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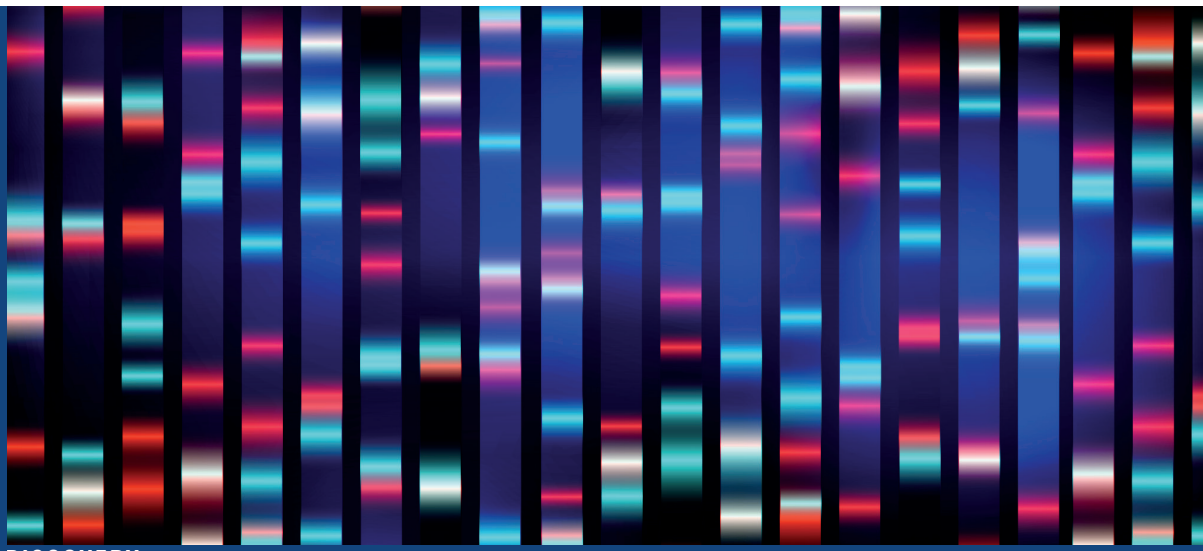
Summary

Gene expression profiling of patient-derived samples can provide actionable data to support drug discovery programs. Our optimized *in vitro* assays assess potential therapeutic candidates in disease-relevant models.



DISCOVERY

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Complex Biology *In Vitro* Assays:

Gene Expression Analysis within the Tumor Microenvironment

Gene expression profiling of tumors with NanoString nCounter® technology is a valuable, versatile tool in the development of immuno-oncology therapies; the method can be used to elucidate immunological mechanisms, identify biomarkers, and assess the ability of novel therapeutics to enhance anti-tumor immune responses.

Gene expression profiling supports deeper interrogation of how novel therapeutics modulate the tumor microenvironment (TME), allowing one to examine the impact on tumor infiltrating lymphocytes (TIL) and identify potential biomarkers for downstream clinical studies. Profiling at a gene expression level can select therapeutics which are likely to drive a robust immune response and help elucidate the immunological mechanisms underlying tumor clearance. Comparisons can be made to gene expression profiles obtained with clinical therapeutics[1]. Gene expression profiling can be used in combination with other platforms such as Luminex multiplexing of cytokine levels on matched tumor samples to determine protein expression levels for a more targeted set of genes.

Our gene expression technology may be used to screen a wide range of cellular, biologic, or small molecule therapeutics targeting anti-tumor immunity in rodent models, humanized patient-derived xenografts (PDXs), and human clinical samples. The technology can measure expression of up to 800 genes with a sensitivity that is comparable to qPCR[2]. The workflow is simple and largely automated with streamlined software analysis and without enzymatic steps which can risk bias. The resulting analysis can be used to focus further downstream analysis such as qPCR or Luminex. Consequently, the technology is a powerful tool for gene expression profiling of tumor samples. This tool has been used to establish which gene expression profiles from baseline tumor samples are likely to indicate a positive immune response by an individual to treatment with anti-PD-1 therapies[1].

We illustrate the power of this technology to assess immune responses within the tumor using a syngeneic tumor model, expressing OVA, and adoptive transfer of OVA-specific TCR transgenic OT-I, $CD8^+$ T cells and TCR transgenic OT-II, $CD4^+$ T cells; the ability of a model anti-tumor vaccine (OVA/Alum) at generating an anti-tumor immune response within the TME is assessed by gene expression analysis using the immune profiling panel.

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EVERY STEP OF THE WAY

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Assay Setup

Ovalbumin (OVA)-expressing tumors were established subcutaneously in mice by injection of EG7-OVA cells. Once tumors were established, the mice were injected with $CD8^+$ OT-I and $CD4^+$ OT-II, TCR transgenic T cells, which recognize known peptides derived from the OVA protein. Animals were then immunized with either alum or OVA/alum. Subsequently, tumors were excised and stored at -80 °C in RNAlater™. RNA was then extracted and analyzed using an nCounter® NanoString with the PanCancer Immune Profiling Panel.

