T cell epitope identification by mass spectrometry: *the future in detail*

T cells play a crucial role in building a specialised immune response and help adapt the immune response to different challenges, whether they are intracellular bacteria, viruses or cancer or extracellular organisms such as blood-borne bacteria and parasites.

By Dr Nikolai Schwabe and Katherine Catchpole

One of the most fundamental parts of the human immune response is that of recognition of epitopes by T cells. T cell epitopes are peptide fragments of pathogenic, other foreign or self- proteins. These proteins are taken up and processed by a cell, and then are bound into the cleft of a specific major histocompatibility complex (MHC) molecule and presented on the outer surface membrane. T cells identify certain fragments as foreign, and either kill the cells directly or stimulate the immune response of B cells to produce antibodies against the originating proteins and pathogens. Large proteins can be broken down by specialised enzymes into hundreds of short peptide fragments, but only very few of these fragments will elicit an immune response. Identifying the epitopes which create a response is vital for research into the functioning of the immune system, and critical in drug and vaccine development.

Understanding T cell responses at the individual peptide sequence level, or the 'molecular signatures' involved, can be very useful in directing the function of the immune system. For example, vaccines constructed that contain only the most effective portions of a pathogen in causing an immune response thus maximise the balance between effectiveness and safety of the vaccine. Equally in allergy and autoimmune diseases, a molecular understanding of what causes such conditions could lead to the design of highly targeted and effective countermeasures. Developments on this level have the potential to be transformative in areas such as rheumatoid arthritis and multiple sclerosis where current therapies only contain disease progression, rather than leading to reversal or cure.

Cancer is a further area where T cell responses are implicated, not only having a crucial effect in disease prevention but also being instrumental in resolving the disease in patients undergoing conventional cancer therapies. In those patients who achieve a complete response to conventional therapy, small amounts of surviving cancer cells are

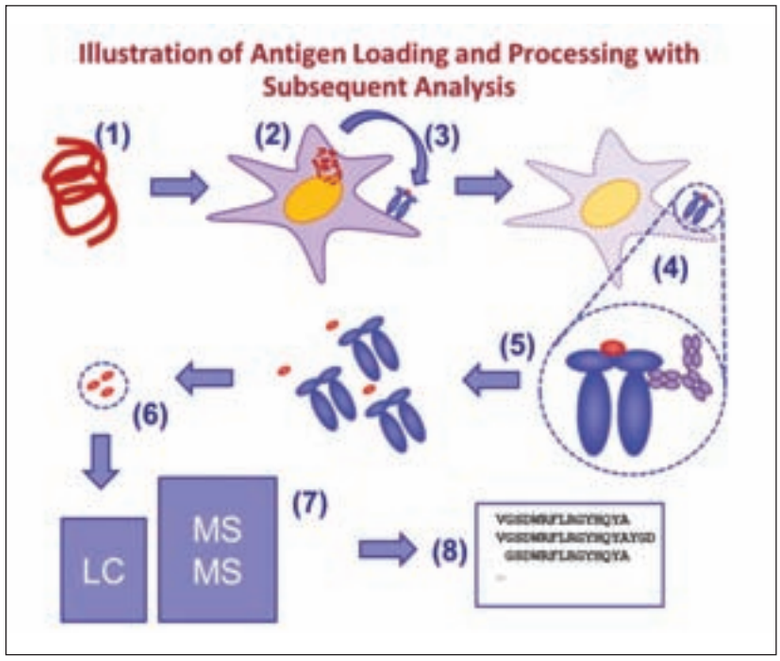

Figure 1
The protein of interest (1) is added to the DC culture (2) whereby it is taken up and undergoes proteolytic cleavage into peptide fragments. Peptide fragments are loaded on to HLA Class II molecules and subsequently presented on the cell surface of mature DCs (3). The cell culture is lysed and Class II HLA peptide complexes are released from the cell (4). The solubilised HLA-peptide complexes are then isolated by an immunoaffinity step which specifically recovers HLA-DR complexes (5). Peptides are eluted from HLA-DR complexes (6) and prepared for sequencing mass spectrometry (7). Mass spectrometry data is subsequently analysed against human proteome peptide fragments patterns and a fragment pattern from the known target therapeutic to determine statistically relevant hit peptides (8)

mopped up by the immune system, which becomes more effective again when the disease burden is reduced[1]. But why are some patients able to mount such a response and others are not? Studying cellular immune responses in cancer is both exciting and confounding; often oncogenes and resulting cancer markers remain uncharacterised and many cancers have evolved to proactively prevent effective immune responses against them[2].

