APPLICATION NOTE

Assessing Functional Quality of Single TILs for Correlative Immunotherapy Biomarkers

Using Single-Cell PSI™ to reveal mechanism in the Tumor Microenvironment

In this application note, we:

- Highlight the challenges in functional characterization of TILs in checkpoint and combination therapies
- Describe the workflow for functional single-cell analysis from both solid tumor TILs as well as patient PBMC
- Predict patient response to checkpoint therapy using PSI as a biomarker in solid tumor TILs, as well as correlate response from these solid tumor TILs with PSI from peripheral blood.

Challenge: Checkpoint combination studies need TILs solutions

Tumor infiltrating lymphocytes (TILs) are not always associated with favorable clinical outcomes because T cells in the tumor tissues are often exhausted and dysfunctional[1,2]. It has been reported that CD8+ melanoma-specific tumor TILs express a high level of PD-1 and are functionally impaired[3], which compromises the effective anti-tumor immune responses. Preventing the exhaustion by blocking the PD-1/PD-L1 pathway in combination with anti-CTLA-4 treatment have been shown to be beneficial for tumor regression and patient long term survival in many clinical trials[4]. Recently, checkpoint inhibitor blockade therapy has rapidly advanced and is now established as a powerful way to manage various

BRUKER

www.brukercellularanalysis.com

malignant tumors. Despite the promising outcomes, there are a significant number of patients who do not respond to these immunotherapies[5-7]. Multiple cytokine producing (Polyfunctional) T cells are the key effectors contributing to the development of potent and durable cellular immunity against various infectious diseases as well as cancers[8-11] (Figure 1). Since TILs directly interact with tumor cells, it is crucial to comprehensively evaluate their effector functions, particularly, their cytokine profiles. Currently, the leading technologies for measuring functional T cell responses are ELISpot and flow cytometry-based assays[12]. The major limitation with these methods is their inability to simultaneously measure all relevant cytokines secreted at live single cell resolution and to identify highly-polyfunctional cell subsets[12,13].Therefore, there is an urgent need for new tools to predict the response to the therapies and decipher the underlying mechanisms of current and future therapies.

Phenotypically identical cells, though functionally highly heterogeneous

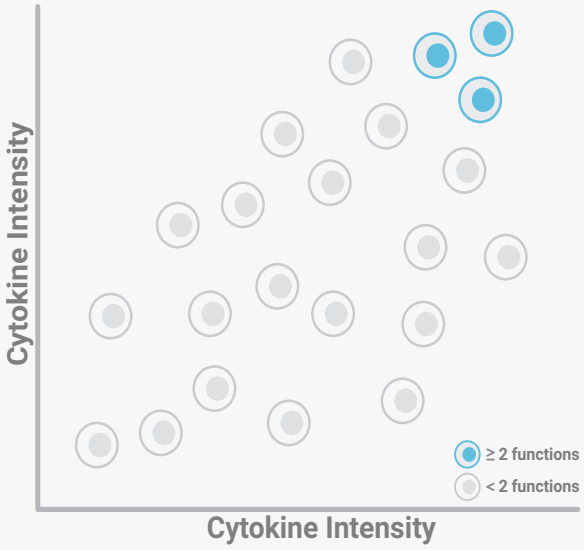

Figure 1 | Highly Polyfunctional cell subsets revealed by Bruker systems enable visualization of immune cells with a high degree of polyfunctional inflammation (cells that secrete two or more cytokines per cell, at a high intensity) that correlate to disease status.

