WHITEPAPER

NanoString Profiling in Immuno-Oncology

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Introduction

Immuno-oncology has significantly progressed as a field in the last decade and improved our ability to harness immune responses in a controlled and rational way to treat cancer. For patients who have robust and durable responses to immunotherapies, it is truly a life-preserving therapy. Unfortunately, those responses are still the minority, and most patients do not experience the benefits immunotherapy can offer. Our ability to predict who will respond to immunotherapy, rationally design novel immunotherapy treatments, and understand the biological underpinnings of cancer are all suboptimal at the present time. Measuring biological activity simultaneously in both the tumor and the immune system is key to addressing these challenges. NanoString Technologies® has pioneered the concept of 3D Biology™ Technology: the ability to measure RNA and protein simultaneously from a small sample using a single detector, which allows for precise characterization of these complex interactions in immuno-oncology research.

Historical Context

Ever since William Coley first treated patients with heat-killed bacteria to elicit an immune response against their tumor, researchers have been trying to use the body's immune system as a natural defense against cancer. However, because of the limited understanding of the immune system's "rules of engagement" with cancer, clinical successes in the quest to develop effective immunotherapies have been elusive. For decades, treatments to control cancer targeted the tumor cell directly. Radiation, surgery, and inhibitors of cellular replication were used to limit growth or kill tumor cells. The immune system was not widely considered to regulate the tumor, although rare reports of spontaneous regression, especially in patients with infections or autoimmune diseases, suggested otherwise.[1,2,3] The cancer immunotherapy revolution began in 2011 with the clinical success of the anti-CTLA-4 monoclonal antibody (ipilimumab) in metastatic melanoma. In patients with tumors refractory to all other therapies, a subset of anti-CTLA-4 monoclonal antibody recipients experienced prolonged survival and control of tumor burden.[4] CTLA-4 belongs to the family of immune checkpoint receptors, which are crucial for fine-tuning the immune response by dampening T cell activation to avoid autoimmunity and the destructive effects of an excess inflammatory response. Checkpoint blockade therapy uses monoclonal antibodies to relieve the inhibition of suppressed T cells, allowing them to be activated and recover their antitumor activity. The promise of blocking immuno-suppressive checkpoints, which started with anti-CTLA-4, has since been confirmed using inhibitors of another important immune-suppressive checkpoint, the PD-1/PD-L1 pathway, in both melanoma as well as advanced non-small cell lung cancer.[5,6,7,8,9] Despite recent clinical success of checkpoint inhibitors as single agents in some solid tumors, outside of melanoma and a subset of Hodgkin's lymphoma, the vast majority of patients with metastatic solid tumors do not respond to checkpoint inhibitors, and combination therapy is more effective but also more toxic and expensive. Furthermore, clinical responses to immunotherapies do not follow the trajectory of conventional treatments; thus, standard criteria for measuring responses are insufficient. Consequently, immunotherapeutic drug development requires biomarker approaches to identify benefiting patient populations and monitor efficacy. Due to the complexity of the immune response and of the tumor biology, it is unlikely that a single biomarker will be sufficiently informative. Next-generation biomarkers need to measure and integrate the responses of the host, tumor, and environment. This requires simultaneous measurement of different molecular entities, including RNA and protein using single samples when possible and using the same units, thus maximizing the amount and type of information collected from precious clinical specimens. This unmet need has driven NanoString to develop 3D Biology Technology—the ability to measure any combination of RNA and protein from the same sample simultaneously on a single system. 3D Biology Technology provides the granularity needed for the next generation of highly predictive biomarkers in immuno-oncology, and it is achievable exclusively on the NanoString platform.

Key Themes in Immuno-Oncology

The immune response to tumors is a complex, multifactorial interaction that is shaped by the host, the tumor, and the environment (Figure 1). This dynamic interplay leads to a constant evolution of the abundance and variety of

neoantigens found on the surface of the cancer cell in a process known as immunoediting (Figure 2). Backed by data from a series of experiments in which tumors were generated in immune-deficient mice and subsequently transplanted into isogenically-matched animals, the immunoediting hypothesis posits that nascent tumors display a diverse repertoire of tumor-associated antigens.[10,11] Upon encountering activated immune cells capable of detecting the tumor, immunogenic cancer cells are detected and destroyed in what is known as the elimination phase. Less immunogenic cancer cells persist in an equilibrium with the immune system where they are neither destroyed nor able to expand. Ultimately, mutations in the tumor will give rise to cells that are not recognized by the immune system and during this escape phase the immune system is no longer able to control the growth of the tumor.[12,13] The immunoediting process is unique for each individual and shaped by tumor intrinsic and extrinsic factors, which are further described below.

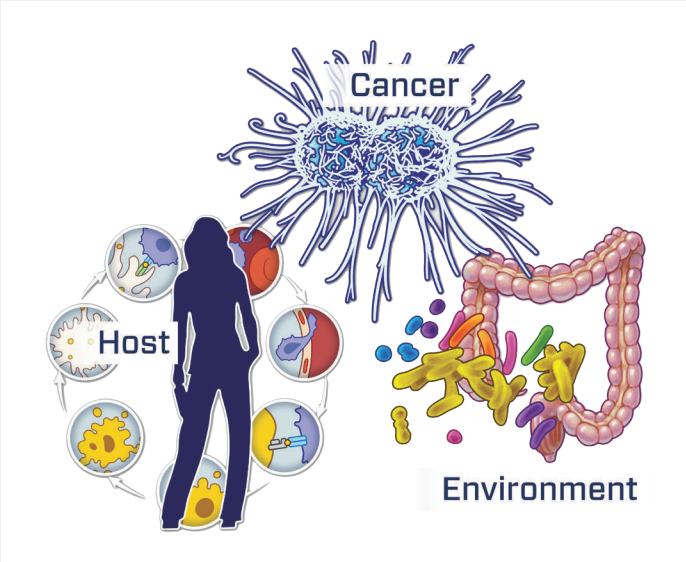

FIGURE 1: Cancer neogenesis and progression involves regulation by the host, tumor, and environment. Due to the complexity of the immune response and tumor biology, an integrated model measuring a combination of RNA and protein may have greater clinical utility.

