Characterisation of high and low avidity peptide specific $CD8^+$ T cells using immunologic, transcriptomic and proteomic tools

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Copyright Statement

Dedication

This thesis is dedicated to my ***parents***, wife ***Anu*** and son ***Adhi*** who have supported me all the way through my studies

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Abstract

One of the hallmarks of successful immunotherapy is the generation of high avidity cytotoxic T cells which can recognise and respond to very low concentration of antigens. This sensitivity of T cells is usually determined by peptide titration ELISpot assays. Even though these assays are generally useful, they are laborious and sample demanding. The assays become even more difficult if the peptide(s) accountable for the generation of vaccine specific responses are unknown such as whole protein or cell vaccines. Therefore, there is a need to identify markers which can quickly and reliably identify a high avidity T cell response in cancer vaccination settings.

To achieve this goal, this study utilised a C57Bl/6J mouse model which could efficiently generate high and low avidity T cell responses, when immunisation was undertaken with two form of vaccines to deliver the target antigens. The antigenic epitopes used for this study were derived from TRP-2 'self' and ovalbumin (OVA) 'foreign' antigens. Immunisation of animals with these antigens in a DNA vaccine format induces a high avidity T cell response, in contrast to the response when these are administered in the peptide vaccine format. However, both the immunisations produced same number of peptide specific $CD8^+$ T cells, which was assessed my multimer staining. When these cells were subjected to *in vitro* stimulations with the target peptides, the functionality of the low avidity T cells was restored whereas the high avidity T cells failed to respond to lower peptide concentrations. This showed the plasticity of antigen specific T cells and their ability to modulate their functionality according to the stimulation they have received.

In order to identify markers that are associated with the high avidity T cell responses *in vivo*, antigen specific $CD8^+$ T cells were isolated from high and low avidity groups using MHC multimer sorting. A global transcriptional profiling was conducted on the mRNA isolated from these cells. Analysis of expression data identified several differentially expressed genes between the groups. Six differentially expressed genes (Granzyme A, Granzyme B, FAS Apoptotic Inhibitory Molecule, Telomerase RNA Component, CD5 Antigen-Like, Spi-C Transcription Factor) were further selected and confirmed using qRT PCR. Expression of three genes were correlated with the microarray gene expression data. Among these, two genes (Granzyme A & B) were further confirmed at the protein level using flow cytometry. Further to this, studies of gene expression activation kinetics of TCR signalling using pentamer sorted cells with anti-CD3/CD28 monoclonal antibody coated microbeads, revealed that ImmunoBody® derived high avidity T cells are more signal competent with rapid up-regulation of genes involving in T cell receptor signalling pathway.

Proteomic characterisation of MHC multimer sorted cells using LC-MS profiling identified several proteins uniquely associated with Peptide or ImmunoBody® pentamer positive T cells. Many of these proteins were associated with important T cell functional properties. Further confirmation of these markers and their role in T cell avidity is required, however these studies were limited by the lack of availability of proteins from the low number of peptide-specific cells and antibodies.

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Chapter 1

General Introduction

1.1 The Immune system

Multicellular organisms, particularly higher order vertebrates, live in a place where they encounter various environmental and pathogenic hazards. Their normal tissue homeostasis is constantly challenged by invading pathogenic microorganisms and their own cellular malfunctions. Adaptability of these organisms to maintain their natural homeostasis against alterations in their natural physical habitat, changes in their normal physiological status are remarkable. This remarkable adaptability is attained from their normal physical adjustments to the changing environmental conditions and also with their advanced immune system evolved over millions of years. The vertebrate immune system coexisted with them during their evolution as a higher tissue grade organism, so it has learned, adapted and evolved with them to meet their needs and deeds over the time. Present day, the immune system of vertebrates is by far the best defence system allowing adjustments to any perturbations in their natural tissue homeostasis (Taniguchi *et al.,* 2009; Barreiro & Quintana-Murci, 2010) and endows them with ultimate survival advantage.

The realisation that the immune system has the ability to detect dangerous substances dates back to the fifteenth century, in the fight against deadly small pox disease and the subsequent use of '*variolation'* (Kindt *et al*., 2007). This was followed by Edward Jenner's use of 'cow pox fluid' which induced a protective immunity against smallpox in humans (Smith, 2011). However, it was Louis Pasteur who conducted the first systematic study in chickens and sheep using cholera and anthrax bacteria to elucidate the body's ability to develop a protective immunity with the use of external agents (Pasteur, 1881, 2002 [republished]). Jenner coined the term 'vaccine' derived from the Latin word '*vacca*' meaning cow. However, Pasteur is widely accepted as the 'father of immunology' due to his systematic study which led to the birth of modern immunology as a systematic discipline (Smith, 2012). Since then the causative components (antibodies first, then the T cells) which endow immunity have been identified and the types of immune cells 'fully' classified. Today, there is an active area of research aimed at entirely understanding the 'sub-subsets' of immune cells and effector molecules

which organise the highly coordinated entity of the 'immune system' in a faultless manner.

Studies conducted in the last century have classified the mammalian immune system into two key categories based on the nature of the immune mechanisms and cells involved - "innate" and "adaptive" immunity (Kindt *et al*., 2007). The innate immune system is widely considered to be the first line of defence against pathogenic organisms, whereas the adaptive immunity is acquired and evolved during the course of one's own life time. The cells and organisation of these two arms of basic immune system are different; however they are interdependent with many signals and functions overlapping between them. The immune system is not exclusively restricted to the eradication of pathogens; it is also capable of the identification and elimination of malignant transformations within the body. Therefore it is important to understand tumour immunity in the context of basic characteristic of these two immune arms, their interdependence and shortfalls.

1.1.1 Innate immunity

The innate immunity is considered to be the most primitive type of defence mechanism with its different layers of first line of immune barriers (Beutler, 2004). The footprints of innate immunity can be traced back to the very early days of evolution up to invertebrate organisms (Boehm, 2012) and even in plants with their pathogen related (PR) proteins (Zvereva and Pooggin, 2012). The basis of the innate immunity starts with the physical barriers which block the entry of any invading organism into the body such as skin and mucous membrane. These physical barriers are usually sufficient enough to protect against the hazardous extrinsic factors such as bacteria and fungi (Gallo and Nizet, 2009). However, there are many occasions when these primary barriers are broken or compromised and the internal spaces of the body are then exposed to the outer environment. Once this has happened, other components, predominantly innate immune cells, will come into play as the next layer of immune defence (Gallo and Nizet, 2009).

1.1.1.1 Cells of the innate immune system

All the cells of the innate immune system originate from myeloid precursor cells of the bone marrow (Figure.1.1). These cells are broadly divided into two major classes as

mononuclear phagocytes (*dendritic cells, mast cells and macrophages*), and polymorpho nuclear phagocytes (*neutrophils, basophils, eosinophils*) depending on the shape of their nucleus. Blood monocytes and macrophages are the key cells with a ubiquitous distribution throughout the body to facilitate the instantaneous detection of

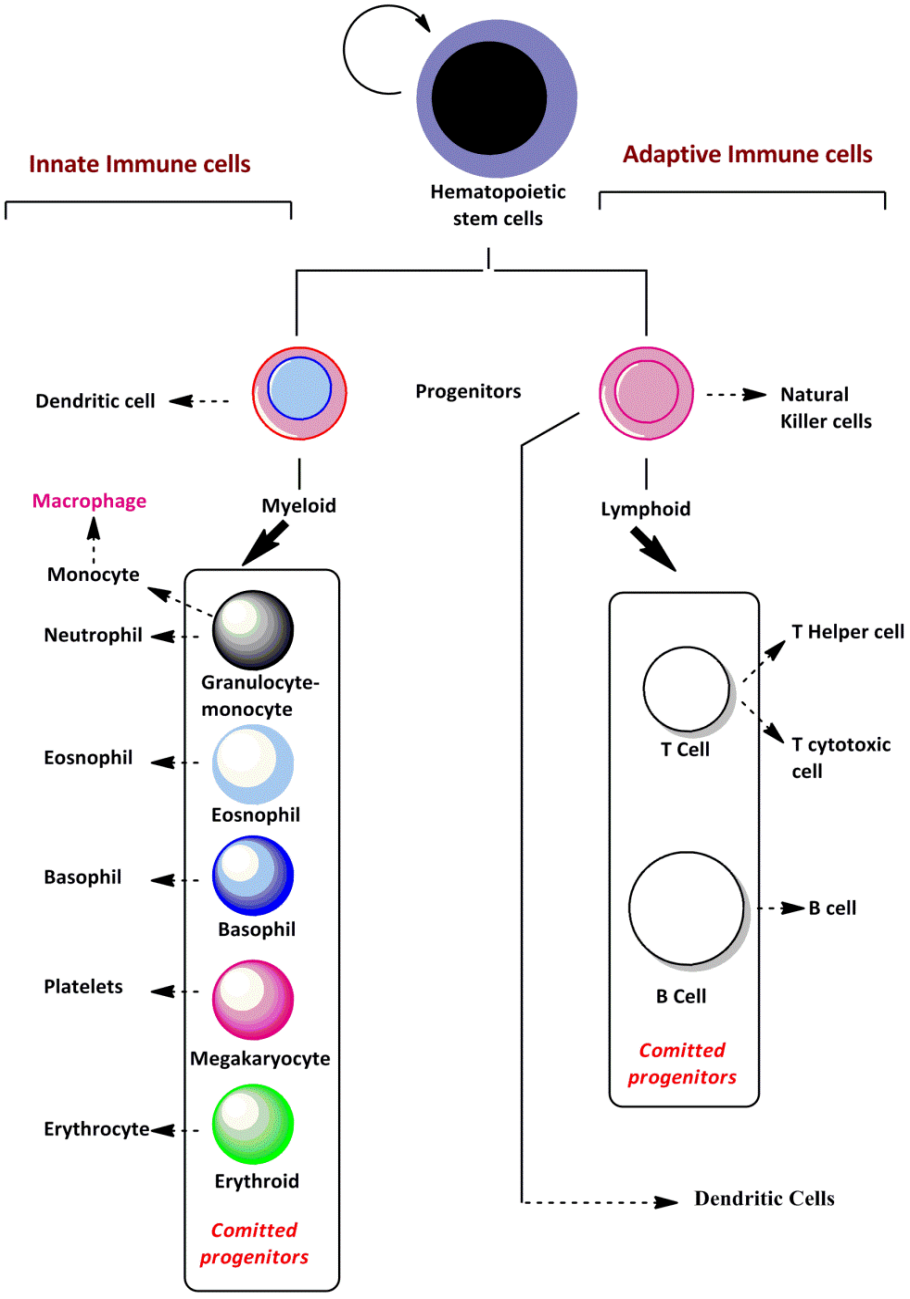

Figure 1.1: Schematic representation of haematopoiesis by which different cells of innate and adaptive immune system is generated in the bone marrow. The left panel shows myelopoiesis in which the cells of the innate immune system are formed and the right panel shows lymphopoiesis where the cells of adaptive immune lineage is generated.

danger signals anywhere in the body. Macrophages are produced in the bone marrow as immature monocytes and circulate in the blood to become mature monocytes. Once they arrive in the tissue environment they become mature macrophages (Sica and Mantovani, 2012). Depending on the tissue or anatomic site in which they reside, macrophages can acquire different morphologic shapes (e.g. Histiocyte and Kuppffer cells in the liver, microglial cells in central nervous system). The main role of macrophages is the management of cellular homeostasis by the detection of danger signals and elimination of them by phagocytises (Biswas *et al.*, 2012).

Macrophages also play an important role in the recruitment of the entire army of immune effector cells of innate and adaptive immunity by secreting chemoattractant when a danger signal is detected. The minority population of mononuclear phagocytes are dendritic cells which were first noticed by Paul Langerhans (1868) and later brought into the limelight by Steinman and Cohn in 1973. Unlike many other innate cells, dendritic cells can originate from both myeloid and lymphoid progenitors of the haematopoietic system in the bone marrow. They are also distributed in the skin, kidney, liver, connective tissues, internal lining of the body and many other organs (Sato & Fujita, 2007).

Among the polymorphonuclear phagocytes, neutrophils are considered to be the key cell type in fighting against infection (Simons, 2010). They are produced in the bone marrow and are capable of migrating into tissues with the capacity to perform phagocytic function. Eosinophil's and basophils mainly fight against parasitic infection and allergic responses, respectively, with the latter being non-phagocytic (Kindt *et al*., 2007).

1.1.1.2 Defence coordination by innate immune cells

One of the hallmarks of innate immunity is the lack of specificity and immunologic memory, mainly due to the lack of specialised receptors such as T cell receptors (TCR) and B cell receptors (BCR). Even though no antigen specific receptors are available on innate cells, almost all of them have a highly conserved and versatile 'generic' family of receptors that are known as toll like receptors (TLR) (Pradere *et al*., 2013), or pattern recognition receptors (PRR) (Wolska *et al.*, 2009; Seya *et al.*, 2010). PRRs are capable

of detecting invading micro-organisms and the pathogens by scanning for danger molecules broadly known as pathogen-associated molecular patterns (PAMPs) (Akira & Hemmi, 2003) expressed by pathogenic organisms or dying self-cells with damage associate molecular patterns (DAMPs) (Krysko *et al.,* 2012; Ahrens *et al.,* 2012).

The cells of the innate immune system continually keep normal cells, tissues and their microenvironment under their watchful surveillance, for the maintenance of tissue homeostasis, and to keep them from diseases. Any perturbations in tissue or cellular homeostasis trigger macrophages and mast cells to secrete cytokines, and other chemo-attractant molecules which enhance the infiltration of further leukocytes to the damaged or malignantly transformed tissue. Neutrophils are the first innate cell to reach the site of such changes. They mainly use TLR2/TLR4 for the detection of a multitude of bacterial lipoproteins and keep the pathogen in check by phagocytic clearance, however the use of these receptors by neutrophils for the detection of cancer cells also has also been reported (Klink *et al.,* 2012). Subsequently, dendritic cells take up the antigens and migrate to the nearest lymphoid organs and present them to the adaptive immune system thereby acting as an interface between innate and adaptive immunity. (DeVisser *et al.,* 2006). Macrophages also have elevated levels of MHC class II expression (Baumgart *et al.,* 1998; Xaus *et al.,* 2000) and can therefore also act as a primary bridge between the innate and adaptive immune system, mainly by communicating with helper T cells (T_H cells).

An outlier in the classic innate cell lineage (myeloid) are the natural killer cells (NK cells), capable of secreting two well-known immune modulatory cytokines tumour necrosis factor (TNF-∞) and interferon-γ (IFN-γ). They are also capable of secreting a variety of chemokines such as Macrophage Inflammatory Protein-1α & β (MIP-1∞ and MIP-1β) and RANTES [Regulated on Activation, Normal T Cell Expressed and Secreted] (Fauriat *et al.,* 2010; Roda *et al.,* 2006). NK cells expresses a variety of non TLR receptors (activation & inhibitory) for detecting danger signal and can discriminate between self and non self (Jaeger and Vivier, 2012). Recent studies revealed that they are capable of developing antigen specific immunologic memory which was previously thought to be an exclusive property of B and T lymphocytes (Sun *et al.,* 2011; 2012). This has resulted in a paradox for the classical definition of innate and adaptive immune system.