T cell epitope discovery and the quest to decipher immune responses is a key frontier in medicine and physiology

Finding which portions of a protein or whole pathogen contribute to a T cell immune response is an active area of research. There are several methods for identifying peptide fragments (or epitopes) recognised by T cells in an immune response. Selecting the most appropriate method depends on the source proteins involved in the response being identified and available to the investigator, and also the accessibility of blood samples from those individuals thought to have an existing response to the protein. Where such information and samples are on hand, a method that is conceptually simple (but laborious nevertheless) is to synthesise each possible peptide fragment generated from the protein of interest and use a functional assay such as ELISPOT[3] to test available blood samples for a memory immune response. In such an assay, T cells that have already encountered a specific peptide fragment are restimulated and if the fragment is recognised, a cytokine response is elicited and can be measured within a short period of time[4].

T cell epitope discovery is, however, most needed in those cases where either the information about the proteins involved in a response is lacking or blood samples from responding donors are unavailable. This is the case for the development of many novel vaccines targeted against complex pathogens, where the proteins involved in potentially generating a beneficial immune response are as yet unknown. Equally important is the discovery of unwanted immune responses which can occur against new drugs, which are themselves proteins. In this case it is desirable at the preclinical stage to find the epitopes that patients could respond to once a drug enters the clinic. At the same time this means that clinical samples with such responses are unavailable at the outset.

In instances where the originating proteins are known, such as in the case of novel drug and existing vaccine candidates, but where patients with known immune responses against such proteins are not, a range of naïve T cell assays and MHC binding assays can be used to define new epitopes with high accuracy. Here it is possible to effectively run clinical trials *in vitro* wherein candidate protein or peptide sequences are tested on blood samples from tens of healthy blood donors and the ability of each donor in producing an immune response to each protein or peptide *de novo* is measured. The main challenge with this technique is cost and throughput especially where many proteins are to be tested.

The advent of sequencing mass spectrometry in the 1990s[5,6], has proven to be a scientifically exciting technology for identifying peptide sequences involved in T cell responses. Using this method, cells that 'present' the processed peptides of interest in their MHC molecules are harvested. The cells are then lysed, MHC molecules purified away from other cell material and the bound peptides isolated. Recovered peptides are then separated by liquid chromatography and sequenced by a technique of sequencing tandem mass spectrometry[7]. In short, this ingenious approach allows peptide fragments present on the surface of a cell and visible to T cells, to be recovered and identified through sequencing[8].

The power of this approach lies in the fact that an artificial antigen presentation system can be developed that simulates the natural immune response and can be followed on a molecular level. This system is also capable of identifying new

sequences that play a crucial role in immune responses, even in cases where the sequence of the originating genes or proteins involved is not known at the outset. A pre-existing immune response in a patient is also not required. In other words, peptides causing immune responses against cancer can be identified in this way without knowing the proteins or even genes involved in the cancer. The antigen presentation system can simply be run by feeding cancer cells or cancer cell lysates to antigen presenting cells in cell culture. Equally, individual key peptides involved in immune responses against complex pathogens can be identified simply by feeding inactivated pathogen to cell cultures.

Understandably, this antigen presentation technology was received with great excitement in the 1990s and generated a range of key publications[5,6]. The only remaining caveats being the techniques involved were extremely complicated and the technologies very difficult to use with any great efficiency. Therefore after the peak of early academic interest had subsided, very little commercial use had been made of the technology.

With the dramatic advance in analytical techniques over the last 15 years, the potential of the approach has been re-recognised by ProImmune, a bioassay service company based in Oxford, UK. The company has now developed an assay platform called ProPresent® for general use in a variety of applications for volume commercial application. The key advantage of the system is that once configured it can be run with considerable time efficiency. Key sequences can now be discovered from what is effectively proteome-sized starting information in physical form, in only a few days.

The principles underlying this assay platform are the same as with prior academic technology: MHC-peptide complexes are recovered from the cell surface from any suitable cell source in an immunoaffinity purification step. Depending on the anti-MHC antibody used, different alleles or allele groups of molecules can be recovered, eg pan=Class I or Class II or HLA-DP, DQ, DR, etc. Peptides are then separated from the MHC molecules and sequenced in tandem mass spectrometry. The mass spec analysis is compared with the genome of the organisms of interest to confirm the identity of peptides through standard algorithmic methods. A particular advantage of the method is that because it is highly sensitive it requires less cell material for analysis, enabling projects that would previously not have been feasible (**Figure 1**).

A critical input for the assay is clearly the source of cells to be used and the assay is very flexible in this regard. In one example the customer can simply provide frozen cell pellets for their samples. The cells can be from any source, such as tumour tissue, cultured and loaded cells including from human and other vertebrate sources.

Alternatively, cohorts of healthy and also disease state donors can be recruited. In a typical study, monocyte-derived dendritic cells are cultured from a number of donor samples and the cells are loaded with antigen, which may be purified protein, protein mixtures or cell lysates, including pathogen lysates. Dendritic cells are then matured to a desired state and harvested for analysis.

