Chapter 12

Identification and Validation of Targets for Cancer Immunotherapy: From the Bench-to-Bedside

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Additional information is available at the end of the chapter

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1. Introduction

The link between immune responses and cancer is evident from findings such as, a compromised immune system resulting in an increased tumour incidence [1] and cancer patient sera evidencing recognition of autologous cancer antigens [2]. The identification of tumour associated antigens (TAAs) plays a central role in our understanding of how cancer cells can inhibit the immune system and how we can overcome this tumour immune suppression to break tolerance and achieve cancer destruction [3]. Antibodies reacting with TAAs on the surface of cancer cells provoke an extremely effective immune response [4] which can be exquisitely specific to the tumour cells present in the body. However not many surface proteins are present on tumour cells and limited otherwise in expression to healthy non-essential tissues.

TAAs are most often proteins which have acquired mutations or have elevated expression levels which are expressed at the sub-cellular level. The ideal immunotherapy targets should also play a role in tumour progression [5]. For example p53 [6] is one of the most desirable targets for immunotherapy – targeting p53 can kill both the evolving tumour cell population and any cancer "stem" cell which harbours this as an early tumourigenesis aberration and supports further tumour growth. In addition, a number of tumour antigens have been shown to be useful biomarkers for cancer diagnosis [7] and survival [8].

In this chapter, we will examine how tumour antigens are identified and characterised to demonstrate their potential as immunotherapy targets and examine their role as biomarkers for treatment response and patient survival, and targets for personalised therapies.

2. Which tumour antigens?

Many of the tumour antigens identified by serological identification of antigens by recombinant expression cloning (SEREX) can be classified into one or more categories which are: cancer-testis, mutational, differentiation, amplified/overexpressed, splice variant and viral antigens [9]. Cancer-testis (CT) antigens [10] have been found to be highly expressed in tumours but not in normal tissues with the exception of immunologically protected sites (those tissues which lack major histocompatibility complex (MHC) class I and therefore do not present self-antigens). CT antigens make attractive targets as due to their limited expression, they should therefore induce specific anti-tumour immune responses and less toxicity to healthy tissue [11]. However some debate remains as to the definition of a CT antigen [12] with some suggestion of differential levels of expression and others suggesting no expression in normal tissues expect those in immunologically protected sites (such as ovary, placenta and testis). TAAs that are frequently found in tumours and provide excellent targets for immunotherapy include Wilms tumour 1 [13] and PRAME [14].

3. Identification of tumour antigens

Strategies are required to help identify potential targets which can be used for cancer immunotherapy. Some of the most commonly used and successful techniques are described as follows:-

3.1. Reverse-Transcription-Polymerase Chain Reaction (RT-PCR) and real-time PCR (RQ-PCR)

Reverse-transcription-polymerase chain reaction (RT-PCR) and real-time PCR (RQ-PCR) has been used to examine known TAA expression in a range of solid and haematological malignancies [15-19]. Although this has provided important expression information and a good starting point to identify potential antigenic targets in a range of cancers, these studies are entirely limited to tumour antigens which had already been discovered.

3.2. Representational difference analysis

Representational difference analysis was developed by Thierry Boon's group and used to discover a number of CT antigens [20, 21] including the MAGE family of antigens, typically from melanoma with one exception, RAGE, from renal cancer. Briefly, total RNA was extracted from normal tissue (driver) and a tumour sample (tester) and used to construct double-stranded cDNA. Both cDNA samples were digested with the restriction enzymes DpnII and ligated to adapters which contained primer binding sites. The fragments were amplified by PCR, the adapters removed and new adapters for unrelated primers ligated to the tester. The tester and driver were then mixed and hybridized leading to three combinations of product: driver-driver (no amplification), tester-driver (linear amplification) and tester-tester (exponential amplification). A further two hybridization and amplification steps generate greater variation in the products which are subsequently cloned and sequenced.

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3.3. Serological identification of antigens by recombinant expression cloning (SEREX)

Serological identification of antigens by recombinant expression cloning (SEREX) provided a much needed boost to the area of antigen identification at a time when few cancer antigen identification options existed [2]. SEREX was not limited to immunogenic cancers such as melanoma and has now been used to identify more than 2,000 antigens [22-23] in a large range of different solid [24-26] and haematological malignancies [27-30]. cDNA libraries are created from tumour samples, cell lines or healthy normal donor cells (such as testes). RNA from these cells were reverse transcribed and inserted as cDNA into phage vectors and expressed as recombinant proteins on the capsid surface of phage which survived on permissive E.coli. Expressed proteins were transferred to nitrocellulose membranes and following the removal of excess E.coli waste, phage plaques were immunoscreened using pre-cleared patient sera. Any positive plaques were isolated, eluted and used for secondary confirmatory screening, prior to cDNA sequencing of phagemid inserts [31].

3.4. Serological proteome analysis (SERPA)

Serological proteome analysis (SERPA) was first described by Klade *et al* in 2001 [32]. Proteins were extracted from primary tumours or cell lines, separated concurrently on two 2D gels and transferred to nitrocellulose membranes. A third gel is stained with Coomassie Blue as a preparative gel. The membranes are incubated with cancer patient's sera and normal control. The two gels are directly compared and any bright spots on the cancer sera membrane were cut from the preparative gel and indentified using mass spectrometry [33, 34].

