APPLICATION NOTE

Applying CyTOF Solutions to Cancer Immunotherapy Clinical Trials

Unique high-content proteomics reveals predictive response, biomarker translation and mechanism of action

Introduction

It is critical for immunotherapy clinical trials to attain a comprehensive understanding of the immune response to disease and to potential treatment. This in-depth knowledge better enables accurate identification of predictive markers, biomarker translation and understanding mechanism of action, all of which are important for efficient and effective clinical trial execution. CyTOF™ technology is uniquely positioned to facilitate this level of understanding.

This application note outlines:

- Overcoming challenges in characterizing immune health states with the CyTOF platform
- Addressing limitations of fluorescence flow cytometry
- The impact of CyTOF technology: four clinical trial case studies
  - Identification of predictive-response biomarkers of combination checkpoint therapy for endometrial cancer
  - Peripheral immune indicators of response and survival in checkpoint therapy for non-small-cell lung cancer
  - Complete-response biomarker analysis of neoadjuvant combination therapy for stage 3 melanoma
  - Mechanism of action driving NKG2D-CAR T efficacy in T cell acute lymphoblastic leukemia (T-ALL) and acute myeloid leukemia (AML)

How CyTOF technology overcomes challenges in characterizing immune function

Not only is immune health complex, but understanding the variation in response across different health states involves more than just identifying cell types.

Both cell type and function are key in determining mechanisms behind immune potency and persistence, immune-related adverse events and immunosuppression. To obtain a complete picture, high content solutions that assess intracellular activity are needed to capture a wide range of cell types and their functions.

The high-parameter capabilities of CyTOF platforms enable the comprehensive measurement of functional profiles for immune cells, including intracellular markers such as cytokines, phosphorylation events and transcription factors.

While intracellular proteins are difficult to detect using fluorescence-based cytometry due to challenges with autofluorescence and spillover that can limit fluorochrome capacity and reduce resolving power for rare subsets, CyTOF technology provides higher signal resolution to allow for the detailed determination of cellular functional diversity and to more clearly understand distinct immune signatures (Figure 1).

The wider coverage and higher resolution of the CyTOF platform are essential to identify more diverse functional subsets and understand true function in immune health

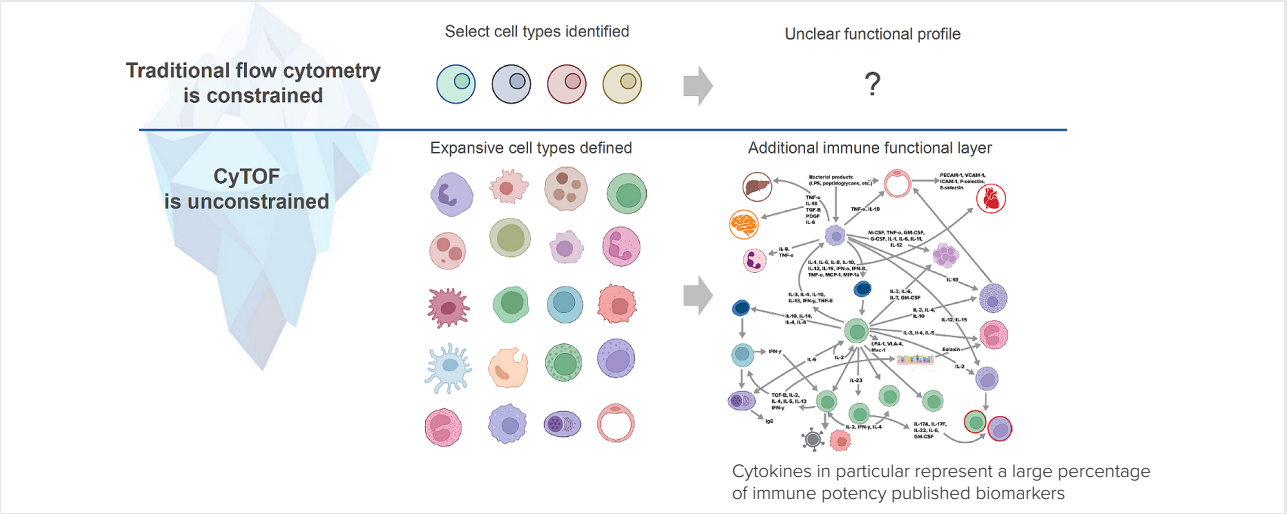

Figure 1. CyTOF technology is the only single-cell platform that can simultaneously and deeply profile a broad range of surface and functional markers on the same cell, capturing functional biology and identifying and analyzing predictive biomarkers.

