PROIMMUNE
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ProImmune REVEAL™ Immunogenicity System

ProImmune's REVEAL™ Immunogenicity System offers a powerful approach to enable developers of biological therapies to minimize the risk of immunogenicity related adverse drug reactions, and aids selection of the best candidates for lead optimization.

It is the only available system that combines unique assay platforms that are best-in-class to manage immunogenicity risk at a pre-clinical stage: ultra-sensitive CFSE T cell proliferation assays, and the most comprehensive range of cell-free HLA-peptide binding assays covering the entire human population. Both assays are set up on optimized high throughput platforms to run in parallel and provide answers in a time-efficient manner.

The assays help to establish the most in-depth profile of the helper T cell immune response to one or more drug leads. If desired, these leads can then be re-engineered to remove unwanted helper T cell epitopes. The best lead from this process can then be tested to validate that its ability to cause T cell proliferation has been reduced.

Working with ProImmune

ProImmune's REVEAL™ Immunogenicity System as a highly customizable service that is delivered on a pure 'fee for service' basis. ProImmune is independently owned and free of any restrictions or tie-ins as a service provider. We do not develop our own therapeutic pipeline and we have not entered into any strategic alliances that would restrict our capacity to work in specific target areas. In practise this means that our customers do not have to worry about potential conflicts of interest or complex downstream milestone and royalty payment structures.

Our teams of experienced scientists have developed sensitive CFSE T cell assays for the prediction of immunogenicity, and leading recombinant MHC technologies, including physical MHC-peptide binding assays for antigenicity prediction. The system is modular and can be tailored to specific project requirements, providing a flexible and cost effective solution as part of the drug development process.

Background to Immunogenicity in Drug Development

The immunogenicity of biological drugs has the potential to be a significant obstacle in the development of successful new therapies. Unwanted immunogenicity can manifest itself particularly through anti-drug antibody (ADA) responses. ADA responses can lead to allergic reactions, reduction or neutralization of the activity of the drug and in some cases cross-reactive immune responses, which could lead to serious adverse events.

Immunogenicity is caused or influenced by a multitude of factors:

- Intrinsic antigenicity of T and B cell epitopes in a drug can cause cellular and humoral anti-drug immune responses;
- Extrinsic factors that influence immunogenicity include the following:
    - The disease setting can pre-dispose for a higher or lower immune response, for example, in many autoimmune settings the immune system is already over-active, whereas in some diseases such as some cancers, patients may be immune compromised or suppressed;
    - The drug itself modulates the immune system, e.g. by killing or modifying the function of immune cells;

- The effector function of the drug, such as complement-dependent cytotoxicity opsonization, may lead to immune stimulation;
- Effective valency of the drug, for example due to process and storage dependent aggregation;
- Formulation based effects, such as unwanted adjuvant effects;
- Amount, frequency and route of administration, for example, a drug administered subcutaneously may have a higher potential for immunogenicity than if it is given intravenously.

Overall, for a drug to be immunogenic it needs to have T cell epitopes, but extrinsic factors such as those mentioned above will also have a very significant effect on immunogenicity, and may outweigh intrinsic antigenicity. However, understanding antigenicity plays an important part in defining an optimal strategy for the development of drugs that are optimized for the avoidance of unwanted immunogenicity.

Disease-associated Bias of Immunogenicity

Many diseases with major unmet medical need such as autoimmune diseases, show a pre-dominant association with certain tissue types, especially HLA class II alleles. For example rheumatoid arthritis (RA) is strongly associated with HLA DR1 and HLA DR4. A significant therapeutic target in RA is the B cell surface antigen CD20. Monoclonal anti-CD20 antibodies have been developed for B-cell non-Hodgkin's lymphoma and B cell leukemia. The most successful anti-CD20 antibody is Rituxan® (Rituximab), a chimeric mouse/human monoclonal antibody developed by IDEC Pharmaceuticals and co-marketed by Biogen Idec, Genentech and Roche. While the implications of the immunogenicity of Rituxan® have not been significant in the original leukemia application, the drug causes significant immunogenicity in the RA indication, where it was also shown to have efficacy. It is thought that DR1 and DR4 restricted T cell epitopes in Rituxan® contribute over proportionally to this observed immunogenicity due to the high incidence of theses alleles in the RA patient population. As one of its key attributes, ProImmune's REVEAL™ Immunogenicity System can clearly identify how the drug antigenicity is distributed across the HLA background and the system can be used to reassess the potential impact of drug antigenicity in relation to an HLA biased disease-state population.