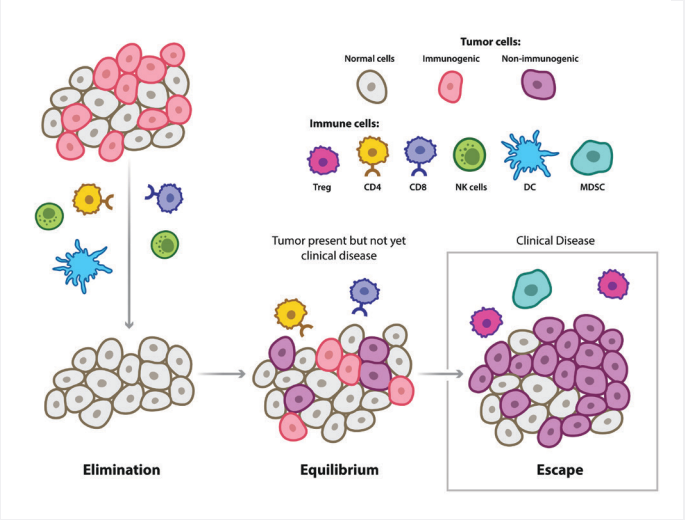

FIGURE 2: Immunoediting promotes outgrowth of less immunogenic cancer cells, which facilitates tumor outgrowth and escape of immune-mediated control.

NanoString has developed a variety of tools described in this White Paper to address aspects of immune (Figure 5) and tumor biology in addition to the panel described below, which captures the complex interplay between the two:

- nCounter® PanCancer IO 360™ Panel - 770-plex gene expression panel that profiles the immune system, the tumor, and the tumor microenvironment. The content of this panel characterizes individual genes and pathways that shape tumor-immune interactions. This panel contains genes composed by the Tumor Inflammation Signature, an investigational biomarker of pembrolizumab response currently under investigation in clinical trials, as well as 14 additional prospective signatures that measure biological activities in the tumor that may also contribute to tumor immune evasion. Any nCounter® Vantage 3D™ Protein panel, with the Vantage 3D Protein Solid Tumor panel content pairing particularity well, or 30 user-selected genes for customized and multi-analyte analysis can be added to this RNA panel.

Role of the Host

An individual's genetic makeup can greatly influence the odds of developing cancer as well as the immune responses against the tumor. The vast majority of somatic mutations are essentially innocuous, yet mutations also arise in genes located within key driver pathways and these mutations can initiate tumorigenesis.[14] For example, germline mutations affecting BRCA1/BRCA2 genes, which are involved in DNA repair, increase the likelihood of developing breast or ovarian cancers.[15] Likewise, patients with familial adenomatous polyposis have mutations in the APC tumor suppressor gene, which leads to the accumulation of polyps on the surface of the large intestine that eventually transform into colon cancer.[16] The immune system plays a vital role in restricting tumor growth, as evidenced by the increased rate of tumor formation and tumor growth in mice lacking one or more

components of the immune system.[17] Chen and Mellman describe the interactions between cancer and the immune system as a series of carefully regulated events that can be self-propagating called the Cancer Immunity Cycle (Figure 3).[18] Each step of this cycle requires the coordination of many factors both stimulatory and inhibitory in nature. Initially, low-level inflammation (from a chronic pathogen, chemical exposure, or an undetermined source) is induced at the site of the tumor. In the absence of secondary pathogen- or cell damage-associated signaling, this inflammation is transient but drives early rounds of tumor proliferation. At the same time, a small amount of tumor cell death will release cancer cell-associated antigens into the tumor microenvironment where they will be processed and displayed by antigen-presenting cells.

Following the initial inflammatory step the antigen-bearing dendritic cells migrate to tumor-draining lymph nodes where they prime circulating cognate T cells (Figure 4). The T cells become activated, adopt effector cell phenotypes, and traffic to the tumor where they exert their physiological effects. In some patients lymph node-like assemblages, called tertiary lymphoid structures, establish in or at the periphery of tumors.[19] The structures are replete with T cells, B cells, and antigen-presenting cells and it is likely that they facilitate anti-tumor immune responses.

Within the tumor, cytolytic T cells (CD8+) detect tumor cells via antigen-specific interactions with the T cell receptor and directly lyse the cognate tumor with a combination of perforin and granzymes. Natural killer cells (NK) are also able to directly lyse tumor cells but unlike cytolytic T cells they are regulated by the balance of engagement of activating and inhibitory receptors on their cell surface. Tumor cells can trigger the activating receptors by displaying cell stress markers on their surface or fail to engage inhibitory receptors by downregulating key antigen presentation pathways to evade T cell-mediated detection. Helper T cells (CD4+) differentiate to a spectrum of phenotypes that produce cytokines to support a variety of immune functions. For example, Th1 helper T cells produce IFNγ to promote cytolytic T cell, NK cell, and macrophage activation. Th2 helper T cells produce IL-4, IL-5, and IL-13, which suppress Th1 responses and may promote suppressor monocyte populations. Th17 helper T cells produce IL-17, which can have immune activating or inhibitory effects. Regulatory T cells (Treg) inhibit antitumor T cell responses by contact inhibition (via CTLA-4 expression), suppressive cytokine secretion (TGFβ, IL-10), or altering the metabolism in a way that is unfavorable to further conventional T cell growth (via adenosine accumulation or tryptophan depletion).

In addition to the inhibitory activities of Tregs, innate suppressive cells also accumulate in the tumor microenvironment and inhibit anti-tumor immune responses. This compartment is made up of a spectrum of cell types, including myeloid-derived suppressor cells, tumor-associated macrophages and neutrophils, and immature dendritic cells,

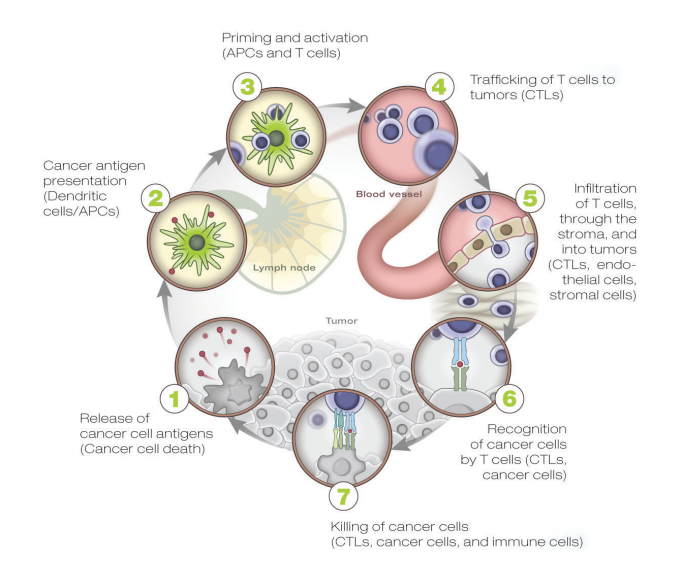

FIGURE 3: The Cancer Immunity Cycle.[18]

