Simultaneous Detection and Functional Characterization of CD4+ and CD8+ Neoantigen-Specific T-Cell Responses Using Multiplexed, Multiparameter Flow Cytometry

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Background
- Neoantigens, which arise in cancer cells from somatic mutations that alter proteins without generating neo-antigenic sequence, are emerging as an attractive target for immunotherapy.¹²
- They are uniquely expressed on tumor cells as opposed to healthy tissue and may be recognized as foreign antigens by the immune system, increasing immunogenicity.¹³
- The NEO-PTC-01 induction process aims to raise memory and de novo CD4+ and CD8+ T-cell responses to patient-specific neoantigens through multiple rounds of ex-vivo T-cell stimulation, generating a neoantigen-reactive T-cell product for use in adoptive cell therapy. Testing and optimizing the many potential variables in this process requires detailed characterization of the stimulated T-cell product, which can be laborious and time-intensive.
- We have therefore developed a unified assay to simultaneously detect antigen-specific T-cell responses and characterize their magnitude and function, combining multiple distinct methods to provide a unique readout:
  - T-cell co-culture with antigen-presenting cells (APCs) to present peptides (nM)
  - Sample multiplexing using fluorescent cell barcoding
  - Lineage gating
  - CD8+ antigen

Purpose
- Peptide-major histocompatibility complex (MHC) multimer staining to identify antigen-specific CD8+ T cells⁴
- Multiparameter intracellular cytokine staining to examine T-cell functionality

Here we describe the optimization of this streamlined assay, and demonstrate its application to study T-cell responses induced from a healthy donor. As an example, we characterize induced T-cell cultures from a healthy donor each containing multiple de novo or memory responses. Further, we compare the magnitude, sensitivity and functionality of the induced T-cell responses.

Materials and Methods
- The induction process for the NEO-PTC-01 personal neoantigen-specific T-cell therapy is under development (Figure 1).
- Briefly, peripheral blood mononuclear cells are cultured with antigen-loaded monocyte-derived dendritic cells (DCs) to preferentially expand (induce) antigen-specific T cells. Samples of the induction cultures are collected at timespoints throughout the process for characterization.
- Induction of memory and naive T-cell responses is modeled using pools of antigens which have been previously characterized and validated for immunogenicity (Figure 2A)²⁴.
- To evaluate the success of a given induction process, we use a recall response assay followed by a multiplexed, multiparameter flow cytometry panel. A sample taken from an induction culture is labeled with a unique two-color fluorochrome cell barcode.²⁴ The labeled cells are incubated on antigen-loaded, or unloaded, DCs overnight to stimulate a functional response in the antigen-specific cells (Figure 2B). The next day, uniquely labeled cells are combined prior to antibody and multimer staining (Figure 2C).

Results

Optimization of recall assay with multiplexed, multiparameter flow cytometry
- We confirmed the ability to fully deconvolute multiplexed samples by labeling samples and then acquiring either separately or as a mixture (Figure 3A). Uniquely labeled samples could be fully resolved with minimal cross-contamination to other barcodes.
- Detection of antigen-specific CD8+ T cells by multimer staining is maintained with sample multiplexing. An induced sample was split, labeled with four unique two-color barcodes, and then combined for multimer staining (Figure 3B). All nine barcodes yield comparable multimer staining pattern and detected frequency of multimer+ cells.

Figure 3. Optimization of Recall Assay with Multiplexed, Multiparameter Flow Cytometry

Optimization of sample multiplexing and detection of T-cell functionality
- The NEO-PTC-01 induction process utilizes multiplexed antigen exposure to antigen-loaded DCs to elicit reactivity in the T-cell product.

Figure 4. Optimization of Sample Multiplexing and Detection of T-Cell Functionality

Conclusions
- The goal of the NEO-PTC-01 personal neoantigen-specific T-cell therapy is to induce multiple enriched T-cell populations against high-priority neoantigens unique to each patient.
- Studies in healthy donor material demonstrate reproducible induction of CD8+ memory responses to a high magnitude and with a predominantly cytotoxic functional profile.
- We also observe reproducible induction of de novo CD4+ responses to multiple antigen targets in the same culture as well as induction of CD8+ responses towards naive targets (data not shown).
- To guide optimization of the induction process, we have developed an assay that allows for the simultaneous analysis of specificity and functionality of both CD4+ and CD8+ T cells.

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Disclosures
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References

Figure 1. Overview of NEO-PTC-01 Induction Process

Figure 2. Methodology for T-Cell Inductions and Analysis

Figure 3. Optimization of Recall Assay with Multiplexed, Multiparameter Flow Cytometry

Figure 4. Optimization of Sample Multiplexing and Detection of T-Cell Functionality

Figure 5. Simultaneous Analysis of Specificity and Functionality of Induced CD8+ Memory Responses

Figure 6. Detection and Functional Characterization of de novo Induced CD4+ Responses with Multiple Specificities in the Same Culture

Figure 7. Optimization of Sample Multiplexing and Detection of T-Cell Functionality