Profiling a Structure Activity Relationship (SAR) Library for Immunological Activity in Human PBMCs: Multiplexed Measurements of Cell Phenotype, Viability, and Cytokine Secretion from a Single High Throughput Screen

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Introduction

The success of immunomodulatory approaches for the treatment of disease has generated tremendous interest in discovery of new immunotherapy based therapeutics. Immunotherapy has the potential of affecting – positively and negatively – the myriad interactions among cells and signaling molecules regulating the immune system, and projects should include high throughput screening campaigns aimed at profiling the effects of compounds on these complex interactions. We describe a no-wash, high throughput screen that provides a multiparameter activity profile for a structure activity relationship (SAR) library of immunomodulatory compounds. Seven known immunomodulatory compounds were used as templates to generate an SAR expansion library of 1438 compounds. The selection utilized a combination of various substructure and similarity search methods combined with the generation of virtual templates. Phytohemagglutinin (PHA) activated PBMCs were treated with the compounds at two doses, 16.7µM and 83.3µM. Immunophenotyping was done by α-CD3 and α-CD8 antibodies and differential toxic effects of compounds on the viability of individual cell subsets plus effects on the secretion of three cytokines were used to generate an immunological profile for each compound tested. We demonstrate the combination of a robust SAR expansion system with high throughput, multiplex screening on primary cells to generate activity profiles that can be used for identifying viable therapeutic candidates.

Peripheral Blood Mononuclear Cells (PBMCs) as a Biological Model of Immune Function

Human PBMCs are a primary cell type comprised of a mixture of immune cells – including but not limited to NK-cells, B-cells, and T-Cells of various subtypes and stages of differentiation. PBMCS are an excellent model for studying effects of potential drugs to the immune system, as the model is able to recapitulate many of the complex immune responses to compound treatment. In addition to the complexity of the cell mixture, activation or suppression of the immune response (immunomodulation) is often seen in concert with coordinated cytokine responses.

Activation of T-cells can be induced by treatment with phytohemagglutinin (PHA), which will trigger the T-cell population to proliferate as well as cause changes to the cytokine secretion profile of the PBMC culture as a whole. T-cells can be identified as positive for the surface marker, CD3. A specific subtype, the cytotoxic T-Cell will also express CD8. Compounds that alter the ability of PHA-stimulated T-cells to proliferate or secrete certain cytokines might be candidate immune-modulatory compounds for further investigation.

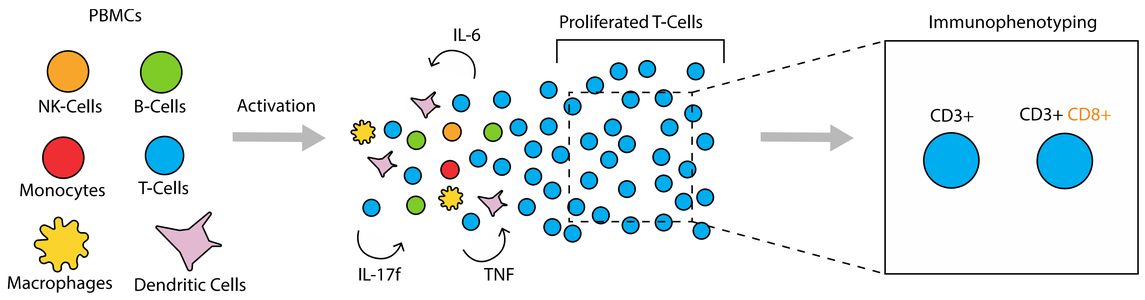


Figure 1: Human PBMCs are a biological model of immune function. The complex mixture of cells can be nonspecifically stimulated by the use of phytohemagglutinin (PHA). PHA stimulation will cause activation of PBMCs, which can be measured in the proliferation of T-cell subsets. Cotreatment of the cells with immunosuppressive compounds and PHA will suppress the induced proliferation. IntelliCyt's MultiCyt reagent panel contains options for multiplexing cell proliferation and viability with commerically available immunophenotyping markers (CD3 and CD8). Additionally, IntelliCyt's QBeads assay can be used for multiplexed detection of cytokine secretion (IL-17f, IL-6, and TNF in the case of this study).