Traditional Animal Models are Insufficient for Predicting Immunogenicity in Many Biological Drugs

New biological drugs are often based on a human background or are humanized (in the case of antibody drugs). This means that they will appear foreign in an animal host and could elicit uncharacteristically high immune responses. The challenge is therefore to find a set of physical testing methods that can be used at a pre-clinical stage to compare the antigenicity of drugs leads that take account for the fact that the human immune system is 'uniquely human'. ProImmune's REVEAL™ Immunogenicity System addresses this through replicating a substantial portion of the human T cell immune pathway in comprehensive *in vitro* assays that span all major human ethnicities. Our T cell assays are carried out on samples derived from a large bank of blood donors, which are fully characterized for their tissue type. Our HLA binding assays replicate the pathway of antigen presentation through recombinant HLA molecules in a cell-free assay.

Prediction of the Relative Antigenicity of a Drug

Evaluating B Cell Epitopes

ADA responses will be directed towards B cell epitopes on a drug. Although it is possible to identify B cell epitopes in any protein through a variety of epitope mapping approaches it can be difficult to quantify the impact of key conformational epitopes that may contribute the majority of relevant B cell antigenicity in a drug lead.

Evaluating T Cell Epitopes

Antibody responses that are of high affinity and sufficient titer require the help of $CD4^+$ T cell immune responses for their maturation. Contrary to the complex three-dimensional B cell epitopes that often depend on secondary and tertiary protein structure, $CD4^+$ T cell epitopes are relatively short linear peptide stretches (typically 11-20 amino acids long). Identifying all potential $CD4^+$ T cell epitopes in the protein content of a drug is therefore a powerful rational approach for assessing and comparing antigenicity of drug leads. The information on such epitopes can be used to identify the best lead from a group of candidates for a new drug. It also raises the possibility that T cell epitopes can be removed in order to avoid unwanted immunogenicity, by mutating key amino acid residues that do not affect drug potency.

There are several approaches for T cell epitope prediction and validation. Publicly available predictive class II HLA binding algorithms are usually free of charge, but the prediction data correlates poorly with physical binding data, leading to a high proportion of false positive and false negative results. In addition, in most cases peptide sequences have to be submitted over the Internet, causing potential concerns about data confidentiality. Assays such as MHC-peptide elution followed by sequencing can assess the peptides presented by the immune system; however, the majority of presented peptides are not immunogenic, and the process of identifying truly antigenic peptides is extremely time and labor intensive.

The ProImmune REVEAL™ Immunogenicity System has been developed to improve assessment of the potential immunogenicity of biologics at a preclinical. The system combines unique proprietary and complementary assay platforms that can identify $CD4^+$ T cell epitopes in any protein sequence quickly and with high confidence. Due to its unprecedented resolving power, it is possible in most cases to identify the unique HLA restriction of epitopes.

The ProImmune REVEAL™ Immunogenicity System in Detail

CFSE T Cell Proliferation Assays

The first component in the system is an ultra sensitive flow cytometric T cell proliferation assay. Unlike traditional T cell assays, which are based on radioactive thymidine incorporation, ProImmune's new assay utilizes powerful flow cytometry methods that have the following advantages:

- Live gating reduces assay noise from irrelevant cells and improves sensitivity;
- The analysis is carried out only on $CD4^+$ T cells which are clearly identified as a subset;
- The percentage of proliferating $CD4^+$ T cells is actually determined by gating;
- Proliferation is measured for the full assay time, and during a time window at the end when relevant responses could be missed;
- Further phenotyping of responses is possible, e.g. to distinguish true naïve from memory responses that may exist to a particular antigen.

Our T cell assays are carried out on collections of donor samples that are untreated with the drug of interest and are therefore presumed to be naïve to the drug antigens encountered. The assay is carried out on a representative donor group, which includes 20-50 individuals chosen to reflect a broad HLA background representing the target population group for the drug. Critically all donors used are HLA-genotyped on DQ, DP and DR loci, enabling a more accurate mapping of responses to four digit HLA sub-type. In our experience, a relevant number of responses can be attributed to epitopes restricted to DP and DQ alleles, the significance of which has often been neglected in the past.

