Multiplexed Cancer Immune Response Analysis

nCounter® PanCancer Immune Profiling Panel *for* Gene Expression

Lucas Dennis, Seely Kaufmann, Patrick Danaher, Christina Bailey and Joseph Beechem

v1.0 | SEPTEMBER 2014
NanoString Technologies, Inc., Seattle, WA 98109

Multiplexed Cancer Immune Response Profiling Using the nCounter® PanCancer Immune Profiling Panel

Introduction

The ability of mutated cells to give rise to pathological cancer relies upon the capability of these cells to evade immune recognition, suppress immune activity, and persist in a chronically inflamed environment[1,2]. Tremendous growth in the understanding of these complex processes is beginning to generate breakthroughs in the treatment of cancer, and "Cancer Immunotherapy" was recently named the "Breakthrough of the Year" by *Science* in 2013[3]. However, our understanding of the immune response to cancer is far from complete. There is a need for tools that enable researchers to explore the tumor microenvironment in new and different ways. This report describes a novel gene expression panel that will enable researchers to create profiles of the human immune response in all cancer types and shows how this panel has the potential to accelerate the development of drugs, therapies, and predictive biomarker signatures for response to immunotherapeutic treatments.

The nCounter® PanCancer Immune Profiling Panel is a highly multiplexed gene expression panel designed to quantitate 770 genes that fall into four functional categories (**FIGURE 1**):

1. **Identifying immune cells**, such as those in a PBMC population or infiltrating into a tumor.
2. **Assessing immunological function** and response to immunotherapy, such as immune checkpoint regulation.
3. **Identifying tumor-specific antigens**, such as cancer-testis (CT) antigens.
4. **Housekeeping genes** that facilitate sample-to-sample normalization.

The nCounter PanCancer Immune Profiling Panel is fully compatible with clinically relevant sample types such as fresh-frozen (FF) tissue, formalin-fixed, paraffin-embedded (FFPE) tumor sections, isolated immune cell populations such as PBMCs, and cell lysates. The panel may be used in conjunction with nCounter® Panel-Plus products for additional flexibility in experimental design.

PanCancer Immune Profiling Panel: 770 Genes

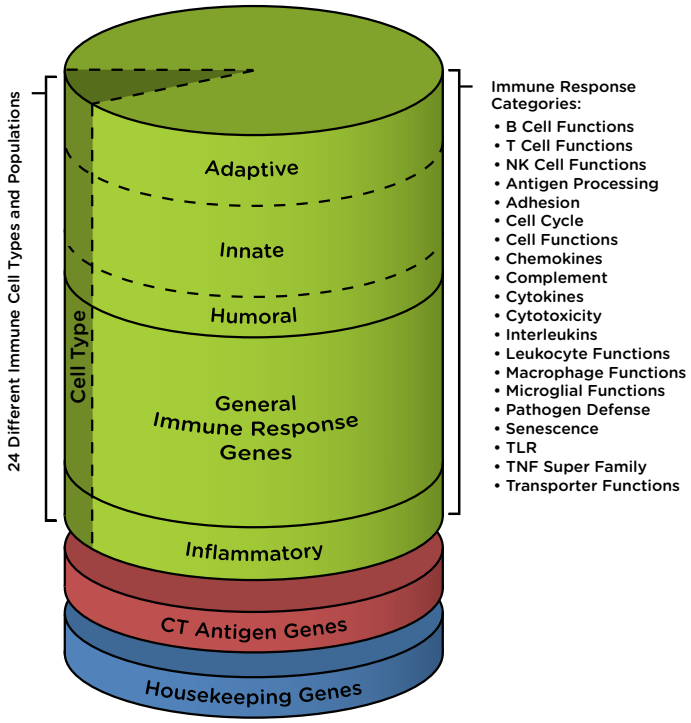


FIGURE 1: Distribution of genes included in the PanCancer Immune Profiling Panel, including genes for identifying Immune Cells (dark green), Immune Response genes (green), CT Antigens (red) and Housekeeping genes (blue). Biological process categories comprising the Immune Response genes are indicated on the right.

Identifying Immune Cell Types in Cancer

Many immune cell types (**TABLE 1**) are found in the tumor microenvironment and interact with a tumor, creating a complex milieu that affects the growth and evolution of cancerous cells by processes such as promoting angiogenesis, inducing immune tolerance, and immunoediting[4-6]. Identifying and observing discrete cellular populations within samples has been a focus of immunological study for decades. Histopathological and flow cytometric analyses have provided ample evidence that variable numbers of infiltrating immune cells are found within the tumor microenvironment[7,8].

The classification and enumeration of immune cells within tumor samples and in the periphery has been shown to be a significant and powerful predictor of patient survival[9,10] and has led to efforts such as Immunoscore, a new approach proposed for classifying cancer pathologies[11,12]. By including markers demonstrated to specifically identify major immune cell populations within cancer samples[13] (**TABLE 1**), the PanCancer Immune Profiling Panel can efficiently define both the immunological activity of these samples as well as identify changes in immune cell populations in response to external stimuli such as immunotherapeutic adjuvants.

