A Chip-based Immunogenicity Risk Assessment Tool for Therapeutic Protein Products

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SUMMARY AND KEY FINDINGS

In this study, we show the accuracy of PEPperPRINT immunogenicity risk assessment pipeline on ranking two well-known PD-L1 inhibitors based on their epitope content.

Our innovative chip-based MAPPs methodology offers rapid identification of immunogenic hotspots of TPPs, and presents several advantages over *in silico* and other *in vitro* MAPPs assays:

- The peptide library representing the TPP undergoes screening with recombinant human HLAs, obviating the necessity for patient samples or scarce biological material.
- Discovery occurs within a cell-free system, mitigating potential interference from cells or other protein-based culture materials.
- Testing of identified epitopes in AIM and Treg assays facilitates the assessment of their contribution to potential immunogenicity, yielding a more precise ranking.
- Peptide-HLA binding affinity data enhances the accuracy of immunogenic peptide ranking and provides guidance for drug development, without reliance on predictions or complex computational methodologies.

In addition to the chip-based MAPPs assay, the data generated with our ADA epitope mapping assay could be instrumental in:

- Assessing the contribution of immunogenicity to clinically observed adverse effects, if present.
- Determining whether the immunogenic response impacts drug efficacy.
- Identifying a unique epitope for patients experiencing specific side effects could aid in identifying individuals at higher risk of developing these adverse events.
- Analysis of pre-existing ADA responses for better patient stratification early in clinical trials

AIM

Given the growing numbers of therapeutic protein products (TPPs) being developed, generation of tools to identify immune responses mounted against them has become significantly important. At PEPperPRINT, we developed a novel pipeline to enable:

- Profiling potential T-cell immunogenicity through a **chip-based MHC associated peptide proteomics (MAPPs) assay**
- Discrimination of **regulatory T cell epitopes** from effectors and assessment of immunogenicity risk based on epitope content
- Identification of immunogenic epitopes recognized by anti-drug antibodies **(ADA)**
- Monitoring of pre-existing and treatment- emergent antibody responses for assessment of treatment risks and **immunogenic adverse effects**

STUDY BACKGROUND

In this study, we adapted our peptide microarray platform technology to assess the immunogenic potential of two extensively studied PD-L1 inhibitors, which exhibit markedly different reported immunogenicities, aiming to validate existing clinical data.

Therapeutic protein products have revolutionized the treatment of several diseases including cancer, chronic autoimmune and inflammatory diseases However, many of these therapeutics are reported to be immunogenic at various levels[1]. This immunogenicity is associated with ADA generation, which can neutralize drug efficacy by altering its pharmacokinetic and pharmacodynamic properties. Identification of ADA epitopes in polyclonal Ab mixtures like sera is usually challenging due to this high diversity. Here, we show that High-density peptide microarrays are powerful tools to simultaneously screen thousands of peptides against serum antibodies in a high-throughput context[2].

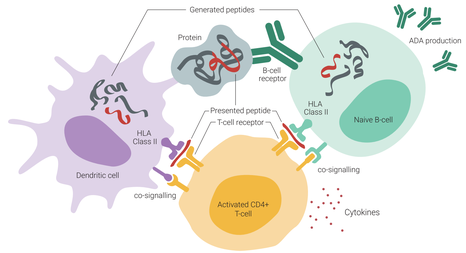
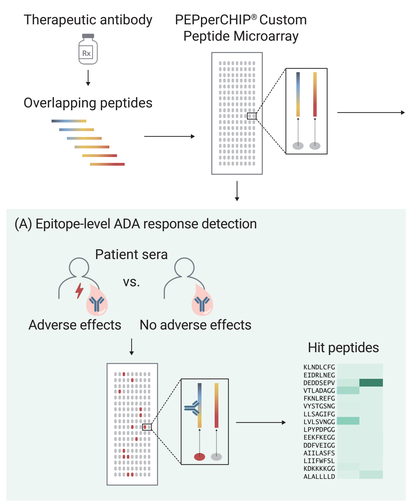


Fig. 1. ADA formation involves an interplay among dendritic cells (DCs), T cells, and B cells. DCs primarily capture antigens through macropinocytosis and receptor-mediated uptake, enabling them to sample extracellular proteins. Conversely, B cells predominantly internalize antigens by recognizing their specific structures through B cell receptors. DC-activated T cells can subsequently activate B cells that are presenting identical sequences.

