PROIMMUNE
www.proimmune.com

Cellular Analysis Services

Are you facing major obstacles to running cell mediated immunity assays in-house? Protocol optimization, lack of experienced operators and the cost and time needed to set up and maintain assays are common limitations to obtaining fast, reproducible results for your research study, or preclinical or clinical trial.

ProImmune's Cellular Analysis Services offer you optimized, rapid and affordable cellular assays to GLP/GCP standards. Our service is designed to help you accelerate your immune monitoring or epitope discovery projects, by saving you the cost and effort of setting up and maintaining these assay platforms in your own laboratory. Services are based on a choice of standard assay formats in order to maintain affordability.

- **ELISpot assays**
- **Intracellular Cytokine Staining**
- **Antigen-specific $CD8^+$ T cell detection with Pro5® Pentamers in flow cytometry**
- **NKT cell detection with CD1d tetramers in flow cytometry**
- **HLA Tissue typing of donors**
- **Isolation and cryopreservation of PBMC from fresh, whole blood**
- **Dendritic cell (DC)-T cell assays with whole proteins**
- **Naïve CFSE T cell proliferation assays with peptides**

To make life easier, outsource your cellular assays to ProImmune. We have developed a straightforward, step-wise approach that allows us to understand your requirements quickly and that sets out clearly what you can expect from us at each stage. The steps include assay design, cell preparation and shipping, HLA tissue typing, assay fulfilment, preparation and delivery of the final report and post-project consultation with a technical expert. As a consequence you can maximize the amount of work outsourced, with minimum inconvenience.

- Free up time for core research and project planning
- Remove the cost and effort of setting up assays in your laboratory
- Optimized protocols to minimize assay variation
- Rapid turnaround of projects
- Order individually or as part of a more complete epitope discovery project
- Outsource to our experienced team

Assay Specifications and Applications

	ELISpot	Intracellular cytokine staining	Flow Cytometry with Pro5® Pentamers	Flow Cytometry with CD1d Tetramers	Peptide CFSE T Cell Proliferation	DC-T Cell Proliferation	HLA Tissue Typing
Assay Specifications							
ProImmune supplied donor cells	•	•	•	•	•	•	•
Customer sourced samples	•	•	•	•	*		•
Whole blood accepted for cryopreservation	•	•	•	•	•	•	
Frozen cells accepted	•	•	•	•			•
Genomic DNA accepted							•
Analysis performed on frozen cells	•	•	•	•	•	•	•
Applications							
Assessment of $CD4^+$ T Cells		•			•	•	•
Assessment of $CD8^+$ T Cells		•	•				•
Immune monitoring	•	•	•	•	•	•	
Cell surface phenotyping		•	•	•	•		
Epitope mapping	•	•	•		•		
Antigen-specific $CD8^+$ T cell detection			•				
Natural Killer T (NKT) cell detection				•			
Proliferation of T cells					•	•	
Batch release assessment					•	•	
Whole protein antigenicity assessment						•	
HLA tissue typing of donor samples	•	•	•	•	•	•	•

* Inquire

Sample Management

All cellular analysis services are carried out at ProImmune's qualified facilities. ProImmune has substantial experience in the shipping of customer cell samples worldwide. We handle the shipping process seamlessly from your facility through our own pre-qualified, specialized sample shipping providers. Alternatively, you can use your own shipper under our instruction.

Samples can be provided fresh or cryopreserved. Fresh samples must be cryopreserved for storage before the assays are performed. We recommend that all fresh samples be processed within 24 hours of collection from the donor. We can supply validated cryopreservation protocols, which help ensure high viability of the samples once they are thawed at our laboratories under optimal conditions. Alternatively, we can process your samples for you. Clinical trial samples can be received under GCP conditions.

All assays are carried out on cryopreserved samples. While cryopreservation reduces cell viability, we have extensively validated that this loss can be limited to only 15-20% by following best practise methods. By taking this controllable effect into account, an accurate and valid picture of the original sample can be formed.