As summarized in Figure 2:

- High content coverage allows for the detection of a larger range of critical analytes necessary to assess patient pathophysiology and response to therapy
- Resolving complex immune profiles with high-parameter data is necessary to reflect true *in vivo* biology, revealing differentiating markers that help elucidate disease mechanisms and serve as predictive biomarkers
- Fluorescence flow cytometry is a disadvantaged biomarker screening tool because challenges with autofluorescence and spillover limit fluorophore choice and coverage capabilities
- CyTOF technology provides the widest coverage available for comprehensive screening, capturing the most relevant immune metrics

Clinical trials demonstrate how high content and coverage with CyTOF reveals critical predictive biomarkers

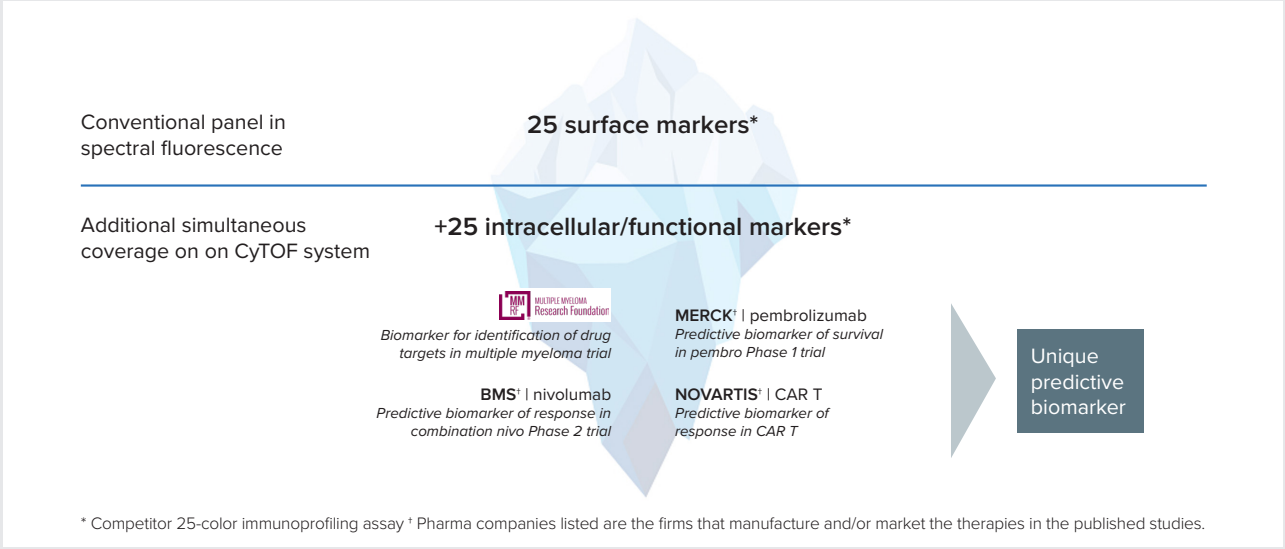

Figure 2. With the ability to look at surface and functional markers simultaneously, CyTOF technology captures unique biomarkers of predictive response, mechanism of action and patient stratification that fluorescence-based flow cytometry misses. The four clinical trial case studies highlighted here are described in more detail in the following sections.

CASE STUDY: Biomarker prediction

CyTOF technology identifies predictive biomarker of response in checkpoint combination therapy

This Phase 2 clinical trial tested the efficacy and safety of a checkpoint inhibitor (nivolumab) and an antiangiogenic agent (cabozantinib) combination therapy versus nivolumab monotherapy in 82 endometrial cancer (EC) patients[1].