NanoString has included 109 genes that define 24 immune cell types and populations. Genes were chosen after careful review of literature that included studies examining expression in purified cell populations[13]. For some cell populations, such as T cells, both pan T cell and lineage-specific markers were included, *e.g.*, observation of CD3E expression in a tumor sample could indicate infiltration of T cells while observation of FOXP3 in the same sample would specifically identify the presence of regulatory T cells, enabling the detection of rare cell populations.

Immune Cell Types

TABLE 1: Immune cell types and populations involved in the response to cancer and corresponding population-specific genes included in the PanCancer Immune Profiling Panel.

	Cell Type		Description	Panel Genes
Adaptive Immune Response	B Cells		Perform several roles, including generating and presenting antibodies, cytokine production, and lymphoid tissue organization.	BLK, CD19 (CD19), CR2 (CD21), HLA-DOB, MS4A1 (CD20), TNFRSF17 (CD269)
	T Cells		Play a central role in immunity and distinguished from other lymphocytes (*e.g.*, B cells) by the presence of a T cell receptor (TCR) on the cell surface.	CD2, CD3E, CD3G, CD6
		Helper T Cells	A subset of CD3+CD4+ effector T cells that secrete cytokines with different activities.	ANP32B (APRIL), BATF, NUP107, CD28 (CD28), ICOS (CD278)
		T_H1	Produce IL-2 and IFNγ and promote cellular immunity by acting on CD8+ cytotoxic T cells, NK cells and macrophages.	CD38, CSF2 (GM-CSF), IFNG, IL12RB2, LTA, CTLA4 (CD152), TXB21, STAT4
		T_H2	Produce IL-4, IL-5 and IL-13 and promote humoral immunity by acting on B cells.	CXCR6 (CD186), GATA3, IL26, LAIR2 (CD306), PMCH, SMAD2, STAT6
		T_H17	Produce IL-17A, IL-17F, IL-21 and IL-22 and promote anti-microbial inflammation.	IL17A, IL17RA (CD217), RORC
		T_{FH}	Trigger the formation and maintenance of germinal centers through the expression of CD40L and the secretion of IL-21 and IL-4, thereby playing a critical role in mediating the selection and survival of B cells.	CXCL13, MAF, PDCD1 (CD279), BCL6
		Regulatory T Cells (T_{reg})	CD3+CD4+ T cells that inhibit effector B and T cells and play a central role in suppression of autoimmune responses.	FOXP3
		Memory T Cells	T_{cm} (central memory)	Educated T cells that rapidly respond to antigen. Central memory T cells express L-selectin and CCR7; they secrete IL-2, but not IFNγ or IL-4.	ATM, DOCK9, NEFL, REPS1, USP9Y
			T_{em} (effector memory)	Educated T cells that rapidly respond to antigen. Effector memory T cells do not express L-selectin or CCR7; they secrete effector cytokines like IFNγ and IL-4.	AKT3, CCR2 (CD192), EWSR1 (EWS), LTK, NFATC4
		Cytotoxic (CD8) T Cells	Effector T cells with cytotoxic granules that interact with target cells expressing cognate antigen and promote apoptosis of target cells.	CD8A (CD8), CD8B (CD8B), FLT3LG, GZMM (MET1), PRF1
		Gamma Delta T Cells (Tγδ)	Express surface antigen recognition complex type 2 and represent a small percentage of the peripheral T cell population. Functions span innate and adaptive immune responses, including direct cytolysis and establishment of memory phenotypes.	CD160, FEZ1, TARP (TCRG)
Innate Immune Response	Cytotoxic Cells	Natural Killer (NK) Cells	Provide a rapid cytotoxic response to virally infected cells and tumors. These cells also play a role in the adaptive immune response by readily adjusting to the immediate environment and formulating antigen-specific immunological memory.	BCL2, FUT5, NCR1 (CD335), ZNF205
			$CD56_{bright}$	Constitute the majority of NK cells in secondary lymphoid tissues. Abundant cytokine producers and weakly cytotoxic before activation.	FOXJ1, MPPED1, PLA2G6, RRAD
			$CD56_{dim}$	Constitute the majority of NK cells in the periphery and are more cytotoxic than $CD56_{bright}$ cells.	GTF3C1, GZMB, IL21R (CD360)
	Dendridic Cells	Conventional (Myeloid) Dendritic Cells (DC)	Cells that process antigen material and present it on the cell surface to T cells, thereby acting as messengers between the innate and adaptive immune systems.	CCL13, CCL17, CCL22 (MDC), CD209 (CD209), HSD11B1
			iDC (immature)	Play a critical role in initiating tumor immunity. Tumor cells can exploit the functional roles of iDCs for tumor progression via release of soluble factors such as VEGF.	CD1A, CD1B, CD1E, F13A1, SYT17
			aDC (activated)	Promote the induction of the adaptive immune response by presenting captured antigen to naive T cells.	CCL1, EBI3, IDO1 (INDO), LAMP3 (CD208), OAS3
		Plasmacytoid Dendritic Cells (pDC)	Similar in appearance to plasma cells and share many characteristics with myeloid dendritic cells. These cells can produce high levels of IFNα.	IL3RA (CD123)
	Macrophages		Scavengers of dead or dying cells and cellular debris. Macrophages have roles in innate immunity by secreting pro-inflammatory and anti-inflammatory cytokines.	APOE, CCL7 (FIC), CD68, CHIT1, CXCL5, MARCO, MSR1 (CD204)
	Granulocytes	Mast Cells	Granulocytes that can influence tumor cell proliferation and invasion and promote organization of the tumor microenvironment by modulating the immune response.	CMA1, CTSG, KIT (CD117), MS4A2, PRG2, TPSAB1
		Neutrophils	Phagocytic granulocytes that act as first-responders and migrate towards a site of inflammation. Typically a hallmark of acute inflammation.	CSF3R (CD114), FPR2, MME (CD10)
		Eosinophils	White blood cells responsible for combating multicellular parasites and some types of infections.	CCR3 (CD193), IL5RA (CD125), PTGDR2 (CD294; GPR44), SMPD3, THBS1