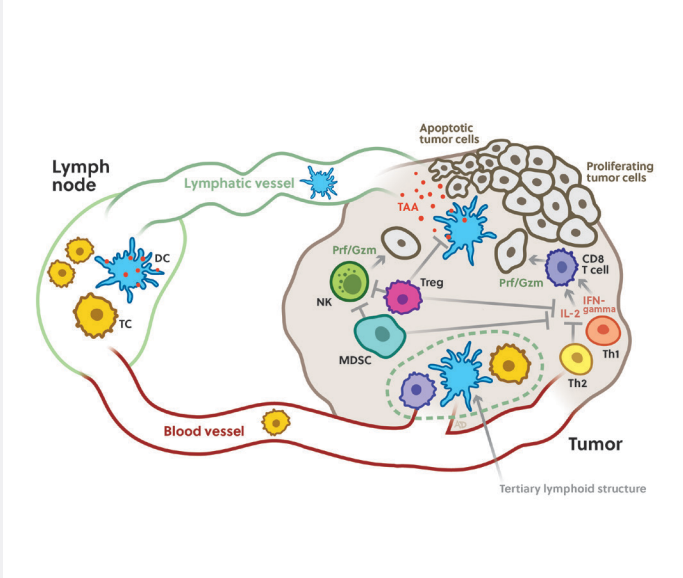

FIGURE 4: Cellular immune response to cancer.

which have some functions in common with Tregs, such as secretion of TGFβ and IL-10, Arginase-I, and Indoleamine 2,3 dioxygenase-I expression.

Given the complexity of the tumor immune response, multiple levels of regulation exist to calibrate the nature and magnitude of the molecular changes that govern the response. Expression of key proteins is controlled transcriptionally, post-transcriptionally, and post-translationally. A thorough understanding of the global immune response to the tumor is required to potentially predict and monitor key changes to the system following therapeutic intervention. NanoString has tools that enable precise monitoring of the tumor immune response through profiling of genotypic background, gene expression (mRNA), and protein expression (Figure 5):

- PanCancer Immune Profiling Panel - 770-plex gene expression panel covering innate and adaptive immune responses, including T and B cell activation and inhibition, inflammation, adhesion molecules, chemokines and cytokines, and pattern recognition receptors. Up to 30 user-selected genes can be added to this panel to customize for specific analyses.

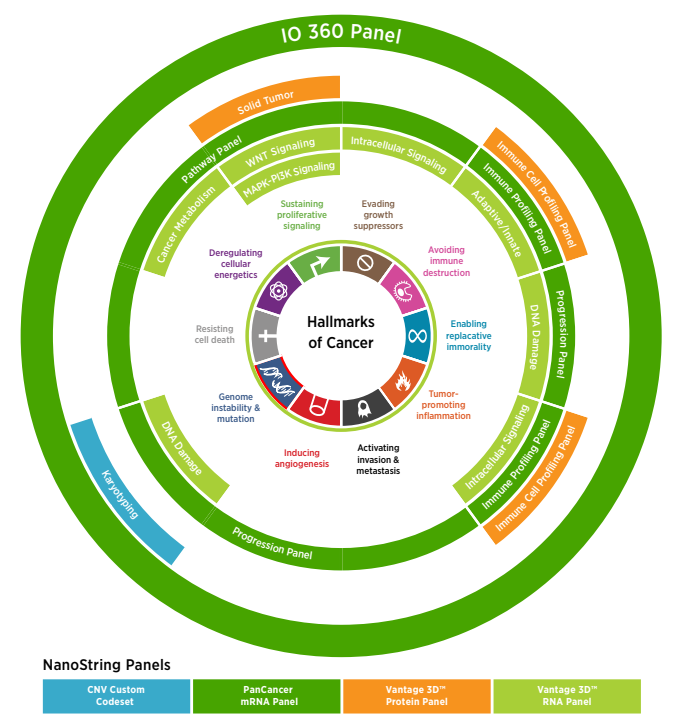

FIGURE 5: The Hallmarks of Cancer adapted from Hanahan, D. and Weinberg, R.A. (2011). The classical cancer driver activities and the NanoString products that address each.

- Vantage 3D RNA:Protein Immune Cell Profiling Assay for cell suspensions - the 770-plex assay described above plus 30 key cell surface immuno-oncology proteins. These 30 proteins are also compatible for use with other Vantage 3D Assays, PanCancer Panels such as PanCancer IO 360, and Custom Codesets. This assay can be customized with 5 additional proteins. Additionally, this assay can be utilized with 3D Flow™ Analysis, which combines flow sorting and 3D Biology Technology in one protocol for multi-analyte analysis of rare cell populations (see Case Study #7).
- Vantage 3D RNA Assays - 192-plex gene expression assays that allow focused investigation of key features of the tumor immune response. Up to 24 custom genes or the 30 key immuno-oncology proteins mentioned previously can be added to this assay for flexible analysis. Specific profiles include: Innate Immunity, Adaptive Immunity, Cellular Profiling, and Intracellular Signaling in addition to others illustrated in Figure 5.
- Myeloid Innate Immunity Panel - 770-plex gene expression panel covering all aspects of the myeloid innate immune response and all major myeloid cell types including neutrophils, eosinophils, mast cells, dendritic cells, monocytes, and macrophages with 19 functional and pathway annotations. Up to 30 user-selected genes or any Vantage 3D Protein Assay can be added to this panel to customize for specific analyses.