1.1.1.3 Innate immunity and its significance in cancer

All the cells of the innate immune system are capable of recognising and interacting with malignant cells (Liu and Zeng, 2012). Among the innate cells, NK cells have attracted much attention recently because of their capacity to detect tumour cells in an MHC independent manner (Le Maux Chansac *et al*., 2005). This has generated a renewed interest in NK cells, since most tumours down regulate MHC molecules as an immune escape mechanism (Maeurer *et al.,* 1996) and thereby become invisible to MHC dependent T cell adaptive immunity. NK cells can therefore provide protection in such instances where cellular immunity fails (Stojanovic and Cerwenka, 2011). The receptors and the mechanism of NK cell detection are now an area of intense study and their clinical utility has been demonstrated in *in vitro* model systems using antibody mediated NK receptor blocking. Huge strides in NK cell biology is evident from the work done by Ahn *et al.,* (2013) via expanding cytotoxic NK cells in a GMP setting which makes them suitable for adoptive transfer into cancer patients and this work has now progressed the use of NK cells as a cellular therapeutic strategy nearer to the clinic. Manipulation of NK killing efficiency was also achieved by classical non vaccine approaches in patients. This includes delivery of mild physiological stress, such as thermal and oxidative stress to the tumours to up-regulate natural NK cells ligands on their surface and thereby increase their susceptibility to NK cell lysis (Dayanc, *et al.,* 2013; Hedlund *et al.*, 2011). These approaches are gaining momentum as an alternative method for tumour control in a less invasive therapeutic approach.

Another key cell type in the tumour microenvironment is the macrophages, which have both pro and anti-tumour capabilities (Bingle *et al.*, 2002). However, some macrophages are found to be more tumourigenic than tumour suppressive by promoting angiogenesis and metastasis (Laoui *et al.*, 2011). In many cases, detection of tumour associated macrophages can be a poor prognosis factor for patients with head and neck cancers, lymphoma and gastric cancer (Cai *et al.*, 2012). However, in certain cancers such as colorectal cancer, macrophage infiltration has been directly correlated with good clinical outcome (Forssell *et al.*, 2007), indicating that the prognostic value of these cells within the tumour.

Neutrophils have not been well studied in the context of tumour progression, possibly due to their short lived nature. However, studies have emerged recently highlighting

that the presence of neutrophils in tumours has been associated with poor prognostic factor (Jensen *et al.*, 2009) including high tumour growth, metastasis and tumour immunoediting (Gregory and Houghton, 2011). Even though the innate immune system has a significant role in the detection of cancers and also alerts the adaptive immune system in a generic way through inflammatory cytokines and in more specialised way through the antigen processing and presentation pathways, it is the adaptive immune system which holds the most clinical promise, if any, in cancer immunotherapy settings. These achievements can be mainly attributed to the discovery of tumour antigens, monoclonal antibodies and antigen specific vaccine in prophylactic and therapeutic settings.

1.1.2 The adaptive immune system

Adaptive immunity is characterised by its receptor specificity (antigen specificity), its ability to memorise a previous antigenic encounter (antigenic memory) and the capacity to discriminate self and non self. Another important feature of the adaptive immunity is the need for antigen presenting cells (APCs) processing and presenting antigens in combination with specialised molecules known as major histocompatibility complexes (MHCs) (Janeway *et al.*, 2001). Above all, the most striking feature of the adaptive immunity is its ability to generate vast receptor diversity in their antigen recognising receptors (B cell receptors on B cells and T cell receptors on T cells). This is believed to be an advanced evolutionary genetic feature in terms of its genetic complexity (Haynes and Wu, 2004) which will be discussed in the following sections.

1.1.2.1 B and T lymphocytes

B and T lymphocytes are generated from committed B or T cell progenitors in the bone marrow via a process of haematopoiesis (Figure.1.1) (Pieper *et al.*, 2013; Koch and Radtke, 2011). Even though these cells derive from a common progenitor they are functionally different in terms of their antigen clearance and their receptor organisations. In B cells, the final effector functions are executed by a secreted receptors, commonly known as secreted antibodies; whereas in T cells, the receptors are always bound to the cells and the effector function (antigen clearance) is directly mediated by T cells themselves, hence T cell immunity is widely known as ‘cellular immunity’. When the B cells encounter a foreign antigen they differentiate into effector cells and memory cells. The effector cells are also known as plasma cells and they produce millions of

copies of antibodies of the same specificity of the antigen against which they have been primed. The detailed development of B cell lineage and antibody generation is well reviewed in Pieper *et al.*, 2013.

The T cells which matured in the thymus can also be broadly classified into two classes of T cells, The cytotoxic T cells (T_C) and the helper T cells (T_H) depending on the membrane glycoprotein found on their surfaces known as cluster of differentiation antigens (CD antigens). T_Cs express a glycoprotein known as CD8 (Cluster of differentiation 8) and T_H express a different glycoprotein known as CD4 (Cluster of differentiation 4). The functional roles of these cells are also different in cellular immunity, although in many occasions they are highly interconnected and interdependent.

1.1.2.2 Evolution of a complex 'anticipatory' receptor repertoire in adaptive immunity

The diversity of antigenic receptors, memory and 'self - non-self' discrimination are considered to be the hallmark of a highly evolved evolutionary feature in adaptive immunity. While innate immunity depends on the TLR, NLR (node like receptors) and scavenger receptors for pathogen recognition, the origins of these sophisticated receptor mechanisms of adaptive immunity can be traced back to 500 million years ago. The rudimentary adaptive receptor mechanisms are believed to be existed in the jawless fish where they used them as a primitive form of 'generic' receptors in their lymphocytes, generally known as variable lymphocyte receptors (VLRs). VLRs are generated with simple recombinational events, which is not as extensive as any recombinational events seen in the higher order vertebrates today (Cooper and Alder, 2006). VLRs are leucine rich repeats (LRRs), capable of offering a moderately wider range of protection to those organisms (Cooper and Alder, 2006). The studies conducted on these receptors suggested that many convergent evolutionary factors were responsible for the generation of TCR and BCR diversity in higher order organisms (Pancer *et al.*, 2004). The origin and evolution of present day BCR and TCR repertoire diversity can also be linked to the identification of two key genes which are conserved throughout the animal kingdom, (which has a defined adaptive immune system) known as recombination activation gene 1 & 2 (RAG-1 and 2) (McBlane, 1995). Although there is no evidence to suggests the existence of these two genes in animals which lack an adaptive immune

system, studies conducted in sea urchins identified two closely related genes known as spRAG1L and spRAG2L, suggesting that these genes might have existed much before the evolution of modern adaptive immune system in some other form and functionality (Fugmann *et al.*, 2006). Mutations in RAG-1 and 2 genes abolishes the entire adaptive immune system and patients with these mutations die due to a complete immune deficiency, thereby further emphasising the role of these genes (Asai *et al.*, 2011) for maintaining a healthy adaptive immune system.

In humans and higher order mammals the TCR and BCR diversity is generated through a process known as V(D)J somatic recombination (Lieber, 1992). This process mainly involves the genes of immunoglobulin family known as variable (V), diversity (D) and joining (J) genes. The recombinational diversity of B cells is generated in the bone marrow and for T cells this occurs in the thymus (Boehm and Bleul, 2007). This recombinational event can generate a theoretical diversity of ~10^{11} antibody receptors (Janeway *et al.*, 2001) and more than 10^{11} - 10^{15} TCRs in both mouse and humans (Sewell, 2012; Robins *et al.*, 2009). Some experimental observations have reported the existence of more than the above estimates (Robins *et al.*, 2010). The recombinational events are facilitated by conserved heptamer sequences known as recombinational signal sequences (RSS) flanking the V and J genes mediated by RAG-1 and RAG-2 enzymes (Grawunder and Lieber, 1997; Swanson *et al.*, 2009). This complexity and diversity of the adaptive immune system is considered to be a highly advanced feature in humans and other higher order vertebrates which complement the primitive innate TLR and VRR receptors and thereby offers immune protection which is adapted to a complex and challenging environmental niche.

1.1.2.3 Peripheral fine tuning of B and T cell diversity

The estimated number of receptors (BCR and TCR) which exists in the adaptive immune system is vast. The previous section discussed the ways in which the structurally similar immunoglobulin gene family can generate such a huge diversity by somatic recombination (V (D) J recombination). In B cells, these diversities can be further enhanced several fold by two other classic processes known as somatic hyper mutations and class switching (Muramatsu *et al.*, 2000). The V(D)J recombination generate the primary antigen independent repertoire for B cells and in the periphery these B cells can undergo an error prone DNA mismatch repair mechanism catalysed by

the enzyme activation induced DNA deaminase for site directed mutations in the antigen binding region of the antibody. This will further increase the affinity of antibodies against the antigens present on the invading pathogens or malignant tissues (Griffiths *et al*., 1984). This process of fine tuning the affinity of antibodies is known as affinity maturation. A second mechanism of further diversification of the antibody repertoire is class switching which help to produce different classes of antibody isotypes with the same specificity (Wysocki *et al.*, 1992).

T cell repertoire diversity can also be generated by the same primary mechanism of V (D) J recombination of immunoglobulin genes. However, there is no evidence of somatic hypermutations or class switching of TCR in the periphery after thymic selection. T cells recognise short peptide fragments presented on the surface of MHC molecules through their complementary determining region (CDR) which has a high similarity to the antibody fab fragment (Bentley and Mariuzza, 1996). The binding diversity of the TCR repertoire is also generated by CDR regions (Janeway *et al.*, 2001). These CDRs detect the top portion of MHC molecule and the peptide which sits in its groove. The T cell diversity is mainly attributed to two categories: structural diversity and functional diversity (Mahajan *et al*., 2005). The generation of the TCR structural diversity has been discussed in the above section, whereas the functional diversification mainly occurs as a consequence of the antigenic stimuli and the cytokines to which they have been exposed to (Buchholz *et al*., 2012). In short, a T cell which has a set number and type of αβ receptors can perform different effector functions depending on the nurturing they have received during their time in the central and peripheral organs. The detailed mechanisms for the generation of T cell lineages and thymic selection are discussed in Chapter 2.

As discussed earlier, the theoretical estimation of the TCR diversity after V (D) J recombination is in the region of $>10^{11}$ - 10^{15} different specificities (Sewell, 2012; Robins *et al.*, 2009). Now the question is whether the theoretical estimate is in line with the actual receptor diversity in the body? No conclusive studies have fully estimated this diversity; however it is possible that using next generation deep sequencing, one day the complete TCR CDR diversity will be assessed.

The TCR is only one component in the functionality of T cells. The abundance of co-receptor molecules and adhesion molecules also contribute towards the fine tuning of

the repertoire within the body during the course of antigen exposure (Chen and Flies, 2013). The allelic polymorphisms found in the MHC molecules also influence the preferential expansion or deletion of certain T cell populations (Walser-Kuntz *et al.,* 1995). Unlike antibody diversification, which is mediated by the affinity of antigens, the T cell populations undergo a different mode of functional diversification which is known as avidity maturation (von Essen *et al*., 2012) (discussed in chapter 2). This process of avidity maturation will mainly diversify the functionality of T cells by keeping all other factors identical for a given population of antigen specific T cell (Slifka and Whitton, 2001). In other words, a T cell population having the same TCR will have diverse proliferative, cytokine and chemokine secretion capability. How this status of functional diversity is achieved is a poorly understood and the genetic and epigenetic factors governing these processes are yet to be dissected.

TCR receptor is an 'anticipatory receptor' with an assumption that the diversity established in the above mentioned processes are good enough to cover all antigenic peptides generated during one's life time (Lo and Allen, 2013). Studies have argued that the maximum available TCR diversity is not sufficient to offer protection against all the possible antigenic peptide fragments produced within the body if it operates in a strict key and lock mechanism (Wooldridge *et al.*, 2011). So a degree of cross-reactivity (termed as TCR promiscuity) in the T cell repertoire adds flexibility to T cells for recognising not exactly but similar epitopes (Wooldridge *et al.*, 2011). The idea of TCR promiscuity is not firmly established yet, but ample evidence is available for the existence of vast cross reactivity in studies conducted with T cell clones in the laboratory. The extent of this cross reactivity in the living organisms has to be established before promiscuity can be considered as an additional mechanism for TCR diversification. For the purpose of this thesis the main literature discussion will be limited to T cells. However, the similarities and contrasts between these two arms of the immune system will be mentioned as and when a cross comparison is required.

1.1.2.4 Mechanism of T cell mediated immunity

T cell immunity is mediated by two structurally and functionally different sets of T lymphocytes known as $CD8^+$ and $CD4^+$ T lymphocytes (Janeway *et al.*, 2001; Kindt *et al*., 2007). The T cell pool which is present in the periphery after positive thymic selection comprises 'naïve T cells' which are always patrolling through the circulatory

and lymphatic system of the body (Kindt *et al.,* 2007). A class of myeloid lineage cells known as dendritic cells take up antigen from the infected site, and present it to the T cell pool after undergoing antigen processing pathways in the secondary lymphoid organs (Kindt *et al.,* 2007). This action by dendritic cells is still believed to be the central link of T cell mediated immunity and due to the nature of their antigen processing machinery and their ability to stimulate T cells, they often portrayed as professional antigen presenting cells along with macrophages and B cells. (Sprent, 1995). Once the selection of a suitable receptor bearing T cell is established from the available naïve pool, they expand clonally and produce large numbers of the effector populations with other co-stimulatory signals received from the dendritic cells. During clonal expansion both $CD4^+$ and $CD8^+$ T cells can develop into an effector population (capable of implementing the effector functions immediately), or as a memory population (which is known as central T cell memory or TCM) (Zhang and Bevan, 2011). The effector cells rapidly mobilise towards the region of infection and clear the antigen immediately by their cytotoxic molecules such as granzymes and perforins (Janeway *et al.,* 2001). These effector cells can be further developed into an effector memory population (TEM) after pathogen clearance (Williams and Bevan, 2007). All these cells can be differentiated by the presence of different markers on their surface (Table 1.1).

Table: 1.1: Phenotypic differentiation of different functional classes of T cells based on the expression pattern of surface and effector molecules.

Marker	Naïve	Effector	TCM	TEM
CD45RO	+	+++	+	+++
CD45RA	+++	-	++	+
CCR7	+++	-	+++	+/-
CD62L	+++	-	+++	+/-
CD95	+/-	+++	+/-	++
GranzymeB	-	+++	-	+/-
CD25	-	+	-	-
CD28	++	-	+++	+
CD127	++	-	++	+

In T cells, functional diversity is more prevalent in $CD4^+$ T cells. Depending on the functional cytokine profile, $CD4^+$ T cells (helper T cells) are divided in to either TH1 or

TH2 subtypes (Mosmann *et al.*, 2009). The TH1 group mainly secrete pro-inflammatory cytokines such as interferon- γ (IFN-γ), IL-2, and TNF-α. TH2 cells mainly secrete IL-4, 5, 6, 10, and 13 are mainly involved in non-inflammatory mediated response (Yamane and Paul, 2013). There are further subdivisions depending on their functional profile as measured by cytokine signature. This polarisation is mainly shaped by the cytokine milieu and the transcription factors (Zygmunt, Veldhoen, 2011) in the region of antigen exposure which is summarised in Figure 1.2. TH-1 is involved in the cell mediated immunity and TH-2 is associated with humoral immunity.