Workflow of IsoLight for detecting secreted proteins from single cells

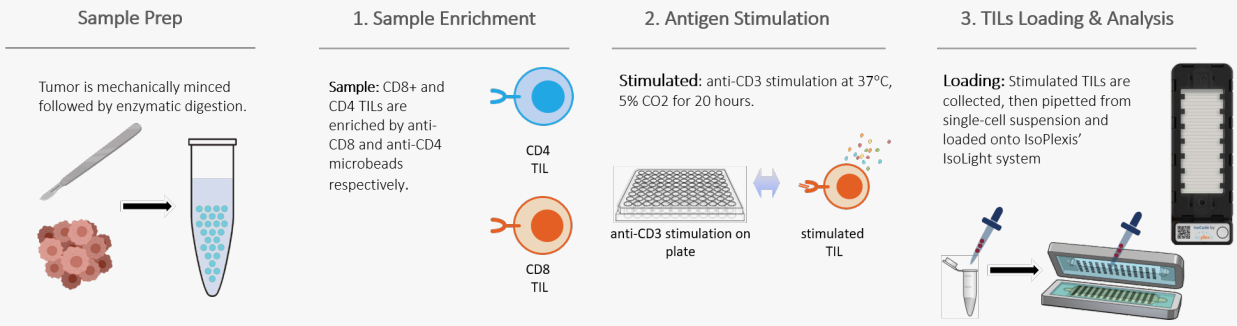

Figure 2 | Key TILs Preparation Steps. Step 1: Cell subsets are separated and enriched by microbeads. Step 2: Cells are stimulated with specific or nonspecific antigen(s). Step 3: After stimulation, cells are stained with fluorochrome-conjugated antibodies or membrane dyes and loaded on an IsoCode chip. Step 4: Cell-loaded IsoCode chip incubation and proteins secretions are completed in the IsoLight automation system.

Isolating TILs is technically challenging and difficult to standardize, given the small amount of available tumor tissues and the low number of TILs from various malignant tissues. To achieve high quantity and quality of single cells, we mechanically minced the tumor and followed by enzymatic digestion. Digested tissues were filtered through a cell strainer to remove undigested large pieces and cell clumps. Single cell suspension was performed positive T cell isolation with anti-CD4 or CD8 microbeads (Figure 2).

As mentioned previously, polyfunctional T cells, defined as single cells co-producing at least two cytokines at the same time, are recognized as key effector cells contributing to the protective immunity. However, it remains challenging to detect the secreted cytokines, because flow cytometry-based assays do not measure true secretion and the data may not reflect the true secretory patterns in vivo.

Bruker PSI: polyfunctionality of a sample combined with the intensity of each cell's secreted cytokines

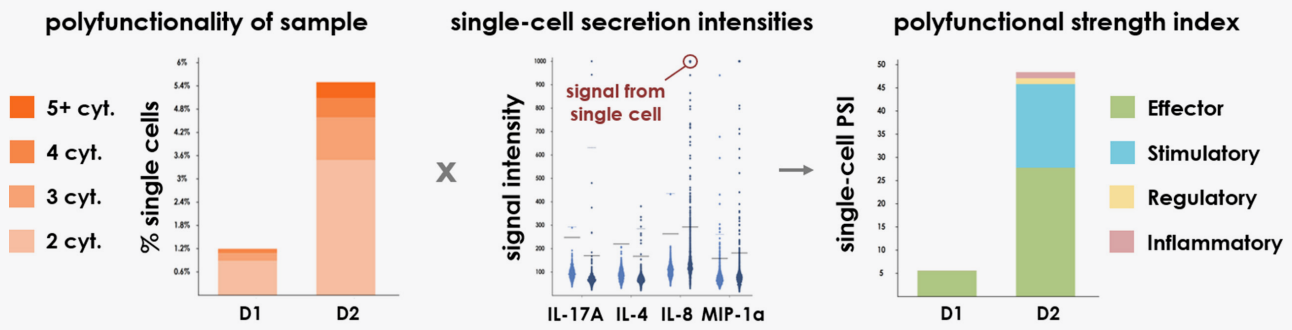

Figure 3 | PSI (Polyfunctional Strength Index) is defined as the percentage of polyfunctional single-cells (secreting 2 or more proteins, i.e. left panel) in a sample, multiplied by the average signal intensity of the secreted proteins from individual functional groups (middle panel) from each cell. Each cell's strength, across 1000+ cells, is then aggregated and simplified into the readout at right. This PSI measurement provides a comprehensible visualization of the potent cell subsets, and the cytokine types driving these potent cell subsets.

To overcome the limitations of traditional methods in the measurement of secreted proteins from single cells[14,15], we took advantage of Bruker's IsoLight proteomic platform to simultaneously detect 30+ secreted proteins from single cells. The IsoLight system employs its IsoCode® chip, a single-cell, highly multiplexed chip consisting of an antibody barcode array by combining sandwich ELISA and fluorescence signal detection. The IsoCode chip measures

Polyfunctional Strength Index (PSI™), a unique single-cell metric defined as the percentage of polyfunctional cells (2+ proteins per cell) in a sample, multiplied by the average signal intensity of the proteins secreted by these polyfunctional single cells (Figure 3).