For each project we will discuss any special requirements for sample handling with you in detail in advance. When shipping, we ensure that all required customs and export regulation information is on hand, and in the unlikely event of any delay in the package clearing customs, we and our shippers ensure that the appropriate coolant is topped up to maintain sample temperature.

Our streamlined service makes outsourcing to ProImmune rapid and affordable compared to other commercial providers. The flow diagram below gives an overview of the processes involved and the specific assays currently offered.

Overview of ProImmune's Cellular Analysis Services

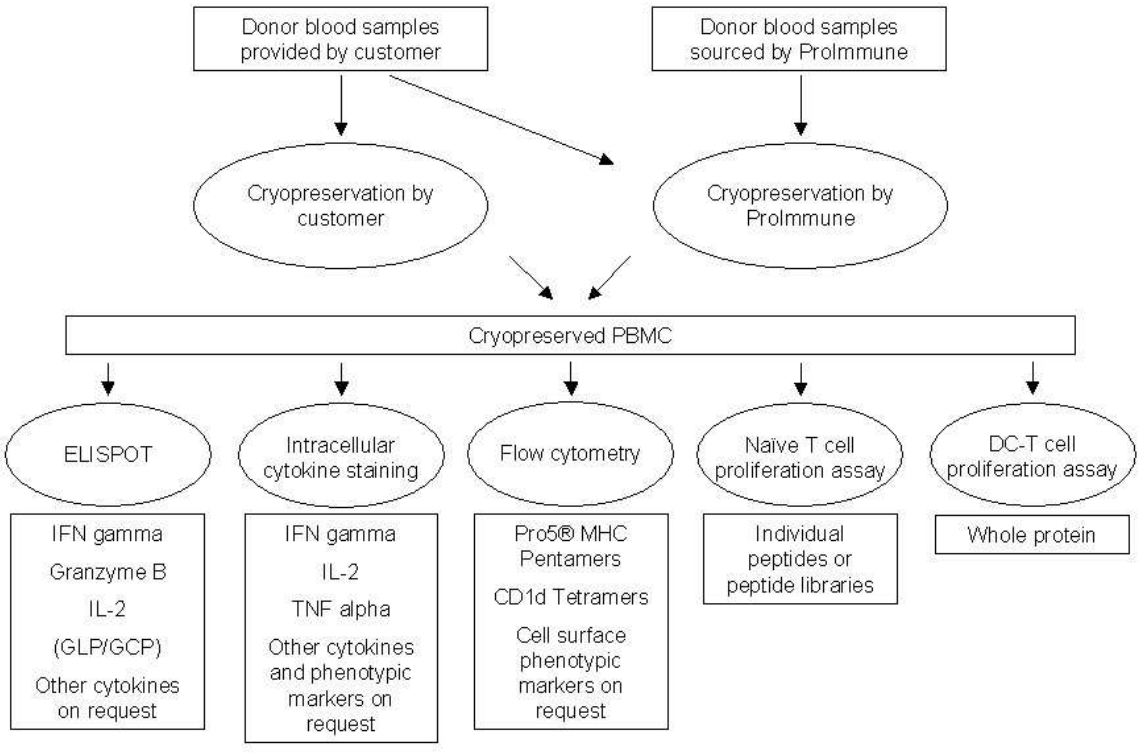


Figure 1: Overview of ProImmune's cellular analysis services.

ELISpot Assay

When set up correctly, the Enzyme-Linked ImmunoSpot (ELISpot) assay is a highly reproducible and sensitive cellular assay, particularly suited to high-throughput analysis of antigen-specific $CD4^+$ and $CD8^+$ T cell immune responses. It can allow detection of a secreted cytokine at the single cell level, as low as 1 cell in 100,000. For these reasons, ELISpot is now a widely adopted assay standard for monitoring T cell immune responses and validating new T cell epitopes.

Carrying out ELISpot assays in-house, particularly with inexperienced staff, can require significant effort and can be time consuming with variable results. You can now save time and resources, and minimize risk by relying on the expertise of ProImmune's experienced team, who perform these assays routinely using optimized protocols.

We have developed a streamlined service that makes outsourcing ELISpot to ProImmune rapid and affordable compared to other commercial providers. Our assays are based on simple standardized formats, which follow a step-by-step process.