Over the past 18 months the technique has been used in the analysis of samples from a broad variety of antigen sources, including from food allergen mixtures, intracellular bacterial pathogens, chemically modified proteins, cancer, viruses and also regular purified proteins. Numerous novel peptide hits have been discovered and these peptides can be considered as part of the potentially most relevant sequences in immune responses against the relevant antigen source.

A number of areas are now discussed in which an antigen presentation assay can be used to further our understanding of T cell responses.

Vaccine discovery

When developing new vaccines the aim is to identify so called 'sub-units' of a pathogen that can be produced, for example, as recombinant proteins or peptides, and can elicit a focused and effective immune response. Ideally, this avoids the need for the traditional approach of producing vaccines that contain attenuated or killed pathogen and alleviates-associated safety concerns, such as the risk of stimulating the disease in immuno-compromised patients. Such protein or peptide vaccines are also an attractive prospect in developing targeted cancer therapies where the challenge is to destroy tumorous cells while leaving healthy cells unharmed[9].

In a recent example antigen presentation technology was used by Ventura and her colleagues at Sanofi Pasteur, in a study of the cytomegalovirus (CMV)[10]. CMV infection is endemic to between 50% and 80% of US adults, and between 10% and 50% of the T cell population of a carrier is specific to the virus. Although often asymptomatic, the pressure this infection puts on the immune system can cause serious health complications in foetuses and immuno-compromised adults. The team at Sanofi investigated which peptide sequence of CMV glycoprotein G (gB) acted as the epitope responsible for stimulating the CD4+ T cell and antibody response.

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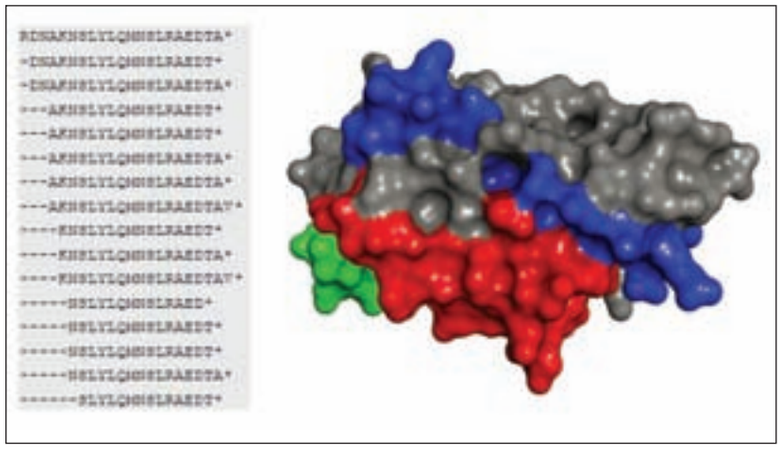

Figure 2
ProPresent® data for Humira® variable heavy chain segment. Left panel shows one of the nested sequence sets identified by mass spectrometry, covering the putative DNAKNSLYLYLQ MNSLRAEDTA epitope. Right panel shows the three epitopes identified for this region, mapped on to the structure of human IgG1k. DNAKNSLYLYLQMNSLRAED TA is shown in blue

The team at Sanofi Pasteur used a T cell reactivity assay, with autologous antigen-presenting cells primed with peptides from the CMV protein glycoprotein B (gB) and CD4+ T cells from CMV-infected donors. ELISpot analysis of repeat peptide fragment screening narrowed down possible epitope identities to an epitope presented by HLA-DR*07:01. As this form of assay has a significant likelihood of responding to sequences that are not the core target epitope, the team used the ProPresent® assay, in an even more direct epitope identification that confirmed the results obtained in the ELISpot assay. Overall the study exemplifies how different complementary assays can be combined in order to discover and confirm important T cell epitopes with high accuracy.

Biological drug development

Unwanted 'off-target' levels of immunogenicity are not only a problem when designing vaccines suitable for people who are immuno-compromised. Several biological drugs currently on the market carry the risk of eliciting dangerous cross reactive immune responses, or, more commonly, simply of creating a response against the drug itself, negating its effects. One example is Humira, with annual sales of approximately $9 billion and one of the leading treatments for rheumatoid arthritis and Crohn's disease. In certain cohorts, Humira can trigger immune responses with repeated dosing a response in nearly all patients receiving the drug[11]. Identifying the exact source of this unwanted immunogenicity could lead to the development of much more universally effective treatments for these significantly disabling diseases in an extremely competitive drug market. Antigen presentation assays can identify quickly which peptide fragments from Humira are likely to be involved in causing a T cell immune response against the drug and once identified such epitopes could be removed through strategic point mutations in the drug sequence (**Figure 2**). Alternatively, when developing follow on generation drugs to Humira, assays can be used to choose the lowest risk candidate drug that should be taken forward into clinical trials.