Cells are labeled with the fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE). Those cells that proliferate in response to antigen show a reduction in CFSE fluorescence intensity, which is measured directly by flow cytometry. Since this is a flow cytometric assay, it accurately determines the percentage of proliferating $CD4^+$ cells, enables detailed phenotyping of T cell responses, and is more sensitive than traditional assays based on radioactive thymidine incorporation.

Cell proliferation is determined by flow cytometric analysis. For naïve T cell assays, a Cell Division Index (CDI) is determined for each stimulated sample, and for DC-T cell assays the percent stimulation above background is determined for each stimulated sample, through comparison with results from an unstimulated sample. The lowest and highest values are discarded and the mean and standard deviations from the intermediate 4 results are calculated. A result is considered significantly positive if the Cell Division Index (naïve assay) is greater than 2x background (untreated) plus 2x standard deviation, or if the percent stimulation above background (DC assay) is greater than 0.02% plus 2x standard deviation. For both assays, a response is considered significant if 2 or more donors give a positive result.

ProImmune offers two types of T cell proliferation assay: Naïve primary T cell assay for peptide epitope screening and DC-T cell assay for whole protein screening.

Naïve Primary T Cell Assay for Peptide Epitope Screening

This assay is used to identify peptide epitope sequences that can elicit helper $CD4^+$ T cell proliferation and therefore potentially cause a helper T cell immune response that may lead to anti-drug antibody (ADA) responses or other unwanted immunogenicity.

$CD8^+$ T cell-depleted and CFSE-labeled donor peripheral blood mononuclear cells (PBMC) are cultured with 5uM of each peptide of interest for 7 days in six replicate wells. Each assay plate includes a set of untreated control wells. The assay also incorporates reference antigen controls, comprising synthetic peptides for known MHC class II antigens, and two potent whole protein antigens.

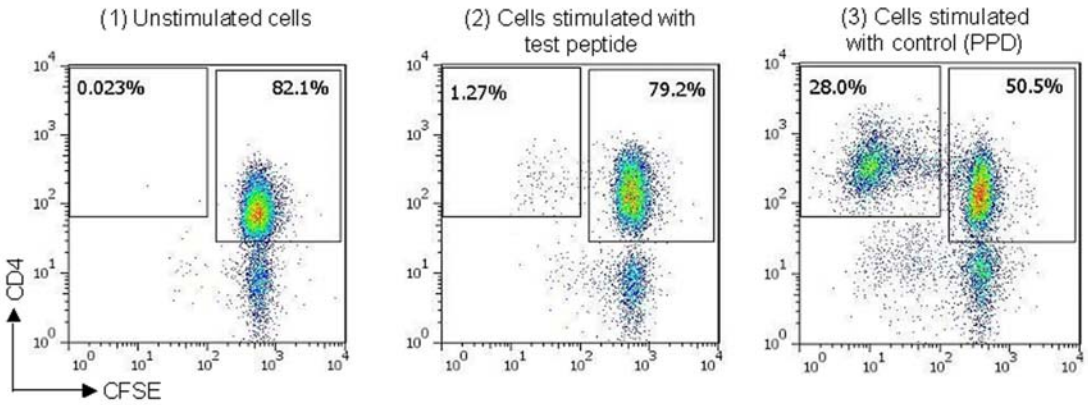

Figure 1: Example staining data from naïve T cell assay, CFSE-labeled T cells cultured (1) in media alone, (2) with test peptide derived from antigen of interest, (3) with control protein, Tuberculin PPD.