Although ADAs have been a standard clinical measure of immunogenicity historically, the root cause is the T cell dependent (Td) immune response generated through presentation of epitopes by subsets of human leukocyte (HLA) class II molecules to CD4⁺ T cells that drive B cell maturation and ADA generation. Here, we present a novel chip-based methodology that allow for the discovery of epitope-specific T cell responses in a remarkably short timeframe and eliminating the requirement for patient material.

METHODOLOGY AND RESULTS



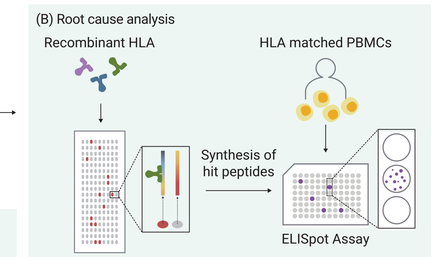


Fig.2. Amino acid sequences of two FDA-approved PD-L1 inhibitors, Durvalumab and Atezolizumab (see reference 4 for immunogenicity data) and generated a high-density peptide microarray by converting these amino acid sequences into 15 amino acid peptides with a maximum peptide-peptide overlap of 14 amino acids for high-resolution epitope data. (A) To detect the anti-drug antibody responses on the epitope level, serum samples from Druvalumab- or Atezolizumab-treated lung cancer patients are screened with PEPperCHIP® Peptide Microarrays. (B) For root cause analysis, same microarrays were analyzed using most common recombinant HLA-DR allotypes in global population, enabling the ranking of two PD-L1 inhibitors by immunogenic potential. Subsequently, identified peptide candidates are synthesized and applied in ELISpot/ FluoroSpot assays with HLA-matched PBMCs.

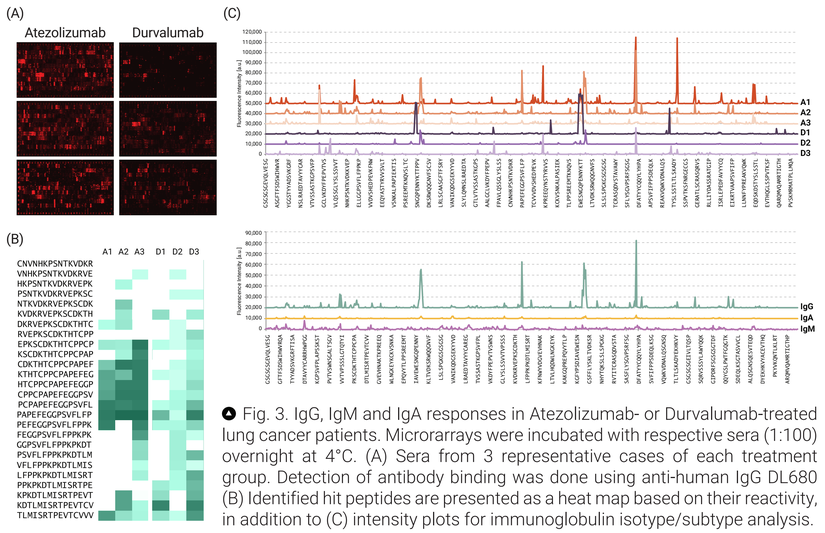
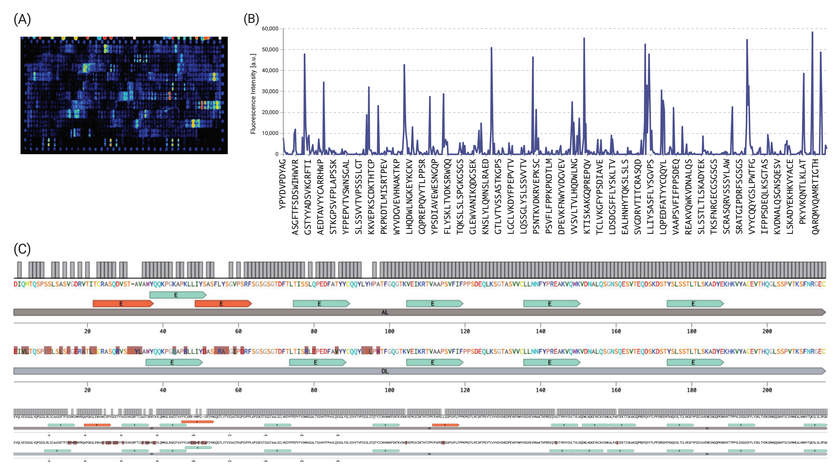


