APPLICATION NOTE

PSI™ Enables Cell Engineering and Therapy from Discovery to Predicting Outcome

Bruker's PSI™ is discovering and revealing the key potent cell subsets that correlate to in vivo outcome in cell therapy throughout discovery and development

- **In this application note, we show the value of Bruker's Polyfunctional Strength Index, PSI™ in cell engineering and therapy; in particular, we:**
- reveal increased NK cell potency, post gene edits, which correlates with in vivo response in a preclinical setting [1],
- use PSI to clearly reveal differences in bioprocessing methods for product manufacturing process optimization [2], and
- predict patient response to a CAR-T therapy using pre-infusion product PSI, demonstrating its potential as a biomarker for clinical outcome [3].

Ensuring the continued success of engineered immune cell therapies in the fight against cancer

CAR-T cell-based immunotherapies have had remarkable success in recent years. CAR T-cell therapy was shown to lead to a 67%- 90% complete remission (CR) rate in adults and children with relapsed/refractory acute lymphoblastic leukemia (R/R ALL) [4-8] while only 29% of adult R/R ALL patients achieved CR with chemotherapy. T cell therapies engineered against tumor antigens have been extensively investigated in patients with blood cancer [9-11] as well as with solid tumors [12-13], and new therapies are constantly being developed for new disease indications and targets.

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

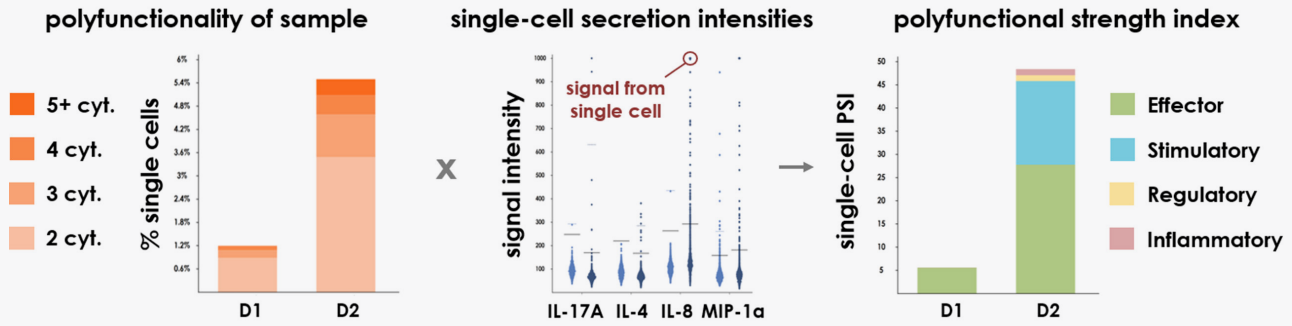

Figure 1 | PSI (Polyfunctional Strength Index) is defined as the percentage of polyfunctional single-cells (secreting 2 or more proteins, i.e. left panel) in a sample, multiplied by the average signal intensity of the secreted proteins from individual functional groups (middle panel) from each cell. 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.

Despite these success stories, challenges remain. Re-engineered T cells are living products whose behavior has been difficult to characterize and predict with existing technologies. Once they are re-infused into the patient, they may result in life-threatening immunotoxicity such as cytokine release syndrome (CRS) and neurologic toxicity [5, 14, 15]. Researchers and clinicians are in urgent need of ways to understand the functional profile of these cell products to accelerate discovery and development, achieve manufacturing consistency, and try to predict which patients will derive the most value from these drugs while minimizing potentially life-threatening side-effects.

In this application note, we discuss how the Bruker Polyfunctional Strength Index (PSI™) can be utilized to address these challenges, in particular, to provide metrics that uniquely relate the cell product response to in vivo pre-clinical and clinical outcome measures.

