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Opportunities in Cancer Immunotherapy

and how to capitalize on them with products and services from ProImmune

Cancer immunotherapy encompasses the approaches and treatments that harness and magnify the ability of the immune system to detect and destroy cancer cells. Based on the central role of the immune system in maintaining human health, cancer immunotherapy aims to exploit the exquisite specificity and capability for memory of the immune system to achieve potentially complete remission of primary tumors or disseminated metastases.

We have gained a much greater understanding of how and why some cancers or patients fail to respond to immunotherapy and to predict which therapeutic strategy is appropriate for individual patients. Nevertheless, developing immunotherapies for cancer that promote long term survival and quality of life with acceptable side effects in a larger proportion of cancer patients still presents major challenges. These challenges include the need to develop a clearer understanding of the molecular mechanisms and their dynamics by which targeted immune therapies can successfully destroy cancer and control long term disease free survival.

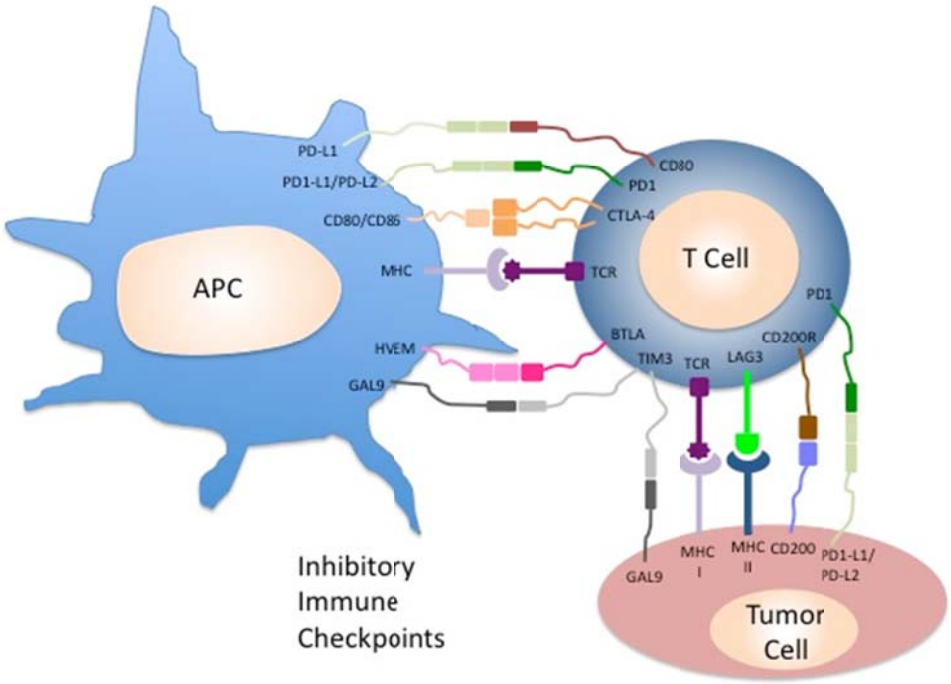
ProImmune provides key tools and technologies for studying the mechanisms of cancer immunology at a molecular and cellular level applicable to all areas of cancer immunotherapy, from antigen discovery and characterization to sensitive information-rich assays that monitor immune responses in clinical trials. We have direct experience working with most of the world's leading organisations in cancer immunology research spanning the full gamut of therapeutic concepts. In this guide we explore how we can help you with our products and services in each of the major areas of immuno oncology and set out our experience to date.

Click on the subject area to learn more about how ProImmune can help you with...

Checkpoint Blockade

The stimulation of an immune response against a tumor is of clear benefit for the prognosis of the majority of cancer patients, where tumor regression is associated with infiltration of anti-tumor CTLs. Following the initial enthusiasm for therapeutic vaccines against cancers, the failure to detect any durable clinical response raised doubts as to this approach. CTLA-4 (Cytotoxic T Lymphocyte Associated protein 4) is a protein expressed on the surface of T cells that, when bound to ligand, transmits inhibitory signals that limit T cell activity. Collectively, these signalling pathways are termed 'immune checkpoints'. Tumor cells use several mechanisms to avoid immune detection and destruction, one being to hijack normal immune checkpoints. Checkpoint blockade uses monoclonal antibodies to block the activity of such inhibitory receptors to release the brakes from the immune system. In recent years this field had been revitalized by demonstrations that a ligand-blocking antibody directed against CTLA-4 reduced significantly the risk of death in patients with advanced melanoma [1, 2]. In addition to CTLA-4, Programmed Death Receptor 1 (PD1) also functions to suppress T cell activity, but in a distinct pathway to CTLA-4 [3]. CTLA-4- and PD1-blockade is now at the forefront of this new era in cancer immunotherapy.

Anti-CTLA-4 antibodies such as ipilimumab, and PD1 inhibitors nivolumab and pembrolizumab, have demonstrated impressive response rates as single agents in initial clinical trials against melanoma, and additional blocking antibodies directed against CTLA-4, PD1 and other negative regulatory molecules are also undergoing clinical development for the treatment of various types of solid tumor [2]. The checkpoint function of CTLA-4 is mostly limited to the activation of naïve T cells and is essential for maintaining T cell homeostasis. In contrast, PD1 is involved in the extrinsic suppression of T cell responses through the induction of Treg cells, suggesting that targeting simultaneously these two pathways in a combination therapy might have synergistic effects on anti-tumor immunity. The demonstrable success of anti-CTLA-4 and anti-PD-1 antibodies in clinical trials is sparked great interest in checkpoint inhibitors. To avoid unwanted side effects due to their expression on normal cells, gene-editing techniques might be employed in the future to delete specifically genes encoding checkpoint inhibitors from



tumor-infiltrating lymphocytes, thereby potentiating their ability to kill tumor cells. In addition to CTLA-4 and PD1, further checkpoint blockade inhibitors are in clinical development.

In addition to their current direct anti-tumor activity, checkpoint inhibitors might also be envisaged to be useful in an adjuvant setting. Checkpoint blockade could be combined with therapeutic vaccines in attempts to boost their efficacy, improve tumor-specific responses to neo-antigens presented on the cancer cell surface, or used together with chemotherapy or radiotherapy [4, 5].

Looking ahead...

Checkpoint blockade has undoubtedly improved the outlook for many patients with a variety of tumor types and is changing the landscape of cancer immunotherapy, but outstanding challenges and questions remain, such as:

- Why do the majority of patients fail to respond to checkpoint blockade?
- Can we gain insight as to why some tumor types respond better than others?
- How best can combinatorial approaches be evaluated?
- What is the importance of the stroma in determining clinical responses?
- Is the diversity of antigens (and neo-antigens) important for predicting clinical response?

How can ProImmune help you?

- Gain access a team of experienced immunologists with expertise in a broad range of immunological applications
- Obtain evidence of checkpoint blockade with our functional assays

Pro5® MHC Class I Pentamers

- Detect, quantify and separate antigen-specific CD8+ T cells
- Analyze antigen specific T cell responses

ProMap™ T Cell Proliferation Assays

- Identify new epitopes that elicit helper T cell proliferation
- Rapid, accurate and detailed phenotyping of T cell responses provided by our flow cytometry methods

ProScern™ DC-T Cell Proliferation Assays

- Highly sensitive functional assay to determine if a candidate protein elicits CD4+ helper T cell proliferation
- Screen whole protein loaded into dendritic cells to determine overall antigenicity

T cell ELISpot Assay Service

- ELISpot assays for IFN-gamma, IL-2, IL-4, IL-10, IL-13, IL-17, Granzyme B, and many others

HLA Tissue Typing Service

- A straightforward, fast and dependable core facility HLA tissue typing service
- Covers a wide range of loci: Class I (A, B, C) and Class II (DRB1, DRB3/4/5, DPB1, DQA1 and DQB1)

ProImmune REVEAL® and ProVE® Class I & II Rapid Epitope Discovery System

- Identify the most likely immunogenic peptides in a protein sequence
- Obtain in-depth information on HLA-peptide binding characteristics of peptides identified in epitope discovery processes
- Assay does not consume precious patient samples
- Gain functional readouts to support *in silico* predictions

Working with ProImmune

Combinatorial approaches enhance cellular immunity.

Combination of CTL-Associated Antigen-4 Blockade and Depletion Of CD25+ Regulatory T Cells Enhance Tumour Immunity of Dendritic Cell-Based Vaccine in a Mouse Model of Colon Cancer.

Saha *et al.*, *Scandinavian Journal of Immunology* 2010, 71:70-82 [6].