Role of the Tumor

Tumors are highly heterogeneous and each patient's tumor likely represents a unique combination of tumor mutations, recruitment of immune cells, and changes to the surrounding stroma and vasculature, with concomitant alterations in metabolism, oxygenation, acidification, and nutrient availability. However, certain key traits must be acquired by all tumors as they escape intrinsic controls that normally limit unchecked cellular proliferation. These traits have been summarized as the Hallmarks of Cancer (Figure 5).[20] Many of these traits are tumor intrinsic activities such as altered metabolism, replicative immortality, and genomic instability. Other traits invoke points of intersection with the immune system[20] including promoting inflammation and avoiding immune destruction. Cancers have recently been recognized to activate signaling pathways that facilitate their evasion of immune-mediated destruction. For example, β-catenin expression in melanoma inhibits dendritic cell and T cell infiltration of the tumor by preventing expression

of CCL4.[21] Likewise, PTEN expression in tumors induces immunosuppressive cytokine secretion, which also prevents T cell infiltration and abrogates response to T cell-mediated immunotherapy such as anti-CTLA-4 and/or anti-PD-1.[22] Finally, epigenetic modifications within the tumor can lead to loss of Th1 trafficking cytokines, T cells, and sensitivity to checkpoint blockade.[23] On the other hand, mutations in DNA repair pathways can also arise spontaneously in tumors leading to significantly increased mutational burden. Mounting evidence suggests that tumors with higher numbers of mutations are more sensitive to immunotherapies due to increased neoantigen display (Figure 6).[24]

NanoString has a wide range of tools that can provide insights into tumor biology through genomic, transcriptomic, and proteomic profiling:

- PanCancer Pathways Panel—770-plex gene expression panel addressing the 13 canonical pathways of cancer, including 124 cancer driver genes. 30 user-selected genes can be added to this panel to customize for specific analyses.
- PanCancer Progression Panel—770-plex gene expression panel that covers epithelial to mesenchymal transition, angiogenesis, extracellular matrix remodeling, and metastasis. 30 user-selected genes can be added to this panel to customize for specific analyses.
- Vantage 3D RNA Assays—192-plex gene expression assays that allow focused investigation of Cancer Metabolism, Wnt Signaling, DNA Damage and Repair, and MAPK-PI3K signaling in addition to others illustrated in Figure 5. These assays are compatible with simultaneous protein profiling and can be customized with the addition of 24 genes.
- nCounter® CNV CodeSets—customizable CodeSets of up to 800 targets to profile replication errors that result in changes to gene copy number.
- nCounter® Gene Fusion Panels - Multiplexed analysis of Lung or Leukemia fusions to directly detect gene fusion events.
- Vantage 3D RNA:Protein Solid Tumor Assay—the 770-plex PanCancer Pathways panel described above plus up to 28 key total and phospho-proteins focused on cancer pathways. The protein is also compatible for use with other Vantage 3D Assays, PanCancer Panels, and Custom Codesets, and can be customized with up to 5 additional proteins.

Role of the Environment

The environment is a critical component of modulating tumorigenesis and host responses against the tumor. Changes induced by the environment are recorded in the genome as epigenetic modifications and are highly regulated by a multitude of chromatin- and histone-modifying enzymes.[25] Epigenetic regulation of transcription can be profoundly dysregulated in cancer, which in some cases can alter immune responses to the tumor. For example, histone methylation and deacetylation can inhibit expression of tumor-associated antigens on the tumor cell surface by blocking peptide loading on the MHC I complex, as well as inhibiting expression of CD80, CD86, and ICAM1 that engage and activate T cells.[26] Another point of intersection of the environment and the immune system is gut microbiota. Numerous studies have shown that sampling of the commensal flora is necessary to establish appropriate thresholds of activation of the immune response.[27, 28, 29] Recent publications are drawing more attention to the

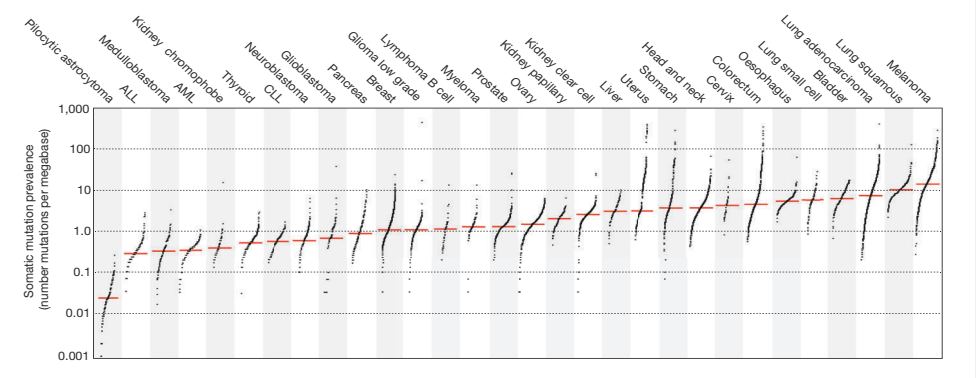

FIGURE 6: Mutational loads across different tumor types correlates with tumor immunogenicity.[24]

specific role of the gut microbiota in regulating immune responses to cancer. Specifically, Vetizou et al. demonstrated that T cell responses specific for Bacteriodes spp. could enhance the efficacy of anti-CTLA-4 antibodies.[30] Furthermore, Sivan et al. showed that certain commensal populations could directly improve control of tumors and work in combination with checkpoint inhibitors to limit tumor growth.[31] Further studies will undoubtedly reveal more and greater interactions between the gut microbiota and the host immune response that alter tumor detection and control in a variety of ways.

Application of the NanoString Platform in Immuno-Oncology

A systems biology philosophy and multi-omics tools are required to understand the complexities of the immune response to tumors. The NanoString platform is actively being used in basic and translational research to elucidate the fundamental biology that drives immuno-oncology. Reducing inherent biological complexity to a single test requires a straightforward, reproducible, and easy-to-use assay that provides consistent results. Ultimately, this type of work will help accelerate the pace of immunotherapy development and allow for a deeper understanding of the interactions between the tumor, host, and environment. Below are seven case studies from the literature and active NanoString collaborations that demonstrate how the nCounter system can reveal the underlying biology of the tumor and host and translate these findings into potential key signatures of clinical activity.

Case Study #1 – Clinical trial with immuno-oncology intervention

The Cancer Immunotherapy Trials Network (CITN) conducted a study treating melanoma with a single agent immunotherapy or a combination of two immunotherapies. Peripheral blood was collected eight days after treatment and profiled with the NanoString PanCancer Immune Profiling Panel. Expression heat maps were generated with nSolver™ analysis software and unsupervised clustering of the data discriminated groups who had received the mono- or combination therapy (Figure 7A). Furthermore, differential expression analysis revealed a seven-gene signature in the combination treated group, of which six out of seven targets were directly related to the mechanism of action of the combination therapy (Figure 7B). These data demonstrate that NanoString technology can identify global changes in gene expression following treatment and those expression patterns could be reduced to a biomarker signature for use in future clinical trials to monitor for efficacy.