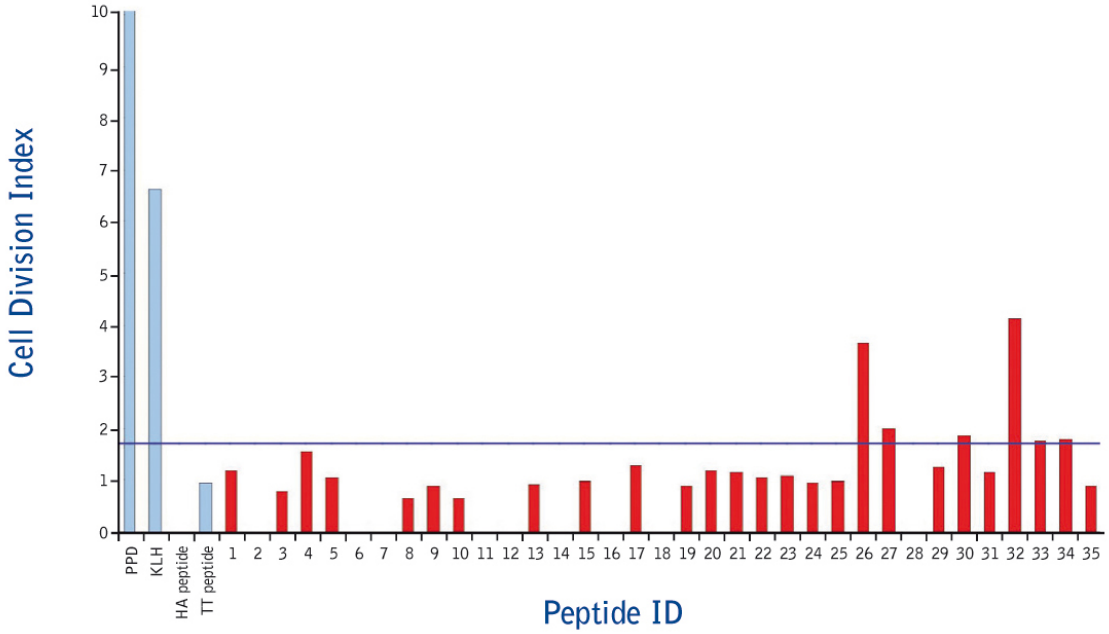

Figure 2: Graphical presentation of the results of the naïve primary T cell assay for one donor for 35 peptides and 4 controls. Five peptides and two controls show a significant cell division index (y-axis, >1.7).

Dendritic Cell (DC)-T Cell Assays for Whole Protein Screening

The 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 and then cultured with the antigen-primed DCs for 7 days in six replicate wells. 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.

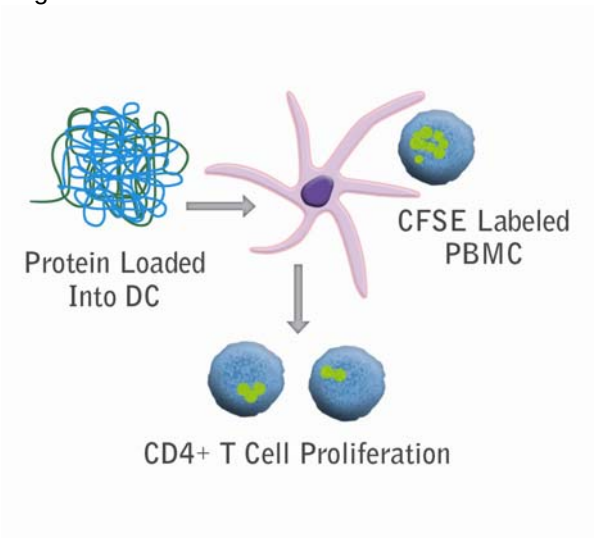

Figure 3: Schematic representation of DC-T cell assay.