This study evaluated the effects of combining CTLA-4 blockade or depletion of Tregs in enhancing the potency of a dendritic cell (DC)

vaccine in a murine model of colon carcinoma, in which the animals were transgenic for both carcinoembryonic antigen (CEA), a tumor-associated antigen overexpressed on many epithelial tumors, and HLA-A2. The findings revealed that *in vivo* depletion of either CD25+ Tregs or CTLA-4 blockade enhanced the potency of the DC vaccine against CEA. The increased in tumor free survival was associated with strong activation of CEA-specific T cell responses directed against a HLA-A2 restricted CEA agonist peptide designated CAP1-6D (YSLGADLNL). An A2Kb/CAP1-6D R-PE-labelled pentamer, a highly sensitive and selective reagent produced by ProImmune, was used to demonstrate a large expansion of CAP1-6D-specific CD8+ T cells in mice treated with the DC-based vaccine in combination with CTLA-4 depletion.

Oncolytic Viruses (OVs)

The field of oncolytic viruses for cancer treatment has seen a recent upsurge in interest; the progress in pre-clinical basic science is now being translated into success in clinical studies, see Seymour and Fisher for a review of this field[7]. Bioengineered OVs are attractive agents to deploy against cancer as they preferentially infect, replicate in and kill tumor cells. The efficacy of OVs is mainly due to their cytotoxic actions that yield a large number of progeny that, upon cell lysis, infect neighboring cells in the tumor microenvironment. Virus-mediated cellular toxicity may also induce immunologic cell death and inflammatory responses [8]. These can activate an anti-tumor immune response through the release of tumor epitopes than can be sampled by antigen-presenting cells. Viral antigens displayed on the surface of the infected cell may also increase the chances of cancer cells being recognized by the immune system.

OVs are now being used in clinical trials targeted against a wide variety of tumor types. Viruses can be administered systemically or injected directly into the tumor mass. A major additional feature of OV therapy is that the virus can be engineered to encode and deliver additional biologic payloads that deliver high-doses of the therapeutic agent - maximizing local activity and minimizing systemic toxicity effects, or diagnostics such as molecules used in imaging. Biologics encoded by OVs include cytokines, antibodies, pro-drug activating enzymes and immune modulators, such as TNFα, anti-CTLA4 and GM-CSF. Further immune modulatory molecules may also be envisaged as effective payloads worthy of investigation, such as novel checkpoint inhibitors, or the co-ordinated delivery of a combination therapy. Strategies that localize the delivery of potent biologics to tumors, while simultaneously avoiding systemic toxicities, are exciting prospects for the clinical management of cancer.

OVs currently being used in pre-clinical studies and clinical evaluation include:

RNA viruses	DNA viruses
• Newcastle Disease virus (NDV)	• Adenovirus (Ad)
• Coxsackie virus	• Adeno-associated virus (AAV)
• Vesicular stomatitis virus (VSV)	• Herpes simplex virus (HSV)
• Measles virus	• Vaccinia virus
• Poliovirus	• Parvovirus
• Reovirus	
• Seneca virus	

Generally, these viruses are themselves non-pathogenic to humans and may only elicit low titres of virus-neutralizing antibodies. However, the bioengineering involved in the production of OVs may lead to the generation of novel and unforeseen anti-vector responses that may blunt their effectiveness. The immunogenicity of OVs may vary from patient to patient and can be dependent upon previous exposure to antigen, the ability of an individual patient to recognize the OV as a foreign antigen, the delivery route and the epitope spectrum of the engineered virus. Generating high-affinity antibody

responses by B cells requires the help of CD4+ T cells that recognize relatively short peptides of about 11-20 amino acids long. The identification of such OV-encoded epitopes from the virus or its encoded payload affords the possibility to further engineer the biologic by mutating specific amino acids without any loss of viability or efficacy.

Looking ahead...

Successful OV therapy may not involve the virus infecting every cancer cell, but instead the most effective results are linked to the virus triggering a more effective anti-tumor immune response and the weakening of immune suppression.

In a patient centric approach, identifying those individuals who respond best to OVs and have the highest chances of sustained disease regression will be critical to the efficacy of virotherapy. A detailed characterization of this patient sub-set in terms of immune responses including HLA profiling, cytokine levels and changes in anti-tumor antibody levels for example, may enable the identification of markers that correlate with tumor regression. Anti-vector responses need to be pinpointed, enabling the discovery and better understanding of the antigen epitopes that are recognized, and characterization of unwanted responses to the vector.

How can ProImmune help you?

ProPresent® Antigen Presentation Assay

- Determine which parts of a virus or payload are visible to the immune system
- Identify directly peptides presented to T cells
- Gain information on HLA restriction of presented peptides

ProStorm® Cytokine Release Assay using the ProArray Ultra® platform

- Assays designed specifically to help predict likelihood of first infusion reactions
- Accurately profile TNFα, IFNɣ, IL-2, IL-4, IL-6, IL-8 and IL-10 levels

T cell ELISpot Assay Service

- ELISpot assays for IFN-gamma, IL-2, IL-4, IL-10, IL-13, IL-17, Granzyme B, and many others

Pro5® MHC Class I Pentamers

- Detect and separate antigen-specific CD8+ T cells

ProT2® MHC Class II Tetramers

- Detect single antigen-specific CD4+ T cells by flow cytometry

HLA Tissue Typing Service

- A straightforward, fast and dependable core facility HLA tissue typing service
- Covers a wide range of loci: Class I (A, B, C) and Class II (DRB1, DRB3/4/5, DPB1, DQA1 and DQB1)

Working with ProImmune

Monitoring anti-viral and anti-tumoral T cell responses in glioma patients

ProImmune worked with a client who was developing and testing a novel oncolytic virus (OV) for the treatment of gliomblastoma multiforme. The virus was capable of replicating in tumor cells, leading to increased ROS, lysosome permeabilization, DNA damage, cell cycle arrest and rearrangement of the cytoskeleton. In clinical studies the OV was either injected directly into the tumor or given by both intravenous and intratumoral routes. The study evaluated the potential benefit for patients by monitoring both an anti-viral- and an induced anti-tumor response, gathering evidence of the detection of T-cell responses in peripheral blood. Two key challenges were identified: (i) to validate the approach undertaken, and (ii) identify anti-cancer specific T cell responses that could be correlated to clinical outcome.

In initial studies, ProImmune's HLA tissue typing service enabled the client to match appropriate cell lines for the study with HLA types typically presented by patients. The cell lines were infected with the OV and then the ProImmune ProPresent® Antigen Presentation Assay was

used to identify viral-encoded or known cancer-associated peptide sequences presented by HLA class I molecules on the surface of the infected cancer cell lines. The ProImmune thinkpeptides service synthesized a range of peptides that were identified by ProPresent®. The peptides were used in IFNγ ELISpot assays to determine antigen-specific responses in post-treatment samples of patient derived PBMCs. Findings showed that the treatment resulted in no significant side effects and that the maximum tolerated viral dose had not been reached. Also included in the ELISpot assays were cancer- and OV-derived peptides identified in the ProPresent® analysis as well as cancer-associated peptides identified in literature searches. ProImmune services not only helped the client to validate the approaches taken, but also to monitor anti-viral- and anti-cancer specific T cell responses to this promising treatment.

First evidence that chemotherapy increased the potency of an oncolytic adenovirus

Immunological Effects of Low-Dose Cyclophosphamide in Cancer Patients Treated With Oncolytic Adenovirus.

Cerullo, V. *et al*., *Mol Ther* 2011; 19.9: 1737–1746 [9].

Cyclophosphamide is a DNA crosslinking agent that is widely used to treat various tumor types. High drug does are required to elicit tumor cell killing, but this results in immunosuppression. Strikingly though, low dose cyclophosphamide improves anti-tumor responses in animal models and in patients with metastatic melanoma. In a first-in-human study, researchers hypothesised that treatment with low dose cyclophosphamide combined with an oncolyitc GM-CSF encoding adenovirus may result in synergistic immunological and clinical effects.

These researchers had previously demonstrated that oncolytic adenoviruses were capable of inducing an adeno- as well as tumor-specific T-cell-mediated immune response. An ELISpot assay was performed to assess T cell functionality after cyclophosphamide treatment. Cells were stimulated either with ProImmunes's HAdV-5 Penton peptide pool that consisted of 140 peptides, each 15 amino acids long and overlapping by 11 amino acids, or a BIRC5 PONAB (Survivin) peptide pool. A major finding of this study demonstrated by these assays was that while cyclophosphamide treatment did reduce Tregs, it did not affect the induction of anti-tumor effector T cells but instead enhanced the efficacy of the virotherapy.

Cross-priming of an adaptive immune response by reovirus

Tumor Infection by Oncolytic Reovirus Primes Adaptive Antitumor Immunity.

