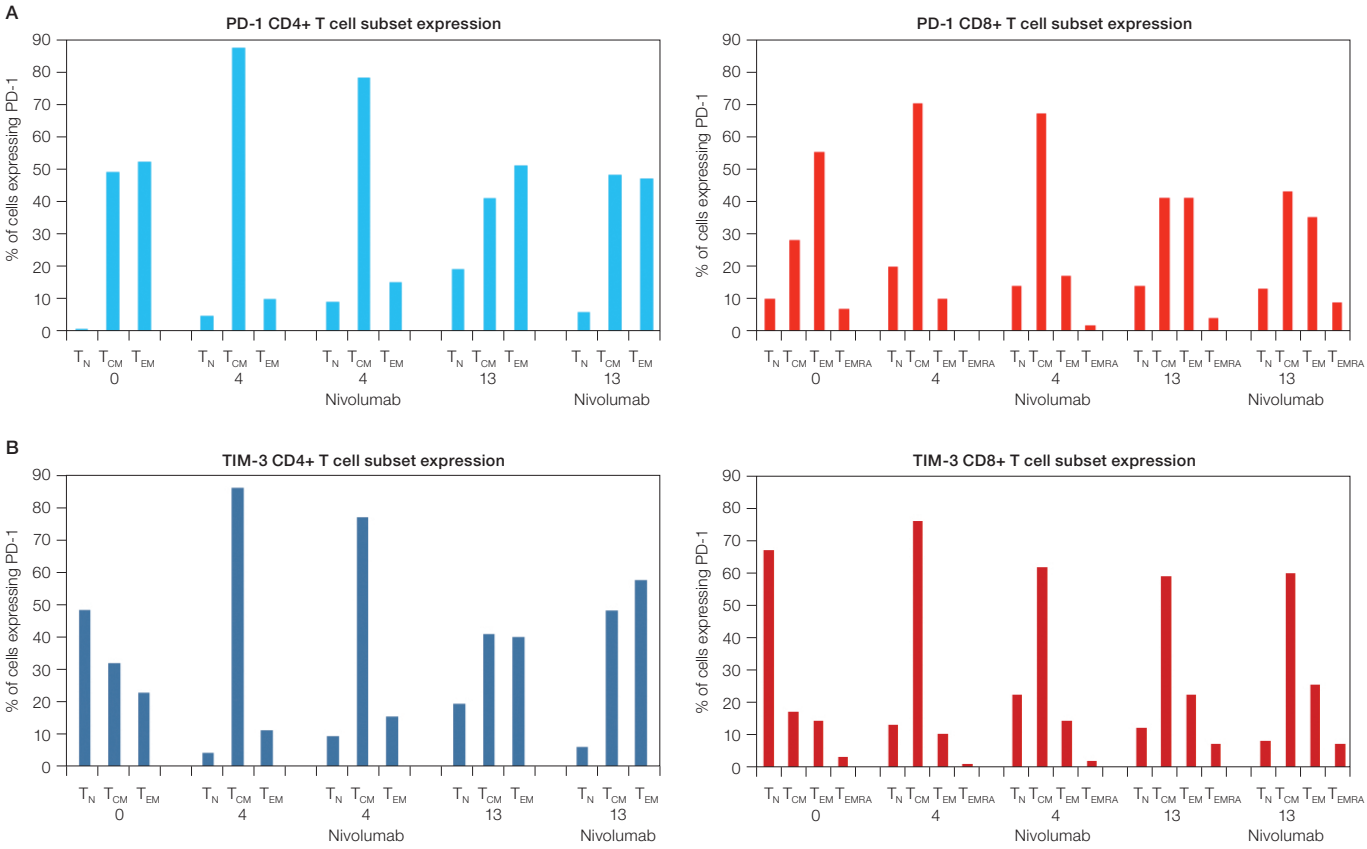

Fig. 3. PD-1 and TIM-3 expression levels. Expression of the checkpoint inhibitors PD-1 (**A**) and TIM-3 (**B**) was measured for CD4+ and CD8+ T parent populations as well as their memory subsets. Expression at 0, 4, and 13 days after stimulation with CD3/CD28 activation beads in the presence or absence of 40 µg/ml nivolumab was measured.