3.5. CDNA microarrays

The differential expression of tumour antigens and/or protein biomarkers between cell and disease subtypes have been directly compared on cDNA microarrays and has allowed our improved understanding of lymphomas [35] and aided our development of personalised therapies [36]. Microarray technology is able to distinguish between different subtypes of a particular cancer as well as identify the expression of novel antigens [37]. Minimal residual disease is a very important tool in the detection of impending relapse in patients who have had some form of treatment. Markers for minimal residual disease in acute lymphocytic leukaemia were identified by gene profiling [38]. cDNA microarray has been used to identify the frequency of elevated tumour antigen expression in acute myeloid leukaemia [28] and also associations between specific cytogenetic abnormalities and relative levels of tumour antigen expression [39]. Micorarray has also been used to elucidate the possible function of tumour antigens such as Synovial Sarcoma X breakpoint 2 Interacting Protein (SSX2IP) in the subversion of cells harbouring cytogenetic abnormalities (t(8;21) associated with mitotic spindle failure and the association between the elevated expression of some tumour antigens (SSX2IP, RHAMM and SURVIVIN) at disease presentation and patient survival [8] in acute myeloid leukaemia.

3.6. Mass Spectroscopy (MS)

Mass Spectroscopy (MS) involves the analysis of peptides eluted from the MHC of antigen presenting cells [40-42] or proteins in serum [43]. This area is reviewed more completely in the

following reviews [44,45]. By using mass spectrometry, it has been demonstrated that as many as 10,000 different peptide species are presented by individual class I MHC alleles [46]. The technique, its strengths and limitations are extensively reviewed [47].

3.7. Protein microarrays

Protein microarrays involve the immunoscreening of protein arrays (approximately 9,000 full length proteins and functional domains) which may be purchased from companies such as Invitrogen, Functional Genomics or Cambridge Protein Arrays. Antibodies in sera from patients [33,48,49] can be detected using generic secondary antibodies (fluorescently conjugated anti-human IgG) and visualised on microarray scanners.

4. Validation of the expression of tumour antigens in tumour cells

Once TAAs have been identified their expression in tumour cells needs to be confirmed. There are a number of assays which can be used to validate the expression of antigens in tumour cells. Many of the most frequently used rely on an available antibody which has been validated [50,51]. Techniques frequently used include:-

4.1. Reverse Transcription (RT-PCR)/Real-time PCR

Total RNA is extracted from cells and used to make cDNA using reverse transcriptase. The cDNA product is amplified by PCR and run on an agarose gel to identify the presence of the transcribed gene in the cell [52]. This technique is sensitive and real-time PCR can provide relative quantitation, however both techniques only indicate the presence/level of gene expression and not protein translation, which can vary greatly between antigens.

4.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Enzyme-Linked Immunosorbent Assay (ELISA) is a straightforward procedure which can be used to detect an antigen using an antibody [53]. The antigen is attached to the bottom of a 96-well plate, or bound by a capture antibody on the bottom of a plate (in the case of a sandwich ELISA). The protein of interest is then incubated with a chemically labelled detection antibody. In most experiments the chemical label is an enzyme and a substrate is added which will produce a colour change detectable by a microplate reader. The technique is sensitive and quantitative when used in conjunction with appropriate protein concentration controls but is better fitted to the analysis of protein in urine and blood, rather than in tissues.

4.3. Immunoblotting

Other systems which use antigen-antibody interactions are techniques such as Western blot. Extracted proteins from tumours or cells are separated by 2-dimensional electrophoretic gels and then blotted onto nitrocellulose membranes. The membranes are incubated with primary and then secondary antibody. The secondary antibody is covalently labelled with an enzyme which reacts with a substrate solution generating colour, which then can be measured [54].

http://dx.doi.org/10.5772/54698

4.4. Immunoprecipitation

The protein of interest can be purified by incubating lysed cell extracts with its specific antibody in solution. Once the antibody has bonded with the protein, the resulting complex can be precipitated using agarose or G Sepharose beads which remove the required protein. The complex can be separated using sodium dodecyl sulphate-polyacrylamide gel electrophoresis [55]. The sample can then be used to determine how much protein is present relative to other cells or other treatment conditions but denaturation is often required and details about sub-cellular localisation are not possible.

4.5. Immunocytochemistry/histochemistry

The antigen of interest can be detected in cells (cytochemistry) or in tissues (histochemistry). The cells or tissue sections are fixed onto slides using a fixative such as paraformaldehyde to immobilise them. They are incubated with the primary antibody and then the secondary which is labelled with a detection molecule. The technique is qualitative informing the user about the sub-cellular localisation of the antigen in tissue and which cell types express it (Figure 1). However quantitation is often lacking and like most methods this requires optimised reagents.