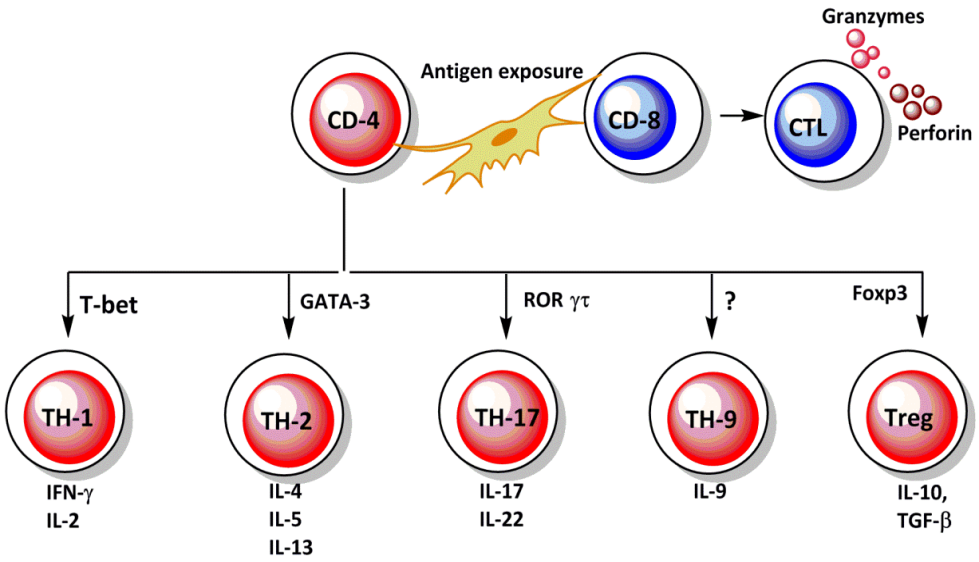

Figure.1.2: Functional diversity of T cells up on antigen stimulation. $CD4^+$ cells can be differentiated into different functional phenotypes depending on the upregulation of transcription factors. Exposure of a naïve T cells to antigen presenting cell results in the generation of CTLs that are capable of performing cytotoxicity function

The direct cytotoxicity of T cells is mainly carried out by antigen activated $CD8^+$ T cells, and is mediated by two major pathways (Janeway *et al.*, 2001; Kindt *et al.*, 2007). Granzyme and perforin mediated (Ca^{2+} dependent) apoptosis, and FASL and FAS mediated apoptosis (Ca^{2+} independent). Once the TCR of an effector cell is engaged with the cognate antigen presented on a MHC class I molecule on the infected cell, it activates the granzyme perforin synthesis pathway and results in the secretion of cytotoxic molecules towards the target cells. As the name indicates perforins are capable of making numerous perforations on the surface of the plasma membrane which

results in an easy access route for the granzymes, a serine protease into the cytoplasm of the target cells. (Figure 1.3)

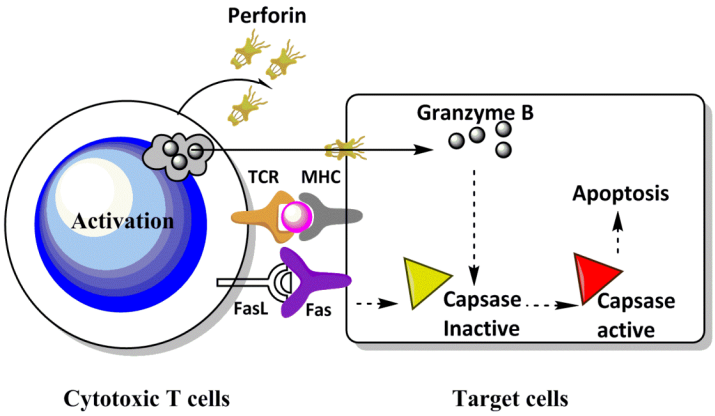

Figure.1.3: Mechanism of CTL mediated cytotoxicity: recognition of a cognate antigen by a CTL leads to the activation and release of effector molecules. Two mechanisms of cytotoxicity is known so far, first one is perforin and granzyme mediated and second is FASL and FAS mediated.

1.1.2.5 Antigen processing and presentation a key factor in T cell mediated immunity

Recognition of peptide antigens mounted on a special molecule known as MHC is a prerequisite for T cell mediated immunity (Janeway *et al.*, 2001). There are two major classes of MHC present in human and they are referred to as MHC class I and II (or human leukocyte antigen, HLA). The MHC class I molecule is a heterodimer made up of a glycoprotein heavy chain and a protein light chain expressed on almost all the nucleated cells with varying levels of expression (Blum *et al*., 2013). MHC class II is only present on professional antigen presenting cells such as macrophages, dendritic cells and B cells. MHC class I is mainly involved in the processing and presentation of endogenous antigens and MHC class II is mainly involved in the clearing up of extracellular pathogens. So for that matter both of the molecules employed different antigen processing pathways known as endogenous pathways for MHC class I molecules and exogenous pathways for MHC class II molecules (Blum *et al*., 2013; Basler *et al*., 2013). Class I and Class II protein chains are synthesised in the cytoplasm and transferred into the rough endoplasmic reticulum (RER) for further folding and

antigen loading (Janeway *et al.*, 2001). MHC class I and II pathways are explained in Figure 1.4.

Even though the term endogenous processing and presentation (also known as direct presentation) are applied to indicate the processing of the endogenously derived antigens in all the MHC expressing cells, sometimes some of the APCs are capable of feeding the peptides derived from exogenous pathways into the class I pathway (cross presentation) (Nierkens *et al.*, 2013). Cross presentation is more prevalent in the dendritic cells and this is of importance in tumour immunology in which dead and inflamed tumour tissues can be mopped up by scavenging dendritic cells, processed and presented to the class I pathway where they potentially trigger both $CD8^+$ and $CD4^+$ arm of the immune system for a better tumour immune surveillance (Brusic *et al*., 2012; Engelhardt *et al*., 2012). Even though this is reported in many cases, the precise mechanism of how cross presentation is happening in what otherwise would be a normal exogenous pathway, still remained to be fully elucidated. The class II pathway is mainly driven by TLR mediated detection and phagocytosis of the exogenous antigens (mostly bacteria and viruses) by macrophages, dendritic cells and B cells (Blanchet and Piguet, 2010). The successful response depends on how early these danger signals (PAMPs and DAMPs) have been picked up by these cells (Kaisho and Akira, 2006).

It is noteworthy that the loading of the peptides onto the MHC molecules has slightly different mechanisms. In the endogenous pathway, antigen loading is carried out by the involvement of TAP and associated molecular chaperones, commonly referred to as peptide loading complexes, whereas in the exogenous pathway, antigen loading is carried outside of rough endoplasmic reticulum (RER) (Leone *et al*., 2013; Hulpke and Tampé, 2013). There is a difference in the peptide binding cleft of both the molecules, in MHC class I, both the sides are closed and it is open for MHC class II molecules. The length of the peptide fragment which can be comfortably loaded on to these two molecules is also different, with a peptide of 8-10 amino acid for MHC class I with anchor residues present at both the ends and 13-18 amino acids for MHC class II with anchor residues distributed possibly throughout the length (Hulpke and Tampé, 2013; van Bleek and Nathenson,

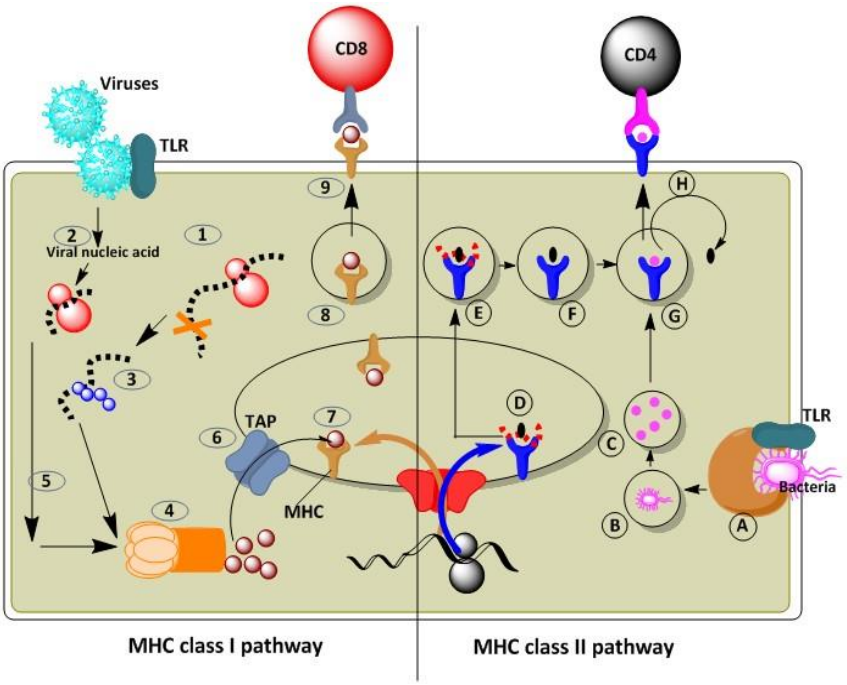

Figure 1.4: The classical antigen processing and presentation pathway. Left panel (endogenous MHC class I pathway). 1, 2 Antigens for this pathway are either endogenously produced a product of DNA transcriptional or mRNA translational error happened in the normal cellular process, or originate from a translated protein product of a viral nucleic acid. 3, 4, 5 the damaged or foreign proteins are immediately tagged by ubiquitin and enter through the proteosomal machinery. 6 The peptide fragments coming out of the proteosomal machinery is then transported in to the rough endoplasmic reticulum with the help of a protein known as transporter associated with antigen processing (TAP). 7. The antigen is now loaded on to a fully folded MHC class I molecule with the help of molecular chaperones calnexin, calreticulin and tapasin. 8, 9 once the peptide is loaded on to the MHC molecule the molecular chaperones dissociates and the peptide MHC complexes leave the RER and transported on to the surface through the golgi complex. Right panel (exogenous MHC class II pathway). A, B Exogenous danger signal is detected by the TLR molecules on its surface and subsequently engulfed by phagocytosis (phagosomes). C. The phagosomes are combined with the lysosome compartment and form the phagolysosomes. D. class II MHC synthesis takes place in a similar fashion as Class I synthesis but are synthesised with an additional invariant chain. This invariant chain prevents unanticipated binding of pepides at very early stages of MHC II formation. E,F,G. The MHC class II depart the RER and pass through the golgi endosome and finally fuse with phagolysosome. As the acidic environment increased in the endosomal compartment the invariant chain is gradually disintegrated to leave only a small fragment in the peptide binding groove known as CLIP (class II associated invariant chain peptide). Finally CLIP is replaced by foreign peptide fragment and move towards the membrane. H. MHC class II is presented on the surface of a professional antigen presenting cell with antigenic peptide mounted on it for $CD4^+$ recognition and expansion.

1993; Rothbard and Gefter, 1991). The deficiency of this antigen processing machinery is a common immune escape mechanism in evading tumour tissue, which will be discussed in the later sections.

1.2 Cancer

Cancer accounts for 13% (7.3 million) of all deaths worldwide in 2008 and it is projected to rise to over 13.1 million by 2030 (WHO, 2013). As with many other developed countries, cancer incidence in the United Kingdom is also on the rise with 521 cases for every 100,000 people (Cancer Research UK, original estimates based on 2010 data and reviewed on December 2012). Cancer can be defined as the anomalous growth of cells developing into a population of cells which can proliferate indefinitely, invade surrounding tissues and spread (metastasise) to distant sites through the blood and the lymphatic system (Ruddon, 2006). Most of cancers originate from single somatic cell and which has acquired many accumulated genetic and epigenetic changes. These changes alter the balance of cellular proliferation and cell death (Ruddon, 2006) and thereby become tumourigenic. Interestingly cancers also contain multiple cell types such as fibroblasts, epithelial cells, cells of innate and adaptive immune system, cells of blood and lymphatic vasculature and mesenchymal cells. This intricate system of cells collectively acts as major cellular supportive machinery and a unique microenvironment for the growth of cancers (de Visser *et al.,* 2006; Hanahan and Weinberg, 2011). Because of this self-contained tissue organisation and the many other immune escape mechanisms that have been developed by the tumours, it is highly difficult to kill every single cancer cell. So for that very reason, attempts to develop very effective cancer vaccines to eradicate cancer still remain a daunting task. However, the broadening of our basic understanding in cancer biology and its interactions with the immune compartment of the body is having a positive impact on the future therapeutic or prophylactic cancer vaccination strategies. The therapies for cancer treatment has been broadly divided into conventional and immunotherapeutics.

1.2.1 Conventional therapies for cancer management

Conventional therapy including surgery, radiotherapy, chemotherapy and hormone treatment represents the mainstay of efficient cancer ‘management’ in clinical settings (Macmillan Cancer Support 2013, “Cancer treatment” [online]). The term

'management' is used here on purpose, since none of the above treatment modalities guarantee complete cure of cancer and the disease can reoccur later on in life.

1.2.1.1 Surgery

The single most important cancer management in patients and its origins trace back to 2000 BC where the ancient Hindus were known to remove abnormal growth of the body (Encyclopædia Britannica 2013, "history of medicine" [Online]). With the latest advancement in surgical procedures it is now possible to precisely locate and remove a tumour with utmost precision. Surgical procedures can be performed with neoadjuvant (Yoshioka *et al.*, 2013) or adjuvant settings (Kruse *et al.*, 2013); in the former the chemotherapeutic agents is given to the patients prior to the surgery to shrink or reduce the tumours and in the latter the same agents are given after the surgical removal of the cancerous tissue to prevent the re-emergence of cancer cells post-surgery (Redden and Fuhrman, 2013; Kruse *et al.*, 2013). Minimal invasive surgery is a common methodology now, where the surgeon uses minor incision in the body to reach the malignant tissues and precisely remove the cancer tissues without destroying much of its normal surrounding tissues. This methodology is highly supported by the advances in cancer imaging and computer modelling (Bharathan *et al.*, 2013; Lamadé *et al*., 2002) to precisely help surgeons to define positive margins around the tumour and use a robotic guided mechanical arm (Bharathan *et al*., 2013) to reach and excise the tumours with accuracy (Venkat and Guerrero, 2013; Randall and Gilbert, 2013). Surgical procedures can be performed in various situations such as preventive, diagnostic, staging, curative and palliative depending on the patient clinical status (Meijers-Heijboer *et al.*, 2003, Khankan, and Al-Muaikeel, 2012). Three major surgical procedures used for the management of the most common cancers are lumpectomy and mastectomy in breast cancer and radical prostatectomy in prostate cancer patients (Karam, 2013; Healy and Gomella, 2013).