Novel therapeutic combinations such as the immunotherapy and antiangiogenic agents reviewed in this publication hold promise as a potential effective treatment against endometrial cancer. Immune profiling using CyTOF provides much needed data on response to treatment, which in this trial demonstrated significantly improved outcomes.

Mass cytometry was critical in identifying the first reported biomarkers for response to therapy, enabling researchers to stratify patients into non-progressors and progressors.

As stated in the publication, "To our knowledge, this is the first pilot study to assess treatment in the post-IO setting, to investigate mechanisms of therapeutic resistance, and to offer a potential treatment option following progression on IO. CyTOF analysis of fresh baseline biopsies provides the first high-dimensional insight into the immune microenvironment of recurrent EC."

CyTOF uniquely stratifies progressors vs non-progressors

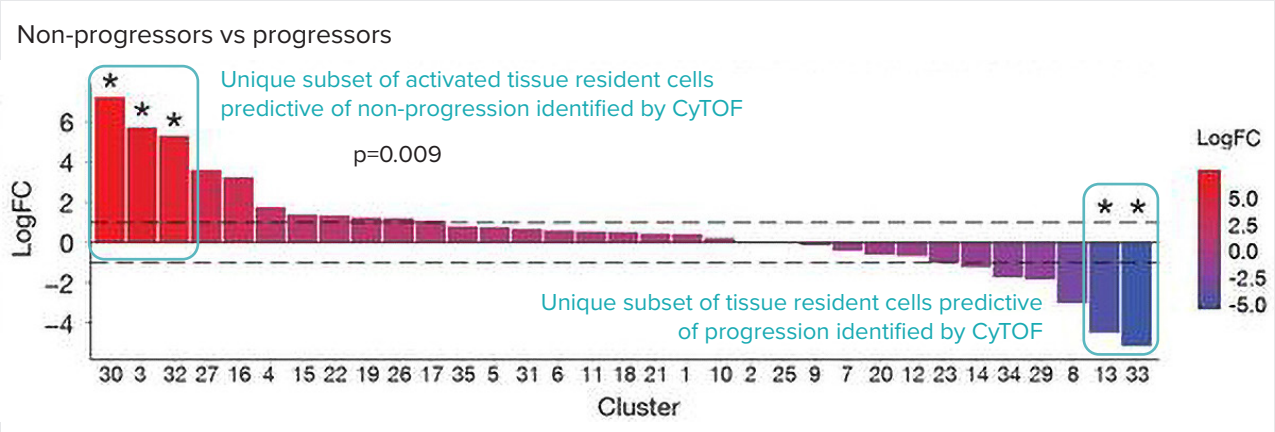

Figure 3. CyTOF analysis identified a unique subset of activated tissue resident (CD103+CD69+) γδ T cells, CD45RA+CD27+ CD8 T cells and CD45RA+CD27+ CD4 T cells predictive of non-progressors. The bar graph shows the differential abundance of each immune cell cluster between non-progressors and progressors.

Study at-a-glance

- A Phase 2 clinical trial demonstrated the efficacy and safety of a combination checkpoint therapy based on immune profiling data in 82 patients
- CyTOF identified a unique subset of activated tissue resident cells that served as predictive biomarkers for non-progression, providing high-dimensional insight into the immune microenvironment of recurrent endometrial cancer

Lheureux, S. et al. "Translational randomized phase II trial of cabozantinib in combination with nivolumab in advanced, recurrent, or metastatic endometrial cancer." *Journal for ImmunoTherapy of Cancer* 10 (2022): e004233.

CASE STUDY: Biomarker prediction

CyTOF technology reveals predictive biomarker of response and survival in checkpoint therapy

This Phase 1 clinical trial tested the safety, tolerability and efficacy of pembrolizumab in 27 non-small-cell lung carcinoma (NSCLC) patients[2].

While immune checkpoint inhibitor (ICI) therapies are positively impacting the treatment of NSCLC, biomarkers that predict efficacy are lacking. The CyTOF platform enabled high coverage evaluation of 31 circulating immune markers that could predict ICI benefit.

Due to the high-parameter capabilities of CyTOF technology, researchers were able to analyze blood baseline frequencies of classical monocytes, NK cells and ICOS+ CD4+ T cells in the same sample and correlate to therapeutic efficacy. CyTOF provided an easy and scalable approach to immune monitoring, generating data that could be used to improve prediction of response in patients with advanced NSCLC.