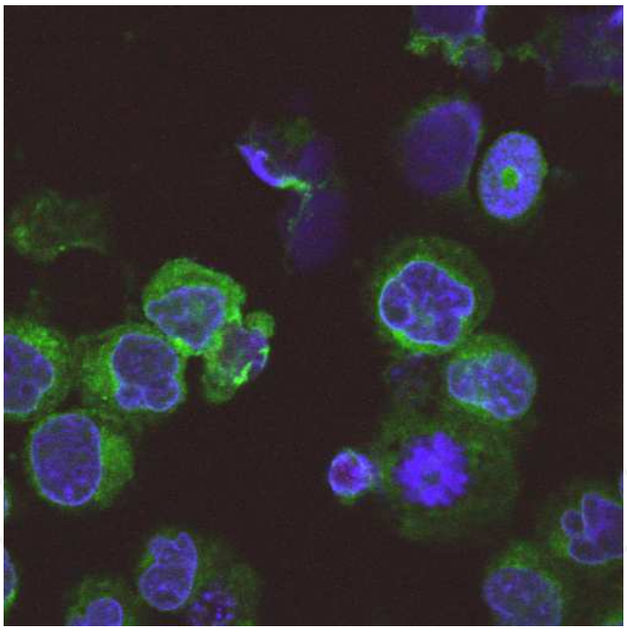

Figure 1. Demonstration of the sub-cellular localisation of the tumour antigen SSX2IP in K562 cells using immunofluoresence microscopy. Cells were air dried for 4-18hours onto glass microscope slides and stored at -20°C wrapped in saranwrap. Cells were defrosted and stained with antigen specific primary and fluorescently labelled secondary antibodies. Using confocal microscopy we detected SSX2IP expression (observed as a green colour by virtue of anti-SSX2IP-fluorescein isothiocyanate) was detectable on the surface of the K562 cells. Cell nuclei were stained blue using 4,6'-diamino-2-phenylindole (DAPI).

The advent of multiple tissue arrays from collaborators or commercial sources provides a screening opportunity once the cancer(s) of interest for the antigen has been defined.

4.6. Flow cytometry

This technique allows the analyses of cells with a variety of parameters such as extracellular or intracellular markers, granularity, size and shape. Cancer cells are labelled with fluorescent antibodies for the required antigen. The cells are passed in a stream and intersected with a laser beam. The intensity of the fluorescence is measured and plotted in the form of dot plots and histograms. This technique is sensitive and informative to allow specific cell types to be "gated" by virtue of size, granularity and detectable protein expression. Machines can measure up to 19 parameters in the most sophisticated machines allowing multiple proteins and cell types to be analysed simultaneously [56]. However the technique requires validated antibodies that have been shown to be appropriate for fluorescence activated cell sorting analysis and enough tumour cells in suspension for analysis.

5. Identification of HLA-binding epitopes — *in vitro* assays

Immune responses in the body can ensure that any foreign matter is eliminated effectively. Class I and II major histocompatability complexes (MHC) are present on the surface of nucleated cells and present processed peptides from proteins inside the cell to T cells. T cells can destroy infected cells if peptides in the context of "danger" are detected [57]. MHC in humans is known as the human leukocyte antigen (HLA) system. MHC class I HLA molecules are highly polymorphic and generally the best defence against infections.

5.1. MHC Peptide binding assay

Peptide antigens are stripped from the HLA class I molecules by mild acid treatment, cells are then incubated with a fluorescent reference peptide together with different concentrations of the peptide of interest. The efficiency with which the required peptide competes for binding to the HLA class I molecules is examined by measuring the amount of HLA-bound reference peptide with fluorescence activated cell sorting analysis [58].

5.2. T2 *in vitro* HLA-A2 binding assay

T2 *in vitro* HLA-A2 binding assay is more frequently used to determine the strength of peptide binding to the most common HLA molecule in Caucasian populations. The HLA-A2 expressing, TAP-1 deficient human T-cell line T2 is used as an assay of HLA-A2 peptide binding efficiency. T2 cells are washed and resuspended in serum-free RPMI media and plated in 96-well microtitre plates. Human β2-microglobulin and often nonamers (nine amino acids long peptides) are added and the cells are incubated overnight at 37°C/5% CO_2. The cells are washed and probed with a HLA-A2-specific monoclonal antibody and appropriate secondary antibody prior to flow cytometry. Only HLA-A2 molecules bound to peptide are stabilised and detectable on the cell surface. Results are reported as a relative mean fluorescence index (MFI), calculated as the MFI of peptide-pulsed T2 cells compared with the MFI of unpulsed T2 cells [59]. Time course assays may be used to indicate how long the peptide remains on the HLA-

A2, indicating how long T cells will have to interact with peptide bound HLA-A2 before the complex falls apart.

6. *In silico* identification of epitopes

There are a number of databases which can be mined to find epitopes which have already been shown to bind to HLA molecules. These have been used to identify established epitopes that may be used in immunotherapy strategies.

6.1. The SYFPEITHI

The SYFPEITHI database allows the prediction of MHC class I and II binding ligands for different mammalian species. When a search is carried out using a protein sequence, a prediction is made based on the amino acids in the anchor and auxiliary anchor positions and other frequent amino acids which can bind to MHC molecules. A score is then calculated which follows certain rules which are: a numerical value of 10 is given to amino acids that regularly arise in anchor positions, the value 8 is set for amino acids occurring in a significant number of ligands, six is for unusual anchors such as auxiliary anchors and less frequent residues of the same set have a value of four. Preferred amino acids have coefficients between 1–4 depending on the signal strength in pool sequencing or the occurrence of individual sequences. Amino acids that are considered as having an adverse effect on binding have a coefficient of –1 to –3 [60]. SYFPEITHI database gets updated regularly and has been used to identify various ligands; p28 peptide as an epitope for the CT antigen PLAC1 in breast cancer [61], p101-111 is the first CTA-derived peptide which induces CD4(+), CD8(+), and B-cell responses *in vitro* [62], p43-57 epitope stimulates T cells in HCA587-derived tumours [63] and PASD1(1) – PASD1(5) [51].