1.2.1.2 Chemotherapy

Chemotherapy is the second most important conventional anti-cancer therapy which can be used either on its own or in conjunction with any other conventional therapies. The term chemotherapeutic agents is broadly used to refer to the substances which can interfere with the growth of cancer cells (Skeel, 2007; Cancer Research UK 2013, "Chemotherapy and cancer" [online]). The specificity of the chemotherapeutic drug is

mainly is confined to their ability to target rapidly dividing cells, and this often causes deleterious side effects in the patients (Skeel, 2007). Haematopoietic cells and hair follicles also divide rapidly and are therefore also affected by this treatment, thereby resulting in the loss of hair and decreased production of immune cells in the patients. Chemotherapeutic agents are classified into several categories depending on their chemical properties and mechanism of action, for example alkylating agents (covalently binds to DNA and leads to the apoptosis of the cells), anti-metabolites (nucleic acid base analogues which resemble nucleosides or bases are incorporated into the DNA strand during the DNA synthesis), microtubule inhibitors (disrupt the assembly and disassembly of the microtubule formation during cell cycle and hence prevent mitosis), topoisomerase inhibitors (inhibit the DNA topoisomerase activity and disrupt the transcription and translational machinery) and other agents (mainly antibiotics having a variety of inhibitory properties in cancer cells) (Skeel, 2007). Chemotherapy also can be used as an adjuvant setting in many cancers in conjunction with other conventional treatment modalities. Major FDA approved chemotherapeutic drugs used in the treatment of cancers are epirubicin (breast cancer), methotrexate (melanoma, breast, head and neck, or lung cancer), docetaxel (breast, prostate and non-small cell lung cancers) cyclophosphamide (leukaemia, lymphoma, lung and breast cancer), mitomycin (stomach, breast, oesophagus and bladder cancers), doxorubicin (various), plaxitaxel (ovarian, breast, non-small cell lung cancer), mitoxantrone (breast, prostate, myeloid leukaemia and non-Hodgkin lymphoma) (Macmillan Cancer Support 2013, "Individual chemotherapy drugs" [Online]).

1.2.1.3 Radiation

Another important conventional therapeutic strategy involves the use of strong, deleterious ionising radiation (mainly photons) to target neoplastic cells (Greco and Zelefsky, 2000). Ionising radiations carefully delivered to the tumour mass can cause two types of DNA damages. Single and double stranded breaks can be induced either directly by ionising the DNA bases or indirectly by creating free radicals that are capable of DNA damage. Several different types of conventional radiotherapy are applied: conventional external beam radiations (Halperin, and Burger, 1985), 3-dimensional conformal radiation therapy (Creak *et al*., 2013), intensity-modulated radiation therapy (Poon *et al*., 2013) and brachytherapy (Park *et al*., 2013).

1.2.1.4 Hormone therapy

The last conventional cancer treatment is hormone therapy. Some cancers such as prostate, ovarian and breast are dependent on hormones for their rapid growth (Jordan and Furr, 2002). Two of the hormones which have a profound effect on tumour growth are oestrogen and testosterone in breast and prostate cancer respectively (Jordan and Furr, 2002). Hormone treatments for these cancers are generally focussed on shutting down the hormone production or blocking the hormone receptors in the tumour. The most widely used hormonal drugs are tamoxifen, zoladex and faslodex in breast cancer (Breast Cancer Care 2013, "Hormone therapy" [online]) and the major hormone therapeutics for prostate cancer are leuprorelin, zoladex, histerillin (leutinising hormone releasing hormone agonist), flutamide, bicalutamide (anti-androgenic) (Cancer Research UK 2012, "Hormone therapy for prostate cancer" [Online]).

1.2.2 The origin and evolution of T cell based immunotherapy

The idea that the immune system is able to fight cancerous cells was first proposed around two centuries ago. The very first recorded observation probably dated back to the 1850's, where German doctors noticed the rapid shrinkage of streptococcal infected tumours. This was followed by William Coley's (1862–1936) famous cancer vaccine nicknamed '*Coley's toxin'*, which used a crude preparation of *Streptococcus pyogenes* to treat cancer patients on a large scale. At the beginning of 20th century, Nobel laureate Elie Metchnikoff and Paul Ehrlich first suggested that the immune system suppresses the growth of tumours (Blair & Cook, 2008), however it took more than 50 years before this was fully acknowledged after the pioneering concept of 'Immunosurveillance' was proposed by Burnett (Burnet, 1970). Frank Macfarlane Burnet (1899–1985) was instrumental in putting the idea of the immune system's capacity to fight against cancer on a firm ground during the 1970's. Burnet proposed the vital role of lymphocytes in the detection and rejection of transformed malignant cell in human body. However, this was later abandoned as a consequence of experiments conducted in athymic nude mice, which showed similar disease incidence in comparison to the wild type animals and suggested that T cells did not have any effect on tumour control (Stutman 1979; Parish, 2003). The real breakthrough came in the 1980s with the observations that some auto-reactive T cells could be demonstrated to escape thymic selection (Sakai *et al*., 1979) and could be recruited later to combat the transformed cells. This led to the dawn of a

new era of T cell based immunotherapy (Topalian and Rosenberg, 1990) which was further fuelled by the identification of tumour associated antigens (TAAs). The first proof of an effective use of T cell based immunotherapy was reported by van Pel & Boon (1982) who used MAGE-1 and demonstrated that T cells located in the periphery are capable of recognising specific proteins expressed on the tumour cells. This opened up novel strategies for targeting specific tumour antigens with T cell mediated immunity induced by vaccination. The important scientists and the landmark discoveries which put immunology in the limelight for fighting cancer are given in Figure 1.5

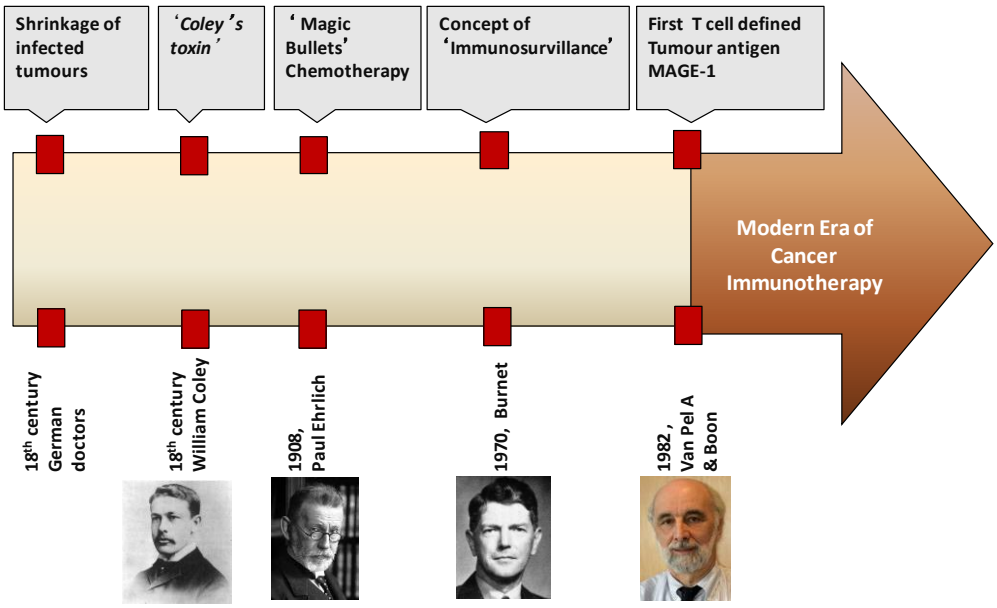

Figure 1.5: The key milestones in the origin and evolution of T cell based cancer immunotherapy over past 200 years

1.2.3 Tumour antigens as a target for T cell based immunotherapy

1.2.3.1 Tumour associated antigens (TAAs)

The formulation of a vaccine which would stimulate the adaptive immune system to control tumour growth is the holy grail of tumour immunologists. The first proof of an effective use of T cell based immunotherapy was reported by van Pel & Boon (1982) with MAGE-1 - they demonstrated that T cells which are capable of recognising specific proteins expressed on the tumour cells (tumour associated antigens, TAAs) can be observed in the periphery. This opened up a novel strategy for targeting specific

tumour antigens with T cell mediated immunity induced by vaccination. One of the problems using these TAAs as an effective target for immunotherapy is their specificity, since most of them are self-proteins and constitutively expressed in almost every cell. As a consequence, the effector cells (T cells) do not recognise them very well because of the phenomenon known as immunological tolerance, which will be discussed later in more details. In short, cancer is a disease which arises from self-mutated cells and in the majority of cases the biochemical alterations in cancer cells are subtle when compared to their normal healthy counterparts. So the successful immune reaction should be capable of recognising these subtle alterations as an altered self and the molecular changes as lethal changes prior to mounting a successful immune response. From a cancer vaccination point of view, it is essential to identify these altered proteins or antigens before devising potential therapeutic strategies. A comprehensive account of major approaches used for the identification of human tumour antigens was reviewed in Li, *et al*., 2005; Mathieu, *et al*., 2009, Coulie, *et al.,* 2014.

1.2.3.2 Classes of tumour associated antigens (TAAs)

Based on the expression status and their function, TAAs are classified into different categories. The first one is "mutated antigens",- these antigens originate from mutations occurring during tumour development, so have huge limitations. Each of these mutations is patient or tumour specific so large-scale patient specific mutation screening is required prior to device any immunotherapy interventions. The second class of TAA are "shared tumour antigens", which are specifically expressed in different kind of tumours and not expressed in the normal tissues. An example includes the cancer testis antigens (CT antigens) which expression is restricted to cancer tissue, placental trophoblast and testicular tissue. Since placental trophoblast and testicular tissues do not express MHC class I antigens they are immunoprivileged and hence immune to T cell mediated immunity. The third group of TAA includes "differentiation antigens" which are expressed in the cancer tissue as well as the corresponding normal tissues. Tyrosinase is an example of a differentiation antigen since it is present in normal melanocytes and melanoma. One of the potential limitations of this type of antigens is autoimmunity. The final group of TAAs are "overexpressed antigens" which have a normal expression in normal tissues, but are overexpressed in malignant tissues. Well studied examples of all these tumour classes are given in Table 1.2.

Table: 1.2. Examples of Tumour associate antigens identified in different classes

Gene	Tumour	HLA	Peptide	Reference
1. Mutated antigens				
BCR-ABL	chronic myeloid leukaemia	A2	SS**KALQRPV***	Yotnda *et al.*, 1998
beta-catenin	Melanoma	A24	SYLDSGIH**F***	Robbins *et al.*, 1996
FLT3-ITD	acute myelogenous leukaemia	A1	YVDFREYEY**Y***	Graf *et al.*, 2007
N-ras	Melanoma	A1	ILDTAG**R**EEY*	Linard *et al.*, 2002
2. Shared tumour specific antigens				
LAGE-1	Shared	A2	MLMAQEALAFL	Aarnoudse *et al.*, 1999
MAGE-A1	Shared	A2	KVLEYVIKV	Ottaviani *et al.*, 2005
NY-ESO-1	Shared	B7	APRGPHGGAASGL	Ebert *et al.*, 2009
SAGE	Shared	A24	LYATVIHDI	Miyahara *et al.*, 2005
3. Differentiation antigen				
gp100	Melanoma	A2	KTWGQYWQV	Bakker *et al.*, 1995
Kallikrein 4	prostate cancer	DP4	SVSESDTIRSISIAS	Hural *et al.*, 2002
PAP	prostate cancer	A2	FLFLLFFWL	Olson *et al.*, 2010
TRP-2	Melanoma	A2	SVYDFFVWL	Kawakami *et al.*, 1998
4. Overexpressed antigens				
HER-2 / neu	ubiquitous (low level)	A2	KIFGSLAFL	Fisk *et al.*, 1995
alpha-fetoprotein	Liver	A2	FMNKFIYEI	Pichard *et al.*, 2008
p53	ubiquitous (low level)	A2	RMPEAAPPV	Barfoed *et al.*, 2000
survivin	Ubiquitous	A2	ELTLGEFLKL	Schmidt *et al.*, 2003
Telomerase	testis, thymus, lymph	A2	RLVDDFLLV	Minev *et al*, 2000

* Red coloured letters indicates the mutations

Although all the antigens mentioned in the Table 1.2 have been shown to be able to generate immune responses in patients, the efficacy of most of these vaccines remains limited. The primary reason for this is the tolerogenic properties that are exerted by tumour cells (Caspi, 2008). In other words, immunotherapy and autoimmunity share a common conceptual ground in the recognition of their target molecules. By using immunotherapy we are altering the fine balance of self-tolerance of T cells and this may lead to catastrophic outcomes such as spontaneous auto-immunity while on treatment. In order to completely understand the fine balance between the auto-immunity and

cancer immunotherapy, one must understand how these cells are generated and educated in the thymus - this is discussed in Section 2.1.

1.2.4 Cancer Immunotherapy

Immunotherapy is a treatment modality which fine tunes our own immune system to recognise and eliminate cancer cells. In the past few decades progress in the field of cancer immunotherapy has led to the production and use of promising therapeutic vaccines such as rituximab (Marcus & Hagenbeek, 2007) in non-Hodgkin's lymphoma and trastuzumab (Hudis, 2007) in breast cancer. However, T cell based therapy has still not achieved its final goal of effective protection for various reasons such as its high similarity to the natural protein within the body, other immune mechanisms operating in the tumour microenvironment etc. Nonetheless, different tumour associated antigens have been identified in various cancers and these could effectively trigger cytotoxic T lymphocyte (CTL) responses in cancer patients (Dougan & Dranoff, 2009). The broad overview of the immune system fighting cancer is depicted in Figure 1.6. The inward investigation into the unsatisfactory performances of many vaccines despite their ability to generate good T cell responses led to the resurgence of other areas of tumour biology, including the study of the tumour microenvironment (TME), immune suppression and tumour immune escape mechanisms. Studies conducted by various groups have identified a range of causative components and they are of profound importance to tumour vaccination strategies, as a better understanding of these will help to increase vaccine efficacy. Therefore, it is essential to mention the suppression mechanism as a part of this literature review in order to fully understand the current status of T cell mediated immune therapy.

1.2.4.1 The three E's of immunosurveillance, the central theme of cancer immunotherapy

A recent revisiting of the concept of cancer immunosurveillance (Zitvogel, *et al*., 2006) has led to a better understanding that the immune system can recognise mutated pre-cancerous cells and 'eliminate' them before the tumour becomes a clinically detectable condition. However, a tumour might bypass these mechanisms by selecting less immunogenic variants of the mutant cells (immunoselection) or by active suppression of the immune system (immunosubversion). Therefore, it is essential to "boost" the immune system with combinatorial vaccination strategies along with the currently

available immunotherapeutic approaches. According to the recent immunoediting hypothesis of Spiotto *et al*.