Measuring Gene Expression Associated with Immunological Function

In their seminal review, Chen and Mellman describe a series of steps that must be initiated and fostered for an anticancer immune response to effectively kill cancer cells, dubbing these steps the Cancer Immunity Cycle[14]. This cycle begins with the release of antigen by cancer cells. Released antigens are processed and then presented, which results in priming and activation of the adaptive immune response. Once activated, T cells traffic to the tumor, infiltrate, and recognize tumor cells, ultimately leading to their programmed destruction. When cancer cells are killed by invading T cells, additional antigen is released into the periphery and starts the cycle anew.

Regulation of these complex immunological processes involves hundreds of genes, many of which function in multiple biological processes. All genes included in the panel were chosen after a careful review of scientific literature and vetting with members of the cancer immunology research community. Each gene is annotated with major immunological function/process information from the Gene Ontology Consortium (**www.geneontology.org**)[15,16]. Over 20 major biological processes are annotated (**FIGURE 2; TABLE 2**), including inhibitory receptors that prevent uncontrolled T cell activation, *i.e.*, checkpoint regulation, which explains the failure of immune protection in many patients[17,18].

A full list of genes found in the PanCancer Immune Profiling Panel and their annotated functions is documented and available at **www.nanostring.com**. In addition to functional annotations, the HUGO Gene Nomenclature Committee (**www.genenames.org**) name and commonly used aliases are described.

Immune Response Categories and Network Map

FIGURE 2: Network map of biological process annotations for Immune Response genes in the PanCancer Immune Profiling Panel. Each biological process is represented by a node, lines between nodes denote shared classifications for a gene, with the thickness of the line representative of the total number of genes with a given shared classification.