Construction of a SAR Library of Immunomodulating Compounds

Based on a Literature search for substances having a differential effect on cytokine secretion, seven compounds were selected (Imiquimod, mycophenolic acid, resveratrol, thalidomide, tomentosin, verapamil and compound C3) and used as templates for running an extensive SAR expansion against the Exquiron Compound Collection of 260,000 compounds, based on a published protocol [1]. After a data fusion step, a total of 1,438 compounds were identified. These compounds were cherry-picked for testing at two different concentrations.

[1] A. Bergner, S. P. Parel, J. Chem. Inf. Model. 2013, 53, 1057

Multiplexed Screening of Immunomodulatory Compounds: Materials and Methods

The iQue Screener was used to screen Exquiron's SAR expansion library for immune-modulatory compounds using a PBMC-based assay (Figure 2). Commercially available, cryopreserved PBMCs from healthy donors were thawed and rested at 37 °C for 24 hours before staining with IntelliCyt's MultiCyt® Cell Proliferation Dye. Cells were then plated directly into 384-well plates containing the SAR expansion library. 1438 compounds were tested in two doses, 16.7 µM and 83.3 µM. In addition to receiving compound treatment, each treated well also received a fixed dose of PHA. Each plate used in the screen also contained unstimulated controls (without PHA or immune-modulator) or a fully simulated control (with PHA, without immune-modulator) which were used as zero percent effect (ZPE) and hundred percent effect (HPE) controls for data normalization.

After 3 days incubation, a 10 µL aliquot of the assay volume was stamped into a new 384 well plate for staining with a dye cocktail containing commercially available CD3-FITC and CD8-PE immunophenotyping antibodies and IntelliCyt's FL3 Membrane Integrity Dye. A 3 µL aliquot of the assay volume was stamped for detection of IL-17f, IL-6, and TNF secretion using IntelliCyt's QBeads reagent. See Figure 2 for a detailed description of the treatment and staining workflow.

No Wash Screening Workflow

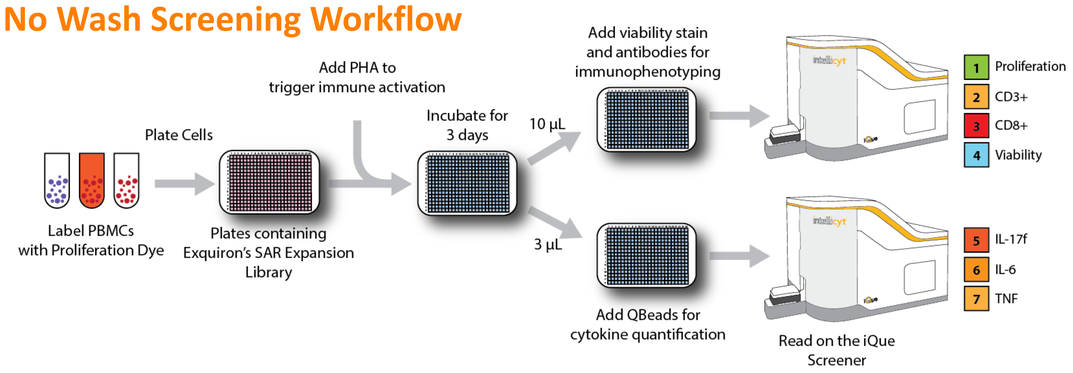
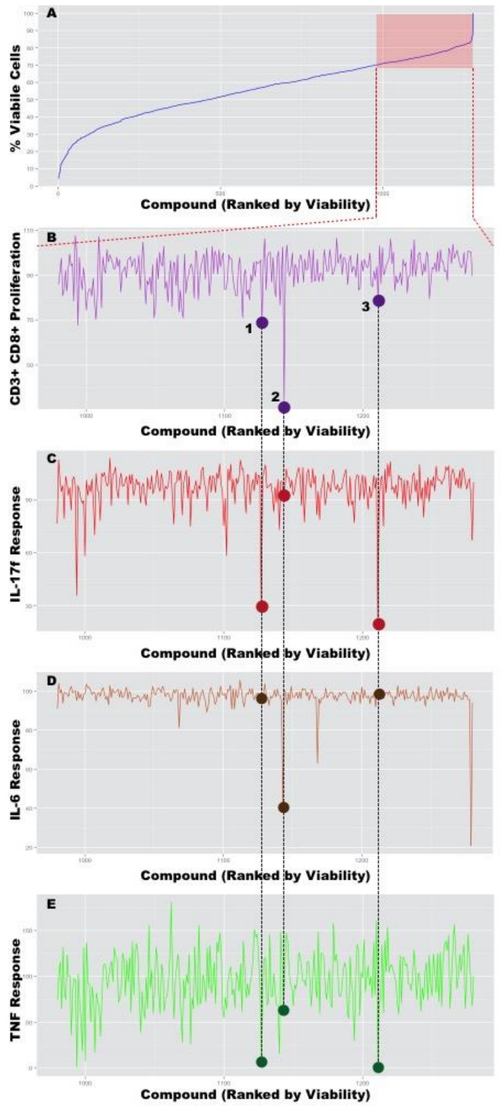