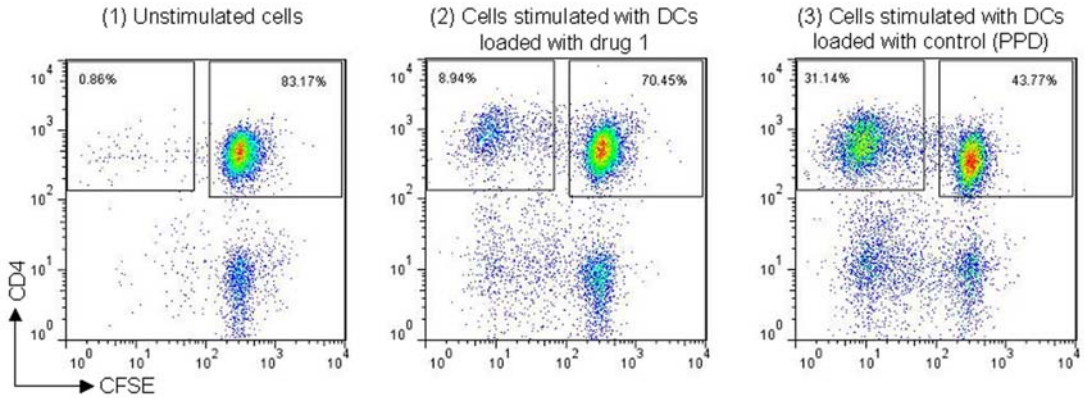

Figure 4: Example staining data from DC-T cell assay, CFSE-labeled T cells incubated with (1) DCs that were not co-cultured with antigen, (2) DCs that had been previously co-cultured with whole protein antigen (drug 1, chimeric antibody), (3) DCs that had been previously co-cultured with control protein, Tuberculin PPD.

When evaluating immunogenicity, it is appropriate to take account of the frequency of donor cell responses across the study cohort. A positive response (percentage stimulation above background >0.02%) in 2 or more independent donor samples is considered indicative of a potential in vivo T cell response. It is also important to consider the strength of positive donor cell responses. This is determined by taking an average of the percentage stimulation above background obtained across accepted donors for each drug. A Response Index is calculated by multiplying the value of the strength of response by the frequency of the donors responding. This index is more representative of the level of immunogenicity than methods of analysis that rely on the frequency of response alone.

If a positive response is observed in a donor sample in either the naïve or DC-T cell assays, then a proliferative immune response has been mounted through at least one of the six HLA class II alleles presented by that donor. If the same peptides are screened using ProImmune REVEAL™ HLA-peptide binding assays the combined results will clarify which HLA alleles are actually involved in the response.

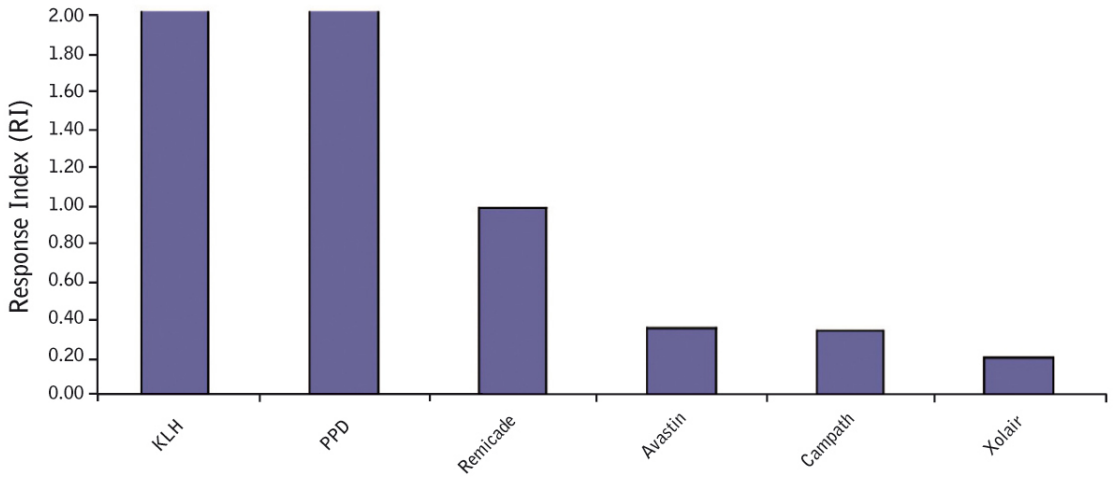

Figure 5: Results of the DC-T cell proliferation assay for 48 donors for 4 whole protein antibody-drugs (Remicade®, Avastin®, Campath®, Xolair®) and 2 controls. Response Index = (% donors responding) x (average strength of response) / 100. Although a successful biologic, Remicade is a chimeric anti-TNF 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.

Different therapeutic proteins will yield specific levels of immunogenicity and this can be directly quantified in the ProImmune DC-T cell assay.

ProImmune REVEAL™ Class II HLA-Peptide Binding Assays

To overcome the inadequacies of class II HLA epitope prediction algorithms, ProImmune has developed a comprehensive, high throughput, *in vitro* class II HLA-peptide binding assay, covering 56 HLA DR, DQ and DP alleles, as well as a complementary assay to determine HLA-peptide binding stability. This range of alleles covers nearly all of the major ethnic groups worldwide. ProImmune's MHC class II HLA binding and stability assays are the only such comprehensive assays offered or even described by any group anywhere.