Crossing over between drug and vaccine development, another recent study carried out at the UK government's Defence Science and Technology Laboratory (DSTL) looks into the development of a virus-neutralising, protective antibody against the tropical disease Venezuelan Equine encephalitis virus (VEEV)[12]. Endemic to horses in southern parts of the American continents, this mosquito-borne pathogen has been developed by several nations as a human-targeting bioweapon. It is considered to be a high risk bioterrorism agent, not least because no medical countermeasures against it in either treatment or prevention currently exist.

The research team at DSTL used a highly effective neutralising mouse antibody against VEEV as the basis for their potential human treatment. In order to make the antibody useful for potential human use DSTL went through a standard approach of humanising the antibody, wherein the mouse antibody complementary determining regions that bind the antigen were grafted on a human antibody constant and variable region backbone that had been suitably constructed. The shape of the beta sheet variable region scaffold of the murine antibody was conserved by making isolated point changes in the human replacement sequence. DSTL were aware however that even humanised or 'fully human' therapeutic antibodies can elicit significant unwanted drug immunogenicity. A further issue in the project was the fact that clinical trails measuring the efficacy of neutralising the disease in humans was not something that would be possible for this agent. In essence the humanised antibody could at best be tested in healthy volunteers for safety. Since DSTL nevertheless wanted to generate early data to understand how successfully the humanised antibody would prevent anti-drug antibodies from occurring, DSTL contracted T cell proliferation assays to be carried out comparing the human T cell response between the humanised and murine antibody variable regions. Using overlapping peptide libraries generated from each antibody sequence T cell assays were carried out for each peptide on tens of

donor samples from a cohort of pre-banked blood donors. Results show a significant removal by the humanisation process of epitopes found in the chimeric antibody sequence which activated T cells that would have potentially caused or aided unwanted immune responses.

Allergy to small molecule drugs

HLA-mediated immune responses can be undermined by certain small molecule drugs, and in some cases this can cause HLA-linked hypersensitivity in the context of specific allele types[13]. In specific cases this leads to immune self-reactivity, where CD8+ T-cells are activated by self-peptides presented by HLA molecules, stimulating an inflammatory immune response.

A recently published example of this is the case-study of abacavir, an HIV replication inhibitor[14]. In this case, a proportion of abacavir-treated patients experience adverse drug reactions. All of the reactions were in patients with the HLA type B*57:01, and approximately 50% of the patients carrying this HLA type are affected. Further investigation revealed that this adverse effect was mediated by abacavir binding to the peptide biding cleft of affected HLA molecules. Abacavir is a molecule small enough to bind within the cleft itself, beneath the C-terminal region, where it is hidden from detection by T-cells but changes the shape and electrochemistry of the cleft region. This influences the range of peptides able to be presented by the HLA molecules[14,15]. As a consequence the peptide repertoire presented in the context of HLA is shifted and new endogenous peptides are selected for, causing a marked alteration in the recognition of 'self' and 'non-self' by the immune system in ways that are comparable to mechanisms involved in autoimmune disease and graft rejection. The role of abacavir and the necessity of self-peptides to this adverse reaction was confirmed using enzyme-linked immunosorbent spot (ELISPOT) assays and also in the antigen presentation assay approach enabled by tandem mass spectrometry.

With reactions as severe as these, early detection prior to commencing clinical trials is clearly desirable, and this example is just another case where the polymorphic HLA types of patients drive relative risk. In the case-study above, whole-blood assays were effective at eliciting the immune response, and these can be carried out for donors covering most of the commonly occurring HLA alleles (**Figure 3**). Other small molecule drugs which induce HLA-linked adverse reactions, such as the antiepileptic drug carbamazepine[16], do so using similar mechanisms, and with an ever-growing list of uncovered HLA-associated adverse drug reactions, it seems likely that *in vitro* testing of small molecule drugs against common HLA allele polymorphisms may well become a standard requirement for preclinical drug development.

Summary

In this article the need for epitope identification and investigation of T cell response as a routine part of the early stages of drug development has been outlined. The methods for doing so are rapidly expanding in both speed and resolution power. In vaccine research epitopes often cannot