Fig. 3. IgG, IgM and IgA responses in Atezolizumab- or Durvalumab-treated lung cancer patients. Microrarrays were incubated with respective sera (1:100) overnight at 4°C. (A) Sera from 3 representative cases of each treatment group. Detection of antibody binding was done using anti-human IgG DL680 (B) Identified hit peptides are presented as a heat map based on their reactivity, in addition to (C) intensity plots for immunoglobulin isotype/subtype analysis.

Result: While our study is based on a limited sample size, PEPperCHIP® Microarrays enabled swift characterization of epitope-specific anti-atezolizumab ADA responses. IgA and IgM mediated immune responses were scarce whereas IgG was found to be the most relevant isotype in ADA analysis. Screening a larger cohort with synchronized sample collection is imperative for comprehensive evaluation.



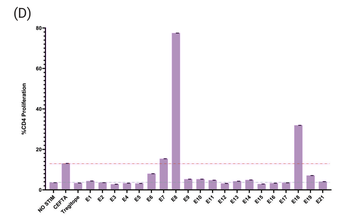


Fig. 4. For root cause/IRA analysis, the same microarrays were screened with recombinant HLA class II constructs, followed by a validation assay.

Due to space limitations, here we show the screening results only for (A) HLA-DRB1:0101 which is known to be associated with the risk of developing ADA, and specifically NAb, in patients treated with Atezolizumab[5]. (B) The intensity plot highlights the interactions of recombinant soluble HLA-DRB1:0101 protein assayed at a concentration of 30 µg/ml.

(C) To be able to visualize the immunogenic regions more clearly, we aligned the heavy and light chains of these inhibitors and integrated the epitope data from our chip-based MAPPS assay (green). We found out 4 additional T-cell epitope candidates (orange) on Atezolizumab amino acid sequence.

(D) To assess the regulatory/activatory potential of candidate epitopes, we performed a CEFTA suppression assay to measure their inhibitory effect on human CD4⁺ T cell recall responses. HLA-DRB1*01:01 PBMCs were labeled with a cell trace dye, plated at 3 × 10⁵ cells/well in RPMI complete medium, and rested overnight. The next day, cells were stimulated with candidate or control peptides, followed by CEFTA peptide mix addition. After six days, cells were harvested, stained for surface and intracellular markers, and analyzed by flow cytometry. The graph depicts CD4⁺ T cell proliferation for each epitope relative to CEFTA stimulation alone. Notably, epitope E8 failed to suppress the CEFTA response, correlating with significantly higher ADA levels. Further validation is ongoing in suppression and activation assays using PBMCs from donors with diverse HLA types.

Result: Our ranking aligns with existing literature, which indicates a significantly higher incidence of ADA formation for Atezolizumab (39.1%) compared to Durvalumab (2.9%)[4]. Inclusion of treatment-naive patients and healthy donors in the analysis would be advantageous for distinguishing preexisting and treatment-emergent ADAs.

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