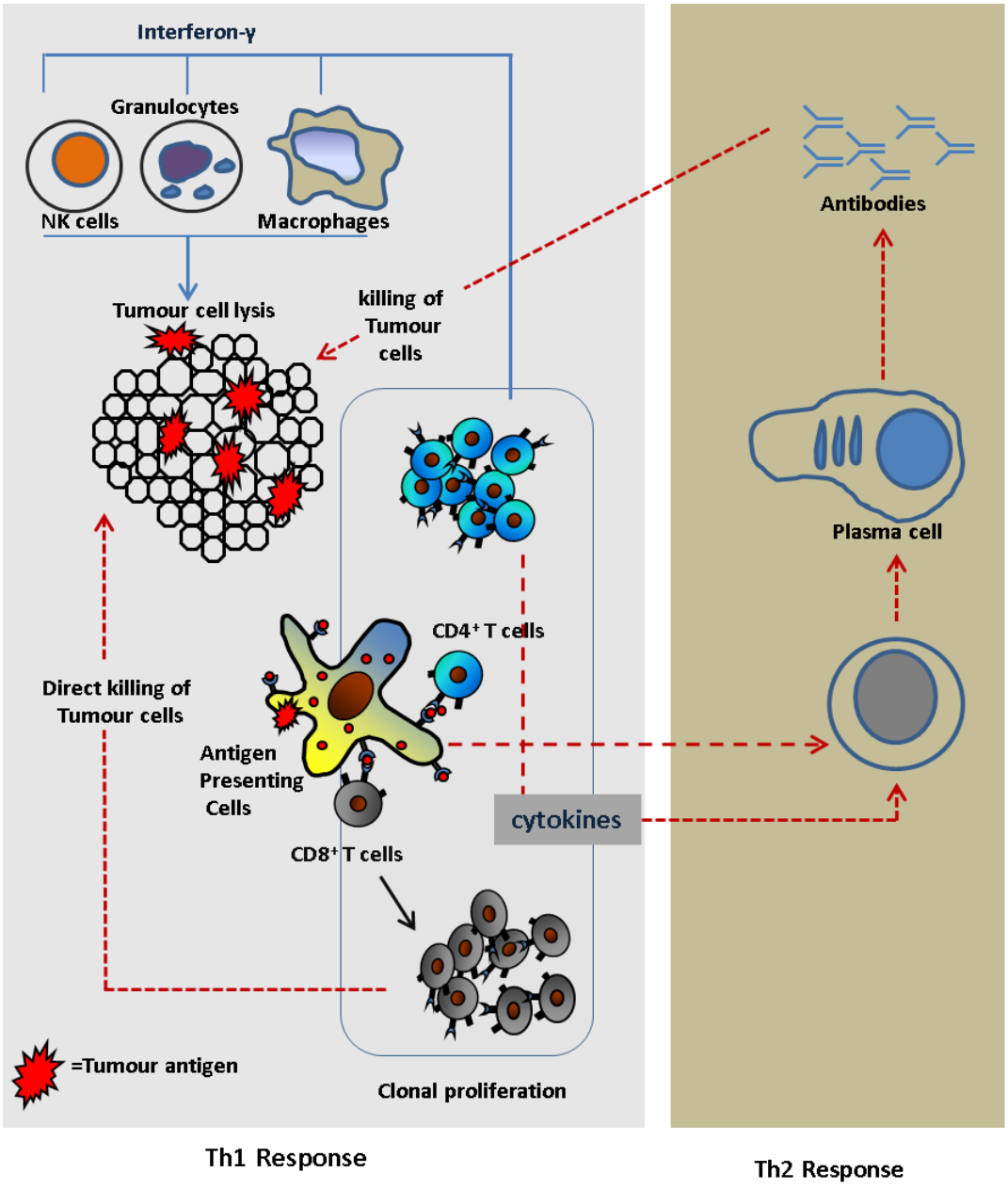

Figure 1.6: A simplified view of the immune system combating a tumour. The most effective way of killing the tumour is $CD8^+$ (CTLs) mediated killing. Interferon- γ secreting $CD4^+$ helper cells also recruit NK cells, granulocytes and macrophages towards the tumour environment and these cells interact with the tumour stroma and interfere with the angiogenesis processes. $CD4^+$ cells are also critical for the maintenance of memory $CD8^+$ cells, so at the time of reoccurrences of the tumour these cells can be sensitised to re-emerging tumours much more quickly, this is known as TH1 response. In the TH2 response antibodies are produced against the tumour antigens and give a much needed humoral immunity support for combating the tumour.

(2004) a perfect balance exists between the immune system and tumour progression, but when that balance is perturbed the immune system can no longer defend itself from tumourigenesis. The strategies employed by the tumour also facilitate the process of keeping the immune system at bay (Figure 1.7.). So the three E's: elimination, equilibrium and escape are commonly known as the mechanisms involved in the process of tumourigenesis.

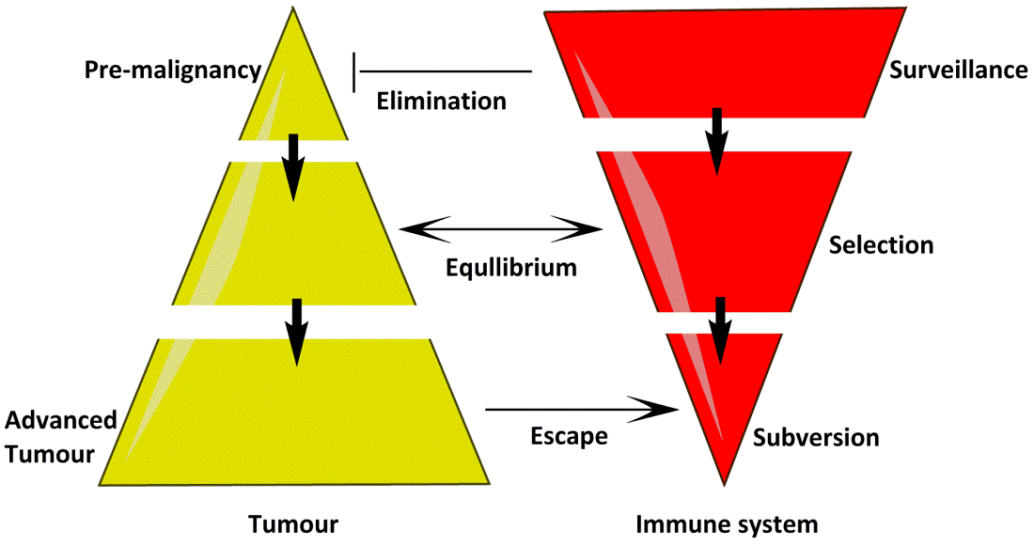

Figure 1.7: There are three separate phases in the crosstalk between tumour progression and immune system defence. In the first phase, the immune system recognises and destroys cancer cells, this leads to a state of equilibrium between the tumour cells and immune cells. During this phase, tumours select less immunogenic variants of the cancer cells and ultimately break the equilibrium and leads to clinically apparent cancers. The yellowish green pyramid indicates tumour progression and the red inverted pyramid represents immune system progression.

(Figure adapted from Zitvogel *et al*, 2006)

1.2.4.2 Tumour escapes mechanisms and immune suppression

The renaissance of Burnet's theory of immunosurveillance in the past few decades has been mainly fuelled by the observations made in patients and laboratory animals, that an immune system can check the growth of an 'immunogenic' tumour. The term 'immunogenic' is not always associated with tumours because of the selection of non-immunogenic variants of tumour cells as discussed above. However, the failure of many clinical trials involving cancer immunotherapy created a re-examination of mechanisms under which tumour specific CTLs execute their effector functions and the factors which prevent it. Over the past decades many tumour derived factors have been identified and they have been broadly classified as tumour micro environment (the collective tumour ecosystem) and tumour cells (at the cellular level).

1.2.4.2.1 The 'tumour microenvironment' and immune suppression

Among many factors identified, the immune suppressive environment that the tumour is creating, during the process of tumour evolution, has been identified as the single major contributing factor for the failures of immunotherapy (Becker *et al.*, 2013). As discussed in the previous section, a tumour is a self-contained system of organisation including cancerous cells, noncancerous stromal cells and most of the immune cells within the tumour environment. This unique ecosystem of cells capable of producing various secretory and cell surface signalling molecules and ligands has been identified to have both pro and anti-tumour characteristics. A classic example of such a molecule is TGF-β, which exhibits both tumour suppressive and tumour promoting activity (Smith *et al.*, 2012; Bachman & Park, 2005). Studies have shown that overexpression of TGF-β leads to increased invasiveness and metastasis by promoting epithelial to mesenchymal transition (EMT) in primary tumour (Miyazono, 2009) which has been attributed to the influence of TGF-β in a SMAD mediation transcriptional regulation in cancer cells (Valcourt *et al.*, 2005). Up regulation of TGF-β has also been reported to cause chemoresistance in colon cancer (Brunen *et al.*, 2013) and also to be negatively influence the immune cells by inhibiting the differentiation of Th1 and Th17 responses

by dendritic cells (Min *et al*., 2012), as well as directly inhibiting cellular cytotoxicity by affecting the expression of cytolytic factors such as granzyme A & B, perforin, Fas ligand and interferon-γ (Thomas, & Massagué, 2005). TGF-β production can also be increased by the production of IL-13 by NKT cells (Terabe *et al*., 2003) which in turn can inhibit CTLs. These molecules can also prevent the maturation of dendritic cells and thereby prevent the complete activation of CTLs which may potentially become tolerogenic (Torres-Aguilar *et al.*, 2010). Targeting TGF-β and its upstream molecules has been found to be a useful clinical strategy for regaining the immune surveillance mechanisms (Fichtner-Feigl, 2008; Takaku *et al.*, 2010).

Another important potent immune suppression molecule which is produced by tumour tissue is IL-10 (de Vries, 1995). IL-10 can down regulate the expression of MHC and other costimulatory molecules on the antigen presenting cells (Halak *et al.*, 1999; Joss *et al*., 2000; Liu *et al.*, 2013) and also directly check the proliferation of T cells. However, a recent study in murine models has shown an increased activation and expansion of tumour residing $CD8^+$ T cells in an IL-10 expressing tumour microenvironment suggesting the potential proinflammatory role of IL-10 in tumour microenvironment (Emmerich *et al*., 2012). Prostaglandin E2 is a small molecule secreted by the tumour cells which has an effect on a wide variety of immune cells. It inhibits the cytotoxic activity of NK cells (Goto *et al.*, 1983), the function of granulocytes (Ballinger *et al*., 2006) and the phagocytic capacity of macrophages (Aronoff *et al.*, 2004). Another immunomodulatory molecule present in the tumour microenvironment is Indoleamine 2, 3-dioxygenase (IDO), a product of tryptophan catabolism. This molecule is expressed by dendritic cells and also by tumour cells (Munn *et al*., 2004). Over-expression of IDO in various cancers has been correlated with poor prognostics (Smith *et al*., 2012).

Other substances produced in the microenvironment include galectin-1, gangliosides (Deng *et al.*, 2000), CCL22 and arginase (Rabinovich *et al.*, 2007). Galactin, a proteoglycan produced by many tumour tissues and stroma is positively correlated with tumour progressions and is now considered as an ideal target for cancer therapy (Laderach *et al*., 2013). It is also believed that galactin inhibits of CTL function in the tumour by promoting apoptosis in those cells (Perillo *et al*., 1995). CCL22 and CCL17 are two chemoattractants produced by tumours and other myeloid cells in the

microenvironment which are playing a key role in the trafficking of regulatory T cells into the tumours (Peng *et al.*, 2012; Rawal *et al*., 2013).

'Immune cells' which are supposed to be the master controllers of the tumour growth can sometimes act as a tumour promoter (Schreiber, 2011). Two important classes of immune suppressive cells present in the tumour microenvironment are regulatory T (Treg) cells and myeloid derived suppressor cells (MDSCs). MDSCs (Young *et al.*, 1987) are cells of myeloid origin, characterised $CD11b^{+}Gr1^{+}$ phenotype in mice, $Lin^{-}HLA^{-}DR^{-}CD33^{+}$ or $CD11b^{+}CD14^{-}CD33^{+}$ phenotype in humans (Gabrilovich and Nagaraj, 2009). They develop during myelopoiesis in the bone marrow and subsequently migrate to different peripheral organs for immune surveillance. In cancer patients, these cells also migrate to the tumour site. In the peripheral lymphoid organ these cells present the tumour antigens to the effector T cells, however they can produce large quantities of reactive oxygen species (Kusmartsev *et al.*, 2004), nitrogen species (Lu and Gabrilovich, 2012) and nitrites such as peroxynitrite (Gabrilovich and Nagaraj, 2009). Interaction of MDSCs with T cells leads to unresponsiveness of T cells (Nagaraj *et al*., 2008). MDSCs are highly plastic and respond to various stimuli which they receive from the tumour microenvironment. Once they enter the tumour, the tumour microenvironment prevents them from maturing and they become immunosuppressive and are broadly known as tumour-associated macrophages (TAMs) (Gabrilovich and Nagaraj, 2009).

TAMs can secrete a myriad of immunosuppressive cytokines such as IL-10, IL-1, IL-6, and TGF-β which are known to have various CTL suppressive activity (Gabrilovich and Nagaraj, 2009; Sica and Mantovani, 2012). One of the well-studied immune suppression mechanisms is metabolic depletion of L-arginine, an essential amino acid present in the tumour microenvironment. TAM produces enzyme arginase in large quantities which catabolise the L-arginine in the tumour microenvironment leading to an increased depletion of this essential amino acid required for optimal T cell proliferation (Munder, 2009). Another mechanism of tumour suppression by MDSCs is the generation of reactive oxygen species within the tumour microenvironment. They can also secrete VEGF and matrix metalloproteinases (MMP) which help tissue repair and angiogenesis, and promote tumour growth (Gabrilovich and Nagaraj, 2009). These cells can adapt to their environment and are very plastic, for this reason it is believed

that many subsets of TAMs are present within the tumour microenvironment, with different roles as tumour suppressors or promoters.

Regulatory T cells (Treg) are $CD4^+$ cells which constitutively express high levels of CD25 on the cell surface molecule and the forkhead box P3 (FoxP3) transcription factor. This population of cells is naturally present in the periphery of normal individuals, but their prevalence is increased in the blood of patients with cancer and also in their tumours. The primary role of these cells is in the maintenance of peripheral immune tolerance and the prevention of autoimmunity in healthy individuals (Goodman *et al*., 2012). There are many ways that a Treg cell can be recruited or expanded in tumours. The first mechanism involves the trafficking of naturally occurring peripheral Treg cells into the tumour. This is driven by CCL17 and CCL22 produced by the tumour and which are ligands for CCR4 and CCR8 receptors present on the Treg cells (Mizukami *et al*., 2008). The presence of immature antigen presenting cells such as MDSCs (especially the M-2 polarised) can trigger the recruitment and expansion of Treg cells in the tumour microenvironment (Biswas and Mantovani, 2010). Several other factors such as the presence of TGF-β (Huber et al., 2004) and IDO (Curti et al., 2007) produced by the tumour cells or the TAMs have been reported to play a favourable role in the Treg cell expansion, especially in the induced Treg cell compartment (Huber et al., 2009). The presence of these cells in the tumour microenvironment has been reported to be associated with poor prognosis in patients with prostate (Flammiger, 2013), breast (Decker, 2012) and many other cancers. Immune suppressive mechanisms in the tumour microenvironment are depicted in Figure 1.8.

1.2.4.2.2 Prevention of tumour killing at the 'point of contact'

Cytotoxic T cells (CTLs) are capable of mounting an efficient anti-tumour effect by direct killing of antigen bearing tumour cells. Even if the tumour microenvironment is favourable for the infiltration, expansion and killing by CTLs, the tumour can directly impair their functionality at the 'point of contact' by carefully orchestrating the expression of inhibitory signals and apoptotic molecules on their surface. This represents another defence mechanism which the tumour can use to evade attack by adaptive immunity (Kerkar and Restifo, 2012; Rabinovich *et al.,* 2007).