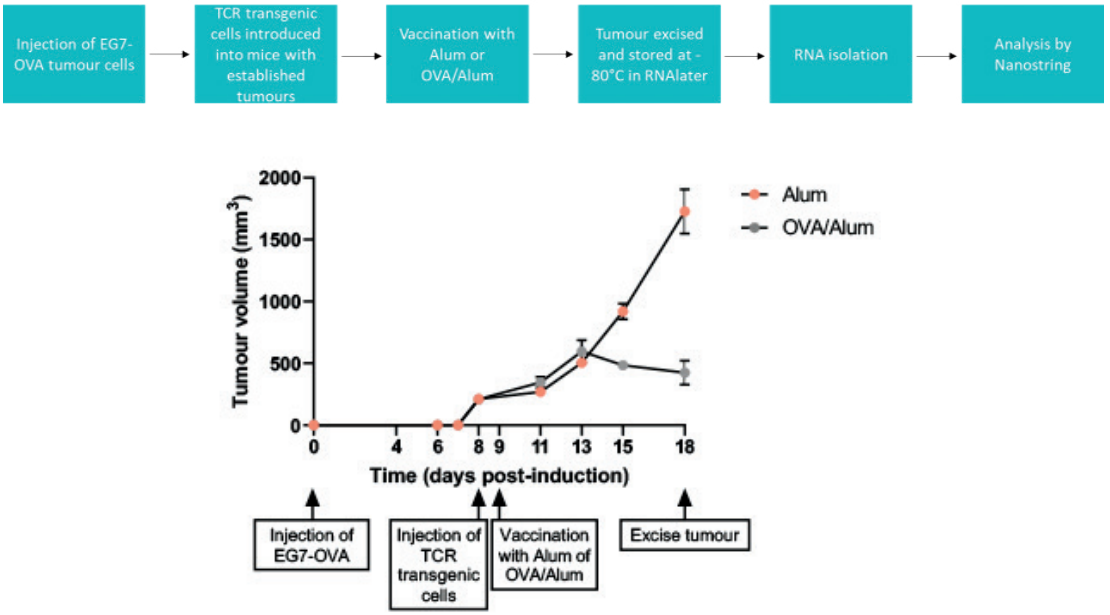

Figure 1: Vaccination of EG7-OVA tumor bearing mice, which had an increased frequency of OVA-specific T cells, with full-length OVA/alum induced a reduction in tumor size.

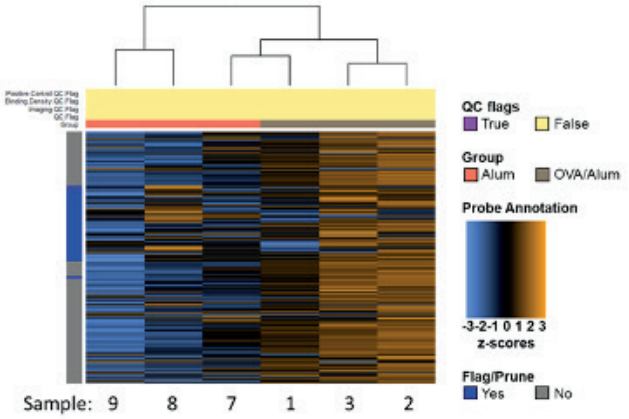

Figure 2: Clustering of the two groups indicates a distinct gene expression profile in the TME driven by OVA/alum vaccination, indicating that vaccination had altered the immune response within the tumor (as indicated by group colouring samples 1, 2 and 3 were vaccinated with OVA/alum and samples 7, 8 and 9 were vaccinated with alum).

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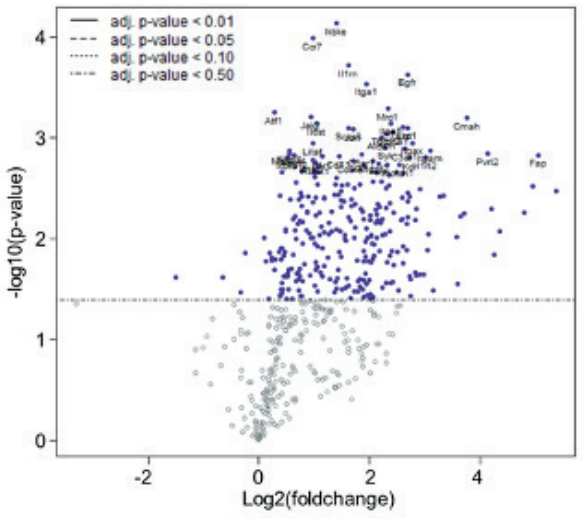

Figure 3: Differential gene expression analysis of tumors treated with alum or OVA/alum allowed identification of genes that were significantly up or down regulated in response to the anti-tumor vaccine (OVA/alum). Volcano plots were generated from advanced analysis on normalized data sets. Data displays each gene's –log10 (p-value) (y axis) and log2 fold change (x axis) with the selected covariate. The 40 most statistically significant genes were labelled on the plot.