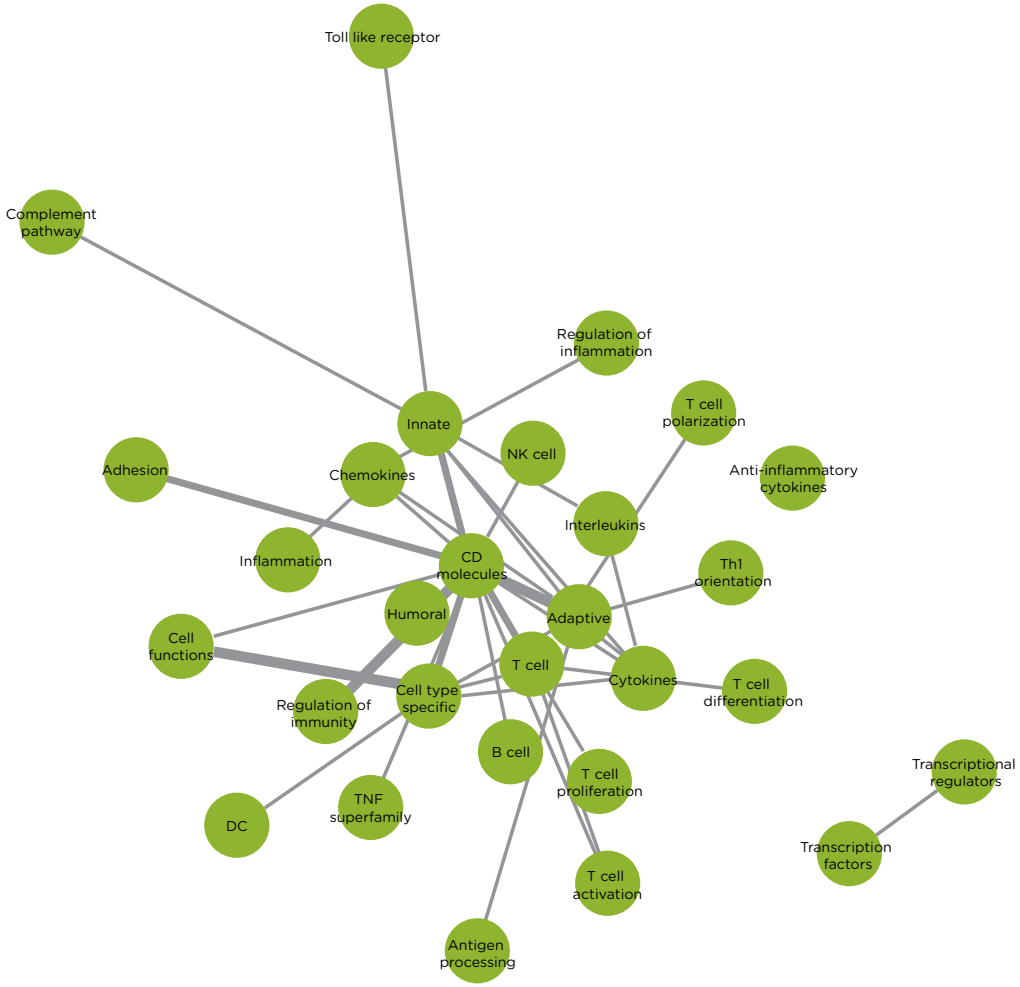


TABLE 2: Annotated biological process categories for the Immune Response genes in the PanCancer Immune Profiling Panel.

Categories	Number of Genes
Adhesion	17
Antigen Processing	155
B Cell Functions	13
Cell Cycle	6
Cell Functions	82
Chemokines	25
Complement	12
Cytokines	22
Cytotoxicity	15
Interleukins	12
Leukocyte Functions	6
Macrophage Functions	82
Microglial Functions	30
NK Cell Functions	56
Pathogen Defense	38
Regulation	99
Senescence	154
T Cell Functions	5
TLR	11
TNF Superfamily	22
Transporter Functions	10

Identifying Tumor-Specific Antigens

Cancer is characterized by the accumulation of genetic alterations and the loss of normal cellular regulatory processes[19,20]. These events have long been known to result in the expression of cancer-specific antigens, which distinguish these cells from their normal counterparts[21]. These unique cancer-antigens are processed by antigen presenting cells, *e.g.*, dendritic cells, and presented as peptides bound to major histocompatibility class I (MHCI) and II (MHCII) molecules to stimulate tumor antigen-specific T cells.

Among all tumor-specific antigens, cancer testis (CT) antigens—their expression is normally restricted to adult testicular germ cells[22]—have been found to elicit spontaneous humoral and cell-mediated immune responses in cancer patients. This makes CT antigens an attractive target for eliciting a specific immune response by immunotherapy[23,24]. The PanCancer Immune Profiling Panel contains probes for 30 of the most frequently studied and currently clinically relevant CT antigens (**TABLE 3**).

Tumor Antigens

TABLE 3: CT Antigens profiled by the PanCancer Immune Profiling Panel.

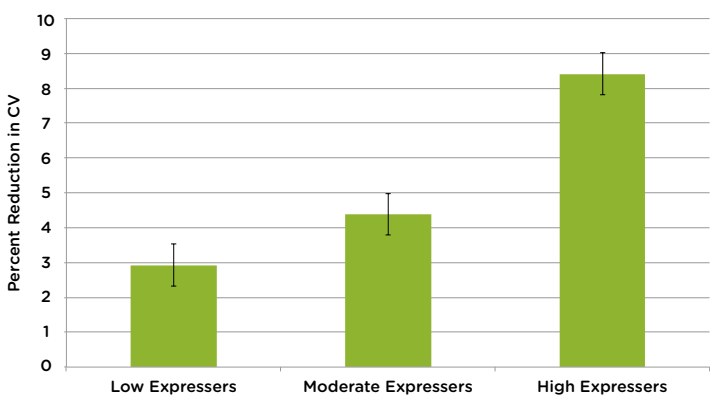
Gene Name	Additional Aliases
BAGE	CT2.1, BAGE1
CT45A1	CT45-1, CT45.1
CTAG1B	CTAG, CTAG1, NY-ESO-1, LAGE2B, LAGE2A, ESO1, CT6.1
CTAGE1	cTAGE-1, cTAGE-2, CTAGE, CT21.1, CT21.2
CTCFL	dJ579F20.2, BORIS, CT27
DDX43	HAGE, DKFZp434H2114, CT13
GAGE1	CT4.1
MAGEA1	MAGE1, MGC9326, CT1.1
MAGEA12	MAGE12, CT1.12
MAGEA3	MAGE3, HYPD, HIP8, MGC14613, CT1.3
MAGEA4	MAGE4, MAGE4A, MAGE4B, MAGE-41, MAGE-X2, MGC21336, CT1.4
MAGEB2	DAM6, MAGE-XP-2, MGC26438, CT3.2
MAGEC1	MAGE-C1, CT7, MGC39366, CT7.1
MAGEC2	MAGEE1, CT10, MAGE-C2
PASD1	CT63
PBK	TOPK, FLJ14385, Nori-3, SPK, CT84
PRAME	MAPE, CT130
PRM1	CT94.1
ROPN1	ODF6, ropporin, ROPN1A, CT91
SEMG1	SEMG, CT103
SPA17	SP17, CT22
SPACA3	ALLP17, SLLP1, LYC3, LYZL3, CT54
SPANXB1	CT11.2
SPO11	CT35, SPATA43, TOPVIA
SSX1	CT5.1
SSX4	CT5.4
SYCP1	HOM-TES-14, SCP1, CT8
TMEFF2	TENB2, HPP1, TR, TPEF, CT120.2
TPTE	PTEN2, CT44
TTK	MPS1, MPS1L1, CT96, MPH1