Case Study #2 – Adaptive immune signatures from early on-treatment tumor biopsies may be predictive of response to checkpoint blockade[32]

To demonstrate that adaptive immune signatures in tumor biopsy samples obtained early on during the course of treatment may be highly predictive of response to immune checkpoint blockade, immune profiling was performed via a 12-marker immunohistochemistry (IHC) panel and targeted gene expression profiling using a NanoString Custom CodeSet containing 795 RNA, 229 of which were immune-related genes and genes pertaining to common cancer signaling pathways (Figure 8). This study also demonstrated differential effects on the tumor microenvironment induced by sequential CTLA-4 and PD-1 blockade. Importantly, potential mechanisms of therapeutic resistance to immune checkpoint blockade were identified. This study demonstrates that immune profiling on both the RNA and protein level of early on-treatment biopsies is potentially predictive of response to PD-1 blockade.

Case Study #3 – Identifying potential immunotherapy targets[33]

In this study, Beard et al. sought to identify potential candidate genes for immunotherapy in metastatic melanoma. Five melanoma cell lines, 59 tumors, and 31 normal tissues were profiled with a Nanostring Custom CodeSet (Figure 9). The group identified seven potential immunotherapy targets that could increase the number of patients potentially eligible for adoptive immunotherapy. CSAG2, MAGEA3, MAGEC2, IL13RA2, PRAME, CSPG4, and SOX10 were highly overexpressed in tumor samples versus normal tissue samples. NanoString was selected to provide a reliable way to test multiple candidate genes at once and select attractive potential targets for further investigation. NanoString analysis also correlated well with IHC, flow cytometry, and qPCR.

Case Study #4 – Identification of immune gene signature predictive for survival of melanoma[34]

Tumor biopsies from patients with stage II/III melanoma were profiled by NanoString technology using a NanoString Custom CodeSet. A 53-gene signature was identified to be predictive of non-progression, including disease-specific survival and recurrence-free survival (Figure 10). This signature was dominated by genes associated with T cell and NK cell activation, migration, and function. This gene signature was subsequently confirmed in an independent cohort, as well as by microarray and immunohistochemistry. Signatures such as these could be used in the future to stratify patients in immunotherapy trials as well as predict patient survival when conventional metrics are ambiguous.

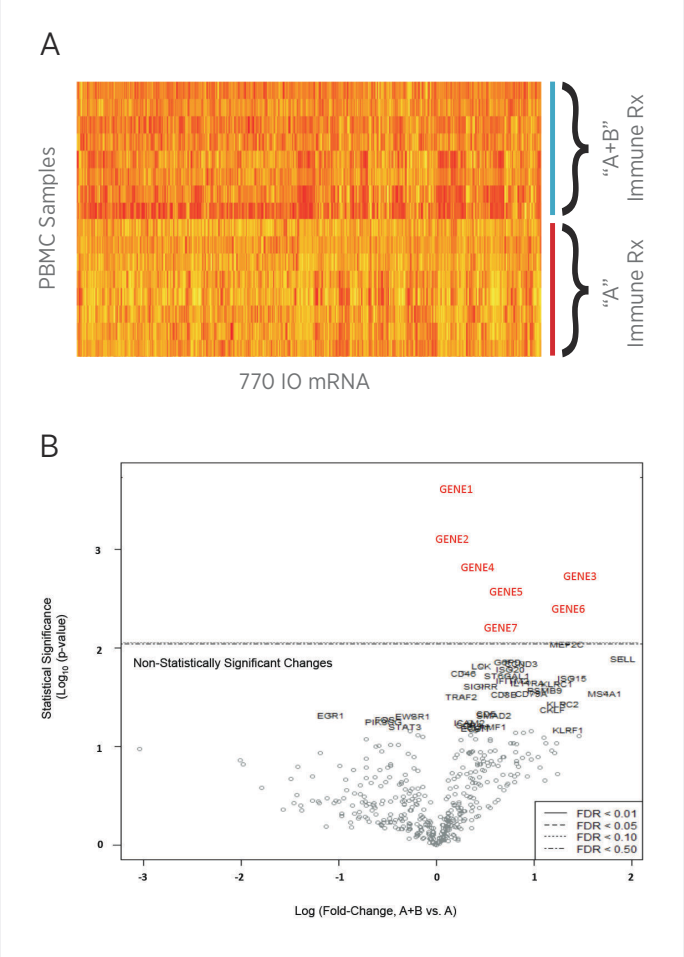

FIGURE 7: NanoString technology differentiates gene expression changes in response to distinct immunotherapies. A) PanCancer Immune Profiling of patient samples following treatment. B) Gene expression signature is identified from NanoString analysis.

Case Study #5 – Commensal bacteria regulation of tumor immunity[35]

In work done by Iida et al. at the National Cancer Institute, the NanoString nCounter system was used to elucidate gene expression changes following immunotherapy in syngeneic colorectal tumor-bearing mice with normal or disrupted gut microbiota. The investigators showed that treatment with the Th1 T cell-promoting regimen of a blocking antibody to IL-10R in combination with the immunostimulatory TLR9 agonist CpG-ODN induced expression of proinflammatory and Th1-associated cytokines, including TNF as detected by NanoString (Figure 11). Treatment with anti-IL-10R and CpG-ODN also induced accumulation of intratumoral myeloid cells and potently suppressed tumor growth. This inhibition was dependent upon the presence of healthy gut flora and detection of the flora via TLR4. These studies confirm the importance of microbiota in regulating anti-tumor immune responses and also demonstrate the power of the nCounter system to broadly elucidate biological pathway activation.

Case Study #6 – IL-10 controls the Th17 response in the tumor microenvironment[36]

IL-10 and Tregs are attractive immunotherapy targets because they suppress cytolytic and Th1 immune responses. Some reports suggest that IL-10 and Tregs can also inhibit Th17 cells in the tumors, which can have both positive and negative effects within the tumor by limiting tumor associated inflammation. In some cancers, this can result in a poor prognosis. Stewart et al. investigated the origin and function of IL-10 producing cells in the tumor microenvironment and discovered that Tregs in the tumors were producing IL-10 in response to IFN signaling, and this IL-10 could negatively regulate Th17 cells. NanoString analysis was performed on flow sorted cells from syngeneic tumor models grown in mice lacking various components of the relevant pathways to uncover that the interplay of type I IFN, Tregs, and IL-10 (Figure 12). Interfering with this network utilizing immunotherapy could have negative consequence of promoting Th17 inflammation and ultimately cancer growth.