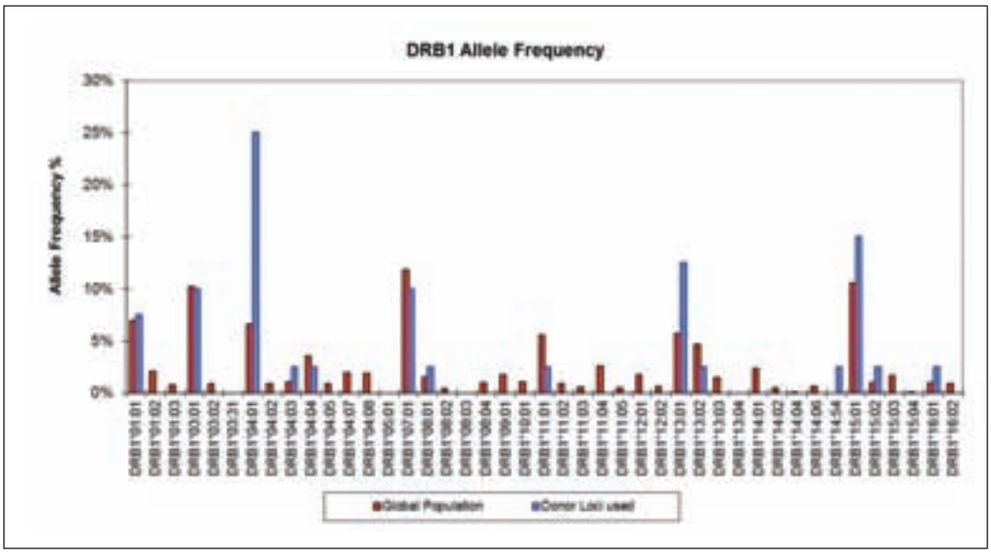

Figure 3: HLA-DR expression patterns in the global population (red) and donor cohort (blue) used in ProPresent®

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be identified directly from a pathogen's genome because of the potential for modification (eg by phosphorylation) of peptide sequences post-translation, and because of the unpredictability of the ways in which protein fragments are processed by immunoproteases, as well as polymorphisms in the pathogen's genetic sequence.

Establishing the baseline immunological response to current commercially available drugs is also an important step in future pharmaceutical development. While biological drugs are still relatively new to the drug market, the field is rapidly expanding. Our ever-increasing knowledge of the rules governing MHC molecule binding, T cell recognition and the complete human immune response have enabled the development of significantly improved assays to measure immune responses and protein immunogenicity *ex vivo*. Identifying drugs with reduced immunogenicity is set to become an area for driving competitive advantage as the use of assays to understand and manage immunogenicity at a preclinical stage become a routine development step.

Highly sensitive *in vitro* assays are critical for research into the immune response. Modular assays using defined sequences can identify sequences within an antigen which have the potential to form MHC complexes in varying HLA types, and naïve T cell assays record whether or not known sequences are capable of provoking T cell responses by using synthetic peptide libraries. However, these modular methods do not take into account the mechanisms of how epitopes are formed from the in-cell processing of proteins. The best way to identify what it is the immune system is 'seeing' comes from highly powerful antigen presentation assays, which use, for example, cultured dendritic cells to naturally produce and present epitopes in the context of HLA complexes that are derived from pathogen or other protein sequences of interest.

The continued development of tandem mass spectrometry analysis techniques allows the identification of presented epitopes from mass and fragmentation patterns with high accuracy. This information can be used to identify the sequence of an unknown epitope or identify immunological effects of sequence variants of a known protein, as in the case of variants of novel biological drugs, new or predicted pathogen strains caused by genetic polymorphism, or cancerous mutations. Using dendritic cells cultured with known HLA types, population biases of epitope responses can also be identified. Overall the power in the MHC-peptide sequencing approach lies in how rapidly it is able to distil sequences of immunological relevance from complex samples and we predict that this aspect will drive the adoption of such assays in many areas of biomedical R&D. DDW

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Katherine Catchpole *works for Dr Nikolai Schwabe's team at ProImmune. Prior to joining ProImmune, Katherine completed her studies in Biological Sciences with a BA from Oxford University.*

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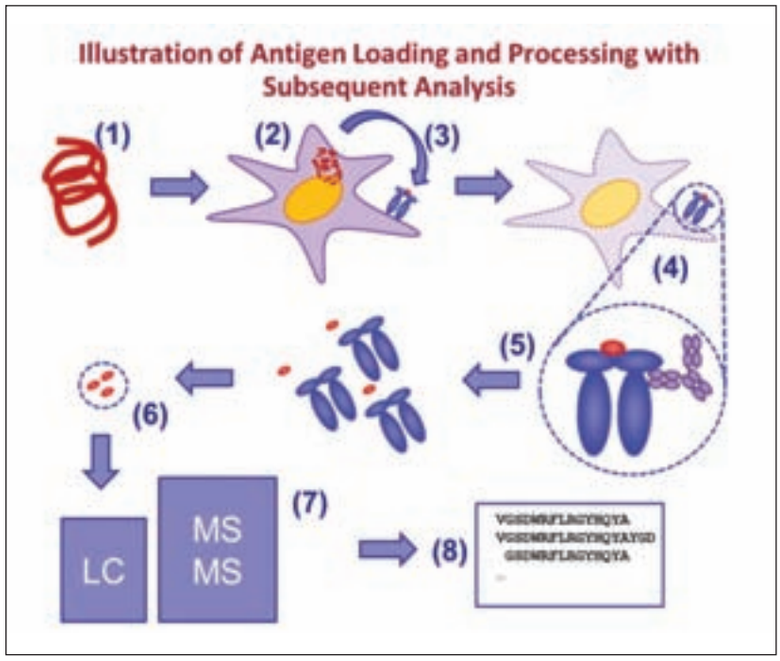