The IsoCode Chip's unique ability to detect vivo correlates from small amounts of TILs

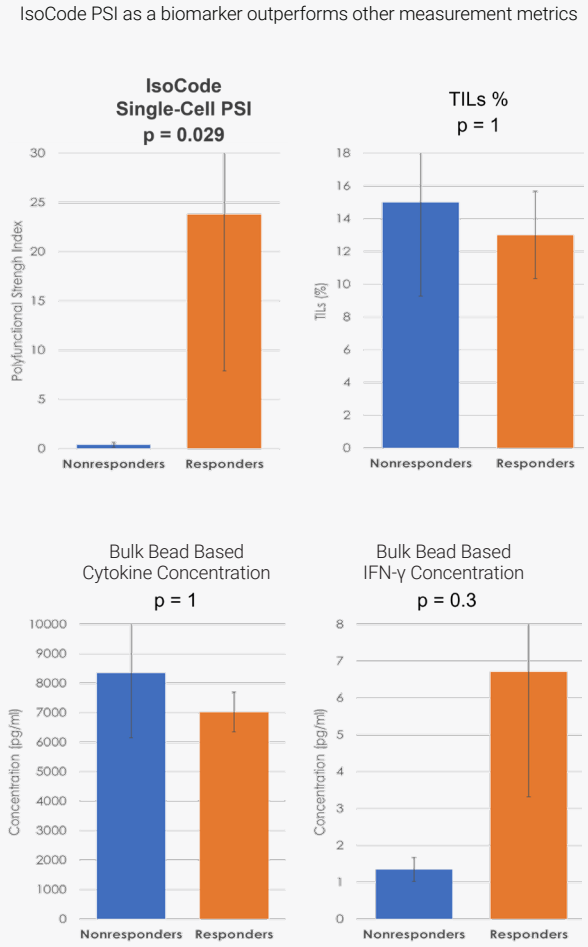

Figure 4 | Single-Cell PSI of CD8+ TILs significantly correlates with outcome of anti-PD-1/CTLA4 treated patients with metastatic melanoma. No significant association was identified in TILs % by histopathological assessment or serum concentration by Bulk Bead Based Cytokine Analysis.

PSI™ alone can significantly segregate responders from nonresponders, where bulk difference is insignificant

Recently, PSI™ has been employed in the anti-CD19 CAR-T cell pre-infusion products manufactured from apheresis of patients with Non-Hodgkin lymphoma (NHL) and demonstrated a statistically positive association with objective response (OR) as well as CRS in these patients after anti-CD19 CAR-T therapy to differentiate responders and non-responders[16]. To fully characterize the polyfunctional responses in CD8+ TILs isolated from tumor tissues of melanoma patients and find if there is any correlation of polyfunctional responses with clinical outcome to anti-PD1/CTLA-4 blockade treatment, we used IsoCode chips to simultaneously measure multiple different secreted proteins from single cells. In parallel, we also performed histological analysis for the percentages of TILs in the tumor tissues and bulk cytokine analysis using bulk serum analysis. Strikingly, our data clearly demonstrated that the single TIL PSI significantly outperformed other bulk-level metrics in the ability to distinguish responders from non-responders (Figure 4).