- IFN gamma, IL-2 and Granzyme B to GLP or GCP quality standard
- Other cytokines available on request
- Detection of $CD8^+$ and $CD4^+$ T cell responses on frozen unmodified PBMC
- Detection of $CD4^+$ T cell responses on frozen PBMC depleted of $CD8^+$ T cells after thawing
- HLA tissue typing of donor samples if required
- Project turnaround in 6-8 weeks, including peptide synthesis and shipment of cells (varies according to exact requirements)
- Assay report and data CD, including images from the automated ELISpot reader
- Project follow-up consultation with technical specialist
- ELISpot assay validation report available on request

Applications

- Clinical immune monitoring
- Epitope discovery
- Determine functionality of preclinical and clinical samples

ELISpot Assay Validation

To be confident that your ELISpot assay is consistently performing well, validation is essential. ProImmune offers assays to GLP- or GCP-accredited standards. We have carried out validation testing to confirm reproducibility between assays and between operators. A sample of our data is shown below.

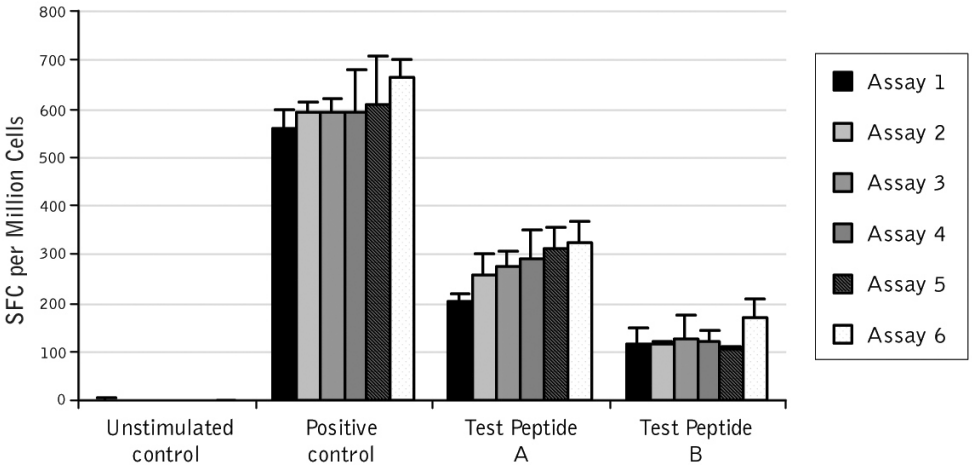
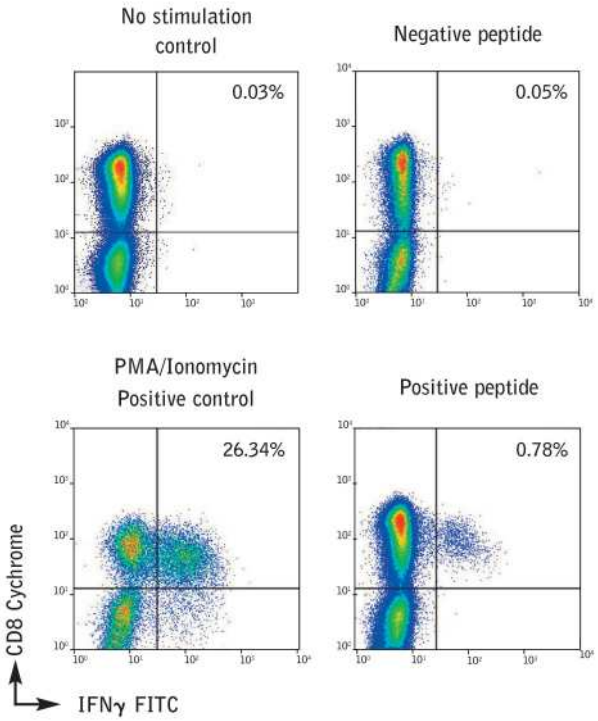


Figure 2: Low inter-assay variability in ProImmune ELISpot Assays: normalized data from two users across six IFN gamma ELISpot assays performed over three days. The data show the consistency of the response for positive control and two test peptides.