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The principles underlying this assay platform are the same as with prior academic technology: MHC-peptide complexes are recovered from the cell surface from any suitable cell source in an immunoaffinity purification step. Depending on the anti-MHC antibody used, different alleles or allele groups of molecules can be recovered, eg pan=Class I or Class II or HLA-DP, DQ, DR, etc. Peptides are then separated from the MHC molecules and sequenced in tandem mass spectrometry. The mass spec analysis is compared with the genome of the organisms of interest to confirm the identity of peptides through standard algorithmic methods. A particular advantage of the method is that because it is highly sensitive it requires less cell material for analysis, enabling projects that would previously not have been feasible (**Figure 1**).

A critical input for the assay is clearly the source of cells to be used and the assay is very flexible in this regard. In one example the customer can simply provide frozen cell pellets for their samples. The cells can be from any source, such as tumour tissue, cultured and loaded cells including from human and other vertebrate sources.

Alternatively, cohorts of healthy and also disease state donors can be recruited. In a typical study, monocyte-derived dendritic cells are cultured from a number of donor samples and the cells are loaded with antigen, which may be purified protein, protein mixtures or cell lysates, including pathogen lysates. Dendritic cells are then matured to a desired state and harvested for analysis.

Over the past 18 months the technique has been used in the analysis of samples from a broad variety of antigen sources, including from food allergen mixtures, intracellular bacterial pathogens, chemically modified proteins, cancer, viruses and also regular purified proteins. Numerous novel peptide hits have been discovered and these peptides can be considered as part of the potentially most relevant sequences in immune responses against the relevant antigen source.

A number of areas are now discussed in which an antigen presentation assay can be used to further our understanding of T cell responses.

Vaccine discovery

When developing new vaccines the aim is to identify so called 'sub-units' of a pathogen that can be produced, for example, as recombinant proteins or peptides, and can elicit a focused and effective immune response. Ideally, this avoids the need for the traditional approach of producing vaccines that contain attenuated or killed pathogen and alleviates-associated safety concerns, such as the risk of stimulating the disease in immuno-compromised patients. Such protein or peptide vaccines are also an attractive prospect in developing targeted cancer therapies where the challenge is to destroy tumorous cells while leaving healthy cells unharmed[9].

In a recent example antigen presentation technology was used by Ventura and her colleagues at Sanofi Pasteur, in a study of the cytomegalovirus (CMV)[10]. CMV infection is endemic to between 50% and 80% of US adults, and between 10% and 50% of the T cell population of a carrier is specific to the virus. Although often asymptomatic, the pressure this infection puts on the immune system can cause serious health complications in foetuses and immuno-compromised adults. The team at Sanofi investigated which peptide sequence of CMV glycoprotein G (gB) acted as the epitope responsible for stimulating the CD4+ T cell and antibody response.

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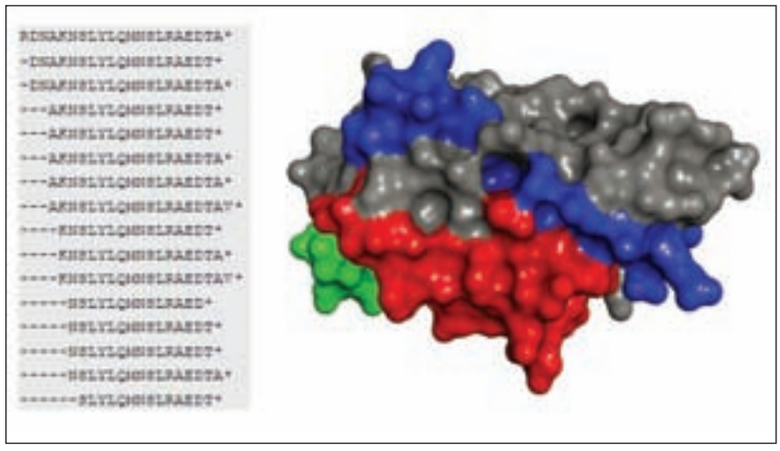

Figure 2
ProPresent® data for Humira® variable heavy chain segment. Left panel shows one of the nested sequence sets identified by mass spectrometry, covering the putative DNAKNSLYLYLQ MNSLRAEDTA epitope. Right panel shows the three epitopes identified for this region, mapped on to the structure of human IgG1k. DNAKNSLYLYLQMNSLRAED TA is shown in blue

The team at Sanofi Pasteur used a T cell reactivity assay, with autologous antigen-presenting cells primed with peptides from the CMV protein glycoprotein B (gB) and CD4+ T cells from CMV-infected donors. ELISpot analysis of repeat peptide fragment screening narrowed down possible epitope identities to an epitope presented by HLA-DR*07:01. As this form of assay has a significant likelihood of responding to sequences that are not the core target epitope, the team used the ProPresent® assay, in an even more direct epitope identification that confirmed the results obtained in the ELISpot assay. Overall the study exemplifies how different complementary assays can be combined in order to discover and confirm important T cell epitopes with high accuracy.