As quoted in the publication, "we report that a baseline immune peripheral score combining these three populations strongly predicts pembrolizumab efficacy."

CyTOF identifies a unique baseline immune peripheral score (BIPS) predictive of survival

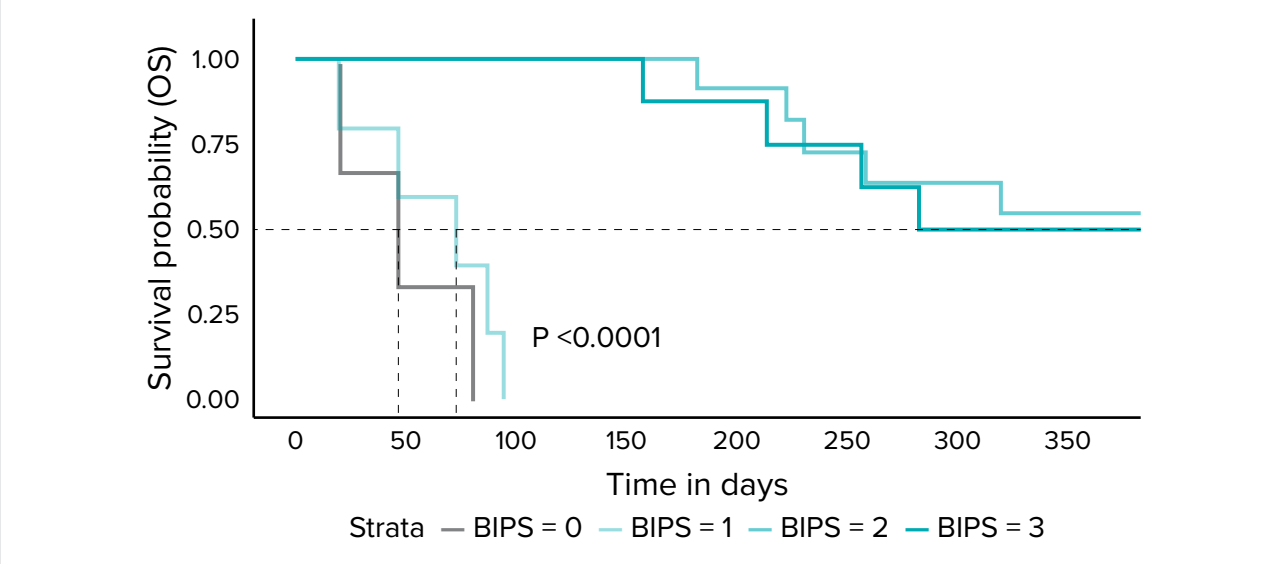

Figure 4. CyTOF analysis demonstrates that blood baseline frequencies of classical monocytes, NK cells and ICOS+ CD4+ T cells are significantly associated with improved objective response rates, progression-free survival and overall survival (shown here). BIPS = a simple score developed by adding one point when the baseline frequency of each cell population was over the pre-cited threshold. BIPS values range from 0-3, with 0-1 identifying progressive disease and 2–3 identifying patients with clinical benefit from treatment.

Study at-a-glance

- A Phase 1 clinical trial utilized CyTOF technology to identify cell types associated with improved outcomes after checkpoint therapy
- Immune monitoring by CyTOF, along with machine learning algorithms, revealed the cells and cell frequencies that strongly predicted pembrolizumab efficacy in 27 NSCLC patients

Rochigneux, P. et al. "Mass cytometry reveals classical monocytes, NK cells and ICOS+ CD4+ T cells associated with pembrolizumab efficacy in lung cancer patients." *Clinical Cancer Research* 28 (2022): 5,136–5,148.

CASE STUDY: Biomarker prediction

CyTOF technology identifies predictive biomarker of response in neoadjuvant combination therapy

This Phase 2 clinical trial tested the efficacy and safety of neoadjuvant vemurafenib, cobimetinib and atezolizumab combination therapy (cohort A) versus cobimetinib and atezolizumab combination therapy (cohort B) in 30 patients with clinically evident resectable stage 3 melanoma[3].