Intracellular Cytokine Staining

Intracellular cytokine staining by flow cytometry is a powerful technique that allows the multiparametric analysis of individual cells in a mixed population. The procedure relies upon the stimulation of T cells in the presence of an inhibitor of protein transport, in order to retain the cytokines inside the cell. Cells are first stimulated with antigen, followed by staining with antibodies specific for extracellular epitopes, such as CD4 and CD8. Intracellular cytokine staining follows fixation and membrane permeabilization. The frequency of cells that produce a particular cytokine is measured using fluorescent antibodies.

ProImmune offers rapid and reliable flow cytometric detection of IFN gamma, IL-2 and TNF alpha cytokine production in $CD4^+$ and $CD8^+$ T cells through its experienced applications team. Successful intracellular cytokine staining requires the design of suitably controlled experiments and a well-maintained facility. By outsourcing your intracellular cytokine staining experiments to ProImmune, you take advantage of our technical proficiency and save time by passing collection and analysis of data to our experts. Multicolor analysis is possible through the use of our 4-color or 8-color flow cytometry instruments with 96-well high-throughput capability.



Intracellular cytokine staining can be used as a monitoring tool to measure the immune response to known antigens, and for epitope discovery, to identify and validate novel T cell epitopes.

Flow cytometric detection of intracellular cytokines allows for simultaneous detailed phenotyping and gating of cells, to select the specific live lymphocyte population.

Figure 3: Example staining for an intracellular cytokine staining experiment.

Our service is based on a standardized format, which follows a step-by-step process.

- IFN gamma, IL-2 and TNF alpha; other cytokines on request
- HLA tissue typing of donor samples if required
- Project turnaround from just 3 weeks, including peptide synthesis and shipment of cells (varies according to exact requirements)
- Assay report and data DVD, including raw data .fcs files.
- Project follow-up consultation with technical specialist

Applications

- Determine functionality of samples
- Measure immunomodulatory effects of test compounds or biologics
- Epitope discovery
- Antigen-specific functional readout when combined with Pro5® Pentamer
- Immune subset phenotyping, e.g. Th17, T regs

Flow Cytometry Testing with Pro5® MHC Class I Pentamers and CD1d Tetramers

ProImmune offers a flexible flow cytometry testing service for measuring antigen-specific $CD8^+$ T cell responses using Pro5® MHC Class I Pentamers, and NKT cells using CD1d tetramers.

MHC Pentamers bind directly to T cell receptors of a single specificity, determined by the MHC allele and peptide combination, and by use of flow cytometry can detect antigen-specific T cell populations as rare as 0.02% of lymphocytes. Staining cells with MHC Pentamers provides a quantitative analysis of lymphocytes that express a specific T cell receptor and in conjunction with other functional assays, such as ELISpot, gives complementary information about an immune response. For increased depth of information, MHC Pentamer staining can be combined with a wide range of phenotyping antibodies.

ProImmune is the only commercial source worldwide for fluorescently labeled human and mouse CD1d tetramers. The tetramers are available pre-loaded with alpha-Galactosyl Ceramide* (α-GalCer) or empty for loading with ligand of choice. Each tetramer also has a relevant negative control. The negative controls are mock-loaded with carrier only (no ligand) and will not bind to NKT cells. Tetrameric CD1d-lipid complexes bind to receptors on NKT cells of a particular specificity (as determined by the lipid ligand used), allowing identification and enumeration of antigen-specific CD1d-restricted NKT cells by flow cytometry. Additional co-staining for intracellular cytokines such as IFN gamma or IL-2, and/or surface markers e.g. CD69 can yield functional data for the antigen-specific subset.

* The alpha GalCer molecule and derivatives thereof are covered by US Patent No.5,936,076, which is owned by Kirin Pharma. The alpha GalCer molecule is licensed to Funakoshi Co. Ltd. for research use worldwide.