MHC down regulation and the defects in antigen processing and presentation pathways are the primary mechanism of immune evasion (Seliger et al., 1998; 2000). MHC down

regulation results in the avoidance of CTL detection by the tumours since this process demands the mounting of antigenic epitopes on the surface of appropriate MHC class I molecules. Some of the defects in MHC molecule expression are reversible with

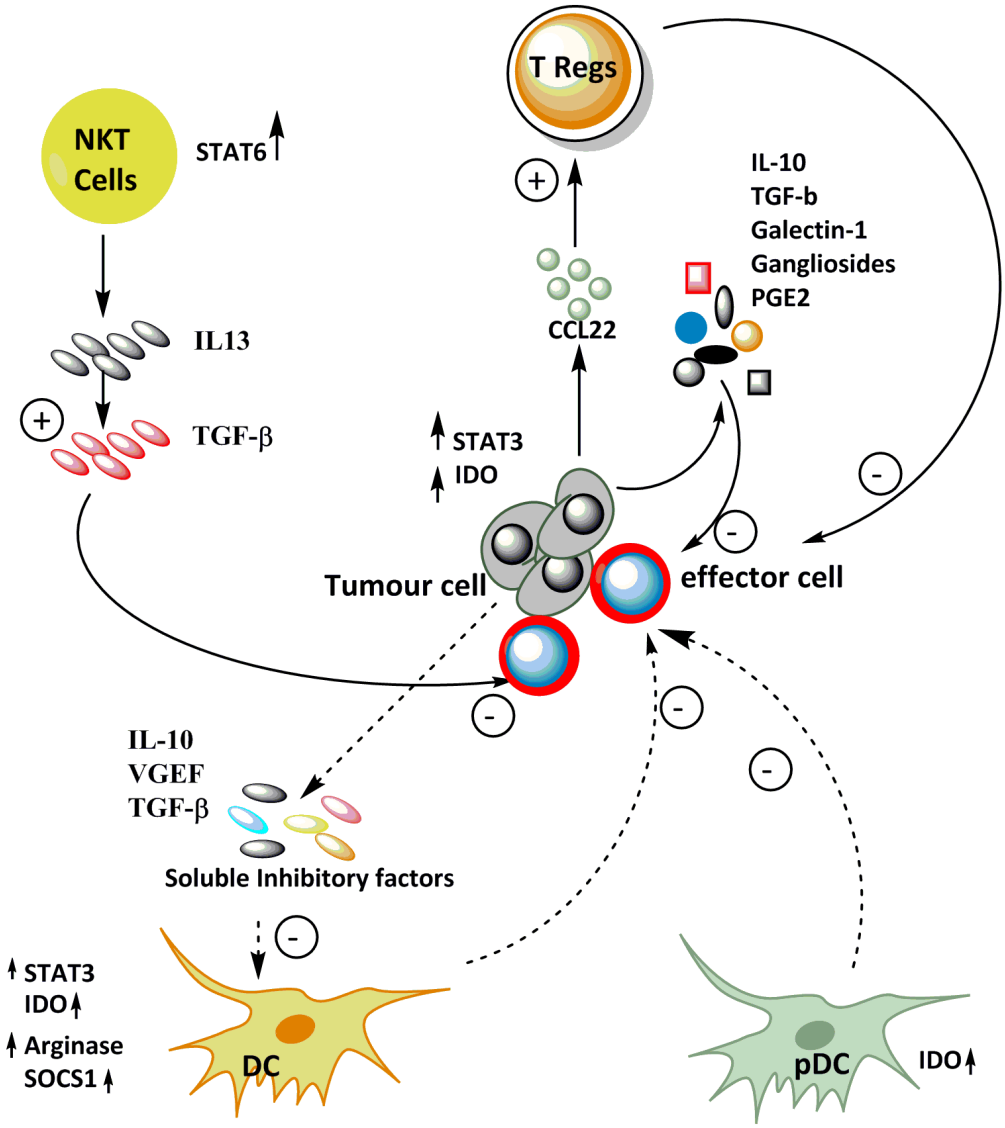

Figure 1.8: Tumour and tumour microenvironment mediated immune evasion is a hallmark of immune resistant tumours. '+' signs indicate a positive influence and '-' sign indicate a negative influence on the target. The substances secreted by the tumour such as IL-10, TGF-β, galectin, ganglioside, PGE2, has a direct negative effect on CTLs, whereas the secretion of VEGF, IL-10 and TGF-β will prevent the maturation of tumour infiltrated dendritic cells which in turn trigger their immune suppressive activity on CTLs by upregulating STAT3, IDO, arginase and SOCS1. Another class of dendritic cells known as plasmacytoid dendritic cells (pDC) can secrete IDO which leads to the degradation of tryptophan an essential amino acid, for T cell proliferation. Other immune cells such as Treg cells and IL-13 secreting NKT cells also have an immune suppressive activity by directly inhibiting the proliferation of CTLs.

interferon-γ or TNF-α treatment since they are mainly originating from epigenetic regulation. However, some of these changes are permanent and irreversible such as the mutations or deletions in the β-2 macroglobulin encoding genes (Restifo *et al.*, 1996).

Another potential escape mechanism involves defects in antigen processing machinery, mainly including the defects in transporter associated with antigen processing (TAP), a crucial element in the transport of antigenic peptides coming out of the proteosomal complex into the endoplasmic reticulum (Leone *et al.*, 2013). Mutations in the TAP genes have also been reported to be a major cause of defective antigen presentation in many tumours (Seliger *et al.*, 2000), resulting in the failure to mount antigenic peptides onto the surface of tumour cells and thereby rendering them invisible to the immune system.

The immune synapse is where T cells make their decision to kill a cell (Franciszkiewicz *et al.*, 2013; González *et al.*, 2013). MHC molecule-TCR interactions trigger many proximal signalling events which are discussed in Chapter 3. These proximal signalling events are supported with many other signals coming from co-receptor ligand interactions in the synaptic complex interface. Tumours can preferentially manipulate these signals for their advantage in order to evade immune attack (Peggs *et al.*, 2008; Pardoll, 2012). Three main classes of negative signals can be generated at this contact point: negative co-inhibitory signals, apoptotic signals and down regulation of proximal signal components in the CTLs (Figure 1.9). The role of these receptors in immune escape is discussed in Chapter 2.

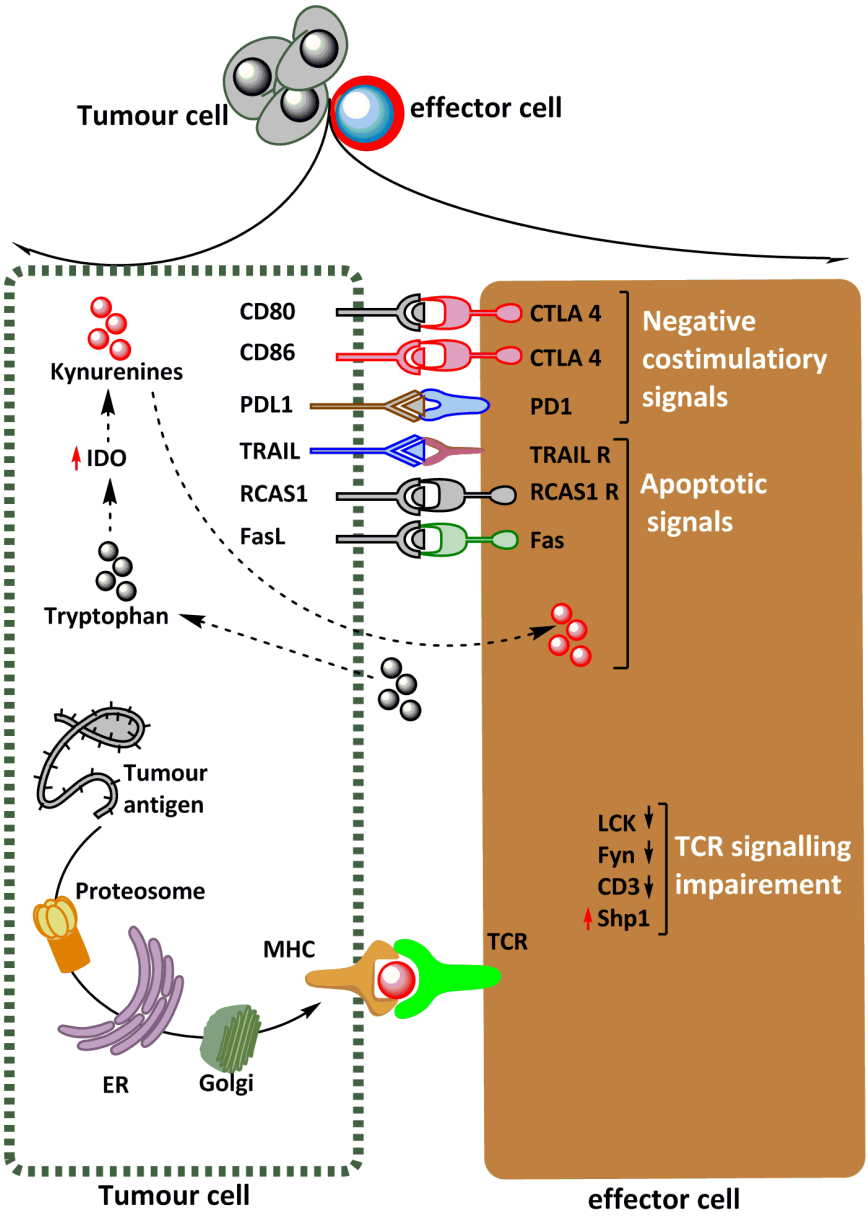

Figure 1.9: Checkpoints of CTL inhibition at the point of contact**.** CTLA-4 is an inhibitory receptor present on T cell which has a high homology to another stimulatory receptor CD28 on T cells. Ligands of both the receptors are CD80 and CD86 on the tumour surface. If the expression of CTLA is higher in T cells, they can bind to the CD80 and CD86 ligands on tumour cells much faster than the stimulatory receptor CD28. This results in the non-functionality of T cells. Similarly, PD-1 present on the T cell which binds to its corresponding ligand PDL1 also leads to inhibition of T cell activation. Major apoptotic signals are generated in T cells via receptors present in the T cell surface such as TRAIL, RACS1 and FAS and also via the by-product of tryptophan catabolism known as kynurenines. The down regulation of proximal signal molecules such as LCK, Fyn, CD3 and Shp1 primarily results in the weakened TCR signalling and leads to the incomplete activation of T cells.

(Figure adapted from Rabinovich et al., 2007)

1.2.5 Cancer vaccines

Surgery, chemotherapy and radiotherapy are still the mainstays of cancer treatment, however these treatments do not always get rid of the cancer completely and even when they do, the disease can come back after many years. Therefore, using vaccination to stimulate one's own immune system for detecting and eliminating cancer cells is an attractive additional strategy to cancer treatment. One potential benefit of immunotherapy compared to traditional treatment options is the detection and elimination of residual cancer cells and the development of immunologic memory in cancer patients. These would then keep a constant vigil on cells which may relapse during life by gaining cancerous properties. So, in a broader definition, cancer vaccines are substances which can either direct or restore the immune system to specifically detect and eliminate pre-malignant and malignant cells and tissues within the body. The cancer vaccination strategies can be prophylactic (protective) or therapeutic (treatment) (Emens, 2008; Frazer *et al*., 2007).

1.2.5.1 Prophylactic cancer vaccination strategies

Two of the recently FDA-approved prophylactic vaccines for the prevention of cervical cancer in women and head and neck cancer in men have recently been launched after the successful use of hepatitis B virus (HBV) vaccine in humans to prevent HBV induced liver cancers in 1981. Gardisil® and Cervarix® are the two recently approved prophylactic vaccines for human papilloma virus (HPV) developed by Merck and Glaxo SmithKline respectively (Lowy & Schiller, 2006; Schmiedeskamp & Kockler, 2006). Gardisil is a mixture of four proteins specific for HPV strains 6, 11, 16 & 18 and acts as a virus like particle (VLP); and also known as quadrivalent vaccine because of the four antigens; (Siddiqui & Perry, 2006). This vaccine has been administered in both males and females between the age of 9 and 26, and in men they proved to be combat genital warts, another papilloma induced condition. Cervarix® is a bivalent vaccine and only contains antigens from two of the most dangerous strains of HPV 16 and 18 (accounts for 70% of cervical cancers) (Schmiedeskamp & Kockler, 2006). It is approved to be given to females aged between 9-25 years.

Apart from the above preventative vaccines approved by the FDA, and the much earlier approved HEPB vaccine (which is mainly against viral induced tumours), prophylactic vaccines against non-virally induced tumour have not yet materialised. It is also worth

noting that some microbes have been shown to be a contributing factor in kaposi sarcoma (hepatitis virus), adult T cell leukaemia (human T cell lymphotrophic virus type-1), stomach cancer (Helicobactor pylori) and bladder cancer (schistosomes).

1.2.5.2 Therapeutic cancer vaccination strategies

Therapeutic vaccinations on the other hand are used in cancer treatment to eradicate an already established tumour. Some are focused on shrinking an established tumour prior to surgery, delaying the tumour progression, preventing tumour relapse and eradicating tumour spread and metastasis. Unlike the above mentioned prophylactic vaccines or any of the conventionally available microbial vaccines, the development of therapeutic cancer vaccines is difficult. The main reason for this is that cancer, which is derived from self, has accumulated many genetic alterations over many years, to which the immune system has been rendered tolerant. Self-tumour antigens are highly tolerated by T cells because of their high similarity to their normal counterpart (immune tolerance and thymic selection of T cell repertoire is discussed in Chapter 2).

So for the above reasons, therapeutic vaccination in cancer patients is at least theoretically a double edged knife. Ideally stimulation of T cells with any of the vaccine should lead to the breaking of tolerance and result in the eradication of the tumour by mounting potential anti-tumour immunity. On the other hand, serious autoimmune reactions are also a real possibility, depending on the expression profile of the target antigen and the potency of the immunity generated. The vaccination strategies can be divided into two main categories, passive therapy and vaccine mediated active immunotherapy.

1.2.5.3 Adoptive therapy

Adoptive immunotherapy is a modality by which tumour antigen/tumour reactive specific cytotoxic T cells are isolated and expanded *ex vivo* and transferred back into the patients. Early studies of adoptive transfer focused on the isolation of tumour infiltrating lymphocytes from the surgically removed tumour from patients and their subsequent expansion before transferring back into the patients (Rosenberg *et al*., 1994). The success of such studies was, however, limited and short-lived. With the identification of tumour derived suppression mechanisms and the immune suppresser cells it is now possible to possible to revisit adaptive transfer and improve the efficacy and outcome.

One of the potential problems of using adoptively transferred T cells to treat an already established tumour is their capacity to infiltrate (Bordon, 2012). This can be due to the poor or damaged vasculature of the tumour due to the harsh hypoxic environment generated by hypoxia inducible factors (HIF) in the tumour microenvironment. Treatment with blood vessel remodelling agents can improve the tumour vasculature and thereby the capacity of adoptively transferred T cells (ACT) to infiltrate the tumours (Bordon, 2012). Studies conducted in pancreatic neuroendocrine tumours have shown that that the presence of interferon-γ in the tumour microenvironment can abolish the tumour vasculature and a low dose of tumour necrosis factor-α (TNF-α) can improve vessel perfusion and thereby increase tumour infiltration of ACT and tumour control (Johansson *et al*., 2012). Apart from this physical barriers, tumour residing suppressive cells such as Treg cells and MDSCs can also influence the effect of adoptively transferred T cells. The presence of Treg cells in the tumour vicinity has been reported to be a limiting factor for a positive clinical efficacy of ACT (Viguier *et al*., 2004) and selective depletion of Treg cells in the tumour has been shown to increase T cell mediated immunity in pre-clinical models (Marabelle *et al*., 2013). Similar to Treg cells, MDSCs also have a negative influence of ACTs, and the depletion of these cells prior to ACT has also demonstrated to result in a positive therapeutic outcome in pre-clinical models (Kodumudi *et al*., 2012).