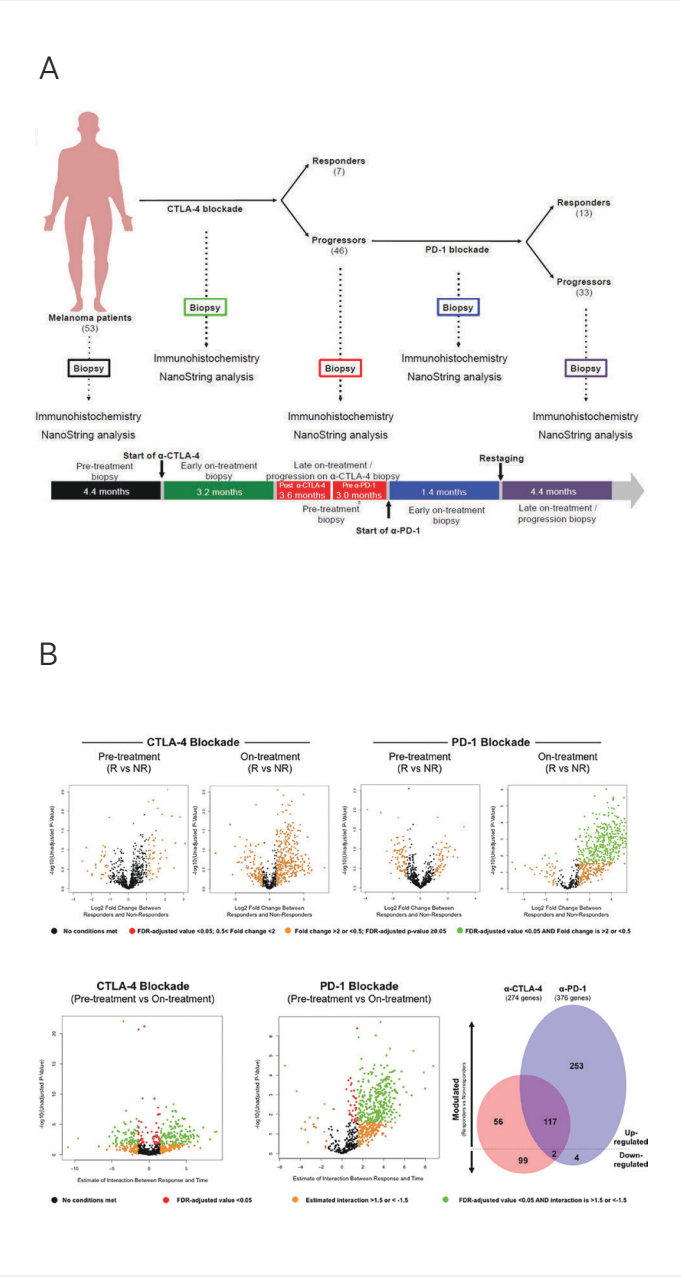

FIGURE 8: A. Illustration of research study design to profile patients with metastatic melanoma treated with CTLA-4 blockade (n=53) and non-responders to CTLA-4 blockade that were then treated with PD-1 blockade (n=46), 13 of which responded. Biopsies were taken at multiple time points for IHC and NanoString analysis. B. NanoString analysis reveals differentially expressed genes for responders and non-responders highlighted in green between different timepoints and treatments. Genes were also identified that showed a significant interaction with CTLA-4 blockade and PD-1 blockade.

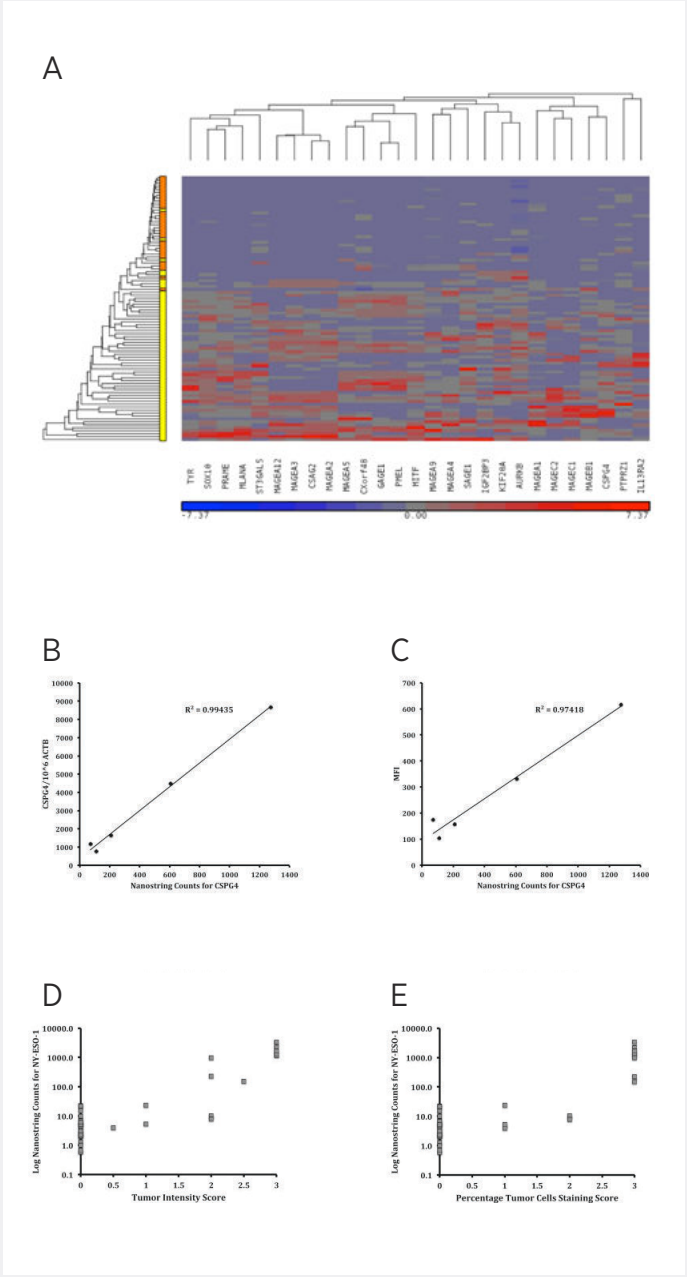

FIGURE 9: NanoString analysis identified A. 27 genes that differentiated tumor (yellow) from normal tissue (orange). Data was benchmarked against B. qPCR, C. flow cytometry D,E. and IHC with D. intensity of staining of 0=no reactivity, 1=weak reactivity, 2=moderate reactivity, 3=intense reactivity, and E. percentage of tumors cells that stained of 0=0%, 1=0-5%, 2=5-50%, 3=>50%.