PSI of engineered cell products indicates cell therapy potency in a manner that correlates to in vivo outcome

Bruker's PSI consolidates high-dimensional, single-cell protein secretion data into a single metric that represents the overall activity of a sample. It captures two critically relevant factors uniquely: the percentage of polyfunctional cells (single cells secreting two or more cytokines) in a sample, and the intensity of all profiled secreted cytokines (Figure 1). Polyfunctional cells are recognized as key effector cells contributing to the development of potent and durable cellular immunity against viral infection, cancer, and other disease [3, 16, 19]. Bruker's ability to capture the range of relevant cytokines from each immune cell represents a unique secreted protein multiplexing capability. See Figure 2 for the Bruker's single-cell polyfunctional strength panel. While the percentage of highly polyfunctional cells on its own is a meaningful indicator of potency, the Bruker system quantitates the intensity of the cytokines secreted by these highly polyfunctional cells. Having both of these key factors in tandem has helped capture the potency of important and highly functional T-cell and other immune cell subsets, which has correlated with in vivo response [3, 19].

Bruker's systems uniquely capture the full range of relevant cytokines from each immune cell

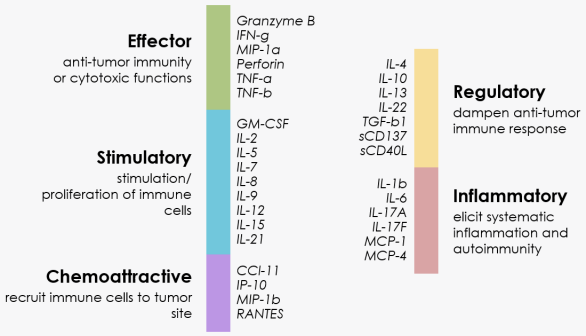

Figure 2 | Bruker's 32-plex single-cell polyfunctional strength panel. The ability to capture the range of relevant cytokines from each immune cell represents a unique secreted protein multiplexing capability.

Discover: Novel Combinations in Solid Tumors: Combining Cytokine Agonist and Cell Therapies in Melanoma

Administration of recombinant human Interleukin-2 (IL-2) was the first cancer immunotherapy approved for the treatment of metastatic renal cell carcinoma (1992) and metastatic melanoma (1998) by the FDA. Clinical data has demonstrated that IL-2 treatment results in complete cancer regression in about 8-10% of patients treated.[1,20] Follow-up data from these patients has indicated that the majority of the patients achieved complete regression lasting for more than 25 years. [1,21] However, given the pleiotropic effects of IL-2 on the immune system, [22] the administration of high-dose IL-2 results in severe hypotension and vascular leak syndrome, which has significantly hampered its use.

Pegylated IL-2 Provides a Powerful Solution in Solid Tumors, yet Further Combinations may Enhance Synergies

Potential side effects restrict optimal IL-2 dosing, further limiting the number of patients who might benefit from this therapy. IL-2 induces not only the desired expansion of tumor-killing $CD8^+$ effector T cells, [23] but also expands immunosuppressive $CD4^+$ $CD25^+$ regulatory T cells, an undesirable side effect. To maximize the efficacy of IL-2 therapy while mitigating side effects, pegylated IL-2 was generated and found to be profoundly more tolerable than its non-pegylated counterpart.

Finding Critical Pre-Clinical Correlates that Reveal T-cell and Immunotherapy Mechanism in Solid Tumors using Bruker's Systems

IL-2 potently activates regulatory T cells (Tregs) by binding IL-2R. NKTR-214, a CD122-biased cytokine agonist, is conjugated with multiple releasable chains of polyethylene glycol (PEG). [24] This pegylation is designed to provide sustained signaling through the IL-2R pathway to preferentially activate and expand effector $CD8^+$ T and NK cells over Tregs in the tumor. In this preclinical study on the pmel-1 mouse model, researchers examined the

Polyfunctionality and PSI reveal synergies and mechanism with novel Combination Therapies