With the development of genetic engineering and the ability to manipulate T cells in the laboratory, many groups have started to create antigen-specific T cells with high affinity T cell receptors widely known as chimeric antigen receptor T cells (CARs) (Turtle *et al.*, 2012). The utility of this technique has been demonstrated using a mutated peptide of p53 (Cohen *et al*., 2005) and NY-ESO-1 (Li *et al*., 2005). The clinical usefulness of these CARs was first demonstrated in patients with metastatic melanoma with significant tumour reduction (Morgan *et al.*, 2006). Another strategy which uses antibodies fused to CD3ζ chain of TCR to mimic the TCR stimulation, and avoid the need for MHC dependent detection of tumour antigens are novel and promising (Chmielewski *et al*., 2013). The drawback of these adoptive transfer treatments is their cost and the expertise needed for the identification and expansion of antigen-specific T cells. Adding to that paradigm, the treatment has to be patient specific and hence it is not always going to be a treatment of choice in the first instance. However, CARs can

overcome the limitation of treating the 'self-derived' tumours by using predefined high avidity ones against the self-antigens without the limitation of tolerance.

1.2.5.4 Active immunotherapy

Active immunotherapy is the manipulation of the patient's own immune system using immunostimulatory agents such as peptides, activated dendritic cells, whole tumour cells and DNA vector vaccines. The advantage of active immune therapy is its low toxicity. In many cases, vaccines are well tolerated in treated patients and will generate immunologic memory (Dillman *et al*., 2007; Winter *et al*., 2011). A comprehensive current status of all the four major active immune treatment strategies is discussed below with relevant examples.

1.2.5.4.1 Peptide and protein vaccines

The identification of tumour antigens and their ability to trigger antitumor immunity generated a new interest in the use of these antigens for mounting immune responses in cancer patients in the past few decades. This approaches often resulted in clinical efficacy in various cancer types (Yamshchikov *et al*., 2001). Various vaccination strategies have been used within this strategy, the most studied of which is the use of short sequences (nonamer, octamer or decamer) for the generation of a $CD8^+$ T cell response or the use of larger fragments (13-18 amino acid long) for the generation of $CD4^+$ T cell response. The use of long peptides over shorter peptides is justified by studies that have shown that vaccine strategies including the long peptides generated stronger responses than the shorter ones (Bijker *et al*., 2007). In many occasions, the whole protein can be used as an immunogen, thereby allowing the patient to process and presenting all the possible antigenic peptides derived from those proteins. However, Melief's group also showed that that long peptide rather than the entire HPV protein generated stronger immune response (Rosalia *et al.,* 2013). The selection of these peptides is usually carried out by the help of bioinformatics tools which predict the binding affinity of the peptide for an MHC.

Numerous vaccine trials have been completed with tumour antigens and still many more are ongoing (Schlom, 2012; Yamada *et al*., 2012). Traditionally, peptide vaccines are injected with different immune modulatory adjuvants such as incomplete or complete Freund's adjuvants (IFA/CFA) in many preclinical studies. These adjuvants in vaccine

formulations act as depot for the antigenic peptide preventing them from immediate diffusion. Now the critical question is "why these vaccines have not showing good clinical efficacy?" In melanoma, peptide vaccines have been tried and tested many times, however the clinical efficacy of these vaccines has not been promising (Stewart & Rosenberg, 2000; Kirkwood *et al*., 2009). That question has been answered by a seminal paper published in Nature Medicine by Hailemichael *et al*., (2013). Their studies demonstrated that vaccine generated T cells can remain localised at the site of injection in a gp100 melanoma tumour model which used incomplete Freund's adjuvant. They also observed a gradual FASL and IFN-γ mediated apoptosis. So the failure rate of the majority of peptide vaccine trials might be attributed to the use of adjuvants.

The first generation peptide vaccines exclusively targeted $CD8^+$ T cell responses and for that reason many of the vaccine trials used short peptides. Recently, studies have been conducted using long peptide vaccines which harbour multiple $CD8^+$ T cell and $CD4^+$ T cell epitopes, and these proved to be more efficient in the generation of durable anti-tumour response (Melief and van der Burg, 2008). These observations correlated with the previous findings using a long ovalbumin peptide sequence (Bijker *et al*., 2007). Authors attributed this observation primarily to the processing and presentation of long peptide prior to the presentation. Diversifying the adjuvants has also proved to increase the efficacy of peptide vaccine, as in the case of TLR agonists like CPG motif (Nava-Parada *et al*., 2007) and Poly IC (van Duikeren *et al*., 2012). The advantage of using whole tumour antigen vaccines rather than long or short peptide vaccines is the ability to generate a broader spectrum of immune response. However, it is difficult to measure the response after therapy since there is no prior knowledge about the responsible peptide. In the case of using natural peptides for the immunisation, one potential problem is their poor immunogenicity.

Different research groups have modified the peptide sequence to enhance the binding affinity to the MHC or to the TCR and found this to be an efficient strategy (Valmori *et al*., 1998). Some of the vaccines in this category, such as an altered gp100 with Montanide ISA 51 have shown positive clinical outcomes in phase III trials in melanoma patients (Schwartzentruber *et al*., 2011). With the knowledge of large number of tumour antigens and their characterised epitopes, it is possible to combine multiple epitopes in a single vaccine provided they are all stable in the mixture. Such

approaches have been carried out in a clinical trial for melanoma in which multiple peptides (up to 12 peptides) were mixed in the same emulsion and injected into the patients with the aim of increasing the breadth of immune reaction. The studies were compared against a peptide mixture of just four and found that there is no significant drop in the overall antigen specific response when more peptides were used for the immunisation (Rosenberg *et al*., 2006). This study discarded the assumption that peptide competition towards the MHC molecule at the site of vaccine injection might reduce the overall immunogenicity of each peptide. These observations are crucial for the development of future multi-epitope vaccines, particularly in the case of tumours. The reason for this is mainly attributed towards the tumour escape mechanisms employed by the tumour which selectively down regulate the antigen targeted by the immune system. So, multi epitope peptide vaccines may hold the future for peptide based vaccine therapy in cancers.

The failures of using single epitope vaccines might be due to several factors other than the overall immunogenicity of the peptide used for the treatment. The short peptide vaccines (8AA length) do not require any antigen processing for presentation and for that reason they can bind directly to the available MHC molecules at the site of vaccine administration. The non-professional cells such as fibroblasts also express MHC molecules resulting in the antigen presentation to T cells without sufficient costimulatory signals, thereby leading to tolerance induction (Rosenberg *et al*., 2006). The stability of the injected peptides within the body is another concern with large quantities of peptidases present in the serum which would degrade the peptides before they can reach the secondary lymphoid organs for antigen presentation (Slingluff, 2011). The final drawback of peptide and protein vaccines might be the affinity of the antigenic peptide. If the peptide does not have sufficient affinity, then it can be displaced from the MHC binding groove by a higher binder which would result in the poor presentation of the antigen (Slingluff, 2011).

The above discussed points highlight the fact that, despite their limitations, peptide vaccines are still a favourite vaccine modality for the treatment of the cancer patient with visible positive clinical outcomes. However, careful design of vaccine formulation and the administration procedures can further improve the efficacy of these vaccines. This is also applied to long synthetic and multi-epitope vaccines where more relevant

clinical and preclinical studies are needed as a rationale for pushing peptide and protein based vaccines to the next level of clinical efficacy.

1.2.5.4.2 Whole cell and dendritic cell vaccines

The use of whole cancer cells to trigger immunity against the cancer has been in practice since the very beginning of cancer immunotherapy (Hanna & Peters, 1978). The vaccination has potential advantage over other vaccines in that the immune response is much broader and eliminates the possibility of tumour antigen escape variants. Mainly two types of whole cell vaccines are in use. The first one is called the autologous whole cell vaccine, derived from the cancer cell removed from the patients own tumour. The second type is the allogeneic whole cell vaccine where the vaccine consists of an irradiated cells derived from different patients and cancer cell lines (de Gruijl *et al.*, 2008). Once the source is identified the cells are irradiated and administered into the patient with appropriate immunostimulatory agents. The former therapy is highly patient specific and it requires the isolation of cancer cells from the patient tumour. Allogeneic therapy is hence more amenable in a hospital setting if the logistics are constrained. Attention must be given to the immune rejection (anti HLA) reactions due to the tissue mismatch by using allogeneic cells in a HLA mismatched patient. However, not many studies have suggested that this is a potential problem (Nemunaitis *et al*., 2006).

There are numerous strategies employed to increase the immunogenicity of cells nowadays, these includes co-injection of GM-CSF, IL2, IL6, TNF-α and IFN-γ as immune-stimulatory agents. The cells can be transfected with a cytokine plasmid which continues to induce the secretion of cytokines, even after irradiation has been tested using a whole cell vaccine (Olivares *et al*., 2011). Most of the tumours were found to produce TGF-β and therefore whole cell vaccines combined with antisense RNA for TGF-β are promising by blocking the production of TGF-β, a potent inhibitor of tumour immunity (Olivares *et al*., 2011; Fakhrai *et al*., 2006). The utility of whole cell tumour vaccines have been demonstrated in various cancers including hormone-resistant prostate cancer (Michael et al, 2005), pancreatic cancer (Laheru *et al*., 2013) and melanoma (Mackiewicz *et al.*, 2012). Another study which used well known prostate cancer cell lines (LNCaP, PC-3) transduced with GM-CSF (known as ‘GVAX’) in human patients has showed positive survival over a period of 26 months (Small *et al.*,

2007). When these cells injected in to the patients the dendritic cells recognise these dying cell and taken them in for processing and presentation. The GM-CSF produced by the cells helps to recruit and mature dendritic cells.

Another therapeutic opportunity exploited by many cancer vaccination trials involves dendritic cells (Schuler, 2010). These are professional antigen presenting cells and the background information on these cells is given in section 1.1.1.3. They originate from the hematopoietic stem cells as $CD34^+$ cells, or monocytes can also be converted into mature dendritic cells depending up on the cytokine stimulus they received. Because of their strong antigen processing and presentation capacity they are considered to be potent cell for the stimulation of cellular immunity. In cancer immunotherapy, dendritic cells have been used extensively for the same purpose and dendritic cells based vaccines have demonstrated their potential in many clinical settings.

The primary objective of any dendritic cells vaccine strategy is to load the cells with sufficient amount of tumour associated antigens (TAAs). There are several strategies that have been employed since their introduction in cancer immunotherapy. There are two main protocols usually followed for the antigen loading into the dendritic cells. The first approach is to target the dendritic cells *in vivo* using specific DNA vectors or molecules to deliver in to TAAs. Secondly, dendritic cells precursors are isolated from patients' blood and induced to differentiate in to dendritic cells *in vitro* prior to loading the antigens and transfer them back into the body after their maturation. Initial studies involving *ex vivo* manipulation of dendritic cells for therapeutic purposes were limited to the use of $CD34^+$ haematopoiesis precursors and were limited by the availability of sufficient number of dendritic cells for antigen loading. Evolution of dendritic cell vaccination and the search for abundantly available dendritic cells leads to the use of $CD14^+$ monocytes to generate dendritic cells from the peripheral blood, and this methodology is become widely accepted method for current dendritic cell vaccination strategies. GM-CSF and IL-4 are the most widely used cytokine for the generation of the immature DCs from blood isolated $CD14^+$ monocytes, however other cytokines such as IFN-α, TNF-α and IL15 have also been used.

The derivation of immature dendritic cells from the monocytes is the initial step of dendritic cell based therapy, complete maturation of the dendritic cells in the presence of suitable agents is crucial, since incomplete maturation of the dendritic cells leads to

the poor expression of the costimulatory molecules which would results in the induction of T cell tolerance and anergy in CTLs instead of stimulation and activation. Inflammatory cytokines such as TNF-α PGE2, R848, IL-1β and IL-6 in current immunotherapeutic settings are the best candidates for the maturation of the dendritic cells *ex vivo*. (Tuyaerts *et al.,* 2007; Skalova *et al.,* 2010). The use of TLR agonists such as Poly-I:C (Polyinosine-polycytidylic acid) or LPS (Lipopolysaccharide) can enhance dendritic cell maturation significantly (Tuyaerts *et al.,* 2007). However, studies have indicated that the use of TLR agonists for dendritic cell maturation has a negative influence on the mobility of mature dendritic cells.

The antigen loading of the dendritic cells can be performed with various methods such as transducing the immature dendritic cells with mRNA or DNA derived from allogeneic or autologous tumour cells or tissues (Cao *et al*., 2013; Chen *et al.,* 2013). The antigen loading can be performed with irradiated dying tumour cells, defined peptides (Oshita, 2012) or whole protein lysates. The use of whole protein lysates (Lasky *et al*., 2013), cells or mRNA is more efficient, since they harbour a wide antigenic repository for the dendritic cells to process and present to the T cells (Bringmann *et al*., 2010). The major breakthrough of dendritic cell derived vaccine came with the first FDA-approved prostate cancer vaccine Sipuleucel-T. This offers a low (4 months compared to placebo), but significant survival advantage to the patients with metastatic hormone-resistant prostate cancer (Gould, 2006; Higano *et al*., 2009, 2010; Schellhammer *et al*., 2013). The vaccine uses autologous dendritic cells and needs leukapheresis of the patients for the loading of antigen to the dendritic cells.

1.2.5.4.3 DNA Vaccine

DNA vaccines are an attractive delivery system for TAAs into the cancer patient. The main advantages of this type of vaccine are the flexibility it offers to tailor the vaccine according to the theoretical and conceptual requirement of the researcher. In general, the vaccines are constructed into a bacterial or viral expression vector backbone with sufficient expression of control element such as optimal eukaryotic promoters and polyadenylation signals (Ghanem *et al.,* 2013). Since it is tailor made, many costimulatory molecules of the adaptive and cellular immunity can also be co-expressed in this backbone in order to facilitate a better immune response (Lan *et al*., 2013). The vaccine delivery has been traditionally carried out with either electroporation or gene

gun mediated route and subsequent incorporation of these vaccines into the skin dendritic cells and also to the normal muscle cells (myocytes) (Rice *et al.,* 2008). The dendritic cells incorporated vaccine translates the tumour antigens and subsequently enters into the class I or class II pathway for its presentation. Another mechanism is cross presentation which is elicited by non- dendritic cells at the vaccine sites. Antigens required for the cross presentation were either produced and secreted from the myocytes if the vaccine design intends to do so, or derived from the dying or dead cells at the vaccination sites. This unique ability of direct and cross presentations with constant supply of the antigens may be an important parameter for the increased immunogenicity of the DNA vaccines in cancer therapy settings (Rice *et al.,* 2008). The clinical utility of DNA vaccines for various cancers has been demonstrated in pre-clinical models and also in Phase I and II clinical trials (Mincheff *et al.,* 2000; Rosenberg *et al.,* 2003; Tagawa *et al.,* 2003; ; Triozzi *et al*., 2005 ; Wolchok *et al*., 2007; Chudley *et al.,* 2012).

An example of DNA based vaccine used for immunotherapy is against melanoma known as ImmunoBody®, by Scancell Ltd. a UK based company. This is capable of generating consistent high avidity T cell responses in cancer patients (Pudney *et al.,* 2010). This study utilises this vaccine as a model system for generating high avidity T cells and hence the details are discussed in the Chapter 2. Some of the representative examples of the above discussed vaccine strategies are given in the Table 1.3.