Since both targeted therapies and immunotherapies improve outcomes in melanoma patients, combination treatments have the potential to drive robust pathologic responses.

CyTOF technology was used to screen these cell populations in the same sample: CD4+ T cells, CD8+ T cells, CD8+ naive T cells, CD8+ central memory T cells (TCM), CD8+ effector memory T cells (TEM), CD8+ effector memory T cells re-expressing CD45RA (TEMRA), B cells, NK cells and γδ T cells. Additionally, functional markers such as PD-L1 were simultaneously analyzed on all subsets.

Flow cytometry used to analyze T cell subsets failed to find associations with response prediction, as stated in the paper: "Using flow cytometry we quantitated three distinct blood immune cell populations: tumor-related T cells, effector cytotoxic T cells (CTLs), and pro-apoptotic T cells. Neither baseline frequencies of any of these populations nor the change in frequency from baseline to post-neoadjuvant treatment correlated with pathologic response."

CyTOF technology enabled quantitative assessment of cell frequencies and revealed associations between specific cell subtypes and pathologic response. For example, data showed an increase in the frequency of CD8+TCM cells at baseline in responding patients, providing insights into new treatment regimens and correlations based on known CD8 + TCM cell activity, indicating their potentially important role in disease eradication in lymph nodes. Further, PD-L1 downregulation on T cells was correlated to PD-L1 binding by atezolizumab.

CyTOF identifies unique subsets of T cells associated with complete pathological response

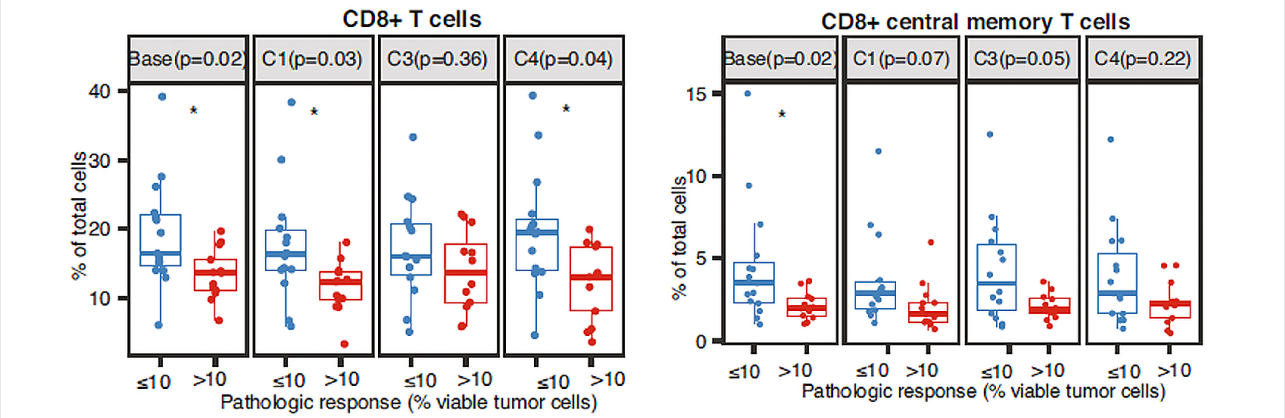

Figure 5. CyTOF technology quantified cell subsets at baseline, after treatment cycle 1 (C1), after completion of neoadjuvant treatment (C3) and after operation (C4) across both cohorts. Frequencies of CD8+ T cells as a percentage of total PBMC are grouped by response status. Increased baseline CD8+ TCM cells were observed in patients with favorable versus unfavorable pathologic responses.

Study at-a-glance

- A Phase 2 trial, NeoACTIVATE (NCT03554083), investigated neoadjuvant combination therapy for melanoma and suggested that the degree of T cell surface PD-L1 downregulation may serve as a biomarker of *in vivo* PD-L1 binding by atezolizumab
- CyTOF technology was used to screen various effector T cell types, along with B, NK and gdT cells, providing a depth of analysis not possible with flow cytometry
- Immune profiling by CyTOF showed peripheral blood CD8+ T cell and CD8+ TCM cell expansion associated with favorable pathologic responses

Hieken, T.J. et al. "Neoadjuvant cobimetinib and atezolizumab with or without vemurafenib for high-risk operable stage III melanoma: the phase II NeoACTIVATE trial." *Nature Communications* 15 (2024): 1430.