Even for researchers experienced in flow cytometry techniques, cell preparation, staining and analysis all require careful planning and execution. By outsourcing flow cytometry testing to ProImmune, you immediately benefit from many years experience from the team that has developed MHC Pentamer technology. Protocols are carried out to optimized procedures to give reliable and consistent results. Our affordable, streamlined service is based on simple standardized formats.

- Pro5® MHC Class I Pentamer staining of antigen-specific T cells
- CD1d tetramer staining of NKT cells
- Additional phenotyping antibodies on request
- HLA tissue typing of donor samples if required
- Project turnaround from 1 week (varies according to exact requirements)
- Assay report and data DVD, including raw data .fcs files.
- Project follow-up consultation with technical specialist

Applications

- Immune monitoring
- Epitope validation

Example Flow Cytometry Testing with Human CD1d Tetramers

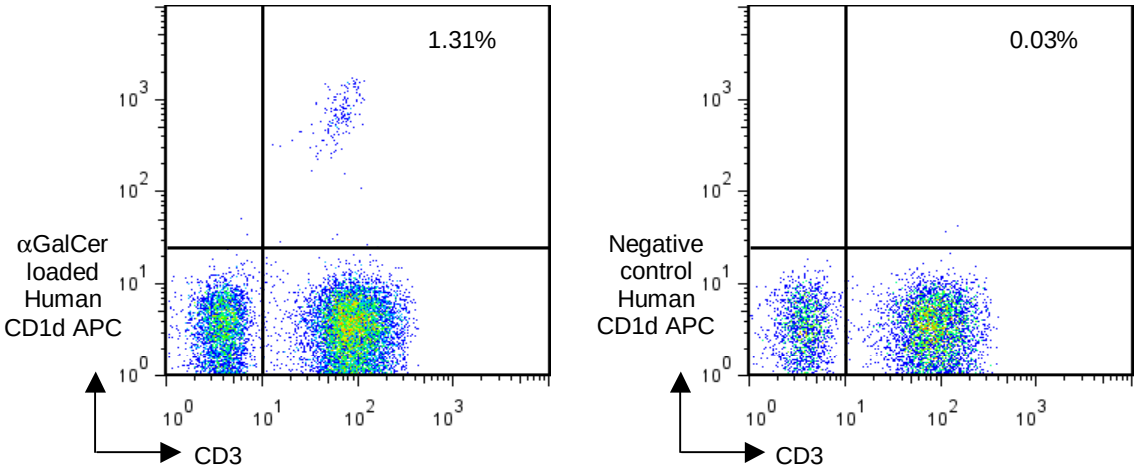


Figure 4: 1 x 10^6 PBMC were incubated with 1 test (0.5 µl) APC labeled, α–GalCer loaded human CD1d tetramer (left plot), or 1 test (0.5 µl) APC labeled, negative control human CD1d tetramer (right plot) for 30 minutes at 4°C. Following a wash step the cells were incubated at 4°C for 20 minutes with anti-CD3 FITC and anti-CD19 PE in 50 µl total volume. Following two further washes the cells were acquired and analyzed by flow cytometry. Non-specific staining was eliminated from the plot by gating on CD19 negative cells before plotting CD3 vs. CD1d tetramer.

T Cell Proliferation Assays

The T cell proliferation assay has been developed to identify the presence or absence of potential T cell epitopes and is useful in preclinical screening of novel peptides or proteins. The assays can address issues of relative antigenicity between structurally similar molecules; for example, they can help to distinguish between different candidates, or can give an indication of the success of protein engineering strategies in deimmunization of a potential immunotherapeutic.

ProImmune offers two types of T cell proliferation assay: naïve primary T cell assay for peptide epitope screening and dendritic cell (DC)-T cell assay for whole protein antigenicity assessment. Cells are labeled with the fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Cells that proliferate in response to antigen show a reduction in CFSE fluorescence intensity, which is measured directly by flow cytometry. This technology enables us to determine accurately the percentage of proliferating $CD4^+$ cells, offers detailed phenotyping of T cell responses, and is more sensitive than traditional assays based on radioactive thymidine incorporation.