6.2. Bioinformatics and Molecular Analysis Section (BIMAS)

Bioinformatics and Molecular Analysis Section (BIMAS) develops computational processes to analyse data generated from molecular biology and genetics research; and provides bioinformatics guidance, support and resources for the collection, management, and display of biological sequence and genomic information for scientists involved in genomics and genetic analysis [64]. Other online software which can be used to identify epitopes includes EpiJen, Rankpep, nHLApred, NetCTL and Multipred [65].

7. Cell based assays — *In vitro* demonstration of T cell reactivity

There are a number of assays which can be used to determine if T cells are activated in response to antigen.

7.1. Carboxyfluorescein diacetate Succinimidyl Ester (CFSE)

Carboxyfluorescein diacetate succinimidyl ester (CFSE) is a cytoplasmic dye which is absorbed by all cell types. Once the CFSE labelled cells divide, the dye is shared amongst the daughter cells equally therefore the fluorescence is halved after each round of the cell cycle. This difference in fluorescence can be measured. The more cells proliferate, the greater the decrease in the fluorescent signal. The fluorescence peaks can be measured by flow cytometry [66]. CFSE labelling is increasingly used to measure target tumour cell killing [67], superseding radiation based assays, as well as T cell proliferation in response to tumour cells *in vivo* [68]. CFSE labelling can also be performed *in vivo* where the dye is injected into the host animal's spleen or lymph nodes, however the labelling is not uniform and it is sometimes difficult to obtain individual peaks once lymphocyte cell division has occurred [66].

7.2. Lymphocyte proliferation assays

Lymphocyte proliferation assays can be used to determine activation of T cells. Peripheral blood mononuclear cells are isolated and cultured in microtitre plates. The specific antigen is incubated with the cells, which causes the T cells to divide and grow. The MTT colorimetric assay is based upon (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazolium salt which gets cleaved by enzymes in the mitochondria to produce blue formazan. Viable, dividing cells will create more formazan which can be quantified using a plate reader. MTT assay is very convenient, however some considerations need to be assessed to avoid false positives such as cell densities, correct culture medium, filtration of media to remove precipitate, optimisation of MTT concentrations and incubation times [69].

7.3. [^{3}H]-Thymidine incorporation assay

[^{3}H]-Thymidine incorporation assay is based on the use of [^{3}H]-thymidine a radioactive molecule which can be incorporated into DNA during the S-phase of cell division. As new DNA is synthesised, occasional thymidine bases are replaced by [^{3}H]-thymidine and subsequently the incorporated radioactivity is measured, following washes to remove unincorporated radioactivity, using a Scintillation Counter [70]. As [^{3}H]-thymidine is radioactive an analogue called bromodeoxyuridine (BrdU) was developed to replace it in assays. BrdU integrates into DNA strands and can be measured using immunohistochemistry and flow cytometry protocols using fluorescent conjugates and can be observed over a longer duration. BrdU is also used to look at the number of cells in each part of the cell cycle by flow cytometry [71]. However BrdU has been found to be more toxic than [^{3}H]-thymidine, possibly because it is structurally very different to the original DNA nucleotides. It also adversely affects cell division, the pattern of cell migration, final position of migrating cells and the fate of labelled cells [72].

7.4. Peptide-MHC (pMHCs)

Peptide-MHC (pMHCs) based assays circumvent issues caused by measuring T cell proliferation. T cell proliferation assays can provide information on whether an immune response has

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been generated but won't determine which T cells, if they are indeed T cells, have been activated. pMHCs, often referred to as tetramers, can be used to identify antigen specific T cells. They are produced through the refolding of β2-microglobulin and heavy chains in MHC molecules with the appropriate epitope of interest. The pMHC is then labelled with biotin using BirA enzyme. A streptavidin molecule conjugated to a fluorescent detector binds to four (tetramers) pMHCs or can used to create multimers (for example dimers, pentamers, dextramers) of these constructed MHC molecules courtesy of the biotin-avidin interaction [73]. T cell populations are added to this mixture and T cells with the specific receptor for the epitope of interest will bind and be measurable by flow cytometry [74]. Shen *et al* [74] have found that cross-reactive T cells i.e. T cells which recognise two different antigens can be identified providing an extra tool in vaccine development. In some cases antigen specific T cells may not bind tetramers due to being undifferentiated and unable to accumulate T cell receptor (TCR) molecules close to the antigen. Another reason could be low affinity between TCR and MHC [75]. Other techniques based on the use of pMHCs include pMHC arrays [76] (Section 7.5), NACS [77] and the combinatorial approach [78,79]. These techniques all provide high throughput analysis of multiple T cell populations with a variety of pros and cons to each technique including issues with background, specificity/binding capacity of individual pMHC complexes, activated induced cell death of pMHC bound T cells, internalisation of pMHCs following T cell binding [80], cost and labour intensity. Sequencing of TCRs (2-3 million every 2-3 days) by companies such as TRON gGmbH (Johannes Gutenberg University Mainz, Germany) and Adaptive Biotechnologies (Seattle, USA) will provide a new way of analysing T cell populations which will be informative with regards to which TCRs are present but not necessarily whether they are present on mature, anergic, activated or functional T cells nor which sub-group of T cells are harbouring them (helper T cells, cytotoxic T cells, Th17 cells or indeed regulatory T cells (Tregs)). This technology allows the first opportunity to examine an extremely large number of TCRs in a very short time and will revolutionise how we examine T cell responses in patients in the future.