Prestwich, R. *et* al., *Clin Cancer Res* 2008; 14: 7358-7366 [10].

Immune-Mediated Antitumor Activity of Reovirus is Required for Therapy and is Independent of Direct Viral Oncolysis and Replication.

Prestwich, R. *et al*., *Clin Cancer Res* 2009; 15: 4374-4381 [11].

Reovirus is a naturally occurring OV that is currently in phase I and II clinical trials for the treatment of solid tumors. Delivered systemically, reovirus selectively infects and is capable of replicating in permissive tumor cells. Virotherapy might be expected to promote an inflammatory environment within the tumor following cell lysis, leading to the release of pro-inflammatory cytokines, Toll-like receptor ligands and infiltration of immune cells. Virally-induced tumor cell lysis can release a wide range of tumor-associated antigens for uptake and presentation by dendritic cells which is critically important in generating an adaptive immune response.

Prestwich and colleagues addressed two related questions. First, did infection by reovirus lead to the generation of adaptive tumor immunity, and, did an anti-tumor response depend on virus replication and oncolysis? Using an *in*

vitro human Mel888 melanoma cell line system, they showed that uninfected Mel888 cells failed

to induce dendritic cell maturation, while reovirus-inected cells matured dendritic cells in a reovirus dose-dependent manner. To address whether the dendritic cell population induced CTL polyclonal expansion, the tumor-associated antigen (TAA) MART-1, which is HLA-A2 restricted in Mel888 cells, was selected for further investigation. CTLs were labelled with the ProImmune MART-1 PE-labelled Pro5® Pentamer (A*02:01 / ELAGIGITLV) or negative control Pro5® Pentamer, counterstained with CD8-FITC and analysed by FACS.

The quality, sensitivity and reliability of ProImmune Pentamers was critical in demonstrating a small but significant expansion of MART-1 specific T cells, indicating that reovirus infection is able to support the priming of an effective CTL response against a defined TAA. Utilizing the ProImmune Pentamer technology further, Prestwich and colleagues then went on to show that an anti-tumor immune response was critical to the efficacy of reovirus, but that this did not depend upon direct viral replication or lysis of the tumor cell.

Potent immune responses induced by a modified vaccinia virus used in combination with chemotherapy.

Vaccination of Colorectal Cancer Patients with Trovax® Given Alongside Chemotherapy (5-Fluorouracil, Leukovorin and Irinotecan) is Safe and Induces Potent Immune Responses.

Harrop, R. *et al*., *Cancer Immunol Immunother* 2007; 13(15 Pt1): 4487-4494 [12].

A team lead by researchers at Oxford Biomedica investigated the safety and immunogenicity of a modified vaccinia virus Ankara (MVA, TroVax®), encoding the 5T4 tumor antigen, in colorectal cancer patients undergoing chemotherapy. The restricted expression of 5T4 on normal tissues and high prevalence on the cell surface of many common cancer types, makes it an attractive target for both CTL and antibody-mediated responses. Over-expression of 5T4 is associated with metastasis and poor prognosis in colorectal, gastric and ovarian cancer patients.

Initial clinical trials in colorectal cancer patients showed TroVax® to be safe, well tolerated and to induce 5T4-specific immune responses in many patients. Harrop *et al* then extended their studies to investigate the effects of TroVax® in patients receiving 5-fluorouracil, leukovorin and irinotecan as first-line therapy. ProImmune Pentamers were used to quantify 5T4-specific CD8+ T cell responses in PBMCs isolated from patients up to 14 weeks post-vaccination. Pentamers specific for HLA-A2 restricted 5T4 CTL epitopes were synthesised: A*02:01 / RLARLALVL (peptide #9) and A*02:01/FLTGNQLAV (peptide #49) and well as a negative control Pentamer. Pentamers were detected using ProImmune Fluorotag by flow cytometry analysis. No negative impact of TroVax® was found on the delivery or activity of the chemotherapy. The exquisite sensitivity and specificity of ProImmune's Pentamers allowed CTL responses to the 5T4 HLA-restricted epitopes to be detected in a large proportion of patients. The extended duration of the response, ~10 months following the first TroVax® vaccination, implied the induction of an effector memory phenotype.

Combining a cancer vaccine such as TroVax® with chemotherapy may have significant benefits for patients. The cytotoxic agent, which reduces tumor load, also results in tumor cell death that could release TTAs such as 5T4 to boost further immunological responses. This strategy may provide the ideal setting of minimal residual disease or metastases whereby efficacy of combinatorial virotherapy can be demonstrated.

Peptide Vaccines

A fundamental and key issue in inducing an immune response is the presentation of antigen. Peptides presented by MHC class I are usually 8-11 amino acids in length. They are generated by proteasomal degradation of cytosolic proteins, fragments of which are taken up by the ER and

loaded onto and presented to the immune system by MHC class I molecules.

Antigen presentation by cancer cells can differ to that of normal cells in different ways [13]. For example, the over-expression of a protein that may drive proliferation of a cancer cell may be processed differently compared to the normal levels of the endogenous protein, leading to a different spectrum of peptide cleavage products for antigen presentation. Cancer cells typically contain a large number of somatic mutations, some of which will occur in the exons of expressed genes, thereby altering the protein sequence and potentially generating novel peptide epitopes (neo-antigens). Such novel epitopes can be considered as tumor specific and can be highly immunogenic, but the number and spectrum of mutations that generate such novel peptide antigens will be different between individual tumor cells and types. Further, HLA-restriction may also limit the presentation of tumor-specific antigens on an individual basis.

While many peptide vaccine strategies have to date been based on predictive motif methodologies, recent advanced in genome sequencing combined with proteomics, represent a major step forward in the discovery of functional epitopes to which peptide vaccines can be designed. These advances will facilitate the development of more personalized vaccine strategies that may combine multiple cancer-specific epitopes with other treatment modalities, such as immune-modulatory agents, that can expand T cell populations and increase the impact of the vaccine [14].

How can ProImmune help?

ProPresent® Antigen Presentation Assay

- Determine the peptides of a neo-antigen that are visible to the immune system
- Identify directly peptides presented to T cells
- Gain information on HLA restriction of presented peptides

ProImmune REVEAL® Class II Rapid Epitope Discovery System

- Obtain in-depth information on HLA-peptide binding characteristics of peptides identified in epitope discovery processes
- Gain functional readouts to support *in silico* predictions

thinkpeptides®: Prospector® Custom Peptide Libraries

- Custom peptide library synthesis, with non-standard amino acids and modifications as required

ProMix™ Pre-Mixed, Purified Peptide Pools

- Clearly defined peptide pools of characterized epitopes of cancer-associated proteins

ProArray Ultra®

- Customizable protein microarrays for ligand binding
- Higher sensitivity and lower cost than ELISA
- Easy and assay quick set-up

Working with ProImmune

Her-2 positive cells targeted by peptides against 'self' antigens

Identification of a Novel Immunogenic HLA-A*02:01-Binding Epitope of HER-2/*Neu* with Potent Antitumor Properties.

Gritzapis, AD. *et al.*, 2008, *J Immunol* 181: 146-154 [15].

Peptide Vaccination Breaks Tolerance to HER-2/Neu by Generating Vaccine-Specific Fasl+ Cd4+ T Cells: First Evidence for Intratumor Apoptotic Regulatory T Cells.

Gritzapis *et al*., 2010, *Cancer Research* 70:2686-2696 [16].

Over-expression of HER-2 is associated with the development of breast cancer where it confers enhanced metastatic potential. In a transgenic mouse model, Gritzapis and co-workers showed that a rare T cell population could recognize specifically an immunodominant epitope from the expressed transgene. They evaluated a vaccine mixture containing two HER-2-derived peptides, representing a CTL and a T helper cell

epitope, as they and others had shown that removal of Tregs led to the generation of anti-tumor immunity, findings that indicated that 'self' tumor antigens can persist *in vivo*. A key finding was that transgenic mice vaccinated with the HER-2 peptides showed long-lasting immunity that delayed the outgrowth of the mammary carcinoma cells used in the study. ProImmune produced a highly specific PE-labelled HLA-A2.1 Pentamer presenting a defined HER-2 peptide that was critical in the demonstration of the presence of increased numbers of functionally active HER-2-specific intra-tumoral CD8+ T cells.

Pentamers used to provide evidence of a peptide-specific CTL response that correlated with patient overall survival

Phase II Clinical Trial of Multiple Peptide Vaccination for Advanced Head and Neck Cancer Patients Revealed Induction of Immune Responses and Improved OS.

Yoshitake, Y., *et al*., 2015, *Clin Can Res* 21(2): 312-321 [17].