Measuring Housekeeping Gene Activities for Sample-to-Sample Normalization Comparison

The Cancer Genome Atlas (TCGA) expression data were examined across twelve tumor types and used to identify a subset of genes with consistent patterns of expression between cancers. These genes were selected as candidate housekeepers and are included in the PanCancer Immune Profiling Panel to aid data normalization. To demonstrate the impact of normalization, six healthy-donor PBMC samples were profiled and gene content was normalized using the geometric mean of all 40 housekeepers. Normalization resulted in reduced expression variance across a range of expression levels with the highest expressing genes showing the greatest reduction (**FIGURE 3**).

In any particular dataset, some housekeepers may not be stably expressed and optimal normalization may be achieved with a subset of the 40, e.g., the housekeepers that rise or fall together by the same amount in the raw data. Algorithms like geNorm[25] and NormFinder[26] can provide a principled means to choose the best housekeepers from among the 40 candidates. In addition to their consistent patterns of expression, these genes were chosen based on their ability to provide coverage of the wide range of expression levels typically observed in experimental datasets.

FIGURE 3: Housekeeping gene normalization results in reduction of expression variance across biological replicates.



Using the PanCancer Immune Profiling Panel

Expression profiling is a powerful tool that can be used for many purposes, including identifying relevant immune resistance mechanisms in the tumor microenvironment[27] and developing predictive biomarkers for active immunotherapy[28, 29]. The examples below provide an outline for how the PanCancer Immune Profiling Panel can be used to address these important needs in cancer research. A final example on page seven demonstrates how the PanCancer Immune Profiling Panel and the PanCancer Pathways Panel may be used together.

Selecting Adjuvants to Support Adoptive T Cell Therapy

Dr. Martin McIntosh, Fred Hutchinson Cancer Research Center — A growing number of cancer patients will be offered some type of adoptive T cell therapy, especially after conventional therapies fail[30]. Some patients have experienced dramatic improvements following T cell therapy, but many patients, even those whose tumors possess the target antigen, have not. Response rates could be improved by modulating the tumor microenvironment toward a state that is more supportive of immune function, and a number of existing tools can do this to some degree[31,32]; including cytokines, interleukins, interferons, checkpoint blockade (PD-1/PD-L1, CTLA-4, *etc.*), and others. Effective modulation strategies may require developing a molecular toolkit (assays and algorithms) that can accurately assess factors that are actionable and is practical for use in a clinical setting.

We plan to use the PanCancer Immune Profiling Panel to identify which actionable factors can be measured adequately using nucleic acid-based assays, with the goal to develop a parsimonious companion diagnostic for T cell therapies. This may be challenging for many reasons, including that solid tumors vary in ways that can confound appropriate interpretation, e.g. variations in both the cellular composition and in the expression level of specific cells in it. To start, we would profile tissues whose cellular composition and immune-environment has also been measured by gold-standard assays such as IHC, and also by clinically impractical flow cytometry, including tissues from patients who are enrolled in T cell therapy trials.

Molecular Stratification of Laryngeal Cancer

Dr. Richard Young, Peter MacCallum Cancer Centre — There are no clinically useful prognostic or predictive molecular markers in laryngeal cancer, and despite recent advances in treatment, the five-year survival is less than 50%[33]. We have a large unique cohort of clinically annotated laryngeal cancer patients for whom we have formalin fixed paraffin embedded tumor tissue blocks. Our aim would be to use the PanCancer Immune Profiling Panel across samples from our cohort, given that immune modulating agents are showing preliminary efficacy in many cancers[34].

We hope to be able to molecularly stratify patients based on expression profiles of immune-related genes and better identify molecules and pathways in the immune response that may have the potential to be therapeutically targeted. Ideally, these efforts will identify novel combinations of checkpoint inhibitors and other immunotherapeutic agents. The gene expression data from this panel has tremendous potential to allow us to more fully understand the interactions between the host immune system and tumor microenvironment and identify novel methods to predict and improve patient outcomes, with the ultimate aim of being able to provide molecular stratification tools of clinical utility in laryngeal cancer and potentially other head and neck cancers.