Naïve Primary T cell Assay for Peptide Epitope Screening

This assay is used to identify epitope sequences that can elicit helper T cell proliferation and therefore potentially cause a helper T cell immune response. The assays are particularly suited to measure the stimulation of naïve T cells. As an example, in order to assess the potential for a peptide to be antigenic in a wider population group, the assays can be carried out on samples provided by ProImmune from healthy donors, who are presumed to be naïve to the antigens encountered. The donor group would typically include up to 50 individuals, chosen to reflect a broad HLA background.

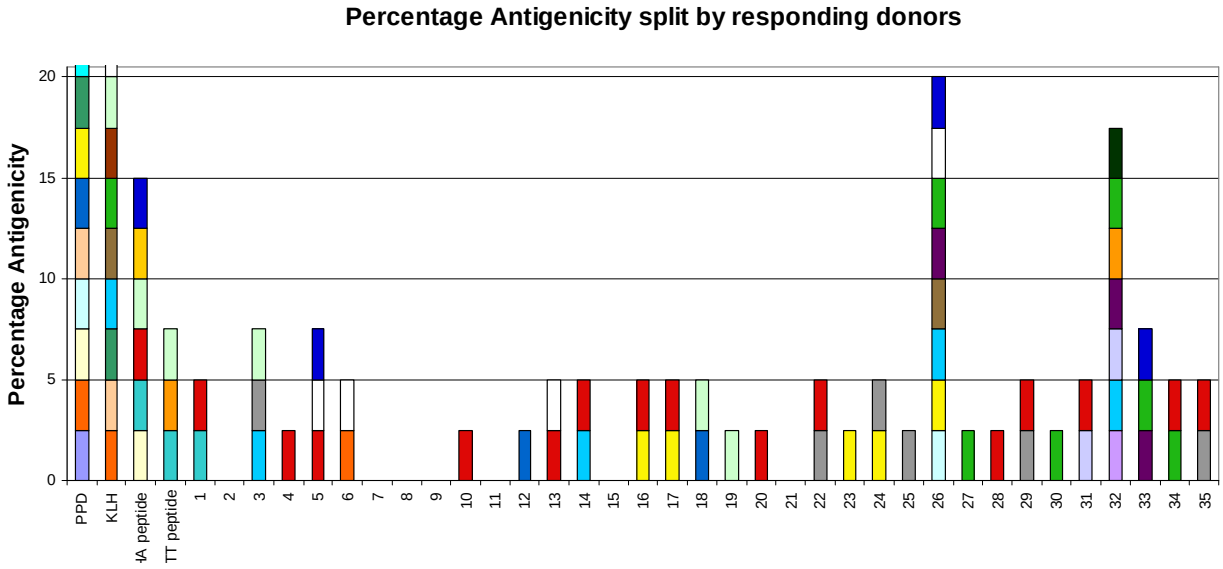


Figure 5: Graph showing the proportion of responding donors out of a total of 41 donors screened against 4 controls and 35 peptides. Each colored segment represents a different donor. A positive response in more than two independent donor samples is considered indicative of a potential T cell epitope. Depending on the size of the study, (20, 40 or 50 donors), this sets a threshold of 10%, 5% or 4% respectively. In this study, a threshold of 4.9% antigenicity was set. Thus, peptides 3, 5, 26, 32 and 33 can be considered as potential T cell epitopes.

Applications

- Immune monitoring
- Validate or confirm epitopes in samples

DC-T Cell Assays for Whole Protein Screening

The dendritic cell (DC)-T cell assay has wide-ranging applications. It allows for an overall comparison of the T cell driven antigenicity of any number of drug candidates at a pre-clinical stage. Crucially, it can also be used for assessing the impact on antigenicity of factors other than protein sequence. Such differences may include a comparison of biosimilars, protein modifications, degradation products, chemical entities given in combination therapies, and other parameters related to manufacturing processes, excipients, drug formulation and stability.

Additionally, in some cases it may not be possible to use the antigen to stimulate PBMC directly, particularly if the antigen involved modifies the function of responding T cells. To avoid such assay interference, antigens can be presented using dendritic cells, allowing the relative antigenicity of different leads to be compared directly.