Figure 2: iQue Screener and MultiCyt Reagents enables a simplified, no-wash assay workflow. PBMCs were batch-labeled with the MultiCyt® FL4 Cell Proliferation Dye before plating into 384-well plates containing compounds from Exquiron's SAR Expansion library. Following addition of cells, PHA was added and co-incubated for 3-days under standard tissue culture conditions. After incubation was complete, a 10 µL aliquot was stamped from each treatment plate into a multiplex of immunophenotyping antibodies (CD3 and CD8) and the MultiCyt FL3 Membrane Integrity Dye. A second stamp of 3 µL from the same motherplate was used for QBeads detection of IL-17f, IL-6, and TNF. Each plate was read on the iQue Screener immediately after staining, without wash steps. Each 384-well plate took about 25 minutes to read.

Multiplex Screening Results for Cell Health, Immunophenotyping, CTL Proliferation, and Cytokine Secretion

Figure 3: Screening results and data analysis strategy. PBMCs were activated with PHA and co-treated with two different doses of compounds from Exquiron's SAR Expansion library targeted for immune modulators. Only data from the high dose treatment (83.3 µM) are presented in this poster. A) Only compounds that resulted in 70% or greater cell viability were included in the analysis. Of the 1280 compounds screened (1 plate was lost due to handling, reducing the original 1438 compounds to 1280), 301 compounds caused less than 30% cell death. B) Next, we used CD3 and CD8 staining to restrict our analysis of proliferation of just the cytotoxic t-cell lymphocyte population (CTL). The extent of proliferation was normalized to ZPE and HPE controls placed on each plate in the screen. The purple dots feature 3 compounds that demonstrated a range of proliferation inhibition, suggesting these compounds vary in their ability to modulate PHA-mediated PBMC activation. Compound 2 demonstrated nearly complete inhibition of proliferation, while Compounds 1 and 3 demonstrated an intermediate effect. C-D) The modulation of IL-17f, IL-6, and TNF varied for each featured compound. Compound 2, which had the strongest inhibition of proliferation, demonstrated little change in IL-17f secretion, a strong inhibition of IL-6, and an intermediate effect on TNF secretion. In contrast, Compounds 1 and 3 had mild effects on proliferation, but near complete inhibition of IL-17f and TNF. IL-6 was unaffected by these two compounds.



Throughput Statistics

Scope of screen	**8 x 384 well plates 3072 total wells**
Total number of compounds screened (2 doses each)	**1280 compounds tested at 2 concentrations each**
Time to read each plate on the iQue Screener	**25 minutes per plate 16 plates (8 for cells, 8 for beads) ~6.6 hours to complete screen**
Number of data points collected*	**18,432**

* 6 endpoints for each compound, each concentration, plus controls: cell proliferation, PBMC immunophenotyping, cell viability, IL-17f secretion, IL-6 secretion, and TNF secretion.

Summary

- The iQue Screener is optimized for rapid screening of compound libraries using assays that require mixtures of suspended objects, such as the suspension cell types found in immunological models like PBMCs.
- The advanced multiplexing capabilities allow multiple, immune-relevant parameters to be gathered in a single plate read. Data gathered in this screen include cell viability, immmunophenotyping markers, cell proliferation, and IL-17f, IL-6, and TNF secretion.
- The low sample volume requirements of the iQue Screener allow precious cell types to be conserved and reduce the amount of reagents required to complete a screen.
- The iQue Screener is able to identify putative immune-modulatory compounds that differ in their immune-modulatory profile based on extent of proliferation and cytokine secretion.

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