CASE STUDY: Mechanism of action

CyTOF analysis confirms NKG2D-CAR T activity in T-ALL and AML and potential enhancement with combination therapy

Following the first in-human Phase 1 study of NKG2D-CAR T safety performed on seven patients, this publication provides comprehensive preclinical evidence of *in vitro* NKG2D-CAR T cell efficacy in T-ALL and AML[4].

To improve typically poor prognosis, CAR T cell approaches that can effectively target AML and T-ALL without off-tumor effects are required.

Mass cytometry was integral in revealing the mechanism of NKG2D-CAR T cell activity: NKG2D-CAR T cells showed significant upregulation of ICOS, CD25, 41BB, OX-40, PD-1 and LAG-3. A decrease in Ki-67 and CXCR3 was seen in TEM and TEMRA CAR subsets and was maintained in TSCM and TCM subsets responsible for an ongoing NKG2D-CAR T cell response.

CyTOF technology detected different characteristics of NKG2D-CAR T cells responding to NKG2D ligand-bearing leukemia cells, as stated in the paper: "[CyTOF] analysis revealed a distinct signature of NKG2D-CAR T cells responding to NKG2D-ligand positive leukemia targets when compared to Empty control T cells or NKG2D-CAR T cells lacking NKG2D-ligand recognition."