In this trial peptides derived from three proteins, LY6K, CDCA1 and IMP3, were used in immunotherapy for head and neck squamous cell cancer (HNSCC). The LY6K 177-186 (RYCNLEGPPI), CDCA1 56-64 (VYGIRLEHF) and IMP3 508-516 (KTVNELQNL) peptides have been shown previously to induce peptide-reactive CTLs. Importantly, these CTL responses are HLA-A*24:02 restricted, as HLA-A24 (at ~60%) is the most common allele in the Japanese population, with 95% of those individuals having an A*24:02 genotype. Pentamer staining assays were used to monitor positive CTL responses in *in vitro* cultured T cells for the LY6K- (85.7%), CDCA1- (64.3%) and IMP3 (42.9%) peptides after vaccination in patients carrying the A24 allele compared to patients lacking the allele. A significantly longer overall survival (OS) was noted for HLA-A*24:02 group of patients that had been vaccinated with either of the peptides and had developed a specific CTL response. Indeed, the OS was longer in those patients that developed a positive CTL response to a larger number of peptides compared to patients that exhibited a CTL response to only one or none of the peptides.

Immunological monitoring of CTL responses using ProImmune Pentamers was critical in this first demonstration that vaccination with multiple peptides correlated with the extent of CTL responses and longer OS in patients with advanced HNSCC.

Cancer Stem Cells

The exact nature and defining characteristics of the cell population that is capable of propagating a tumor is still a matter of conjecture. This makes the critical tumor cell sub-population that is required for immune targeting elusive, but several promising approaches for cancer immunotherapy are under investigation. In the longer term it is envisioned that combination therapy, including both pharmacologic cytoreductive agents with acceptable toxicity, together with immunologic targeting of malignant stem cell populations, may provide an effective curative approach for the treatment of cancer.

The Cancer Stem Cell (CSC) Hypothesis

Tissue specific-stem cells have been described in many tissues, including those most prone to cancer such as breast, prostate, lung and intestine. A defining characteristic of such stem cells is that they are exceptionally long-lived and can self-renew. Stem cells generate progenitors that are more restricted in their developmental potential, but these cells divide more rapidly and differentiate, giving rise to tissue-specific cells. The longevity of stem cells and resistance to the effects of standard chemotherapy and radiotherapy infers that they can accumulate more cellular mutations than a shorter-lived more differentiated cell. Such characteristics have clear implications for disease initiation, progression and approaches to treatment [18].

While the existence of tumor initiating cells has been described for many tissues, the exact origin of the tumor-initiating cell remains controversial. Different models regarding the origin of CSCs have been proposed, these include:

Hierarchical CSC Model
- CSCs exist as rare cell populations in a tumor.
- Cells can be purified on basis of cell surface markers.
- Display a high capacity for self-renewal.

Clonal Evolution Model
- Many tumor cells capable of self-renewing.
- Evolution of tumor cells driven by microenvironment, and by the genetic and epigenetic variation within the cell population.

Clonal Succession Model
- Creation of a dominant clone via serial mutation that leads to the acquisition of new phenotype, such as the capacity for self-renewal.

CSC markers are not clearly defined for most tumor types, indeed some studies report large numbers of CSCs following cell sorting. Such a method of defining 'stem-ness' may well underestimate the number of tumor initiating cells. In poorly differentiated tumors, cells with some stem cell functions may constitute the majority of tumor cells.

Which CSC model prevails may be dictated by the tissue of origin of the tumor. Some systems, it has been proposed, are more likely to follow one model than another. Hematopoietic cancers, where differentiation pathways have been well-characterized, may follow a hierarchical pattern. In contrast, a stochastic pattern may be followed by solid tumors. Such tumors are typically heterogeneous diseases that are more reliant on supporting infrastructure of endothelial cells, fibroblasts and blood vessels that may provide paracrine factors required to support cell viability and growth. While such models may appear to be mutually exclusive, attempts to integrate these different standpoints may offer new insights that may guide future approaches to study design [19]. The epithelial-mesenchymal transition (EMT) program has been proposed to be a major driver of tumor malignancy, where cell plasticity and the activation of EMT may serve as a mechanism to generate new CSCs [20, 21]. The differences in these models have important implications for targeting strategies. The hierarchical model of CSCs suggests that efficacy by targeting a small population of cells can be effective, while other models require that all tumor cells must targeted because any cell can acquire stem-like functions.

Immune Privilege and Tumor Immunoediting

The ability of a cancer cell to seed a new tumor depends, essentially, on the ability of a tumor cell to escape destruction by innate or adaptive immune responses. Three phases have been proposed for a process termed 'tumor immunoediting':

- Elimination
- Equilibrium
- Escape

In the **elimination** phase tumor cells are detected and destroyed by innate/adaptive immunity. If cells escape elimination as a result of a failure in the immunoediting phase, then a dynamic **equilibrium** of tumor growth and immunologic elimination may be established where tumor outgrowth is still contained. However, as elimination is incomplete, this allows cells to accumulate further mutations where the cells enter a latency phase that may last for decades. A clone may then emerge that is capable of **escaping** from a functional immune system. Here, latency requires a sub-pool of cancer cells that are poorly immunogenic, otherwise they would be eliminated by activated CTLs. Survival of the CSCs may well depend on the stem cell niche and micro-environment that may also prevent mutated CSCs from growing into larger tumors [18].

Immunologic properties of CSCs

Our knowledge of the immunologic properties of CSCs is still very limited. The over-expression in tumor cells of key anti-apoptotic proteins such as BCL2, BCL-XL and Survivin, has also been

reported to increase resistance to immune effectors such as T or NK cells [22].

The PI3K/AKT pathway, that is important for proliferation and cell survival, is also a mediator of chemoresistance, and may also be involved in CSC renewal and in the immune escape of tumor cells. HER2, a receptor and important target in breast cancer therapy that is also activated PI3K/AKT, also interferes with antigen processing and presentation [23, 24]. HER2 has also been reported to be critical for CSC maintenance in breast cancer [25]. Similarly, BMX, a tyrosine kinase that when over-expressed confers apoptotic resistance to prostate, breast and colon cancer cells [26], is also required for CSC maintenance in glioma [27].

Immune evasion is recognized as being a hallmark of a cancer cell [28]. CSCs may also actively suppress immune responses. For example, breast [29] and glioma [30] CSCs secrete more TGFβ, whereas colon CSCs secrete more IL-4 [31] that promotes drug resistance and inhibits anti-tumor responses. Tumor immune-surveillance molecules include IFNγ, IL-12, perforin, TRAIL (an apoptosis inducing ligand that binds to DR4/5 receptors), recombination activating genes (RAG1 and RAG2) and activating NK cell receptor NKGD2 [18]. The loss of expression of these proteins results in more frequent or faster spontaneous or carcinogen-induced cancer initiation and progression.

Antigen Discovery and Targeting of CSCs

Effective strategies are urgently needed to discover antigens that enable targeting of CSCs for elimination. Target discovery has proved a difficult challenge – at present, no current approach shows high selectivity for CSCs over non-malignant stem cells. In part, this is related to the potential different routes by which CSCs may be generated, as well as immune evasion and tolerance issues. As antigens may be expressed at different stages of differentiation pathways, targeting of an antigen may therefore not eliminate all the tumor-initiating cells, as these may arise via different routes.

Advances in sequencing technologies mean that the sequencing individual cancer genomes is now a reality. Genome sequencing of cancer cell lines has revealed about 50,000 somatic mutations, with a few hundred of these affecting coding sequences. The spectrum of changes and genes affected can be expected to be different for each cancer type. As CSCs are potentially long lived and pass mutations on to their progeny of amplifying cells, they are more likely to accumulate mutations. Of the changes detected in coding sequences, it is estimated that there may be around 7-10 new and unique MHC binding peptides per HLA allele.

The proteome of the CSC is highly likely to differ from normal tissue stem cells and from that of more differentiated progeny, driven by changes in gene and miRNA expression and epigenetic profiles. Proteins necessary for CSC maintenance or function are likely to be over-expressed and there is much effort being expended to discover these potential targets from a biomarker as well as therapeutic intervention perspectives. Protein over-expression can however lead to changes in the way that the protein sequence is recognized and cleaved during protein turnover. Changes in cleavage patterns can lead to the production of different peptides that can be presented by the MHC. DNA translocations and mutations are frequent in cancer cells and may generate fusion proteins or mutant forms that, when cleaved, generate novel peptide sequences that will be specific to the tumor cell, so called cancer neo-antigens.

Alternate approaches to targeting CSCs can also be envisaged, including

- targeting of the stroma (stem cell niche): deprivation of support required for CSC maintenance.
- containment of CSCs in equilibrium phase: does not eliminate the tumor cell but may permit long-term patient survival.