Exploring Oncogenic Hepatitis C Virus-mediated Immune Dysregulation

Dr. Ravi Waldron, Stanford University — Chronic Hepatitis C Virus (HCV) infection is the leading cause of hepatocellular carcinoma (HCC) in the US and predisposes carriers to lymphoma and cholangiocarcinoma[35-37]. Although HCV is a small RNA virus and does not integrate directly into the host genome, it likely promotes tumorigenesis over time by attenuating anti-carcinogenic host immunity in order to evade the host immune system. Prior research has suggested that HCV induces PDCD-1 in peripheral blood mononuclear cells (PBMCs), decreasing host adaptive immunity by attenuating T cell and macrophage function[38,39]. Other literature also indicates that the innate immune system is modified by HCV in a way that promotes oncogenesis[40]. The host interferon response produced by PBMCs is attenuated by the virus via downregulation of STAT1 and upregulation of SOCS1[38,39]. PBMC mediated IL17 pathways appear to be activated but ineffective at producing cytolysis[41,42]. In addition, TLR8 appears to be upregulated, but the pro-apoptotic effector molecules it signals, MyD88 and IRAK1, appear to downregulated by viral infection[43]. A full picture of the complex web of how HCV manipulates the immune system to promote both chronic infection and oncogenesis remains undescribed and may be invaluable to the early detection and chemotherapy of both HCC and HCV.

The new PanCancer Immune Profiling Panel provides a multiplex assay to comprehensively describe how virus induced modification of host immunity drives oncogenesis. This tool can assess gene expression in human PBMCs collected from HCV infected patients with HCC as compared to both HCV infected patients and uninfected patients. This will allow us to further elucidate the differential regulation of these pathways in the context of both HCC and HCV infection. We can then evaluate the hypothesis that oncogenic attenuation of host immunity occurs via virally induced dysregulation, which may indicate new biomarkers for early detection of tumor or drug targets for therapy.

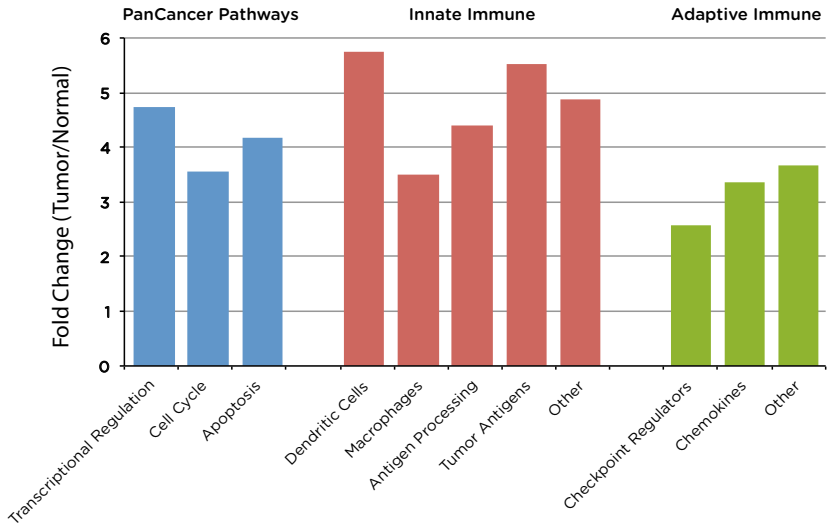
Combining Data from the PanCancer Immune Profiling Panel and PanCancer Pathways Panel

Combining data from the nCounter PanCancer Immune Profiling Panel and the nCounter PanCancer Pathways Panel provides an extraordinary window into the biology of cancer. While the Immune Profiling Panel provides a means to assess the response of the host to the presence of a tumor, the Pathways Panel examines the deregulation state of cancer-specific pathways within the tumor. Together, these two panels can provide a holistic survey of a tumor and its microenvironment and serve to enhance insights gathered via classical immunological techniques such as immunohistochemistry and FACS-based approaches as well as mutational status assessed by next generation sequencing. The breadth of genes profiled between these panels (>1300 unique transcripts) enables efficient discovery of high-information-content gene signatures that may ultimately be translated into clinical assays.

In order to illustrate the types of insights capable of being gleaned from combining the PanCancer Immune and Pathways profiles, 60 ng of extracted RNA from matched colorectal tumor and normal colon infiltrating immune cells were assayed with both the PanCancer Immune Profiling Panel and PanCancer Pathways Panel. Raw data was imported into nSolver for subsequent normalization and annotation. A subset of the housekeeping genes was used to normalize data for each panel. Upregulation greater than 2-fold in tumor samples was observed for 76 genes in the Immune Profiling Panel and 54 genes in the Pathways Panel, respectively; 47 genes in the Immune Profiling Panel and 52 genes in the Pathways Panel were downregulated greater than 2-fold.

Expression profiles of tumor samples assayed with the two PanCancer Panels (**FIGURE 4**) were consistent with previous observations made in colon cancer, where the link between inflammation and cancer is well established[44,45]. From the PanCancer Immune Profiling Panel, upregulation of innate immune genes—including macrophages, dendritic cells, and antigen processing genes—highlighted the inflammatory response, whereas the increase in checkpoint regulators[18], implied a suppressed adaptive anti-tumor immune response. Common tumor antigens were also detected and suggest that PanCancer Immune Profiling could be used as a method to identify tumor-specific immune targets. From the PanCancer Pathways Panel, significant upregulation of oncogenes involved with transcription regulation, cell cycle and apoptosis, and the mitogen-activated protein kinase cascade were observed. Altogether, these findings demonstrate that combining information about cancer pathways, checkpoints, tumor antigens, and inflammatory cells could be used to establish biomarkers that guide combination cancer therapy.