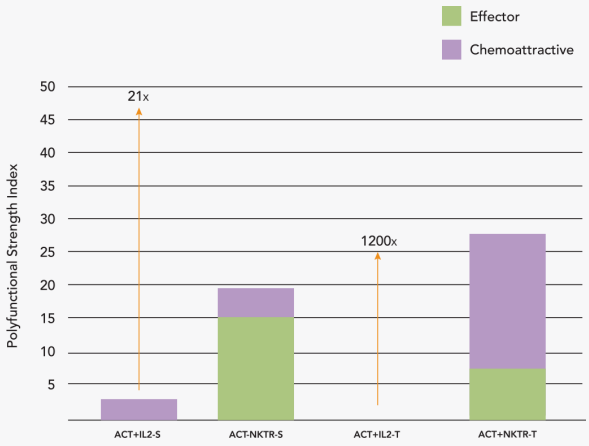

Figure 3 | Polyfunctionality and PSI reveal synergies and mechanism with novel Combination Therapies. The PSI of CD8+ T cells from mice after ACT-NKTR-214 combination therapy was found to be significantly higher than the PSI of CD8+ T cells from mice after ACT-IL-2 control treatment in both spleen and tum

effects of both Interleukin-2 (IL-2) and NKTR-214 on the polyfunctionalities of T cells in both TILs and the spleen. [9]. They found that combination therapy with adoptive cell transfer (ACT) and NKTR-214 provided a robust antitumor response in the aggressive B16F10 melanoma model. [24] The PSI of $CD8^+$ T cells from mice after ACT-NKTR-214 combination therapy was found to be significantly higher than the PSI of $CD8^+$ T cells from mice after ACT-IL-2 control treatment in both spleen and tumor (see Figure). Critically, this type of knowledge of T cell potency of the treatment, driven by the PSI, which correlates to in vivo performance in mice, can help reveal lead choice in a variety of combination immunotherapy contexts.

Polyfunction is Being Used to Advance Discovery of Novel and Potent Immunotherapies Against Solid Tumors

Results suggest that not only in immunotherapies against solid tumors, but also when combining multiple complex immunotherapies to achieve eradication of tumors, PSI can help to reveal new mechanisms that enhance lead choice. With better understanding of the underlying mechanisms of combined immunotherapies, the design of immunotherapy strategies will improve, and further leaps will be made to eradicate cancer.

Optimize: PSI provides the ability to reveal subtle differences in bioprocessing for product manufacturing process optimization

We used PSI to help evaluate a new bispecific CD19/CD22 CAR-T bioprocessing method. Specifically, we compared the polyfunctional response to CD19 and CD22 antigen stimulation of CAR-T cell products generated with the original method (OM) and a modified method (MM). See Figure 4. Both $CD4^+$ and $CD8^+$ samples produced with MM showed significant increases in PSI, clearly demonstrating that MM significantly improved the overall quality of the CAR T cell products compared to the OM [2]. Moreover, this data allowed us to better understand the drivers of this polyfunctional upregulation, for better characterization of the products. This study show that PSI is able to provide an effective method for characterizing cell therapy products and allow better understanding of their potency.

PSI reveals clear superiority of new bispecific CAR-T manufacturing method in responding to CD19 and CD22 antigen stimulation

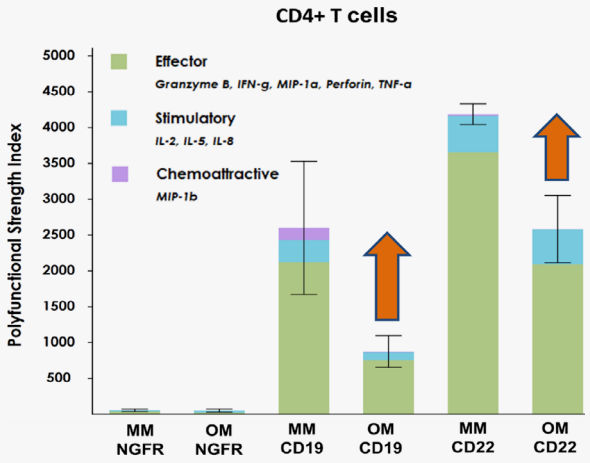

Figure 4 | The PSI of bispecific CD19/CD22 CAR T cell products manufactured using an original method (OM) and a modified method (MM). Both $CD4^+$ and $CD8^+$ cell products manufactured using the MM had a superior polyfunctional response to both CD19 and CD22 antigen stimulation. These results provide a clear indication that the MM manufacturing method significantly improved the overall quality of the CAR T cell product [2].