Donor PBMC are used as a source of monocytes that are cultured in defined media to generate immature dendritic cells. Dendritic cells are loaded with test antigen (whole protein), and are then induced into a more mature phenotype by further culture in defined media. $CD8^+$ T cell-depleted donor PBMC from the same donor sample are labeled with CFSE then cultured with the antigen-primed DC. Each DC-T cell culture includes a set of untreated control wells. The assay also incorporates reference antigen controls, comprising two potent whole protein antigens.

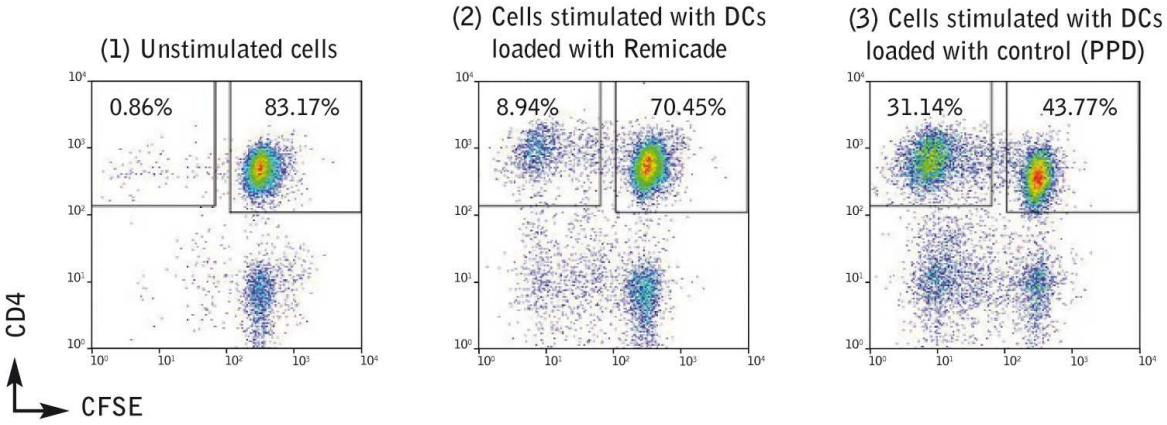


Figure 6: In a study at ProImmune, the DC-T cell proliferation assay was used to compare the relative antigenicity of a number of whole protein antibody drugs. Example staining data from DC-T cell proliferation assay: CFSE-labeled T cells incubated with (1) DC not co-cultured with antigen, (2) DC previously co-cultured with whole protein antigen (Remicade®, a chimeric antibody), (3) DC previously co-cultured with control protein, Tuberculin PPD.

When evaluating antigenicity, both the strength and frequency of a response should be considered. The strength of positive donor cell responses is determined by taking an average of the percentage stimulation above background obtained across accepted donors for each drug. A Response Index is determined by multiplying this value for strength with the frequency of the donor cell responses, and this index is more representative of the level of antigenicity than methods of analysis that rely on the frequency of response alone.