Only IsoCode PSI™ P value is significantly associated with clinical outcome

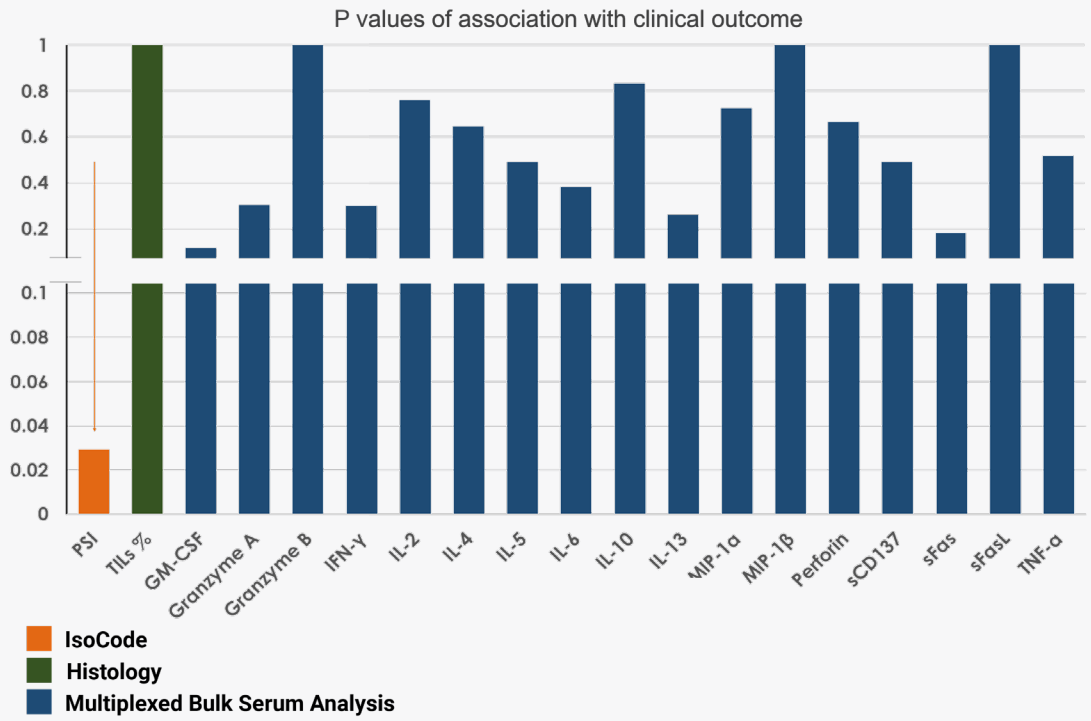

Figure 5 | Comprehensive comparison of P values cross different measurements. P-values were calculated for the association with clinical outcomes. IsoCode: n = 11, Multiplexed Bulk Serum Analysis: n = 10, TILs %: n = 8.

Single Cell PSI reveals mechanism and correlates from small TILs samples

To further understand the correlative values of single cell PSI of CD8 TILs and other individual secreted proteins measurements at bulk levels, we compared 17 secreted cytokines from serum of patients by Multiplexed Bulk Serum Analysis. Our data demonstrated that only single cell PSI of CD8+ TILs can be used as a prognostic tool for anti-PD1/CTLA-4 treatment in melanoma patients and significantly correlated with clinical outcomes.

Comprehensive comparison of p values of all the metrics (Figure 5) we have measured indicates that only single TIL PSI data reveal unique differences at protein level between the responders and non-responders, suggesting other functional bulk readouts are not as sensitive as single TIL PSI to use as a predictive biomarker for differentiating responders from non-responders to checkpoint combination therapies. These data further emphasize that enhancing antigen-specific local anti-tumor immune response will positively impact on the prognosis of malignant solid tumors.

TILs data and PBMC data correspond

In general, anti-melanoma immunity is considered as a more localized immune response in the tumor tissues rather than a systemic immune response in circulating peripheral blood[17,18]. However, some recent studies suggest that T cell responses in peripheral blood might have some correlative values in melanoma as well as certain solid tumors[19-22]. To investigate whether polyfunctional responses of CD8+ T cells in the peripheral blood have similar correlative values as we have observed in the CD8+ TIL samples, we isolated CD8+ T cells from patient PBMCs and performed single cell IsoCode chip analysis for characterizing the secretion profile. Our data demonstrated that the polyfunctionality and PSI of peripheral CD8+ T cells were also increased in responding patients, which are similar to TIL samples, although the differences didn't reach a statistical significance (Figure 6, top and bottom panels). Interestingly, unlike how polyfunctionality in TILs was only driven by effector protein secretions, the polyfunctional responses in CD8+ T cells from PBMCs were driven by a more diverse secretion profile, leading by effector secretion and in combination with chemoattractive and stimulatory secretions. In combination with CD8+ TIL polyfunctional data, peripheral blood can also provide some unique aspects of systemic anti-tumor immune responses in patients receiving checkpoint inhibitor therapies.