ProImmune can help you meet your challenges in cancer stem cell immunotherapy

ProImmune REVEAL® and ProVE® technology platforms

- Rapid epitope discovery and antigen characterization technology
- Study the immunogenicity of variant proteins

ProScern™ DC-T cell Assays

- Compare overall T cell antigenicity of proteins
- Antigens loaded into, processed and presented by dendritic cells
- Test for functional T cell epitopes

ProMap™ T Cell Proliferation Assays

- Identify peptide epitopes that can elicit helper CD4+ cell proliferation
- Cell proliferation determined by sensitive flow cytometry

Cancer Neo-Antigens

Mounting evidence has highlighted an important role for neo-antigens (tumor specific novel antigens) in mediating immune recognition of tumor cells. As tumors develop and grow, they acquire mutations, a proportion of which occur in exons and can change the coding sequence of a gene, thus altering the encoded protein forming a potential neo-antigen. Neo-antigens are both tumor- and patient-specific and, as they are not found in the normal human proteome, these neo-epitopes represent an important class of tumor rejection antigens. The genetic changes that generate mutant proteins that lead to malignant outgrowth can also be targeted by the immune system. Since tumor-restricted antigens can be targeted, such a personalized cancer treatment holds the promise of being highly specific and safe.

Early clinical studies with ipilimumab, that targets the checkpoint inhibitor CTLA-4, demonstrated the potential clinical benefits of the infusion of *ex vivo* expanded tumor-infiltrating CTLs. Emerging data suggest that it is the revelation and recognition of such neo-antigens to the immune system by checkpoint inhibitors is a major factor in their clinical effectiveness.

Next-generation sequencing of exons of expressed genes from tumor cells, combined with mass spectrometry, can be used to identify non-synonymous changes in the proteome. The number of somatic mutations acquired varies between tumor types; current estimates in melanoma patients suggest that these tumors can harbour about 10 somatic mutations per megabase of DNA, which equated to ~150 non-synonymous mutations in expressed genes[32]. Clearly, a mutated protein must be processed and an altered peptide presented to the immune system, thus only a small fraction of the mutated proteins will generate peptides that will be recognized by CD4+ or CD8+ T cells. Even then, not all neo-antigens expressed by tumor cells will induce a T cell response; this may be due to inefficient cross-presentation, the immune-dominance of a particular epitope, or escape from tumor immunoediting. The repertoire and clonality of neo-antigens expressed by tumor cells can also influence T cell reactivity and clinical outcomes [33]. This may be reflective of the mode of mutation induction and the tumor cell population (stem or more differentiated cells) that expresses the neo-antigen.

ProImmune can help you discover and characterize novel cancer antigens

ProPresent® Antigen Presentation Assay

- Determine which parts of a neo-antigen are visible to the immune system
- Identify directly peptides presented to T cells
- Gain information on HLA restriction of presented peptides

ProImmune REVEAL® Class II Rapid Epitope Discovery System

- Obtain in-depth information on HLA-peptide binding characteristics of peptides identified in epitope discovery processes
- Assay does not consume valuable patient samples
- Gain functional readouts to support *in silico* predictions

ProMap™ T Cell Proliferation Assays

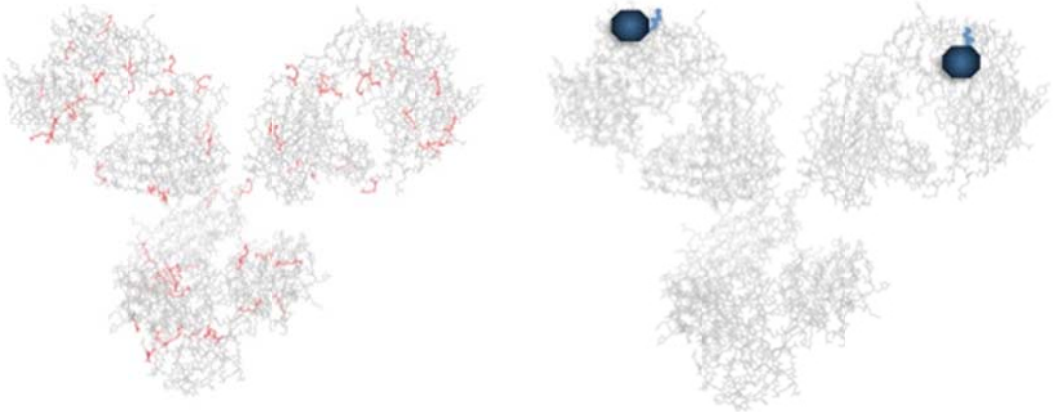
- Identify new epitopes that elicit helper T cell proliferation
- Rapid, accurate and detailed phenotyping of T cell responses provided by our flow cytometry methods

ProScern™ DC-T Cell Proliferation Assays

- Highly sensitive functional assay to determine if a candidate protein elicits CD4+ helper T cell proliferation
- Screen whole protein loaded into dendritic cells to determine overall antigenicity

Antibody Drug Conjugates (ADCs)

The development of a successful ADC brings together the latest advances in bioorthogonal chemistry, protein engineering and drug development. The combination of the specificity of antibodies coupled with the potency of small molecules is a powerful strategy to create targeted drugs. Early ADCs were prepared by conjugation of drug to lysine or cysteine residues on the antibody, yielding heterogeneous product mixtures with varying drug to antibody ratios (DARs). Recent advances in protein engineering and bioorthogonal chemistry mean that ADCs that are site-specific and homogeneous can be produced (see figure, adapted from [34]). Conjugation of drug may be targeted to cysteine residues, glycans, unnatural or non-canonical amino acids that have been incorporated into the antibody, or the modification of peptide tags.



Left panel: Human IgG showing all lysine residues, highlighted in red (pdb structure 1IGY), indicating the many potential sites of non-specific conjugation with activated esters. Typically the products of such reactions are heterogeneous with variable DARs. *Right panel:* Engineered antibody having only two sites where the conjugate (shown in blue) can be specifically incorporated into a lysine residue. A fully conjugated antibody will have a DAR of 2.

Biopharmaceutical companies have used protein engineering tools extensively over the past decades and are now incorporating bioorthogonal chemistry as the applications of ADCs progress into clinical use, reviewed in [34]. ADCs continue to be developed with refinement of the chemistry and protein engineering techniques that will improve stability and potency. The site of conjugation, linker sequence and the small molecule drug all influence the potential immunogenicity of an ADC. As peptides presented by the MHC are generated by proteolysis of source proteins, the modifications incorporated into the ADC may alter the cleavage patterns of the molecule, leading to the presentation of novel peptides that may be recognized as foreign. The modifications introduced into ADCs may also increase their potential for self-aggregation in their production that facilitates uptake by dendritic cells where it may be processed to form novel B or T cell epitopes.

Importantly, the evaluation and characterization of the immunological responses to the biotherapeutic will be critical to help understand the clinical value of any ADC under development [35]. ProImmune offers a range of products and services for use in characterizing immune response to ADCs and to monitor quality control during manufacture.

ProImmune can help with immune monitoring of your ADC

ProPresent® Antigen Presentation Assay

- Identifies which portions of the ADC is visible to T cells by the immune system
- Identify directly peptides presented to T cells
- Gain information on HLA restriction of presented peptides

ProScern™ DC-T Cell Proliferation Assays

- Highly sensitive functional assay to determine if a candidate protein elicits CD4+ helper T cell proliferation
- Screen whole protein loaded into dendritic cells to determine overall antigenicity
- Uses fully formulated ADCs, providing an excellent *in vitro* comparison of the relative antigenicities of proteins produced in different formulations, or as batch controls during manufacture

Working with ProImmune

ProImmune was enlisted by a leading US biotechnology company to track anti-tumor responses to a proprietary ADC in patients who has been diagnosed with a range of solid tumor types. Blood was collected at from around 30 patients during the course of the trial and processed by a CRO in the US, before shipment of the cryopreserved samples to the UK. Samples of individual time points from the donor cohort were assembled and shipped for analysis. ProImmune sourced and supplied control samples and assisted the client with a prioritization strategy for sample analysis. Experienced ProImmune staff performed a work-up of the samples for analysis; noting the lower than expected cell yields, ProImmune staff worked with the CRO and suggested changes to the cryopreservation protocol that improved cell yield.

Following peptide design and synthesis by ProImmune, the ProImmune team carried out IFNγ ELISpot assays requested by the client to determine to which region of cancer antigen the donor had mounted a response.

ProImmune facilitated data transfer in a format requested by the client as well as the standard ProImmune format; the client complimented ProImmune on the quality and clarity of the report provided when compared to other CROs they had engaged previously.