The assays can be used to compare drug candidates for the purpose of lead optimization. For example, where several human antibody candidate leads need to be evaluated, the predicted antigenicity of peptides spanning any non-human or low frequency human protein content (such as hypervariable portions) can be compared, quickly and efficiently. Critically the ProImmune REVEAL™ assays can show peptides that bind to many class II alleles, implicating them as pan-DR, pan-DQ or pan-DP binding sequences that could be antigenic over a significant percentage of the population.

Table1. Alleles available for class II HLA-peptide binding and stability assays

Name	Allele	Name	Allele	Name	Allele
R01	DRA1*01:01 + DRB1*01:01	R32	DRA1*01:01 + DRB1*15:02	P01	DPA1*01:03 + DPB1*01:01
R02	DRA1*01:01 + DRB1*15:01	R33	DRA1*01:01 + DRB1*15:03	P02	DPA1*01:03 + DPB1*02:01
R03	DRA1*01:01 + DRB1*03:01	R34	DRA1*01:01 + DRB1*16:01	P03	DPA1*01:03 + DPB1*03:01
R04	DRA1*01:01 + DRB1*04:01	R35	DRA1*01:01 + DRB1*16:02	P04	DPA1*01:03 + DPB1*04:01
R05	DRA1*01:01 + DRB1*11:01	R36	DRA1*01:01 + DRB3*02:02	P05	DPA1*01:03 + DPB1*04:02
R06	DRA1*01:01 + DRB1*13:01	R37	DRA1*01:01 + DRB3*03:01	P06	DPA1*01:03 + DPB1*05:01
R07	DRA1*01:01 + DRB1*07:01	R38	DRA1*01:01 + DRB5*01:01	P14	DPA1*02:01 + DPB1*01:01
R08	DRA1*01:01 + DRB1*01:02	Q01	DQA1*01:01 + DQB1*05:01	P15	DPA1*02:01 + DPB1*02:01
R11	DRA1*01:01 + DRB1*04:02	Q02	DQA1*05:01 + DQB1*02:01	P16	DPA1*02:01 + DPB1*03:01
R13	DRA1*01:01 + DRB1*04:04	Q03	DQA1*01:02 + DQB1*05:02	P17	DPA1*02:01 + DPB1*04:01
R14	DRA1*01:01 + DRB1*04:05	Q06	DQA1*01:02 + DQB1*06:02	P18	DPA1*02:01 + DPB1*04:02
R15	DRA1*01:01 + DRB1*04:07	Q08	DQA1*03:01 + DQB1*03:02	P19	DPA1*02:01 + DPB1*05:01
R16	DRA1*01:01 + DRB1*04:08	Q09	DQA1*01:02 + DQB1*06:04	P20	DPA1*02:01 + DPB1*06:01
R19	DRA1*01:01 + DRB1*08:04	Q10	DQA1*05:01 + DQB1*03:01	P21	DPA1*02:01 + DPB1*09:01
R20	DRA1*01:01 + DRB1*09:01	Q11	DQA1*02:01 + DQB1*02:02	P22	DPA1*02:01 + DPB1*11:01
R21	DRA1*01:01 + DRB1*10:01	Q12	DQA1*03:01 + DQB1*03:01	P23	DPA1*02:01 + DPB1*13:01
R22	DRA1*01:01 + DRB1*11:02	Q15	DQA1*02:01 + DQB1*03:03	P24	DPA1*02:01 + DPB1*14:01
R23	DRA1*01:01 + DRB1*11:03	Q16	DQA1*03:03 + DQB1*03:03	P25	DPA1*02:01 + DPB1*15:01
R24	DRA1*01:01 + DRB1*11:04			P26	DPA1*02:01 + DPB1*17:01

A peptide library of overlapping 15mers is synthesized to cover the protein segments of interest from the candidate lead to be evaluated. All peptides are then tested for binding against any or all, of the panel of class II HLA alleles. The assay includes a set of positive and intermediate controls for each HLA allele. The results are also presented to show the contribution of peptide binding weighted by the incidence of each allele in the world population or relative to the allelic distribution in a disease setting to be investigated.