Predict: PSI of pre-infusion CAR-T product correlates to clinical outcomes in study

Predicting patient response to cell therapy has been a major challenge. Biomarkers that allows clinicians to stratify patients into responders and non-responders and to identify those with an increased risk of suffering from adverse side effects, such as immunotoxicity, is therefore urgently needed.

Here, we present evidence that cell polyfunctionality, captured as PSI, may be a powerful predictive tool for objective response (OR) to cell therapy.

Pre-infusion CAR-T product PSI uniquely correlates with clinical outcome to CD19 CAR-T therapy

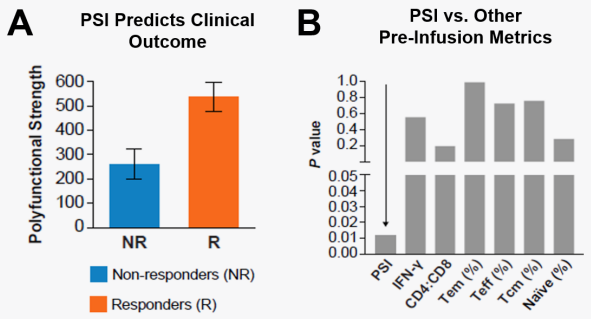

Figure 5 | PSI is shown to be superior over other pre-infusion CAR-T product potency metrics in associating with objective response. Pre-infusion CAR T cell product PSI outperformed other analyzed pre-infusion metrics, incl. IFN-γ co-culture cytokine intensities, $CD4^+$:$CD8^+$ T cell ratio, and various T cell phenotype frequencies, which did not show statistically significant associations with clinical response [3].

PSI uniquely correlates with CD19 CAR-T cell therapy clinical outcome.

To determine whether PSI is correlated with clinical outcome, we profiled pre-infusion $CD4^+$ and $CD8^+$ CAR-T cell samples from 20 of the 22 patients in a clinical trial [3]. Of these 20 patients, 14 had shown an objective response to CAR-T cell therapy. Using Bruker's systems, we found the PSI of pre-infusion CAR-T cells stimulated with CD19-K562 target cells.

We found that the PSI of the pre-infusion CAR-T cell products, which combined $CD4^+$ and $CD8^+$ CAR-T response, showed significant correlation with the OR of patients (Figure 5A). Specifically, we found that the average PSI of the responder subgroup was more than twice as high as the PSI of the non-responders, a difference that was shown to be statistically significant ($p = 0.0119$).

In addition, we showed that PSI outperformed other pre-infusion metrics, including IFN-g co-culture cytokine intensity, ratio of $CD4^+$ to $CD8^+$ T cells, and various T cell phenotype frequencies (Figure 5B). PSI was the only metric that statistically differentiated responding from non-responding patients. The correlation of PSI with clinical outcome indicates the metric's potential as a biomarker for guiding personalized CAR-T cell treatments and potentially predicting therapeutic efficacy.

PSI requires Bruker's highly multiplexed cellular cytokine panel, which allows teasing out subtle differences in response.