7.5. pMHC arrays

pMHC or tetramer arrays [76,81] (Figure 2) provides a strategy to determine which specific $CD8^+$ T cell populations are present in the peripheral blood of patients. Antigens identified by the techniques described already can be used to help expand the pMHC array for future studies. In addition, the pMHC array provides a means to investigate epitope spreading and changes in T cell specificities with disease progression. The technique benefits from the low number of purified $CD8^+$ T cells required for each array ($0.5\text{-}2 \times 10^6$), which can be purified from 20ml of patient peripheral blood using StemCell $CD8^+$ negative isolation beads providing "untouched" T cells (Bonney, Guinn *et al*, in preparation). The purified $CD8^+$ T cells are then lipophyllically dyed with DiD (Molecular Probes), washed and incubated with the pMHC array. The pMHC array has a detection limit of 0.02% matching the sensitivity we can reproducibly achieve with flow cytometry when analysing patient samples. Where sample availability permits, pMHC array data should be validated by flow cytometry [82] using the same pMHC tetramers as in the pMHC array. The pMHC array has the added advantage that it can be used for the ini-

tial screening of a relatively small number of $CD8^+$ T cells against a large number of pMHCs on the array, and a short-list of T cell populations which are shown to exist on the pMHC array can then be quantitated by flow cytometry (limiting the amount of sample required in subsequent studies). The pMHC array can be used to analyse patient samples at a number of disease time-points (presentation, post-treatment (surgery and/or radiotherapy) and with disease progression) to examine how T cell responses to tumour antigens change with treatment, to examine epitope spreading and to correlate changing immune responses with clinical responses.

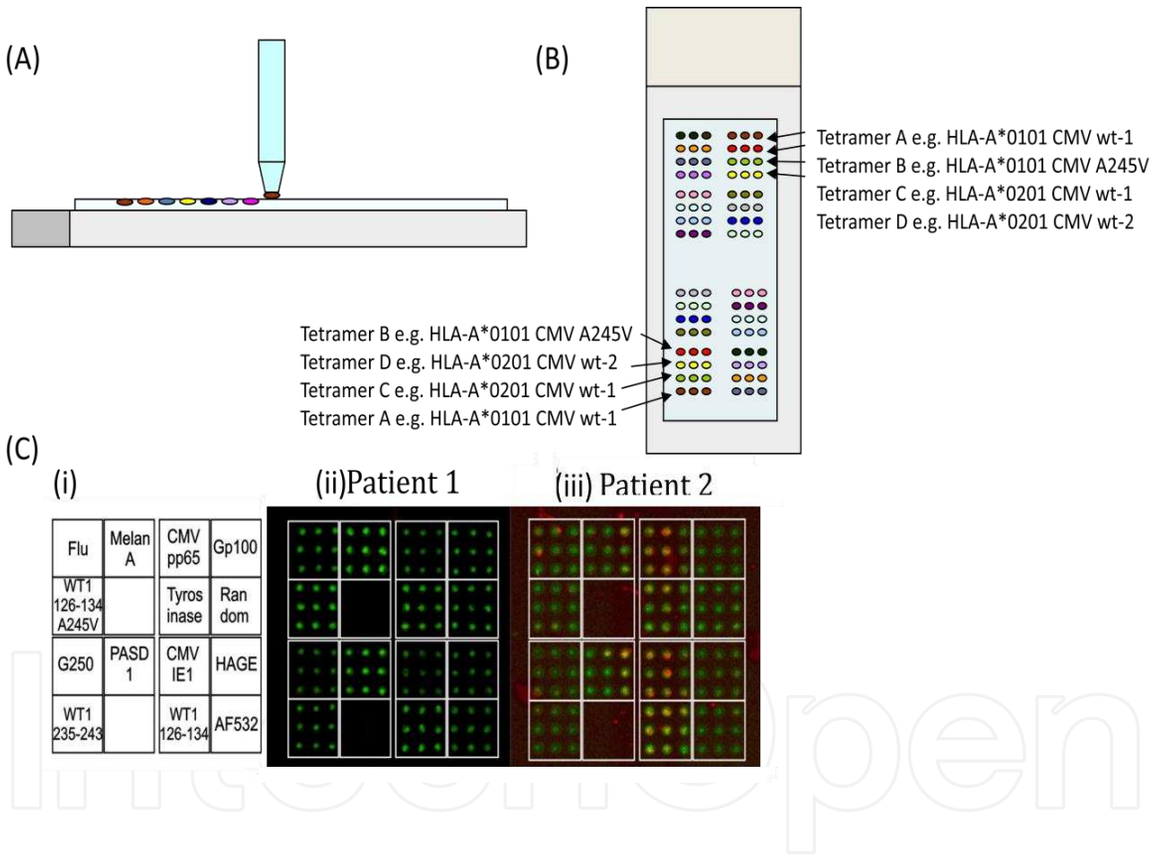

Figure 2. pMHC arrays for the simultaneous detection of T cell populations in patient peripheral blood. (A) Using a QArray^2 printer and HPLF 0.3mm solid tip pins (Genetix) we printed multiplexed AF532-conjugated pMHCs at a concentration of 0.5mg/ml so that 1ng was placed in each spot (shown as coloured ovals). Each spot is 400uM wide with a 700uM inter-spot distance. (B) At least six spots per pMHC are applied in each of two independent sites. Each hydrogel slide can hold up to 1,000 spots of tetramer in total. (C) We applied 0.8-1.5 x 10^6 lipophillically-dyed $CD8^+$ T cells/slide (seen in red). pMHCs included a range of HLA restrictions and had already been shown to work in flow cytometry studies,(i) pMHCs are spotted across the gel, (seen as green spots) (ii) even some HLA-A2 positive patients show no reactivity with any pMHCs (iii) while others show the presence of multiple T cell populations which recognise tumour antigens. Based on figure in [144].