In a pilot study ProImmune scanned the variable region protein sequence of Remicade® using HLA-peptide binding and stability assays. Remicade® is an anti-TNF-alpha, chimeric antibody, with a human constant region and mouse variable regions. It us used in the treatment of rheumatoid arthritis and Crohn's disease and other autoimmunity-related diseases. Remicade® is known to be a relatively immunogenic antibody, due to its mouse variable region, and shows relatively high ADA responses.

ProImmune generated a peptide library of overlapping 15mers representing the mouse variable region of Remicade®, excluding the human germline sequences. The peptides were screened for binding against a panel of 56 HLA DR and DQ alleles with corresponding controls. A sample of the results is presented in figure 6, showing the contribution of peptide binding weighted by the incidence of each allele in the world population.

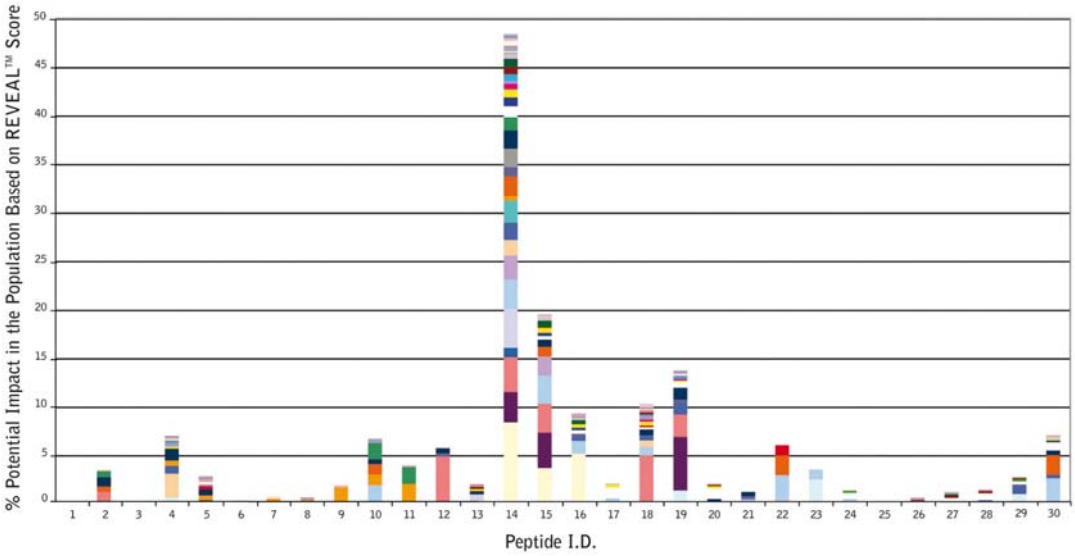

Figure 6: The potential antigenic impact in the population of overlapping peptides representing part of the mouse variable region of Remicade® analyzed with the ProImmune REVEAL™ binding assay.

Results from MHC-peptide binding assays cannot determine T cell epitopes alone. Binding peptides may not be presented by antigen presenting cells in practise, due to a lack of appropriate protein processing, or other factors. Our experience with the peptide-binding assay at ProImmune indicates that most proteins will have an appreciable number of class II HLA binding sequences, irrespective of whether or not the sequence is human derived. However, this is not unexpected, since the function of MHC molecules is to provide a broad-spectrum antigen display mechanism.

Nevertheless, in order to be a T cell epitope a peptide sequence has to bind to an HLA allele. The specificity and distribution of binding of such a sequence across the full complement of human class II HLA determines whether a peptide can or can not be an epitope. The HLA-peptide binding assays determine the exact binding sequence and HLA restriction of that sequence, and provide key information for epitope ranking and modification.

The Powerful Combination of HLA-Peptide Binding and T Cell Proliferation Assays

While each of the two components of the process has its own features and advantages the system gains its significant predictive power from the combined interpretation of these two complementary assays. Epitopes that are seen in the T cell assays can be compared with the profile of HLA binding. Given the high resolution of our donor panel and the wide range of alleles used we often identify very clear hits where several donors for a hit peptide are positive for the HLA sub-type, and the same profile is seen for HLA binding. Such high-confidence results usually also give a clear guide to the key residues involved in HLA binding, which would be targets for further protein engineering if required.

The ProImmune REVEAL™ Immunogenicity System can be tailored to the individual needs of the researcher. Contact us for a specific quotation for your project, email enquiries@proimmune.com.