While overall PSI correlated with clinical outcome, we wished to also better understand which cellular and cytokine subsets of the CAR-T product PSI associated mostly strongly with outcome. Since CD8⁺ T cells are known to have effector and tumor-killing properties, it was hypothesized that the CD8⁺ subset would drive the increase in PSI. However, the CD4⁺ CAR-T PSI was in fact more highly correlated with objective response of the patients (p = 0.0117, Figure 6A) than the CD8⁺ CAR-T PSI (p = 0.1528, Figure 6B). We further determined that the higher PSI in responding patients' CD4⁺ CAR-T samples was driven by multiple, non-redundant cytokines, including the effector/anti-tumor cytokines IFN-γ, and MIP-1a, stimulatory cytokine IL-8, and inflammatory cytokine IL-17A. Stimulatory IL-5, chemoattractive MIP-1b, and effector cytokine Granzyme B also had notable contributions to the increased PSI (Figure 7).

CD4⁺ CAR-T product PSI shows correlation with clinical outcome to CD19 CAR-T therapy, while CD8⁺ CAR-T PSI does not

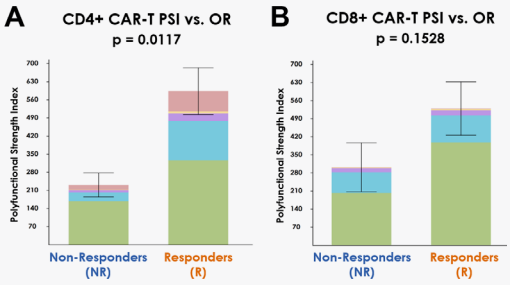

Figure 6 | The PSI of CD4⁺ CAR-T cells pre-infusion correlated with patient response to the CD19 CAR-T therapy (p=0.0117); by contrast, CD8⁺ CAR-T cell PSI did not significantly correlate with outcome. Since CD8⁺ T cells are known to have effector and tumor-killing properties, we initially believed the CD8⁺ subset would drive the increase in PSI. However, the CD4⁺ CAR-T PSI was in fact more highly correlated with objective response of the patients [3].

CD4⁺ CAR-T cells produced multiple cytokines that drove correlation of pre-infusion PSI to therapy outcome

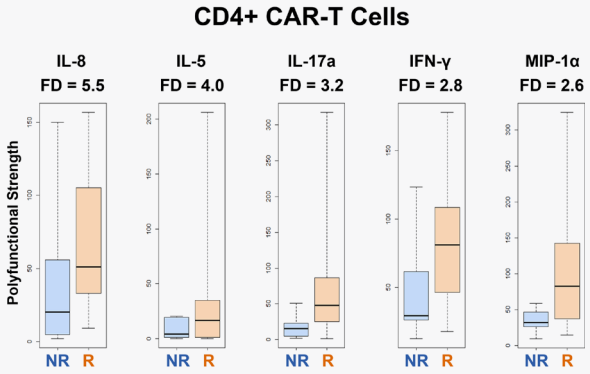

Figure 7 | Multiple non-redundant cytokines produced by CD4⁺ CAR-T cells in response to CD19 stimulation correlated with clinical outcome. While Bruker's single-cell data allows such observations to be made, PSI acts as a powerful overall potency metric, which does not rely on prior knowledge or assumptions about which cytokines most strongly associate with outcome

This study shows the potential power of PSI as a pre-infusion biomarker for in vivo clinical outcome of CAR T cell treatment in patients, where other tested metrics have not associated with outcome. Given the range of contributing cytokines noted, PSI is effective as an overall, aggregated cellular potency metric that does not require knowing the specific subsets that are most correlated with outcome. Moreover, PSI lets you tease out subtle differences in response, further narrow down what drove these differences in PSI and identify additional biomarkers that strengthen the correlation with clinical outcome.

Conclusion

PSI is a powerful and unique metric, with a wide range of applications in engineered immune cell therapy research and development, spanning:

- preclinical discovery, such as demonstrating increased NK cell potency, post gene edits, which correlates with in vivo response [1],
- product and manufacturing optimization, where PSI can reveal distinct differences in bioprocessing methods for process optimization [2], and
- clinical outcome correlates, including the effectiveness of PSI as a potential pre-infusion biomarker for CAR-T therapy patient outcome [3]

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