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7.6. Intracellular cytokine staining assay

Intracellular cytokine staining assay detects particular cytokines released by immune cells which can provide a useful insight into the responding T cell populations. Such cytokines could include interferon-gamma (IFNγ), interleukin-2 (IL-2), IL-4 and tumour necrosis factor-α. Cells are plated and incubated with the antigen to stimulate cytokine production. To prevent the cytokine from exiting the cell a transport inhibitor is added e.g. brefeldin A. The cells are then fixed in paraformaldehyde and permeabilized to allow the anti-cytokine antibody to bind. The results are analysed by flow cytometry. The use of intracellular cytokine staining assay to detect the cytokine IFNγ shows high reproducibility and linearity with little background [83]. Duration of culture prior to antigen stimulation, as well as the cytokine accumulation period, are critical parameters of these methods. In both murine and cattle models, following 2-6 hours in culture, T cells produced a mixture of cytokines IFNγ, IL-2 and tumour necrosis factor-α, however following 6-16 hours of culture only IFN-γ cytokine was found [84].

Using multiple peptides from distinct TAAs to stimulate immune cells may prove the most effective for peptide vaccines. A cocktail of four multiple myeloma antigen peptides were used to stimulate T lymphocytes from HLA-A2 positive people to induce IFNγ production, cell proliferation and cytotoxicity against HLA-A2 positive multiple myeloma patients' cells [85]. Indeed long peptides may offer the advantage of allowing the immune system to choose the epitope(s) it can best process and present from a peptide sequence and induce an effective cytotoxic T cell response in the presence of longer $CD4^+$ helper motifs [86]. Conversely sometimes longer proteins can inhibit $CD8^+$ T cells responses [87] but this may vary depending on the constituents of individual protein sequences.

8. Cell based assays — *In vivo* assays

There are a number of approaches that can be taken when using mouse models to detect T cell immune responses. Animals can be used in transplantable tumour (xenografts) models or genetically engineered tumour models. In xenograft models human tumour cells are taken and injected into immunodeficient mice so that complex immune responses involving multiple cell types can be investigated. In contrast, in genetically engineered models, genes known to cause cancer are activated or tumour suppressor genes are "switched off" to allow their effects on tumour growth to be examined. In addition transgenic mice can be used to examine T cell responses to epitopes presented on MHC molecules as described in this section.

8.1. Immunodeficient mice

Immunodeficient mice such as athymic nude mice, severely compromised immunodeficient (SCID) mice and non-obese diabetic severe combined immunodeficiency mice will accept xenografts of human cells [88]. Depending upon the number of cells injected, or

the size of the tumour transplanted, the tumour can develop over weeks to months and the response to appropriate therapeutic agents studied *in vivo* [89]. Indeed such models have been used to examine the effectiveness of various immunotherapeutic strategies including whole cell vaccines [90], dendritic cell (DC) vaccines [91], peptide vaccines [92] and DNA vaccines [67,87,93].

8.2. Genetically modified mice

Genetically modified mice may have genes which are overexpressed or deleted and the effects of these genes on tumour development can be studied. Examples include p53 null and heterogenous mice [94,95] demonstrated that these genes can act as oncogenes and lead to tumour development. Possible therapies for these oncogenes/tumour suppressor genes are tested for their response in an *in vivo*, full organism context [89] and examples include Ad-p53, AAV-HGFK1 [96].

HLA-A2 transgenic mice have genes inserted into the DNA so they will express the HLA molecules known in mice as H-2. In order to prevent the presentation of murine H-2-restricted cytotoxic T lymphocyte (CTL) epitopes in HLA-A2 (AAD) transgenic mice, HLA-A2.1 transgenic/H-2 class I knockout mice (HHD mice) were created [97]. In HHD mice, the H-2 class I gene is knocked out, and a chimeric HLA-A2.1 monochain (HHD) is produced by linking the C terminal of the human β2-microglobulin (unit of the class 1 MHC) covalently to the N-terminus of the chimeric HLA-A2 heavy chain (which contain the α1&2 domains of HLA-A2.1 and the α3 domain of H-2Db) by means of a peptide bond. This guarantees the sole expression of the HHD molecule on the cell surface, making sure that any identified CTL epitopes are HLA-A2 restricted [98]. HHD mice allow epitopes which are presented on human HLA-A2 to be examined for their ability to induce T cell responses in a variety of studies; for example STEAP, a prostate tumour antigen has been shown to be targeted by anti-tumour T cells [99] and DNA vaccines encoding Wilms tumour antigen 1 induce cytotoxic responses in mice [100] using this model system.

9. Modes of immunotherapy

One of the biggest debates in cancer immunotherapy remains which mode will be the most effective. The National Cancer Institute have suggested that immunotherapy studies should focus on a limited number of antigenic targets to maximise the chances of success [101]. However for some cancers effective immunotherapy targets have yet to be discovered (i.e. ovarian cancer, adult acute lymphocytic leukaemia) and better targets may yet be determined.