Bone Marrow Transplantation

Following on from the first successful bone marrow transplants in the 1960s, the field of hematopoietic stem cell transfer (HSCT) has advanced considerably. HSCT is a potentially curative treatment that has increased the survival of patients with a range of diseases, including hematological and other malignancies [36], such as melanoma for example. While many children with acute lymphoblastic leukemia can be treated successfully with chemotherapy protocols, allogenic HSCT was shown to be beneficial to children who had relapsed following chemotherapy.

A major barrier to the efficacy of HSCT is the onset of graft-versus-host disease (GVHD), that accounts for ~20% of fatalities. Acute GVHD emerges in 20-70% of patients [37] and chronic GVHD in >50% of patients [38]. New immunomodulatory approaches are in development to combat the pathogenesis of GVHD, including targeting inflammatory cytokines such as IL-21, inhibiting T and B cell signalling, altering immune cell trafficking and inhibiting PKC activity [36]. Other strategies to tackle GVHD include the use of the proteasomal inhibitor bortezomib [39-41] that decreases IL-6 production, and expansion of the Treg population by low-dose IL-2 [42].

To enable the safe introduction of these new therapies that hold the promise of being able to avoid the requirement for generalized immune suppression, appropriate immune monitoring strategies are required to identify early signs of rejection or GVHD.

How can ProImmune help with your immune monitoring needs?

T cell ELISpot Assay Service

- ELISpot assays for IFN-gamma, IL-2, IL-4, IL10, IL13, IL-17, Granzyme B, and many others

Pro5® MHC Class I Pentamers

- Detect antigen-specific CD8+ T cells
- Enrich for specific cell populations using tagged Pentamers

Working with ProImmune

Rapid isolation of EBV-specific donor T cells for adoptive transfer in post-transplant lymphoma

A Novel Haplo-Identical Adoptive CTL Therapy as a Treatment for EBV-Associated Lymphoma After Stem Cell Transplantation.

Uhlin, M. *et al*., *Cancer Immunol Immunother* 2010, 59(3): 613-633 [43].

Pro5® MHC Pentamers were central to the development of this novel and successful strategy used by Uhlin and co-workers for the treatment of EBV-associated tumors following stem cell transplantation. The rapidity by which the EBV-specific CD8+ T cells could be isolated was a crucial factor in developing this life-saving procedure when other standard treatment options had failed.

A patient presented whose CT scans revealed had developed a life-threatening EBV-associated lymphoma, together with high EBV titres in blood and major organs, 3 months after cord blood transplantation for AML. Having exhausted other lines of treatment, an adoptive EBV-specific CTL transfer from the patient's mother was performed. Uhlin and co-workers turned to ProImmune's Pro5® MHC Pentamers for the rapid detection and isolation of EBV-specific CD8+ T cells from the donor. Two Pro5® MHC Pentamers were used for cell enrichment: A*02:01 / CLGGLLTMV, and A*02:01 / GLCTLVAML. These APC-labelled Pro5® MHC Pentamers are highly specific enabling the detection of small number of positive donor cells; the APC fluorophore also being used for cell separation by magnetic beads.

Analysis of polymorphic markers confirmed that the infused cells were not rejected and after only 36 hours post infusion, Pentamer-positive EBV-specific T cells had expanded *in vivo* from 0.3% to 4.4% of total CD8+ T cells. Strikingly, EBV titres fell back to normal levels only days after transfusion. The clinical effects of the EBV-specific T cells on the lymphoma tissue were profound. At day 189 post-CTL infusion, CT scans revealed that all organ-associated tumors had vanished; remnant streaks were seen in the thorax and an enlarged left adrenal gland had returned to normal size. The patient was admitted again to hospital 12 months later with EBV in the tonsils, blood and colon. After a second infusion with EBV-specific CTLs isolated using the Pro5® Pentamers, the tonsillitis was cured within 24 hours and the patient became EBV-PCR negative in peripheral blood after two weeks.

Pro5® MHC Pentamers enable rapid isolation and enrichment of bi-specific TCR cell populations

Genetic Engineering of Virus-Specific T Cells with T-Cell Receptors Recognizing Minor Histocompatibility Antigens for Clinical Application.

Griffioen, M., *et al*., *Haematologica* 2008, 93(10): 1535-1543 [44].

Infusion with donor lymphocytes is an effective form of adoptive immunotherapy for haematological malignancies following allogenic stem cell transplantation. However, patients can often develop graft-versus-host disease (GVHD) due to the recognition of minor histocompatibility antigens. In this study,

Griffionen and colleagues developed an efficient technique of transferring T cell receptors recognizing hematopoiesis-restricted minor histocompatibility antigens into CMV- or EBV-specific T cells to generate long-lived bi-specific T cells for use in clinical gene therapy.

A range of CMV and EBV virus-specific Pro5® MHC Pentamers were constructed with a biotin tag and used to isolate antigen-specific T cells from PBMCs of healthy donors by streptavidin-coated magnetic bead separation. The Pentamers enabled highly purified (85-97%) virus-specific T cells to be isolated that were subsequently transduced with retrovirus encoding a TCR. This efficient method of generating and selecting T-cell receptors-engineered virus-specific T cells supports the use of surface expression of transgenic TCRs in clinical approaches to gene therapy.

Cytotoxic & Targeted Therapies

Chemo- and radiotherapy remain as front-line interventions for the clinical treatment and management of many tumor types. Many agents used in these therapies induce DNA damage directly, and the failure to repair these genotoxic lesions leads to cell death. The beneficial effects of radio- or chemotherapy may largely depend on immune responses by generating 'danger' signals and by the liberation of antigens from damaged or dying tumor cells. The term 'immunogenic cell death' (ICD) is commonly used to indicate a specific type of programmed cell death that engages an adaptive immune response. Features of ICD often manifest features of apoptotic cell death and may depend in part on components of the apoptotic machinery. Only a few of the cytotoxic chemotherapies currently in clinical use have the ability to trigger ICD. These include specific anthracyclines (doxorubicin, epirubicin and idarubicin), mitoxantrone, oxaliplatin, cyclophosphamide (an alkylating agent) and the proteasomal inhibitor bortezomib.

The induction of ICD relies on establishing adaptive stress responses that promote the co-ordinated release of specific molecules that bind to receptors on bystander cells, including those of the adaptive and innate immune system. A number of key steps and molecules have been identified that are critical in mediating ICD [45], reviewed by Zitvogel *et al* [46]:

1) The exposure of the ER protein calreticulin on the plasma membrane,
2) Secretion of ATP,
3) The release of HMGB1 into the intracellular space where it can bind to toll-like receptor 4 (TLR4) on surrounding cells, and
4) Production of type I interferon.

The mechanism of tumor cell death may also be critical for the presentation of antigens to the immune system. Necrotic cell death liberates inflammatory molecules, whereas apoptotic cell death eliminates cells without causing an inflammatory response. On the other hand, autophagy is a regulated pathway that is responsible for the bulk degradation of portions of the cytoplasm and organelles, which are sequestered in double-membrane bound vesicles, called autophagosomes [47]. Autophagosomes fuse with lysosomes to generate autolysosomes in which the proteins and organelles are degraded. Autophagy can be induced in tumor cells where it can promote cell survival in response to starvation, hypoxia or other stresses thereby having a tumour promoting activity in established cancers. Autophagy also functions in the host's intrinsic, innate and adaptive immune response against viruses, where some viruses have evolved virulence factors that evade or counteract the execution of autophagy. Recent evidence demonstrated that only tumour cells competent for autophagy, when treated with ICD inducers *in vitro*, can induce a tumour-specific immune response *in vivo*.

In contrast to the relatively non-specific cytotoxic agents used in chemotherapy, targeted therapies are designed to block essential biochemical reactions, signalling pathways or mutant proteins that are required for the

survival and proliferation of tumor cells in specific groups of cancer patients. Combinatorial strategies combining immunotherapy with chemotherapy or targeted therapy may be translated into clinical benefits for patients [48-50]. Small molecule inhibitors to the kinases EGFR, BRAF, KIT, ALK and HER2 are effective in attenuating tumor growth in selected patients, but their effect is limited by the emergence of resistance mechanisms [51]. In addition to these small molecules, the development of specific monoclonal antibodies, such as trastuzumab and cetuximab that target HER2 and EGRF respectively, have yielded encouraging results [52]. Many targeted antibody therapies also have immunomodulatory effects. In addition to attenuating signalling from receptors, trastuzumab and cetuximab also augment antigen presentation through the formation of immune complexes that enhances the production of tumor-specific T cells [53]; for example DC cells express Fc receptors that bind and internalize antibody-antigen immune complexes.