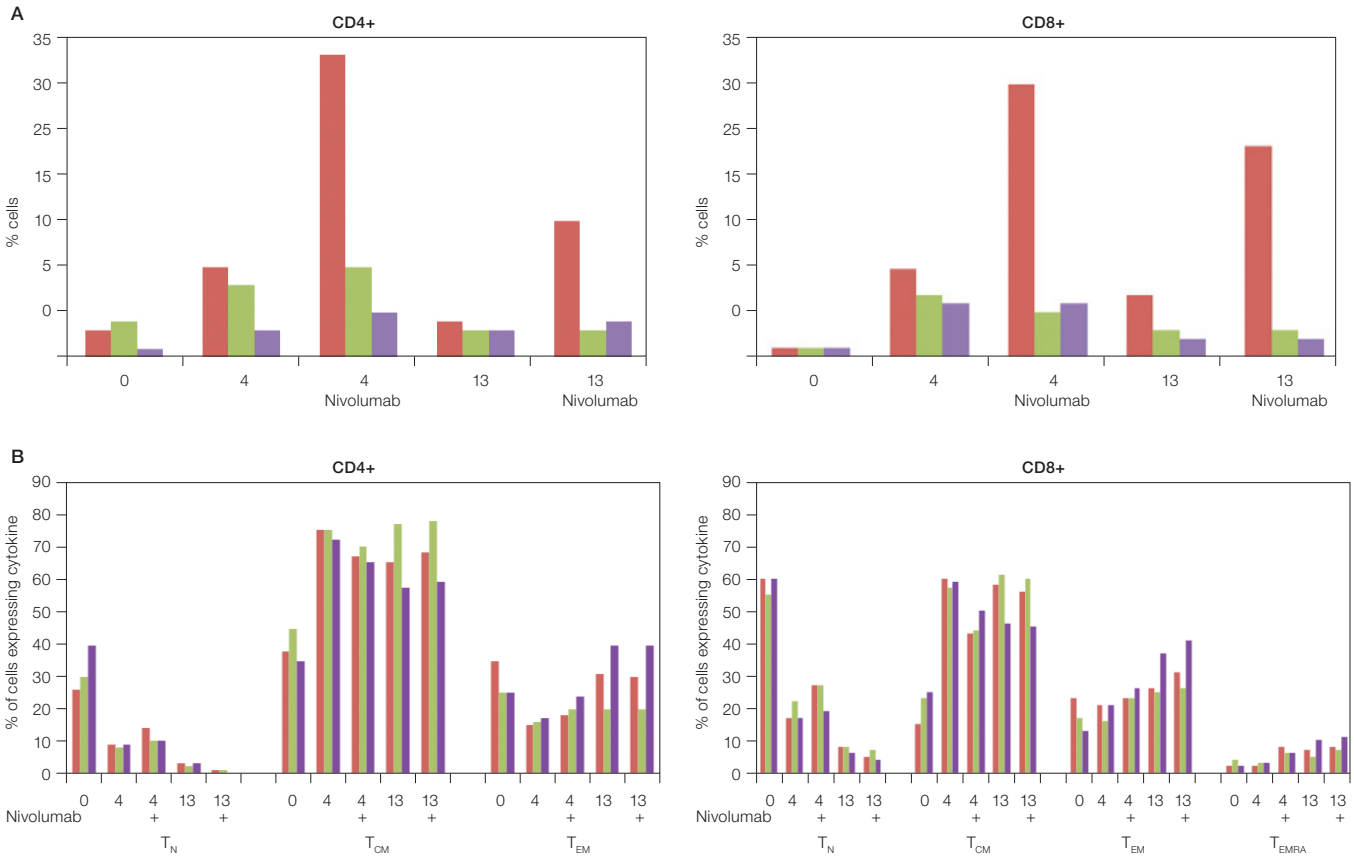

Fig. 4. Cytokine expression levels. Expression of cytokines IL-2, TNF-α, and IFN-γ was measured for CD4+ and CD8+ T parent populations (**A**) as well as their memory subsets (**B**). Expression at 0, 4, and 13 days after stimulation with CD3/CD28 activation beads in the presence or absence of 40 µg/ml nivolumab was measured. IL-2 (■); TNF-α (■); IFN-γ (■).

Conclusion

In this study, our most significant findings, measured by concurrent monitoring of the expression levels of several cell surface proteins and cytokines, were that treatment of CD3/CD28 bead–activated pan T cells with the anti–PD-1 monoclonal antibody nivolumab, resulted in a possible delayed T cell exhaustion phenotype as well as changes to the expression levels of the checkpoint inhibitors PD-1 and TIM-3 and the cytokine IL-2. In addition, we were able to track changes to T cell subpopulations undergoing T cell exhaustion transitioning through T_N, T_{CM}, and T_{EM} following chronic activation with the CD3/CD28 beads.

The use of this multicolor flow cytometry with the ZE5 Cell Analyzer allowed 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. Our in vitro model of T cell exhaustion and treatment with nivolumab resulted in an abundance of data generated by simultaneously staining for 16 parameters in each sample. Without the ability to analyze such a large panel on the ZE5 Cell Analyzer more sample would be needed, time would be wasted on multiple staining experiments, and comprehensive analyses of discrete cell populations would be much more difficult. More important, had the panel been split into two panels separating PD-1 and TIM-3, the co-signaling interaction between these checkpoint inhibitors would have been missed. Thus, having the ability to expand or combine panels into a larger panel on the ZE5 Cell Analyzer aids discovery and validation.