Biological drug development

Unwanted 'off-target' levels of immunogenicity are not only a problem when designing vaccines suitable for people who are immuno-compromised. Several biological drugs currently on the market carry the risk of eliciting dangerous cross reactive immune responses, or, more commonly, simply of creating a response against the drug itself, negating its effects. One example is Humira, with annual sales of approximately $9 billion and one of the leading treatments for rheumatoid arthritis and Crohn's disease. In certain cohorts, Humira can trigger immune responses with repeated dosing a response in nearly all patients receiving the drug[11]. Identifying the exact source of this unwanted immunogenicity could lead to the development of much more universally effective treatments for these significantly disabling diseases in an extremely competitive drug market. Antigen presentation assays can identify quickly which peptide fragments from Humira are likely to be involved in causing a T cell immune response against the drug and once identified such epitopes could be removed through strategic point mutations in the drug sequence (**Figure 2**). Alternatively, when developing follow on generation drugs to Humira, assays can be used to choose the lowest risk candidate drug that should be taken forward into clinical trials.

Crossing over between drug and vaccine development, another recent study carried out at the UK government's Defence Science and Technology Laboratory (DSTL) looks into the development of a virus-neutralising, protective antibody against the tropical disease Venezuelan Equine encephalitis virus (VEEV)[12]. Endemic to horses in southern parts of the American continents, this mosquito-borne pathogen has been developed by several nations as a human-targeting bioweapon. It is considered to be a high risk bioterrorism agent, not least because no medical countermeasures against it in either treatment or prevention currently exist.

The research team at DSTL used a highly effective neutralising mouse antibody against VEEV as the basis for their potential human treatment. In order to make the antibody useful for potential human use DSTL went through a standard approach of humanising the antibody, wherein the mouse antibody complementary determining regions that bind the antigen were grafted on a human antibody constant and variable region backbone that had been suitably constructed. The shape of the beta sheet variable region scaffold of the murine antibody was conserved by making isolated point changes in the human replacement sequence. DSTL were aware however that even humanised or 'fully human' therapeutic antibodies can elicit significant unwanted drug immunogenicity. A further issue in the project was the fact that clinical trails measuring the efficacy of neutralising the disease in humans was not something that would be possible for this agent. In essence the humanised antibody could at best be tested in healthy volunteers for safety. Since DSTL nevertheless wanted to generate early data to understand how successfully the humanised antibody would prevent anti-drug antibodies from occurring, DSTL contracted T cell proliferation assays to be carried out comparing the human T cell response between the humanised and murine antibody variable regions. Using overlapping peptide libraries generated from each antibody sequence T cell assays were carried out for each peptide on tens of

donor samples from a cohort of pre-banked blood donors. Results show a significant removal by the humanisation process of epitopes found in the chimeric antibody sequence which activated T cells that would have potentially caused or aided unwanted immune responses.

Allergy to small molecule drugs

HLA-mediated immune responses can be undermined by certain small molecule drugs, and in some cases this can cause HLA-linked hypersensitivity in the context of specific allele types[13]. In specific cases this leads to immune self-reactivity, where CD8+ T-cells are activated by self-peptides presented by HLA molecules, stimulating an inflammatory immune response.

A recently published example of this is the case-study of abacavir, an HIV replication inhibitor[14]. In this case, a proportion of abacavir-treated patients experience adverse drug reactions. All of the reactions were in patients with the HLA type B*57:01, and approximately 50% of the patients carrying this HLA type are affected. Further investigation revealed that this adverse effect was mediated by abacavir binding to the peptide biding cleft of affected HLA molecules. Abacavir is a molecule small enough to bind within the cleft itself, beneath the C-terminal region, where it is hidden from detection by T-cells but changes the shape and electrochemistry of the cleft region. This influences the range of peptides able to be presented by the HLA molecules[14,15]. As a consequence the peptide repertoire presented in the context of HLA is shifted and new endogenous peptides are selected for, causing a marked alteration in the recognition of 'self' and 'non-self' by the immune system in ways that are comparable to mechanisms involved in autoimmune disease and graft rejection. The role of abacavir and the necessity of self-peptides to this adverse reaction was confirmed using enzyme-linked immunosorbent spot (ELISPOT) assays and also in the antigen presentation assay approach enabled by tandem mass spectrometry.