When T cells were found to be able to identify cancer cells [102] it was thought that T cell therapies would be the most effective with the aim being to stimulate $CD8^+$/CTL cells to kill tumour cells. This can be achieved by a number of ways such as through the use of DCs [103], peptide vaccines [85], DNA vaccines [104] and natural killer cells [105]. In recent years

monoclonal antibodies (mAb) have become standard treatments for cancer. Ultimately if there is an antibody and it recognises a surface antigen solely on cancer and non-essential cells then this will likely be the most effective way to cause tumour destruction. mAbs are derived by vaccinating an animal with the target antigen and testing to see if the B cells are producing antibodies against it. Then the B cells are extracted from its spleen and infused with myeloma cells to produce hybridomas. Hybridomas divide perpetually and produce the mAb to the antigen in large amounts [106].

Rosenberg *et al* [107] showed that only 2.6% of immunotherapy clinical trials had worked and therefore an overhaul was needed in the practice of immunotherapy. Subsequently the same group showed that adoptive T cell therapy could be very promising with cell numbers being returned to the patient [108] and their status – activated but not mature [109] and cell numbers being the main issues. It is also likely that the best strategy may include a combination of conventional and immunotherapy techniques [110] or even a combination of immunotherapy techniques as demonstrated in increasing numbers of mouse models [111] and clinical trials [112].

DCs are antigen presenting cells therefore they have received some attention for possible use in cancer immunotherapy. DCs pulsed by peptide and injected into the skin showed a response rate of 28%. This percentage increased to 35.7% when immature DCs are injected straight into the tumour and even higher to 40% for advanced pancreatic cancer [113].

Tumour-infiltrating T cells (TIL) therapy has been used in stage IV melanoma patients. TILs are obtained from the blood, lymph nodes or from a tumour tissue biopsy. TILs are isolated, activated and expanded using IL-2 *in vitro*. The patient undergoes lympho-depleting chemotherapy prior to the T cells being injected back in to the blood [114].

When a tumour antigen is secreted into the circulation in high levels immune tolerance can be induced in the thymus. $CD8\alpha^{-}Sirp\alpha^{+}$, a subset of DCs, are able to capture tumour antigens in the blood, which can induce tolerance through Tregs or negative selection. Tregs are cells which are part of the tolerance system which prevents autoimmunity [115, 116]. Simultaneous Treg depletions (using anti-CD25 antibodies for instance) may aid the effectiveness of immunotherapy in some cancer types where Treg infiltration into the tumour is rife [117,118].

There are a number of reviews in this area of research which aim to look into effective immunotherapy strategies for the future. These include cellular immunotherapy [119], whole call vaccines [120], multidrug resistance [121] and DCs [122]. Targeted therapeutic strategies along with ever improving designs in clinical trials pave the way for further success [123].

10. Clinical trials

Clinical trials are undertaken after a large amount of data has been obtained on the antigen of interest in the lab. This data is required to ensure treatment safety and efficacy as far as is

possible. It remains imperative in most countries that treatments have been tested on live animals prior to first-in-man clinical trials and that favourable results are apparent in order for treatments to be taken into clinical trials. People, often patients, are recruited as compensated or full volunteers. The drug is given to participants initially to show that it is safe and then that it is effective. Dose escalation is also important so that an effective and safe dosage in humans is used.

Clinical trials have four phases, very basically as follows: I – evaluation of safety, II – safety and efficacy (with Phase I/II often including dose escalation), III – efficacy in a large cohort of patients and IV – post-approval studies. Phase I trials look at the safety and the best dose of the drug to administer. Such trials often involve 13 patients or less, and these patients are often have late stage cancer and are refractory to all other treatments with little chance of recovery. Phase II trials start to look at the efficacy of the medicine and often involve 20 patients with late stage disease. Phase I and II trials have to be completed successfully in order for testing to proceed to Phase III. Current "best practise" treatment is compared to the new drug being tested in phase III trials. Only if the new drug offers an improvement over best practise does the new medicine have a chance of becoming the standard treatment. At this time the drug will need to be licensed and approved by the authoritative body e.g. the US Food and Drug Administration, and once licensed, phase IV trials investigate the long term benefits and unexpected side effects. In some cases the drug may go through one of the phases more than once before moving forward and even then may get rejected [124].

It is very important that trials follow certain rules for the results to be considered legitimate. Phase III trials need to be randomised i.e. a computer randomly puts people into one of two groups. These can also be double-blind trials so that neither the patient nor the investigator knows which treatment is being given, thus avoiding any bias. In some cases there may not be any treatment to compare a new therapy against, in which case a placebo (inactive treatment) is given to one group [125]. In the UK the Medicines and Healthcare products Regulatory Agency is responsible for the regulation of medicines and medical devices and equipment used in healthcare, and the investigation of harmful incidents as well as overseeing the use of blood and blood products [126].

Cohen *et al* [127] have created an online website called BreastCancerTrials.org which matches patients to current trials taking place depending on the information they provide. This provides a valuable source for cancer patients who may want initial guidance on which clinical trials may be beneficial to them.

11. Assays to demonstrate efficacy of the response

Assays tend to reflect the immunotherapy strategy employed with the efficacy of antibody therapies being measured by tumour destruction, ertumaxomab destroys tumour cells expressing HER2/neu [128], bispecific antibodies represent a new class of anticancer therapeutics [129] and antibody-targeted delivery of a vaccine can improve tumour cell killing [130].