FIGURE 4: Upregulation of genes associated with indicated activities in tumor samples for both the PanCancer Pathways Panel and the PanCancer Immune Profiling Panel (Innate and Adaptive Immune response).



Conclusion

The immune system is the body's natural defense against cancer. This system is a complex collection of intricate pathways interwoven with dozens of different cell types that activate or inhibit a response to disease or infection. A previous white paper, *Multiplexed Cancer Pathway Analysis Using nCounter PanCancer Pathways Panel*, described how cancer can be defined using 13 separate canonical pathways influenced by driver genes that regulate cell division and cell fate. It is known that cancer must evade the immune response in order to survive[2,4,6], and the complex interactions between tumors and their microenvironment remain to be elucidated[13]. Researchers studying both cancer and the immune system are challenged with the complexity of understanding their interaction. In the present white paper we have discussed the importance of identifying immune cell types that infiltrate the tumor microenvironment, the role tumor antigens play in initiating the Cancer Immunity Cycle, and outlined the need to assess the entire spectrum of immunological function and responses.

The nCounter PanCancer Immune Profiling Panel is a unique gene expression tool that covers many important features of the immune response in the tumor microenvironment to facilitate rapid development of gene expression profiles across any cancer type. The panel and the nCounter Analysis System are ideally suited for use with clinically relevant samples such as FFPE tissue, PBMCs, whole blood, cell lysates, urine, and saliva. Selected genes were specifically chosen to identify and elucidate the complex immunological responses that occur in cancer and in response to external stimuli including immunotherapies. We also showed that when used in conjunction with the PanCancer Pathways Panel, the PanCancer Immune Profiling Panel could deliver insights into the biology of both a tumor and the immune response. Our example highlights the ability of these panels to evaluate the immunosuppressed, highly proliferative, and genomically unstable nature of a colorectal cancer sample.

With contributions from several scientists, we have also touched upon many ways the PanCancer Immune Profiling Panel can be used, including understanding the effects of virus-mediated immune dysregulation, selecting adjuvants to support adoptive T cell therapy, and identifying predictive and prognostic biomarker signatures. The PanCancer Immune Profiling Panel may yet have many additional uses to further our understanding of how the immune system responds to cancer and its treatment.