PBMC T cell PSI™ P value reflects differences seen in the TILs

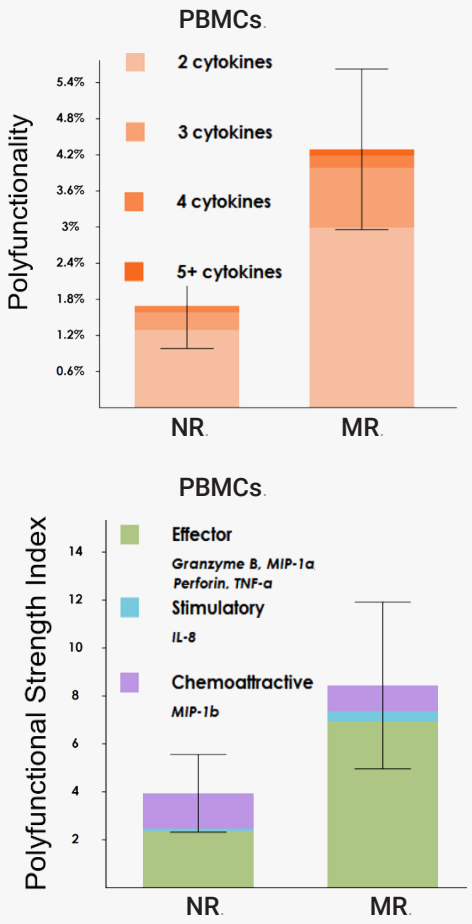

Figure 6 | PBMCs from responding patients show similar overall trend of upregulation in single cell response, but distinct secretion profiles. Single-cell PSI of PBMC from the same patients was determined by using single cell proteomic analysis of a panel of 17 secreted proteins. Responding patients (MR) showed elevated polyfunctional profiles than nonresponders (NR) in PBMC-derived CD8+ T cells. top, polyfunctionality. bottom, polyfunctional strength index (PSI).

Applying Bruker Systems to next generation checkpoint studies

We have demonstrated that the polyfunctionality of TILs measured by PSI is significantly correlated with clinical objective response of patients receiving anti-PD-1/PD-L1 and anti-CTLA-4 therapies. However, apart from well-studied PD-1/PD-L1, there are additional checkpoint inhibitory receptors which are also highly expressed on the dysfunctional or exhausted T cells[23]. Indeed, co-expression of immune checkpoint receptors PD-1, TIM-3, and LAG-3 characterizes chronically activated and exhausted tumor-infiltrating T cells (TILs) in several pre-clinical tumor models, suggesting their targeting may have applicability for the treatment of multiple cancer types. Our preliminary data demonstrated that triple combination treatment dramatically amplified cytokine release from TILs isolated from ovarian cancer tissues, indicating more effective TIL reinvigoration. To further understand the differential effects of triple combination treatment over PD-1 blockade, we analyzed antibody-treated ovarian cancer TILs on a single cell level using IsoCode chip technology. In contrast to PD-1 monotherapy, the triple combination treatment significantly increased the PSI of TILs (data not shown). This data further supports the concept of triple combination checkpoint blockade as a treatment option for ovarian cancer.

Taken together, IsoCode technology uniquely addressed the limitation of traditional methods and deeply deciphered the heterogeneously polyfunctional profile of single TILs, indicating the comprehensive analysis of anti-tumor immune responses at single cell can be a very promising biomarker for the prediction of clinical responses and prognosis. IsoCode technology will also help with the design for the next generation combination therapies in a more scientific manner and achieve synergistic efficacy while controlling for unwanted immune related side effects.

Conclusion

PSI is a powerful and unique metric, with a wide range of applications in checkpoint and combination therapy research, including:

- PSI as a biomarker of response in checkpoint therapy
- Utilizing PSI in biomarkers for solid tumor and peripheral blood applications in checkpoint and combination immunotherapies
- Next generation checkpoint therapies as well as combination therapies and the application of these biomarkers in various cancer types

References

1 Zarour, H. M. Reversing T-cell Dysfunction and Exhaustion in Cancer. *Clin Cancer Res* **22,** 1856-1864, doi:10.1158/1078-0432.CCR-15-1849 (2016).

2 Rosenberg, S. A. *et al.* Tumor progression can occur despite the induction of very high levels of self/tumor antigen-specific CD8+ T cells in patients with melanoma. *J Immunol* **175**, 6169-6176 (2005).

3 Ahmadzadeh, M. *et al.* Tumor antigen-specific CD8 T cells infiltrating the tumor express high levels of PD-1 and are functionally impaired. *Blood* **114**, 1537-1544, doi:10.1182/blood-2008-12-195792 (2009).

4 Zou, W., *et al.* PD-L1 (B7-H1) and PD-1 pathway blockade for cancer therapy: Mechanisms, response biomarkers, and combinations. *Sci Transl Med* **8**, 328rv324, doi:10.1126/scitranslmed.aad7118 (2016).