The discovery of small molecules, antibodies and peptides that reveal tumor cells to the immune system will be critical in the development of novel immunotherapies.

How can ProImmune help with your immune monitoring needs?

ProPresent® Antigen Presentation Assay

- Identifies which portions of the ADC is visible to T cells by the immune system
- Identify directly peptides presented to T cells
- Gain information on HLA restriction of presented peptides

ProImmune REVEAL® Class II Rapid Epitope Discovery System

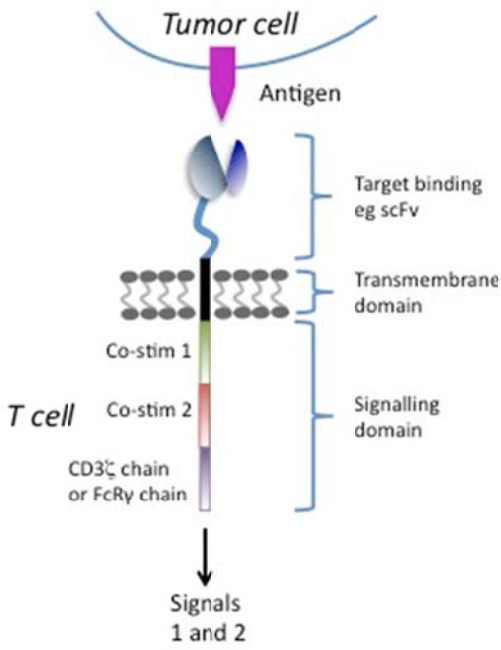
- Obtain in-depth information on HLA-peptide binding characteristics of peptides identified in epitope discovery processes
- Gain functional readouts to support *in silico* predictions

T cell ELISpot Assay Service

- ELISpot assays for IFN-gamma, IL-2, IL-4, IL-10, IL-13, IL-17, Granzyme B, and many others

CAR T Cells

T cells play a critical role in cell-mediated immunity, but tumors can evade the host immune response through, for example, the creation of an immunosuppressive environment. Chimeric antigen receptor (CAR) T-cell therapy involves genetically engineering the patients' own T cells to recognize specific antigens present on cancer cells. CARs combine antibody-like recognition of tumor antigen (for example through an engineered scFv) with T-cell activating and co-stimulatory functions [54]. CAR-T-cells can be engineered to recognize a variety of antigen types including proteins, carbohydrates and glycolipids expressed on the tumor cell surface. Unlike T-cell receptor recognition, the tumor antigen does not need to be processed and presented by the MHC, meaning that the same CAR can be used in all patients presenting the same tumor antigen, regardless of HLA type. Potential safety risks have been identified for CAR T-cell therapies, the most critical being related to on-target off-tumor activity. The modified T cells might trigger a response against host tissues that may express the target antigen at low levels, trigger cytokine release syndromes, or display off-target reactivity where



the antigen target sequence is present in an unrelated protein.

How can ProImmune help?

ProStorm® Cytokine Release Assay

- Uses fresh whole blood from healthy HLA-typed donors
- For measurement of TNFα, IFNγ, IL-2, IL-4, IL-6, IL-8 and IL-10.

ProPresent® Antigen Presentation Assay

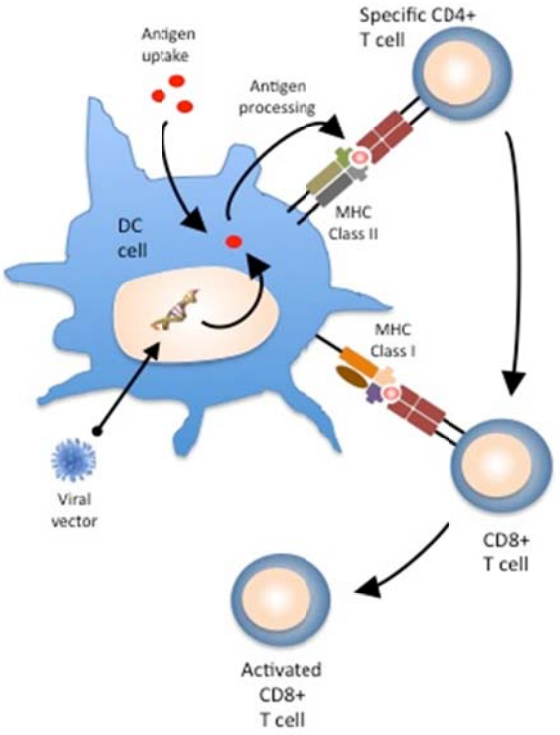
- Determine the peptides of a neo-antigen are visible to the immune system
- Identify directly peptides presented to T cells
- Gain information on HLA restriction of presented peptides

Cell Based Therapy

In essence, cellular immunotherapy harnesses cells from the innate or adaptive immune system to elicit an anti-tumor response. In passive immunotherapy approaches, cells are often activated or genetically modified *ex vivo* to enhance their anti-tumor properties, before being injected back into the patient. Active immunotherapy aims to activate the endogenous immune system, either by cells modified *ex vivo* or by vaccines, an approach that can elicit longer term anti-tumor effects. T cells, dendritic cells (DC) and natural killer (NK) cells are most commonly used in cell based therapies.

T cells are often used in cancer therapy as they can be easily modified to express tumor-specific antigens that improve targeting to the cancer. One interesting approach uses chimeric antigen receptors (CARs) that combine the ζ chain of a T cell with the selectivity of recombinant antibody binding domains, leading to increased binding between T cell and target antigen. An important feature of CAR T cells is that the antigen does not need to be processed and presented to the T cell receptor via the normal routes of antigen processing.

DCs are the most potent of antigen presenting cells. Once a resting DC had been loaded with antigen and activated, it migrates to a lymph node where it encounters and activates T cells. DC therapy aims to 'train' the host's immune system to recognize antigens on tumor cells and



kill them [55]. DCs express both MHC class I and class II on their cell surface, and hence are able to sensitize CD8+ and CD4+ T cells to all acquired antigens and induce a long-term immune response. DCs can be generated from a variety of sources, including separation from whole blood and by differentiation of pluripotent stem cells [56]. Antigens for loading into DCs can be derived from different sources, including whole tumor cell lysates where a defined tumor antigen has not been identified, peptides, or full-length protein produced *in vitro*. Viral vectors have also been used to transduce cells to express the specific antigen of interest.

NK cells are defined as CD3-CD56+ lymphocytes and play an essential role in combating tumor cells, reviewed in [57]. An array of either activating or inhibitory cell surface receptors either stimulates or dampens NK activity. Inhibitory receptors include the killer-cell

immunoglobulin-like receptors, while activating receptors include cytokine and chemokine receptors. NK cells also express death ligands FasL and TRAIL, that bind either to the Fas or TRAIL receptor DR5 respectively, to initiate an apoptotic cascade that kills the target cell. In addition, NK cells also express FcγRIIIA or CD16, a receptor that exerts antibody-dependent cell-mediated cytotoxicity (ADCC).

How can ProImmune help with your immune monitoring needs?

ProScern™ DC-T Cell Proliferation Assays

- Highly sensitive functional assay to determine if a candidate protein elicits CD4+ helper T cell proliferation
- Screen whole protein loaded into dendritic cells to determine overall antigenicity

Working with ProImmune

Rapid isolation of EBV-specific donor T cells for adoptive transfer in post-transplant lymphoma

A novel haplo-identical adoptive CTL therapy as a treatment for EBV-associated lymphoma after stem cell transplantation.

Uhlin, M. *et al*., *Cancer Immunol Immunother* 2010, 59(3): 613-633 [43].

Epstein-Barr virus (EBV)-related malignancies, such as post-transplant lymphoproliferative disease (PTLD), are major complications that can arise following cell or organ transplantation. In immunosuppressed transplant recipients EBV-specific immunoreactive T cells are often depleted or absent, contributing to the development of solid, organ-associated, EBV lymphomas. First-line treatments for PTLD are a dose reduction in immunosuppressive drugs and chemotherapy. If these approaches fail, then adoptive transfer of EBV-specific T cells is an option, but slow and complicated isolation procedures mean that their administration to the patient is often too late to be beneficial.

Michael Uhlin and colleagues from the Karolinska University Hospital were presented with a patient whose CT scans revealed had developed a life-threatening EBV-associated lymphoma, together with high EBV titres in blood and major organs, 3 months after cord blood transplantation for AML. Having exhausted other lines of treatment, an adoptive EBV-specific CTL transfer from the patient's mother was performed. Uhlin and co-workers turned to ProImmune's Pro5® MHC Pentamers for the rapid detection and isolation of EBV-specific CD8+ T cells from the donor. Two Pro5® MHC Pentamers were used for cell enrichment: A*02:01/CLGGLLTMV, and A*02:01 / GLCTLVAML. These APC-labelled Pro5® MHC Pentamers are highly specific enabling the detection of small number of positive donor cells; the APC fluorophore also being used for cell separation by magnetic beads.