Case Study #7 – 3D Flow™ Analysis for T cell profiling

To understand immune biology, it is often necessary to analyze pure cell populations in addition to heterogenous mixtures of cells. NanoString 3D Flow Analysis integrates 3D Biology Technology with flow cytometry cell sorting, a common method to isolate cells of interest. Two experiments were performed to demonstrate the multi-omic capabilities of this workflow to profile T cell biology. First, to look at molecular profiles across multiple T cell subsets, 5,000 cells from four T cell populations were sorted from bulk PBMC directly into lysis buffer (Figure 13A). Each of these sorted T cell populations were analyzed with the Vantage 3D RNA:Protein Immune Cell Profiling Assay (Figure 5), which includes 30 cell surface targets and 770 RNA. This experiment highlights the similarities and differences between these distinct populations using minimal sample input (Figure 13A). To profile stimulation induced changes, CD3+ T cells were sorted from bulk PBMC pre- and post-stimulation with CD3 and CD28 and analyzed using the Vantage 3D RNA:Protein Immune Cell Profiling Assay to reveal key RNA and protein targets modulated by stimulation (Figure 13B).

Conclusions

The immune system has great potential for specific destruction of tumor cells with no toxicity of normal tissue and for long-term memory that can prevent cancer recurrence. Perhaps, for the first time, the promise of teaching the immune system to recognize, eliminate, and maintain surveillance against cancer is attainable. Immunotherapy has the potential to be more effective and its efficacy more durable than what is obtainable with surgery, radiation, or targeted therapies. However, efforts to modulate the effects of the immune system in the tumor environment are imperfect and incomplete as we do not yet have a thorough understanding of how the tumor, host, and environment interact with each other. These interactions are spatially and temporally dynamic, further complicating analysis. A significant effort is dedicated to understanding the nature of these interactions and predicting, controlling, and monitoring the consequences of modulating the immune response. New treatments and new combinations of existing treatments should further enhance our ability to direct immune responses against the tumor and elicit novel immunological signatures of efficacy for each patient. NanoString provides a platform uniquely designed to address key questions in immuno-oncology and reduce biological complexity to relevant signatures that can be identified and developed to potentially make cancer a manageable disease. The ability to utilize 3D Biology approaches to generate biomarker signatures that can measure mRNA changes, protein changes, and post-translational modifications—all simultaneously—ushers in an entire new era of precision oncology and may greatly accelerate the pace with which these new and exciting therapies come to market.

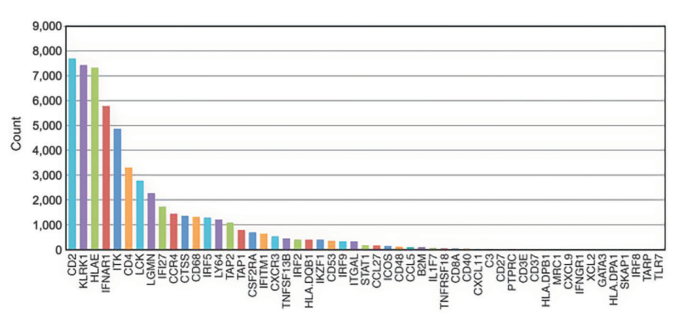

FIGURE 10: Gene signature associated with survival in melanoma.

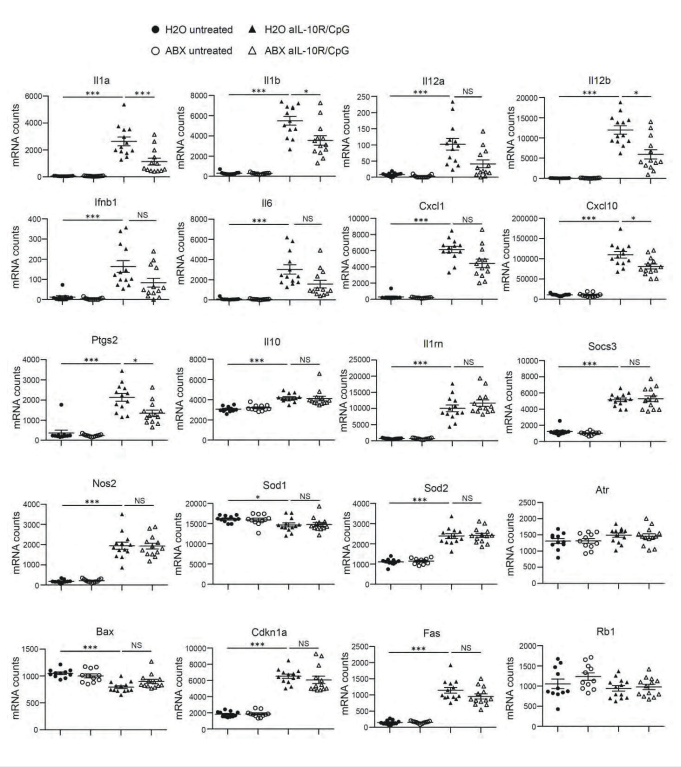

FIGURE 11: NanoString analysis reveals effects of microbiota on immunotherapy. Data shows wide-scale gene expression changes following antibiotic and/or Th1 immunotherapy.