References

1. Cavallo F, De Giovanni C, Nanni P, Forni G, Lollini PL. (2011) in *Cancer Immunology, Immunotherapy*, pp 319–326.
2. Hanahan D, Weinberg RA. (2011) Hallmarks of cancer: The next generation. *Cell* 144:646–674.
3. Couzin-Frankel J. (2013) Breakthrough of the year 2013. Cancer immunotherapy. *Science* 342:1432–3.
4. Koebel CM *et al.* (2007) Adaptive immunity maintains occult cancer in an equilibrium state. *Nature* 450:903–907.
5. Schreiber RD, Old LJ, Smyth MJ. (2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 331:1565–1570.
6. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. (2011) Natural innate and adaptive immunity to cancer. *Annu Rev Immunol* 29:235–271.
7. Grivennikov SI, Greten FR, Karin M. (2010) Immunity, Inflammation, and Cancer. *Cell* 140:883–899.
8. Talmadge JE, Donkor M, Scholar E. (2007) Inflammatory cell infiltration of tumors: Jekyll or Hyde. *Cancer Metastasis Rev* 26:373–400.
9. Galon J *et al.* (2006) Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 313:1960–1964.
10. Mlecnik B *et al.* (2011) Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* 29:610–618.
11. Ascierto PA *et al.* (2013) The additional facet of immunoscore: immunoprofiling as a possible predictive tool for cancer treatment. *J Transl Med* 11:54.
12. Galon J, Angell H, Bedognetti D, Marincola F. (2013) The Continuum of Cancer Immunosurveillance: Prognostic, Predictive, and Mechanistic Signatures. *Immunity* 39:11–26.
13. Bindea G *et al.* (2013) Spatiotemporal dynamics of intratumoral immune cells reveal the immune landscape in human cancer. *Immunity* 39:782–795.
14. Chen DS, Mellman I. (2013) Oncology meets immunology: The cancer-immunity cycle. *Immunity* 39:1–10.
15. Ashburner M *et al.* (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. *Nat Genet* 25:25–29.
16. Lovering RC, Camon EB, Blake JA, Diehl AD. (2008) Access to immunology through the gene ontology. *Immunology* 125:154–160.
17. Mullard A. (2013) New checkpoint inhibitors ride the immunotherapy tsunami. *Nat Rev Drug Discov* 12:489–492.
18. Pardoll DM. (2012) The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 12:252–264.
19. Vogelstein B, Kinzler KW. (2004) Cancer genes and the pathways they control. *Nat Med* 10:789–799.
20. Vogelstein B *et al.* (2013) Cancer genome landscapes. *Science* 339:1546–58.
21. Coulie PG, Van den Eynde BJ, van der Bruggen P, Boon T. (2014) Tumour antigens recognized by T lymphocytes: at the core of cancer immunotherapy. *Nat Rev Cancer* 14:135–146.
22. Simpson AJG, Caballero OL, Jungbluth A, Chen Y-T, Old LJ. (2005) Cancer/testis antigens, gametogenesis and cancer. *Nat Rev Cancer* 5:615–625.
23. Scanlan MJ, Gure AO, Jungbluth AA, Old LJ, Chen Y-T. (2002) Cancer/testis antigens: an expanding family of targets for cancer immunotherapy. *Immunol Rev* 188:22–32.
24. Caballero OL, Chen YT. (2009) Cancer/testis (CT) antigens: Potential targets for immunotherapy. *Cancer Sci* 100:2014–2021.
25. Vandesompele J *et al.* (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3:RESEARCH0034.
26. Andersen CL, Jensen JL, Ørntoft TF. (2004) Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. *Cancer Res* 64:5245–5250.
27. Gajewski TF, Fuertes M, Spaapen R, Zheng Y, Kline J. (2011) Molecular profiling to identify relevant immune resistance mechanisms in the tumor microenvironment. *Curr Opin Immunol* 23:286–292.
28. Melero I *et al.* (2014) Therapeutic vaccines for cancer: an overview of clinical trials. *Nat Rev Clin Oncol.*
29. Beard *et al.* (2013) Gene Expression Profiling using Nanostring Digital RNA Counting to Identify Potential Target Antigens for Melanoma Immunotherapy. *Clin. Can. Res.* 19(18):4941-50.
30. Pedrazzoli P, Comoli P, Montagna D, Demirer T, Bregni M (2012) Is adoptive T-cell therapy for solid tumors coming of age? *Bone Marrow Transplant* 47:1013–1019.
31. Tey SK, Bollard CM, Heslop HE. (2006) Adoptive T-cell transfer in cancer immunotherapy. *Immunol Cell Biol* 84:281–289.
32. Dougan M, Dranoff G. (2009) Immune therapy for cancer. *Annu Rev Immunol* 27:83–117.
33. Corry J *et al.* (2011) Larynx preservation with primary non-surgical treatment for loco-regionally advanced larynx cancer. *J Med Imaging Radiat Oncol* 55:229–235.
34. Topalian SL et al. (2012) Safety, Activity, and Immune Correlates of Anti–PD-1 Antibody in Cancer. *N Engl J Med* 366:2443–2454.
35. Bosch FXX *et al.* (2004) Primary liver cancer: Worldwide incidence and trends. Gastroenterology 127:S5–S16.
36. Zignego AL, Giannini C, Gragnani L. (2012) HCV and lymphoproliferation. *Clin Dev Immunol* 2012.
37. El-Serag HB *et al.* (2009) Risk of hepatobiliary and pancreatic cancers after hepatitis C virus infection: A population-based study of U.S. veterans. *Hepatology* 49:116–123.
38. Frazier AD *et al.* (2010) Programmed death-1 affects suppressor of cytokine signaling-1 expression in T cells during hepatitis C infection. *Viral Immunol* 23:487–495.
39. Ma CJ *et al.* (2011) PD-1 negatively regulates interleukin-12 expression by limiting STAT-1 phosphorylation in monocytes/macrophages duringchronic hepatitis C virus infection. *Immunology* 132:421–431.
40. McGuinness PH, Painter D, Davies S, McCaughan GW. (2000) Increases in intrahepatic CD68 positive cells, MAC387 positive cells, and proinflammatory cytokines (particularly interleukin 18) in chronic hepatitis C infection. *Gut* 46:260–269.
41. Hao C *et al.* (2014) Imbalance of regulatory T cells and Th17 cells in patients with chronic hepatitis C. *Immunology*.
42. Kondo Y *et al.* (2014) HCV infection enhances Th17 commitment, which could affect the pathogenesis of autoimmune diseases. *PLoS One* 9.
43. Chen Y *et al.* (2013) HCV-Induced miR-21 Contributes to Evasion of Host Immune System by Targeting MyD88 and IRAK1. *PLoS Pathog* 9.
44. Terzić J, Grivennikov S, Karin E, Karin M. (2010) Inflammation and Colon Cancer. *Gastroenterology* 138.
45. Candido J, Hagemann T. (2013) Cancer-related inflammation. *J Clin Immunol* 33.

NanoString Technologies, Inc.

530 Fairview Ave N
Suite 2000
Seattle, WA 98109 USA

Toll-free: **1 888 358 6266** | Fax: 206 378 6288
www.nanostring.com | info@nanostring.com

LEARN MORE

Visit www.nanostring.com/pancancer_immune to learn more about nCounter® PanCancer Immune Profiling Panels.

SALES CONTACT

United States: **us.sales@nanostring.com**
Europe: **europe.sales@nanostring.com**
Other Regions: **info@nanostring.com**

nanoString TECHNOLOGIES®

FOR RESEARCH USE ONLY. Not for use in diagnostic procedures.