With reactions as severe as these, early detection prior to commencing clinical trials is clearly desirable, and this example is just another case where the polymorphic HLA types of patients drive relative risk. In the case-study above, whole-blood assays were effective at eliciting the immune response, and these can be carried out for donors covering most of the commonly occurring HLA alleles (**Figure 3**). Other small molecule drugs which induce HLA-linked adverse reactions, such as the antiepileptic drug carbamazepine[16], do so using similar mechanisms, and with an ever-growing list of uncovered HLA-associated adverse drug reactions, it seems likely that *in vitro* testing of small molecule drugs against common HLA allele polymorphisms may well become a standard requirement for preclinical drug development.

Summary

In this article the need for epitope identification and investigation of T cell response as a routine part of the early stages of drug development has been outlined. The methods for doing so are rapidly expanding in both speed and resolution power. In vaccine research epitopes often cannot

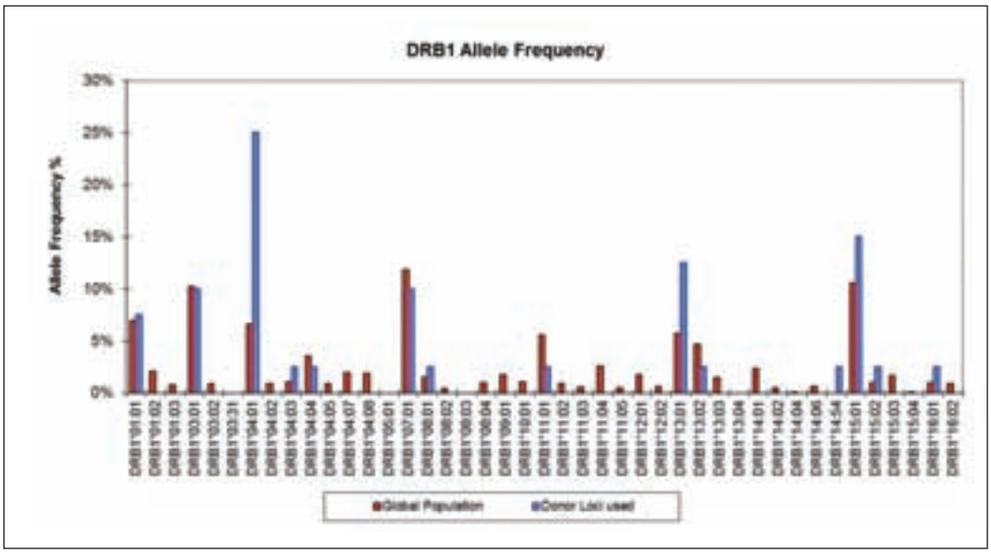

Figure 3: HLA-DR expression patterns in the global population (red) and donor cohort (blue) used in ProPresent®

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be identified directly from a pathogen's genome because of the potential for modification (eg by phosphorylation) of peptide sequences post-translation, and because of the unpredictability of the ways in which protein fragments are processed by immunoproteases, as well as polymorphisms in the pathogen's genetic sequence.

Establishing the baseline immunological response to current commercially available drugs is also an important step in future pharmaceutical development. While biological drugs are still relatively new to the drug market, the field is rapidly expanding. Our ever-increasing knowledge of the rules governing MHC molecule binding, T cell recognition and the complete human immune response have enabled the development of significantly improved assays to measure immune responses and protein immunogenicity *ex vivo*. Identifying drugs with reduced immunogenicity is set to become an area for driving competitive advantage as the use of assays to understand and manage immunogenicity at a preclinical stage become a routine development step.

Highly sensitive *in vitro* assays are critical for research into the immune response. Modular assays using defined sequences can identify sequences within an antigen which have the potential to form MHC complexes in varying HLA types, and naïve T cell assays record whether or not known sequences are capable of provoking T cell responses by using synthetic peptide libraries. However, these modular methods do not take into account the mechanisms of how epitopes are formed from the in-cell processing of proteins. The best way to identify what it is the immune system is 'seeing' comes from highly powerful antigen presentation assays, which use, for example, cultured dendritic cells to naturally produce and present epitopes in the context of HLA complexes that are derived from pathogen or other protein sequences of interest.

The continued development of tandem mass spectrometry analysis techniques allows the identification of presented epitopes from mass and fragmentation patterns with high accuracy. This information can be used to identify the sequence of an unknown epitope or identify immunological effects of sequence variants of a known protein, as in the case of variants of novel biological drugs, new or predicted pathogen strains caused by genetic polymorphism, or cancerous mutations. Using dendritic cells cultured with known HLA types, population biases of epitope responses can also be identified. Overall the power in the MHC-peptide sequencing approach lies in how rapidly it is able to distil sequences of immunological relevance from complex samples and we predict that this aspect will drive the adoption of such assays in many areas of biomedical R&D. DDW

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