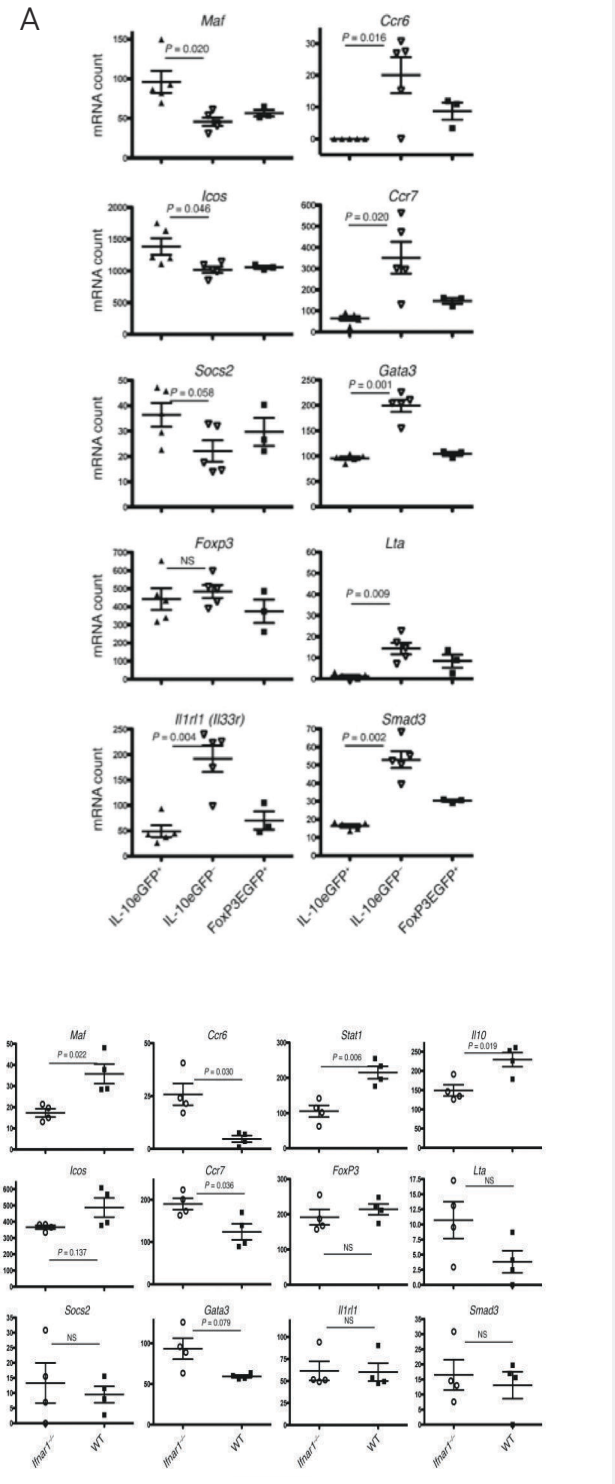

FIGURE 12: A. NanoString analysis of flow sorted Tregs from an MC38 tumor in VERT-X and FoxP3-EGFP mice validates expression differences that indicate IL-10 expression by Tregs associates with T cell activation. B. NanoString analysis of flow sorted Tregs from Ifnar1$^{-/-}$ or WT mice demonstrates to role of Type I IFN signaling for Treg activation.

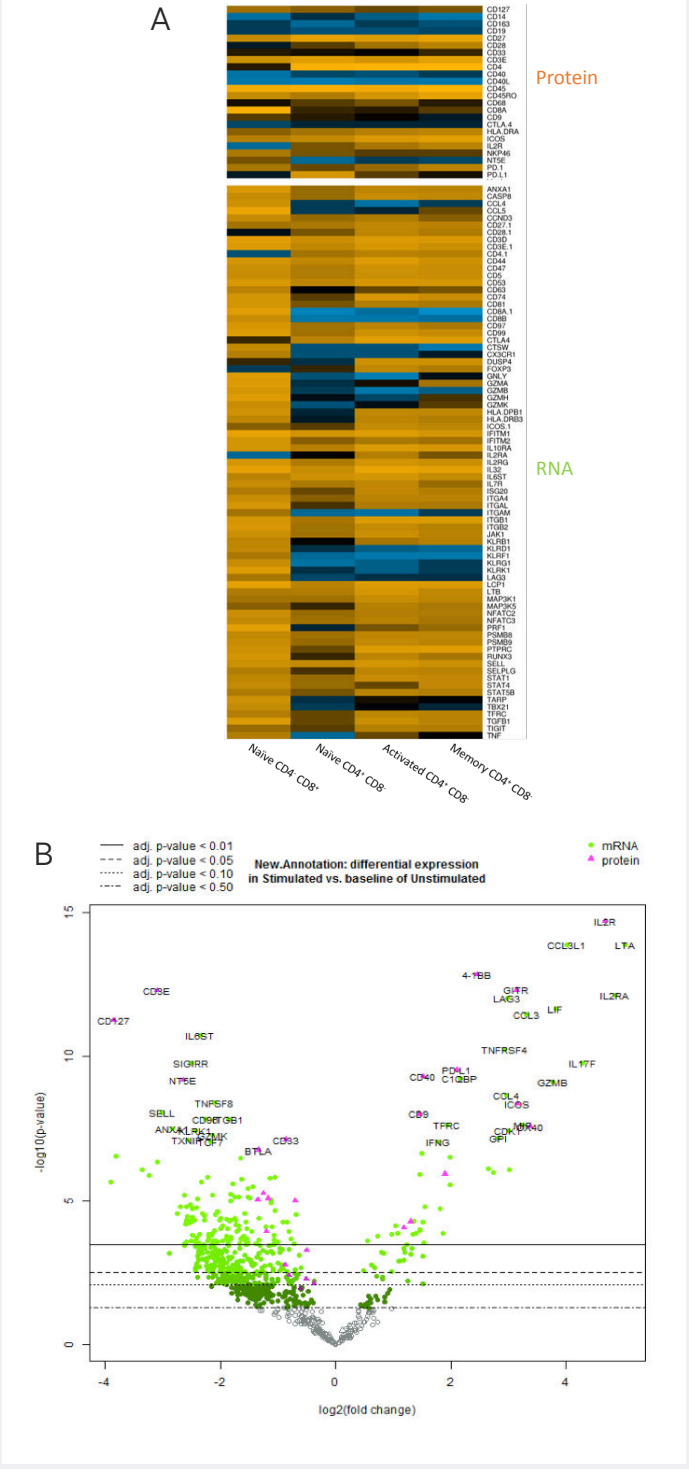

FIGURE 13: A. Heatmap of RNA and protein targets detected in 5,000 flow sorted naïve CD4-/CD8+ T cell, naïve CD4+/CD8- T cell, activated CD4+/CD8- T cell, and memory CD4+/CD8- T cell populations. Analysis shows high correlation of activated and memory cell populations. B. Analysis of 5,000 sorted CD3+ T cells from CD3 and CD28 stimulated PBMC show distinct stimulation induced changes in protein (pink) and gene expression (green).

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