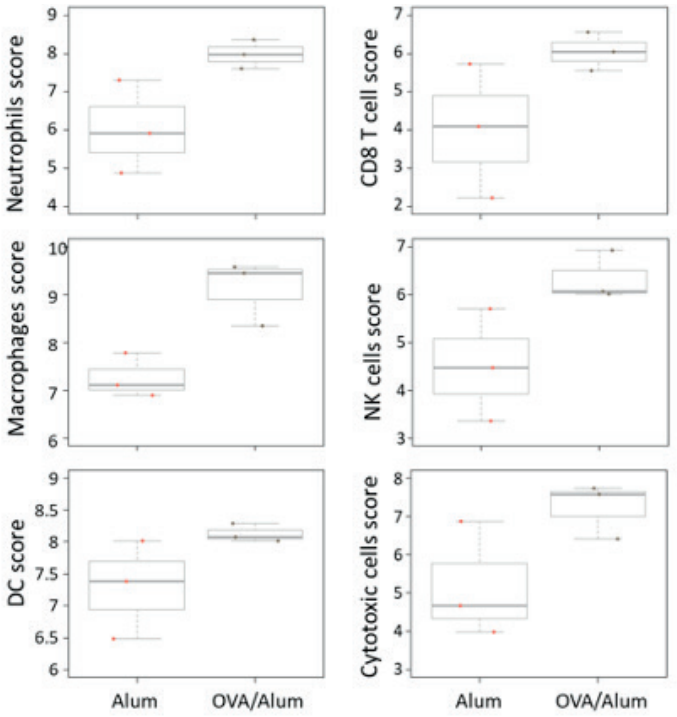

Figure 4: Genes characteristic of individual cell populations were used to measure the abundance of each cell type within the TME with immune cells being more prevalent in the TME following vaccination with OVA/alum.

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Summary

Here we used gene expression technology to elucidate differing gene profiles in a syngeneic tumor model with the aim of demonstrating the utility of gene analysis to assess mode of action and efficacy of novel anti-tumor therapies. Advantages of using this technology for gene expression analysis include the lack of an amplification step which can bias data. By limiting expression analysis to ~800 genes relevant to the therapeutic area, we can cast the net wide enough for screening and enable identification of potential biomarkers, but remain focused enough that the data does not require the same level of bioinformatics required for RNAseq analysis. The PanCancer panel used in this assay interrogates both tumor-associated gene expression as well as immune cell-associated gene expression, profiling the immune cells within the tumor microenvironment (TME). Panels can be chosen to suit the target of a therapy and can be further customized for specific targets of interest.

In the example model shown here, OVA was expressed by the tumor as a novel tumor antigen and the frequency of tumor-specific T cells (OT-I $CD8^+$ and OT-II $CD4^+$ transgenic T cells) were increased by adoptive transfer. This allowed additional *ex vivo* characterization of naïve T cells responding specifically to the tumor by flow cytometry (data not shown) alongside gene expression analysis. The effect of a model tumor vaccine (OVA/alum) on the immune response within the tumor was compared to the response elicited by tumor expressed antigen alone.

Vaccination of animals with OVA/alum drove a reduction in tumor size (figure 1). This was associated with a change in gene expression compared to alum alone. A heatmap of the normalized data, scaled to give all genes equal variance, was generated via unsupervised clustering (figure 2); Orange indicates high expression and blue low expression. Samples were clustered according to their gene expression profile (figure 2) which was distinct for the two groups (OVA/alum vs. alum alone). Volcano plots were generated from advanced analysis on normalized data sets. Data displays each gene's –log10 (p-value) (y axis) and log2 fold change (x axis) with the selected covariate. The 40 most statistically significant genes were labelled on the plot (figure 3). Those animals vaccinated with OVA/alum upregulated immune-related genes compared to animals vaccinated with alum alone, demonstrating enhanced presence of immune cells within the TME. The genes of greatest significance included those implicated in immune cell signalling and regulation as well as known cancer-associated genes.

Advanced analysis was then performed to determine the abundance of immune cell types in the TME. Genes characteristic for individual cell populations were used to measure the abundance of each cell type in the tumor. Cell type scores are shown as the mean of the log2 expression for each set of unique cell-specific marker genes (figure 4). Several immune cell types were found to be more prevalent in tumors from animals vaccinated with OVA/alum compared to alum alone, indicating an increased immune cell infiltrate. The cell type profiling analysis can be assessed in conjunction with TIL analysis by flow cytometry or protein expression by Luminex multiplexing. This provides a powerful set of information on whether a novel therapy is acting on target *in vivo* and helps identify the mechanisms by which it is acting to drive an anti-tumor response.

Further applications include using gene expression analysis to identify biomarkers in clinical samples; NanoString nCounter® technology may be used in clinical trials alongside Luminex analysis of lysates from matched tumors to test novel therapeutics. Complex analysis such as this allows for comparison of gene expression profiles to protein expression and the deconvolution of potential immunological mechanisms driving anti-tumor immunity.

References

[1] Ayers, et al, 2017, *J Clin Invest*, Vol 127(8), p2930-2940

[2] Geiss et al, 2008, Nat Biotech, Vol 26, p.317–325

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