Analysis of polymorphic markers confirmed that the infused cells were not rejected and after only 36 hours post infusion, Pentamer-positive EBV-specific T cells had expanded *in vivo* from 0.3% to 4.4% of total CD8+ T cells. Strikingly, EBV titres fell back to normal levels only days after transfusion. The clinical effects of the EBV-specific T cells on the lymphoma tissue were profound. At day 189 post-CTL infusion, CT scans revealed that all organ-associated tumors had vanished; remnant streaks were seen in the thorax and an enlarged left adrenal gland had returned to normal size. The patient was admitted again to hospital 12 months later with EBV in the tonsils, blood and colon. After a second infusion with EBV-specific CTLs isolated using the Pro5® Pentamers, the tonsillitis was cured within 24 hours and the patient became EBV-PCR negative in peripheral blood after two weeks.

Pro5® MHC Pentamers were central to the development of this novel and successful strategy used by Uhlin and co-workers for the treatment of EBV-associated tumors following stem cell transplantation. The rapidity by which the EBV-specific CD8+ T cells could be isolated was a crucial factor in developing this life-saving procedure when other standard treatment options had failed.

Persistence of memory to tackle melanoma

Establishment of antitumor memory in humans using *in vitro*-educated CD8+ T cells.

Butler, M. *et al*., *Sci Trans Med* 2011, 3 (80): 80ra34 [58].

Patients with advanced stage melanoma have a poor prognosis, on average surviving for less than one year. Adoptive T cell therapies for melanoma have been developed, where CTLs specific for previously characterized tumor antigens are transferred into patients whereupon they traffic to and destroy tumor cells. Since the CTLs are patient-derived the therapy has minimal toxicity. However, a major limitation of this approach has been keeping the transferred CTLs alive in the patient without the need for extra patient manipulation; typically the lifespan of the transferred cells is short, so without repeated rounds of treatment the clinical benefits are short lived.

In previous work Butler and co-workers have used what they term an 'artificial antigen presenting cell' (aAPC) system to generate anti-tumor T cells that behave as effector memory cells *in vitro*, having a surface marker phenotype CD45RA-, CD45RO+, CD62L+/-. They then evaluated the persistence of these cells *in vivo*. CD8+ T cells specific for the well-characterized melanoma antigen MART-1 (ELAGIGILTV) were generated and expanded, and then infused into patients. ProImmune MART-1 Pro5® MHC Pentamers A*02:01 / ELAGIGILTV were used to monitor responses; sustained increases in the frequency of circulating MART-1 specific CD8+ T cells was noted for up to a year, in the absence of other therapies such as CTLA-4 blockade. Post-infusion tumor biopsies showed that the infused MART-1 cells trafficked to the sites of disease, as shown by DNA analysis of CDR3 sequences that was used as a cellular marker, where they medicated biological and clinical responses.

Further studies indicated that the percentage of peripheral MART-1 specific T cells with a CD45RA- CD62L+ central memory phenotype had increased, indicating that the CTLs generated *in vitro* persisted *in vivo*.

Simplifying the production of dendritic cell (DC) vaccines

Antigenically modified human pluripotent stem cells generate antigen-presenting dendritic cells.

Zeng J. *et al*., *Sci Rep* 2015; 5: 15262 [56].

DCs play a pivotal role in the induction of antigen-specific T cell responses and are commonly used in clinical trials. Currently, the majority of DC vaccines are produced from the patient's own blood, hence the therapy can be considered as personalized. A critical step in DC vaccine production is antigen loading that defines the specificity of the vaccine. DCs can be loaded with tumor lysate, peptides, proteins, DNA/RNA or by viral transduction, but these methods are not without their drawbacks; each approach requires the production of clinical-grade material, time consuming and often detrimental cell manipulations, and the limited amount of time during which the antigen is presented before further loading is required.

Zeng and colleagues sought to simplify and speed up DC vaccine production by eliminating the need to load the DCs with antigen. In a novel strategy, they bring together DC biology and the application of human pluripotent stem cell (hPSC) technology. hPSCs were induced to express either full length tumor antigen MART1, or a minigene encoding a ubiquitin sequence that was placed before four MART1 epitopes (ELAGIGILTV) enabling proteasomal targeting. DCs derived from the hPSCs using standard differentiation protocols were shown to express the tumor antigen. Importantly, they then showed that the hPSC-derived DCs efficiently primed antigen-specific T cell responses. DCs were co-cultured with HLA A2+ PBMCs from healthy donors, then MART1-specific T cells identified in FACS assays using ProImmune Pentamers (R-PE labelled A*02:01/ELAGIGILTV) and a ProImmune FITC labelled anti-CD8 antibody. The antigen-specific CTLs could be expanded, the process being monitored by Pentamer staining. Further characterization of the expanded CTL population showed that the

possessed predominantly central memory or effector memory phenotypes.

ProImmune Pentamers helped show that hPSC-derived DCs continuously express tumor antigen, a critical factor in DC immunogenicity.

BiTE®: Bi-Specific T Cell Engager

Recombinant BiTE antibody molecules are constructed by linking the minimal binding domains (V_H and V_L) derived from single chain fragment variables (scFvs) of antibodies for the T cell marker CD3, with those of a tumor-specific antigen via a short linker. This bi-specific antibody platform has the ability to directly link cytotoxic T cells with the tumor target cell. The modular design of the construct allows straightforward adaptation to recognize many different antigen types. By linking directly the target cell and CTL, the BiTE leads to the forced formation of an immunological synapse; this occurs in the absence of TCR specificity, co-stimulation or peptide antigen presentation. Following synapse formation, the T cells are able to kill the target cell directly through apoptosis, induced by the release of granzymes and perforins. Once the BiTE construct has bound to target antigen expressed on the surface of the target cell, binding of CD3 also leads to activation and polyclonal expansion of the CTL population.

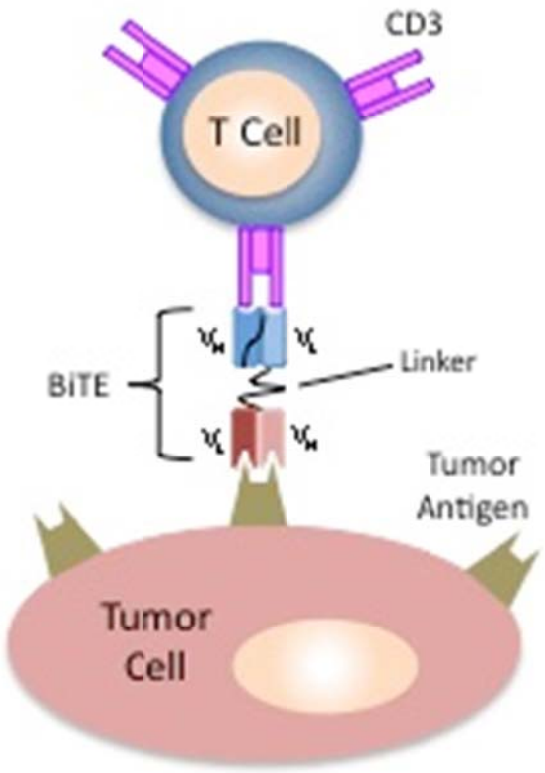
BiTE antibody constructs have entered into clinical trials [59]; they display a short half-life *in vivo* and toxicities that relate to T cell activation such as cytokine release syndromes, making continuous infusion challenging to patients. At present, there are no current guidelines for immunogenicity assessment of such emerging and potent therapeutic approaches [35]. Such novel constructs can contain structural motifs that may carry uniquely associated immunogenicity risks.

In vitro assessment of cellular activation, such as proliferation and release of relevant cytokines (IL-2, IL-6, IFNγ and TNFα), can be monitored in either whole blood or PBMCs. Such investigations are important assessment tools that can inform as to whether the product may have the potential to induce such toxicities in the clinic - factors noted specifically in FDA Guidance for Industry information.

(BiTE® is a registered trademark of Amgen Inc.)

How can ProImmune help?

ProImmune offers a range of assays for investigating drug immunogenicity and risk assessment:



ProStorm® Cytokine Release Assay

- For measurement of TNFα, IFNγ, IL-2, IL-4, IL-6, IL-8 and IL-10

T cell ELISpot Assay Service

- Human IFNγ, IL-2, Granzyme B
- Monitor CD8+ and CD4+ T cell responses
- For immune monitoring or epitope discovery/validation

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