5 Binnewies, M. *et al.* Understanding the tumor immune microenvironment (TIME) for effective therapy. *Nat Med* **24**, 541-550, doi:10.1038/s41591-018-0014-x (2018).

6 Roh, W. *et al.* Integrated molecular analysis of tumor biopsies on sequential CTLA-4 and PD-1 blockade reveals markers of response and resistance. *Sci Transl Med* **9**, doi:10.1126/scitranslmed.aah3560 (2017).

7 Yee, C. Adoptive T cell therapy: points to consider. *Curr Opin Immunol* **51**, 197-203, doi:10.1016/j.coi.2018.04.007 (2018).

8 Bercovici, N., *et al.* New methods for assessing T-cell responses. *Clin Diagn Lab Immunol* **7**, 859-864 (2000).

9 Seder, R. A., *et al.* T-cell quality in memory and protection: implications for vaccine design. *Nat Rev Immunol* **8**, 247-258, doi:10.1038/nri2274 (2008).

10 Haining, W. N. Travels in time: assessing the functional complexity of T cells. *Proc Natl Acad Sci U S A* **109**, 1359-1360, doi:10.1073/pnas.1119856109 (2012).

11 Anastasopoulou, E. A., *et al.* MHC class II tetramer analyses in AE37-vaccinated prostate cancer patients reveal vaccine-specific polyfunctional and long-lasting CD4(+) T-cells. *Oncoimmunology* **5**, e1178439, doi:10.1080/2162402X.2016.1178439 (2016).

12 Heath, J. R., *et al.* Single-cell analysis tools for drug discovery and development. *Nat Rev Drug Discov* **15**, 204-216, doi:10.1038/nrd.2015.16 (2016).

13 Ma, C. *et al.* A clinical microchip for evaluation of single immune cells reveals high functional heterogeneity in phenotypically similar T cells. *Nat Med* **17**, 738-743, doi:10.1038/nm.2375 (2011).

14 Yao, Y. *et al.* CyTOF supports efficient detection of immune cell subsets from small samples. *J Immunol Methods* **415**, 1-5, doi:10.1016/j.jim.2014.10.010 (2014).

15 Chen, G. & Weng, N. P. Analyzing the phenotypic and functional complexity of lymphocytes using CyTOF (cytometry by time-of-flight). *Cell Mol Immunol* **9**, 322-323, doi:10.1038/cmi.2012.16 (2012).

16 Rossi, J. *et al.* Preinfusion polyfunctional anti-CD19 chimeric antigen receptor T cells are associated with clinical outcomes in NHL. *Blood* **132**, 804-814, doi:10.1182/blood-2018-01-828343 (2018).

17 Weinstein, D., *et al.* Diagnostic and prognostic biomarkers in melanoma. *J Clin Aesthet Dermatol* **7**, 13-24 (2014).

18 Wargo, J. A., *et al.* Monitoring immune responses in the tumor microenvironment. *Curr Opin Immunol* **41**, 23-31, doi:10.1016/j.coi.2016.05.006 (2016).

19 Yao, X. *et al.* Levels of peripheral CD4(+)FoxP3(+) regulatory T cells are negatively associated with clinical response to adoptive immunotherapy of human cancer. *Blood* **119**, 5688-5696, doi:10.1182/blood-2011-10-386482 (2012).

20 Kamphorst, A. O. *et al.* Proliferation of PD-1+ CD8 T cells in peripheral blood after PD-1-targeted therapy in lung cancer patients. *Proc Natl Acad Sci U S A* **114**, 4993-4998, doi:10.1073/pnas.1705327114 (2017).

21 Choi, J. *et al.* Diagnostic value of peripheral blood immune profiling in colorectal cancer. *Ann Surg Treat Res* **94**, 312-321, doi:10.4174/astr.2018.94.6.312 (2018).

22 Subrahmanyam, P. B. *et al.* Distinct predictive biomarker candidates for response to anti-CTLA-4 and anti-PD-1 immunotherapy in melanoma patients. *J Immunother Cancer* **6**, 18, doi:10.1186/s40425-018-0328-8 (2018).

23 Anderson, A. C., *et al.* Lag-3, Tim-3, and TIGIT: Co-inhibitory Receptors with Specialized Functions in Immune Regulation. *Immunity* **44**, 989-1004, doi:10.1016/j.immuni.2016.05.001 (2016).