11.1. Enzyme-Linked Immunosorbent Spot (ELISPOT) assay

Enzyme-Linked Immunosorbent Spot (ELISPOT) assay was developed by Cecil Czerkinskdy in 1983 [131] and shows most similarity to the ELISA technique. It is based on the use of a 96 well plate with a polyvinyl-difluoride membrane to which antigen specific monoclonal "capture" antibodies are attached. The cells are grown in media on the capture antibody coated membrane usually for several hours to overnight and secreted protein (often cytokines such as IFNγ) bind to the capture antibody. A second "detection" antibody specific to the protein is used. This is often conjugated to an enzyme allowing a chemical reaction to occur. Black spots form on the membrane wherever protein is present and these can be counted by an ELISPOT reader [131]. ELISPOT assays are one of the most sensitive *ex vivo* detection methods available with low detection thresholds in peripheral blood. ELISPOT is also able to identify patients with allergies through the detection of drug-specific T cells in their blood [132].

11.2. Cultured ELISPOT

Cultured ELISPOT measures memory T cells. Cells are stimulated and plated on a 24-well plate. After an incubation period half of the cell culture supernatant is removed and replaced with Lymphocult (an IL-2 containing growth factor supplement). Fresh Lymphocult is added again on day 7. On day 9, the cells are incubated overnight. On day 10, around $2.5x10^4$ of the originally plated cells are plated for a standard ELISPOT assay. Cultured ELISPOT assays revealed the existence of longer-lasting T cell memory responses [133].

12. Conclusions

This chapter has focussed predominantly on the identification of epitopes within tumour antigens and their validation as they enter clinical trials. Focus on clinical trials using antibody therapies, DCs, natural killer cells, and adoptive therapy among many other options are the focus of other excellent reviews in the field [134-136].

Cancer immunotherapy is a vital area of research that continues to progress at a pace. Our understanding of the immune response and its potential to recognise and kill tumour cells with mans guidance offers hope to the patients for whom few other treatment options exist. Many tumour antigens have been identified, but some cancers still lack antigen targets that are expressed in the majority of the cancer cells by the majority of cancer patients. New techniques to extend antigen discovery will allow the improvement of immunotherapy strategies while the identification of new biomarkers will assist in the development of personalised therapies. Personalised therapies will decrease the cost (quantitative and qualitative) of treatment on patients who are unlikely to respond to it, allowing patients to avoid unpleasant and harmful side effects while maximising patient quality of life.

Target antigen	Mode of immunotherapy	Patient group	Phase of clinical trial	Outcome	Reference
CD22	Monoclonal antibody conjugated to calecheamicin	Refractory and relapsed acute lymphocytic leukaemia	Phase 2	18% patients had complete response, 39% had marrow complete response, 39% had resistant disease, and 4% died within 4 weeks of starting treatment	[137]
TG4010 targeting MUC1 & IL-2	Poxvirus (modified vaccinia virus Ankara) in combination with first-line chemotherapy	Advanced non-small-cell lung cancer	Phase 2B	6-month progression-free survival was 43·2% in the TG4010 plus chemotherapy group, and 35·1% in the chemotherapy alone group	[138]
LY6K and TTK	peptide vaccines in combination with CpG-7909	Metastatic oesophageal squamous cell carcinoma	Phase 1	Vaccination with peptides in combination with CpG-7909 increased and activated pDC populations and NK cell populations	[139]
CTLA-4	Monoclonal antibody with glycoprotein 100 (gp100) peptide vaccine	Previously treated metastatic melanoma	Phase 3	The median overall survival was 10.0 months among patients receiving ipilimumab plus gp100, as compared with 6.4 months among patients receiving gp100 alone	[140]
Prostate-specific antigen	Poxviral vaccines	Prostate cancer	Phase 2	The primary end point was progression-free survival which was similar in the two groups (treated, controls). However, at 3 years post study, treated patients had a overall survival rate of 30% compared to 17% of controls	[141]
CD20	Monoclonal antibody	Relapsed or refractory follicular lymphoma	Phase 1/2	Treatment caused immediate and profound B-cell depletion, and 65% of patients reverted to negative BCL2 status.	[142]
CA-125	Abagovomab, an anti-idiotype antibody	Ovarian Cancer	Phase 1	Improved CA125-specific cellular cytotoxicity might indicate that longer vaccination (nine injections) would be preferred to short (six injections)	[143]

Table 1. Examples of completed clinical trials showing the cancer antigen targets. Representation of the various modes of immunotherapy employed to date, cancer patients treated and the outcome of the trials.

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Abbreviations

BIMAS: Bioinformatics and Molecular Analysis Section; BrdU: bromodeoxyuridine; CFSE: Carboxyfluorescein diacetate succinimidyl ester; CTL: Cytotoxic T lymphocyte; CT: cancer-testis; DC: dendritic cell; ELISA: Enzyme-linked immunosorbent assay; ELISPOT: Enzyme-linked immunosorbence assay; HLA: human leukocyte antigen; IFNγ: interferon-gamma; IL: interleukin; mAb: monoclonal antibodies; MFI: mean fluorescence index; MHC: major histocompatibility complex; PCR: polymerise chain reaction; pMHC: peptide and major histocompatibility complex; SEREX: Serological identification of antigens by recombinant expression cloning; SSX2IP: Synovial Sarcoma X breakpoint 2 Interacting Protein; TAA: tumour associated antigens; TCR: T cell receptor; TIL: Tumour-infiltrating T cells; Tregs: regulatory T cells.

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