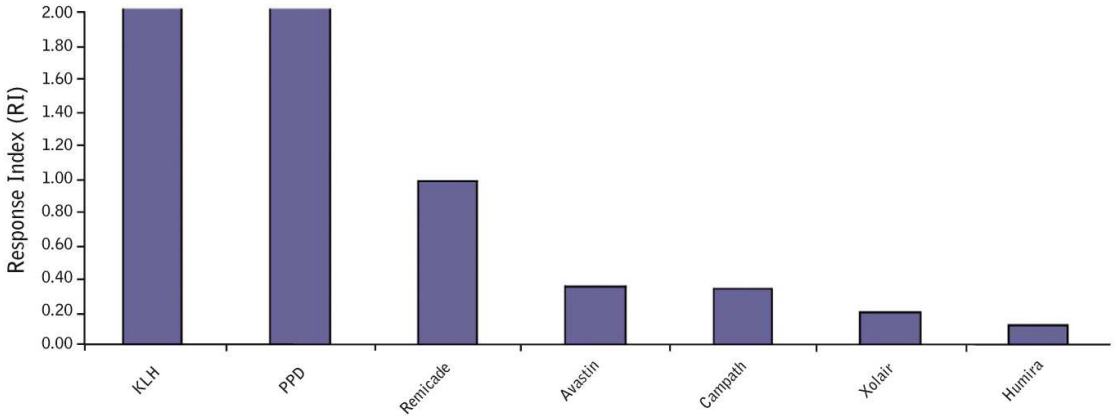


Figure 7: Results of the DC-T cell proliferation assay for 48 donors for 5 whole protein antibody-drugs (Remicade®, Avastin®, Campath®, Xolair® and Humira®) and 2 controls. Response Index = (% donors responding) x (average strength of response) / 100. Although a successful biologic, Remicade is a chimeric anti-TNF alpha antibody that elicits relatively high levels of clinical antigenicity in patients receiving the drug (as measured by anti-drug antibody responses). Avastin, Campath and Xolair have been developed with the approach of antibody humanization. Humira is a fully human antibody also targeting TNF alpha, with low levels of clinical antigenicity.

Although extrinsic factors such as antibody target and disease status of the patient play a key role in the ultimate immune response to a drug, it is crucial in the course of biologic development to have a rational design-led strategy to select protein therapeutic candidates on the basis of low T cell epitope content. ProImmune's new DC T cell assay forms a key element in such a rational approach. It is a ready to use tool that delivers a clear competitive advantage to drug development programs.

Applications

- Measure whole protein antigenicity
- Compare antigenicity of
    - Proteins with different post-translational modifications
    - Biosimilars
    - Degradation products
    - Chemical entities given in combination therapies
    - Parameters related to manufacturing processes, excipients, drug formulation and stability

HLA Tissue Typing Service

In advance of starting ELISpot, T cell proliferation assays or flow cytometry testing, the HLA tissue type of sample donors should be determined. If you do not know the tissue type of your samples and you do not already have convenient access to a tissue typing facility, ProImmune can carry out this service for you. HLA Tissue Typing for Class I (A, B, C) and Class II (DR, DP, DQ) alleles is offered.

- Tier 1 Typing using PCR-SSOP, resolves major allele groups to 4 digits, with some degeneracy
- Tier 2 Typing using PCR-SSP or PCR-sequencing for higher resolution
- Order any or all of the following loci: Class I (A, B, C) and Class II (DRB1, DRB3/4/5, DPB1, DQB1)
- Detailed results report typically sent by email in 15-20 working days

Tier-1, Class I and Class II

Typing by PCR-sequence specific oligonucleotides (PCR-SSOP) to resolve major allele groups to 4 digits, with some degeneracy e.g. HLA-A*23:01 /03/ 05/ 06). PCR-SSOP: The genomic DNA is amplified using PCR, then incubated with a panel of different oligonucleotide probes, which have distinctive reactivities with different HLA-types. The Luminex xMAP® technology is used, where oligonucleotide probes are individually attached to up to 100 distinctly fluorescent microspheres. This allows the measurement of 100 different reactions in a single tube.

Tier 2, Class I and Class II

Can be carried out if needed following Tier 1 typing to achieve higher resolution. Typing by PCR-sequence specific primers (PCR-SSP) or PCR-sequencing, will usually resolve to a specific 4-digit allele, with only occasional degeneracy. PCR-SSP: the PCR reaction is used to define whether the targeted HLA allele is present or absent by using reagents in the PCR reaction specific for individual HLA alleles. PCR-sequencing: the DNA sequence of the HLA allele can be directly analyzed by performing nucleotide sequence analysis of the amplified DNA.

Sample types and order handling

We accept genomic DNA, fresh whole blood or frozen cells, and will provide you with details of how to ship each of these to us. There is an order handling charge for samples sent as whole blood or frozen cells due to the additional processing needed for these sample types.

We offer a worldwide service, available for one sample or hundreds - our high throughput service enables us to process tens to hundreds of samples at a time. You can send your samples with confidence from any location worldwide using our experience in shipping globally.

Discuss your requirements with our customer service team of highly trained immunologists. They will work out the best service for you, and manage the whole process from sample handling to delivery of the final report.

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