References

Lee J et al. (2015). Reinvigorating exhausted T cells by blockade of the PD-1 pathways. 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.

Visit **bio-rad.com/ZE5** for more information

BIO-RAD

Bio-Rad Laboratories, Inc.

Life Science Group

Web site *bio-rad.com* ***USA*** *1 800 424 6723* ***Australia*** *61 2 9914 2800* ***Austria*** *43 01 877 89019* ***Belgium*** *32 03 710 53 00* ***Brazil*** *55 11 3065 7550* ***Canada*** *1 905 364 3435* ***China*** *86 21 6169 8500* ***Czech Republic*** *36 01 459 6192* ***Denmark*** *45 04 452 10 00* ***Finland*** *35 08 980 422 00* ***France*** *33 01 479 593 00* ***Germany*** *49 089 3188 4393* ***Hong Kong*** *852 2789 3300* ***Hungary*** *36 01 459 6190* ***India*** *91 124 4029300* ***Israel*** *972 03 963 6050* ***Italy*** *39 02 49486600* ***Japan*** *81 3 6361 7000* ***Korea*** *82 2 3473 4460* ***Mexico*** *52 555 488 7670* ***The Netherlands*** *31 0 318 540 666* ***New Zealand*** *64 9 415 2280* ***Norway*** *47 0 233 841 30* ***Poland*** *36 01 459 6191* ***Portugal*** *351 21 4727717* ***Russia*** *7 495 721 14 04* ***Singapore*** *65 6415 3188* ***South Africa*** *36 01 459 6193* ***Spain*** *34 091 49 06 580* ***Sweden*** *46 08 555 127 00* ***Switzerland*** *41 0617 17 9555* ***Taiwan*** *886 2 2578 7189* ***Thailand*** *66 2 651 8311* ***United Arab Emirates*** *971 4 8187300* ***United Kingdom*** *44 01923 47 1301*

Bulletin 7125 Ver A US/EG *18-0471 0219 Sig 0119*