CyTOF identifies distinct functional subsets of NKG2D-CAR T cells

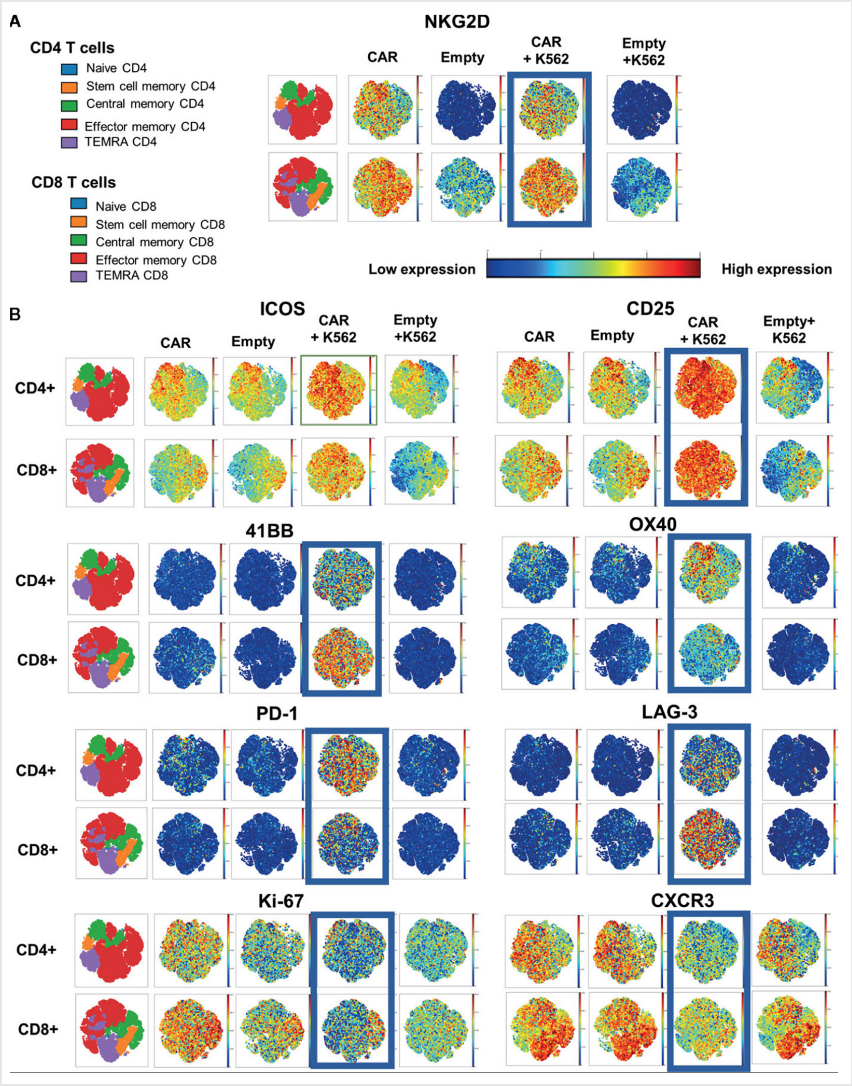

Figure 6. Evaluation of NKG2D-CAR T cells when interacting with NKG2D-ligands on tumor cells. NKG2D CAR T cells or empty control T cells were co-cultured with or without K562 tumor targets and their respective expression profile assessed by mass cytometry. Highlighted boxes show enhanced expression in response to tumor cell presence.

Study at-a-glance

- A Phase 1 study demonstrated that selective NKG2D-ligand upregulation enhances NKG2D-CAR T efficacy against AML, providing a possible strategy for combination therapy
- CyTOF uniquely revealed a distinct signature of NKG2D-CAR T cells responding to associated leukemia targets, informing that NKG2D-CAR T cell response could be further enhanced in combination with checkpoint blockade or genetic modification strategies
- Immune profiling by CyTOF confirmed that even low NKG2D-ligand expression leads to a robust functional response mediated by NKG2D-CAR T cells

Driouk, L. et al. "Chimeric antigen receptor T cells targeting NKG2D-ligands show robust efficacy against acute myeloid leukemia and T-cell acute lymphoblastic leukemia." *Frontiers in Immunology* 11 (2020): 580328.

CyTOF technology simultaneously analyzes phenotype and function to capture critical biomarkers of patient response

These case studies show how CyTOF can reveal mechanism of action, predictive response and relevant biomarkers across a variety of therapeutic areas and indications better than other cytometric technologies.

CyTOF analysis identified:

- A unique subset of activated tissue resident T cells predictive of non-progressors
- A baseline immune peripheral score predictive of objective response, progression-free survival and overall survival
- Unique subsets of CD8+ T cells and CD8+ TCM cells significantly associated with a complete or near complete pathologic response
- Mechanism of persistent NKG2D-CAR T cell response

Offering uniquely wide coverage of surface and functional markers, CyTOF technology is the only solution that can deliver comprehensive data in a highly reproducible, high-content fashion across sites in ways that other platforms cannot accomplish. This is critical to improving immunotherapies by mitigating risks of off-target effects and understanding patient stratification and immune-response biomarkers.

Summary

- Determining both cell type and function simultaneously are key in characterizing immune health state
- CyTOF is the only single-cell proteomics platform that captures highly multiplexed surface and functional markers simultaneously, uncovering unique biomarkers
- CyTOF technology provides the widest coverage available (50+ markers) for comprehensive screening, capturing the most relevant immune metrics
- This is demonstrated in case studies in which CyTOF:
    - Identifies biomarkers for response to therapy, enabling patient stratification based on progression
    - Enables analysis of multiple cell populations in the same sample to reveal associations with therapeutic outcomes
    - Generates quantitative data for multiple cell types at once and correlates to pathologic response
    - Provides new insights into augmenting treatments through combination therapy

References

1. Lheureux, S. et al. “Translational randomized phase II trial of cabozantinib in combination with nivolumab in advanced, recurrent, or metastatic endometrial cancer.” *Journal for ImmunoTherapy of Cancer* 10 (2022): e004233.
2. Rochigneux, P. et al. “Mass cytometry reveals classical monocytes, NK cells and ICOS+ CD4+ T cells associated with pembrolizumab efficacy in lung cancer patients.” *Clinical Cancer Research* 28 (2022): 5,136–5,148.
3. Hieken, T.J. et al. “Neoadjuvant cobimetinib and atezolizumab with or without vemurafenib for high-risk operable stage III melanoma: the phase II NeoACTIVATE trial.” *Nature Communications* 15 (2024): 1430.
4. Driouk, L. et al. “Chimeric antigen receptor T cells targeting NKG2D- ligands show robust efficacy against acute myeloid leukemia and T-cell acute lymphoblastic leukemia.” *Frontiers in Immunology* 11 (2020): 580328.

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