Table: 1.3. Representative example of immunotherapeutic drugs used in cancer

Vaccine	Type	Cancer	Stage/clinical trial	Reference
Rituximab	MAb‡	Lymphoma	FDA	Anderson *et al.*, 1997
Tositumomab	MAb	Lymphoma	FDA	Vose *et al.*, 2000
Alemtuzumab	MAb	Leukaemia	FDA	Lemery *et al.*, 2010
Gemtuzumab	MAb	Leukaemia	FDA	Bross *et al.*, 2001
Trastuzumab	MAb	Breast	FDA	Traynor, 2013
Cetuximab	MAb	Colorectal	FDA	Vincenzi *et al.*, 2010
Bavacizumab	MAb	Colorectal,	FDA	Cohen *et al.*, 2007
Gardasil (HPV)	Prophylactic	Cervical	FDA	Ghazal-Aswad, 2008
Cervarix (HPV)	Prophylactic	Cervical	FDA	Schmiedeskamp *et al.*, 2006
Imiquimod	Adjuvant	Baselcell	FDA	Tinelli *et al.*, 2012
Sipuleucel-T	Therapeutic (DC †)	Prostate	FDA	Gould, 2006
IFN-α	Cytokine	Melanoma,	FDA	Tarhini *et al.*, 2012
IL-2	Cytokine	Melanoma,	FDA	Dillman *et al.*, 2012
TNF-α	Cytokine	Melanoma	FDA	Lejeune *et al.*, 1994
DCVax	Dendritic cell	Brain	Phase III	Fishman 2009
Rindopepimut	Peptide	Brain	Phase III	Babu & Adamson, 2012
GV-1001	Peptide	Lung	Phase III	Nava-Parada *et al*., 2007
PROSTAVAC	DNA	Prostate	Phase III	DiPaola *et al.*, 2006
Trovax	DNA	Renal	Phase III	Harrop *et al.* , 2011
SCIB1	DNA	Melanoma	Phase I & II	Pudney *et al.*, 2010

***** Renal cell carcinoma, † Dendritic cell, ‡ Monoclonal antibody,

1.2.5.5 The quantity and quality of T cell responses

To study T cell functionality it is important to understand its properties at the immunological, transcriptional and protein level. Not much work has been carried out on the characterisation of high avidity T cells at the molecular level. Here we summarise the techniques available for the characterisation of these cells. The earlier assays used for the functional studies of T cells and estimating the overall response to immunotherapy included delayed type hypersensitivity (DTH) *in vitro* proliferation assays for $CD4^+$ cells and *in vitro* cytolytic assays for $CD8^+$ cells, or measuring the humoral responses by enzyme linked immunosorbant assays (ELISA). These assays

have limitations such as low sensitivity and the need for *in vitro* manipulations which makes them inferior to the modern techniques. Modern T cell studies are broadly classified into two types, structural based assays and functional based assays.

1.2.5.6 Structure based assays

1.2.5.6.1 MHC Multimeric complexes: Multimeric complexes used for the identification of peptide-specific T cells were first devised by Altman *et al.*, in 1996. Like antigen antibody specificity, the principle of this method is also based on the affinity of the T cell receptor towards its cognate antigenic peptide presented on MHC molecules. The construction of these multimeric (dimer, tetramer, pentamer and dextramer are most widely used classes of MHC multimers) complexes involves the use of Class I or Class II MHC monomers joined together by fluorescent conjugated avidin. The peptide of interest can be used for tracking peptide-specific $CD4^+$ or $CD8^+$ cells after immunisation using flow cytometry (Wooldridge *et al.*, 2009). This can also be used for the isolation of those cells for further *in vitro* studies or biochemical and molecular characterisations (Duplan, *et al.*, 2007; Callan, *et al.*, 1998).

1.2.5.6.2 Real time quantitative PCR: Unlike conventional PCR which depends on gel-based techniques for product detection, real time PCR combines the target amplification and product detection into a single step (Higuchi *et al.*, 1993; Heid *et al.*, 1996). This real time detection is achieved mainly because of the development of different fluorescent dyes (SYBR Green®, BEBO®) which can be used to relate the increase in product accumulation and fluorescent intensity quantitatively. Quantification is mainly done by the measurement of 'Ct values', which is the number of cycles required for the fluorescence values of each sample to rise above the background fluorescence (Wong and Medrano, 2005). The relationship between copy number and Ct value is inversely proportional, i.e. high initial template copies results in more products accumulation and therefore more fluorescence resulting in a lower Ct value.

In an immune response, if the hypervariable complementary determining region (CDR) of the T cell is known, then real time PCR can be used for the quantitative measurement of that T cell specificity using clone specific primers that flanks the unique CDR (Ochsenreither *et al*., 2008; Seo *et al*., 2001).

1.2.5.7 Functional based assays

1.2.5.7.1 Real time quantitative PCR: Antigen specific immune responses can be measured by studying the antigen driven gene expression of cytokines such as interferon-γ (INF-γ), granulocyte macrophage colony stimulating factor (GM-CSF), tumour necrosis factor-α (TNF-α) and interleukin-2 (IL-2). This approach provides a clear picture of antigen specific functional property of the T cells when used with appropriate controls.

1.2.5.7.2 Flow cytometry cytokine analysis: Possibly, the most widely used technique to isolate cells is fluorescent activated cell sorting. It is possible to analyse or sort cells occurring at low frequency (Russell, *et al*., 2013; Ho *et al*., 2013). Sorting of cells are based up on the labelling with antibodies conjugated with fluorophores having different spectral characteristics (Bendall *et al*., 2012; Chattopadhyay and Roederer, 2012). The fluorescent signal can be obtained using a flow cytometer instrument if the antibody binds to the specific cellular markers. fluorescent activated cell sorting is widely used for the analysis of antigen-specific T cells. T cells subjects to a brief *in vitro* stimulation of 10-12 h with the desired antigenic peptide with protein transport blocking reagents such as brefeldin A or monensin. This helps in the retention of any cytokines produced upon antigen stimulation within the cells. The cells are then fixed and permeabilised using different reagents to detect the cytokine produced. Antibodies against the corresponding cytokines can be used by a process commonly referred as intra cellular cytokine staining (ICS). One drawback of this technique is that the permeabilisation kills the cells (Yee and Greenberg, 2002). A modified version of this analysis known as Cytokine Capture Analysis (CCA) capture the secreted cytokines immediately using an antibody bound to the CD45 receptor of the T cells (Becker, *et al*., 2001; Campbell *et al.*, 2011). The advantage of flow cytometry over ELISpot assays are the opportunities to include multiparameter analysis using different surface phenotypic markers for the complete characterisation.

1.2.5.7.3 Enzyme Linked Immuno SPOT (ELISpot). This is most widely used functional assay for following the functionality and responsiveness of the T cells (Camisaschi *et al.*, 2013; Diaz *et al.*, 2013). Isolated splenocytes or peripheral blood mononuclear cells (PBMCs) are stimulated in an ELISpot plate which contains a membranous surface pre-coated with an antibody against the desired cytokine. Cells are

stimulated for 24-48 h at which time, captured cytokine is detected using a detection antibody which is labelled with chromogenic substances. The resultant spots are then counted on an automated plate reader based on its intensity. The number of spots is then expressed as the spots per total number of T cells that were put on the plate, thereby providing quantitative estimates of the T cell functionality. This assay is very useful to catch anergic cells when combined with structural assays such as multimer staining.

1.3 Aim

The main aim of this study is to identify biomarkers which would allow the detection of high avidity T lymphocytes in the blood or tissue of cancer patients after immunotherapy, irrespective of the peptides used for the immunisation. Recent studies using functional and structural assays on CTLs revealed that a highly diverse functionality of T cells exists with same specificity. The molecular mechanisms revealing this functional heterogeneity are not well studied. There are arguments that T cell functionality is decided during the early encounter of a naive T cell with an antigen presenting cell. The kinetics and the duration of signals received during that first encounter prior to clonal proliferation decide the function of the T cell (Sykulev, 2010). The large scale study of these peptide-specific cells are now feasible with the high through put platform such as high density cDNA Microarrays and LC-MALDI based mass spectrometry (liquid chromatography-matrix assisted laser desorption ionisation). To achieve this main objective the entire project is subdivided in sections

- Establishment of two mouse model systems for studying the generation of high and low avidity CTL responses using immunologic methods.
- Isolation of pentamer positive $CD8^+$ T lymphocytes from high and low avidity mouse models.
- Generate a peptide mass fingerprint (PMF) for high and low avidity T lymphocytes and identify molecular biomarkers associated with high avidity T cell responses.
- Creation of complete transcription profiles for high and low avidity T lymphocytes using cDNA microarrays/quantitative real time PCR.

- Deciphering the genetic and protein interaction networks operating in high and low avidity T cells.
- Confirm and validate the markers and the pathways in a second mouse model and then in human immunotherapy settings.

Chapter 2.

Immunological characterisation of high and low avidity T cells

2.1 Introduction

Identification and characterisation of tumour associated antigens (TAAs) and its ability to generate potent anti-tumour immunity has presented tumour immunologists with a new opportunity to design and develop an effective vaccine against cancer. Although vaccination strategies have been shown to be capable of stimulating the adaptive arm of immune system, especially cellular immunity, in experimental settings, many clinical trials remained unsuccessful (Kudrin and Hanna, 2012). In the majority of the cases, the responses have been detected in the patients on the basis of detecting of antigen-specific CTLs or increased tumour infiltrations of lymphocytes (TILs) (Gajewski, 2007). This poor clinical efficacy, in the context of generating antigen-specific T cell responses has perplexed many in the field and a myriad of limiting factors which check the functionality of the effector cells have now been identified (Gajewski, 2007; Abastado, 2012; Bellone and Calcinotto, 2013). This includes several escape mechanisms and immune suppression mechanisms operating within the tumour microenvironment or by immune cells such as Treg cells and macrophages. Targeting these limiting factors of vaccine efficacy is therefore an active area of research at the present (Sounni and Noel, 2013; Samples *et al.,* 2013).

Although monoclonal antibodies against major tumour antigens have been effective in the delivery of cytotoxic payloads and triggering humoral immunity (Sliwkowski and Mellman, 2013) in patients, T cell based vaccine therapy has been more attractive to the tumour immunologists for two reasons. First, the majority of the identified tumour antigens are intracellular proteins that are either produced as a consequence of erroneous molecular events in the tumour cells, as a consequence of which they are often presented to immune system via an MHC class I or II MHC molecule (Borghaei *et al.,* 2009). Secondly, the capacity of cellular immunity to establish immunologic memory can provide an additional level of long-term protection to patients, post treatment (Caserta *et al.,* 2012).

The different vaccination strategies discussed in the Chapter 1 are capable of generating different types of T cell responses. Peptide vaccines or epitope defined multivalent

vaccines are capable of generating a more defined T cell antigen specific repertoire and it is relatively easy to measure the efficacy of the vaccine by different immune monitoring techniques such as ELISpot® or multimer staining. On the other hand, whole cell and protein vaccines generate a highly polyclonal T cell response against different antigenic targets that are available in the whole cell preparations or the number of antigenic peptide derived from a single whole protein (Keenan and Jaffee, 2012; Kageyama *et al.,* 2013). If this is the case, then immune monitoring is highly difficult mainly because of the lack of information about the responsible peptide(s).

Apart from the immune suppression exerted on vaccine generated T cells, another major limiting factor is the quality of the effector cells (Viganò *et al.,* 2012). The number of tumour-antigen specific CTLs is not necessarily a good indication of a quality response, rather it is the ability of these cells to execute the effector function in a fool proof way. This efficiency can be measured *ex vivo* by its ability to secrete effector cytokines in a peptide-specific manner. This *ex vivo* data can be used as a surrogate marker for predicting the *in vivo* vaccine efficacy in clinical trials (Malyguine *et al.,* 2012; Malyguine2007; Ogi and Aruga, 2013).

T cell avidity and functional avidity are two major indicators of vaccine efficacy. Avidity is the overall binding strength of the T cells to the target antigen and functional avidity is the ability of vaccine generated T cells to respond against its cognate antigen in a dose dependent manner. The detailed discussions of these topics are given in the later sections of this chapter. In short, a general consensus among tumour and viral immunologists is that a clinically beneficial vaccine response is one which is capable of generating a high functional avidity T cell response (Viganò *et al.,* 2012; Mothe *et al.,* 2012).

Unlike foreign antigen based vaccinations, tumour vaccinations are mainly depending on the self-antigenic targets. This is an additional challenge because of the fact that the vaccines are attempting to target a T cell repertoire which is highly tolerant to the self-proteins. Therefore, it is ideal at this point to understand the generation of T cells within the body, careful thymic selections which avoid the high avidity self-reactive T cells and their tolerance mechanisms. This is particularly important because understanding these mechanisms are crucial for appreciating the availability of the total T cell repertoire in the periphery for vaccine targeting.

2.1.1 Ontogeny of T lymphocytes: careful nurturing of T cells in the thymus

The life cycle of a T cell is a journey of careful differentiation, specificity development and a period of intense education, where it learns to recognise self from non-self. For any immunologist, it is important to understand elements of this selection process which take place in the thymus. During this process of thymic selection some autoreactive T cell clones can escape the negative selection and escape into the periphery (Mohan *et al.*, 2010, Enouz *et al.*, 2012). The possible mechanisms for generation of these cells are discussed in the later sections of this chapter.

The remarkable ability of T cells to discriminate self and non-self is not an acquired character in their late stages of development. The trustworthy T cell repertoire is carefully monitored in the thymus before they are released in to the peripheral organs for immune surveillance. Each T cell originates from haematopoietic stem cells in the bone marrow, as committed progenitors known as thymic seeding progenitors (TSP) (Vicente *et al*., 2010). These TSPs then move in to the thymus through blood vessels, and known as early thymocytes. At this stage they lack the expression of CD4, CD8 or T cell receptor molecules on their surface and are known as double negatives (DN). This DN population can further be divided into four subcategories by the presence of other cell surface markers: DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$) and DN4 ($CD44^{-}CD25^{-}$) (Vicente *et al.*, 2010). At late DN stages (DN3 and DN4) the emergence of T cell receptor - CD3 complex [α/β, γ/δ] takes place and is characterised by the expression of a non-rearranged Tα and a rearranged Tβ chain. In the next step, the rearrangement of the Tα chain also completes and the cells enter into the double positive (DP) stage of their thymic lifecycle expressing αβ TCR, CD8 and CD4 molecules on their surface (Haars *et al.*, 1986).

This is followed by the development of $CD8^{+}$ and $CD4^{+}$ single positive T cells by the interaction of double positive T cells with MHC class 1 or 2 molecules present on the cortical epithelial cells of the thymus which are loaded with self-peptides (Anderson *et al.*, 2009). DPs binding to MHC class 1 molecules become $CD8^{+}$ single positive (SP) and conversely those which binds to MHC class II become $CD4^{+}$ single positive (SP) T cells. Major signalling pathways such as Notch (Carlin *et al.*, 2012) and hedgehog (Rowbotham *et al*, 2008) play an important role in the above differentiation and lineage

commitments in the thymus. The detailed development of T cell ontogeny is depicted in Figure 2.1

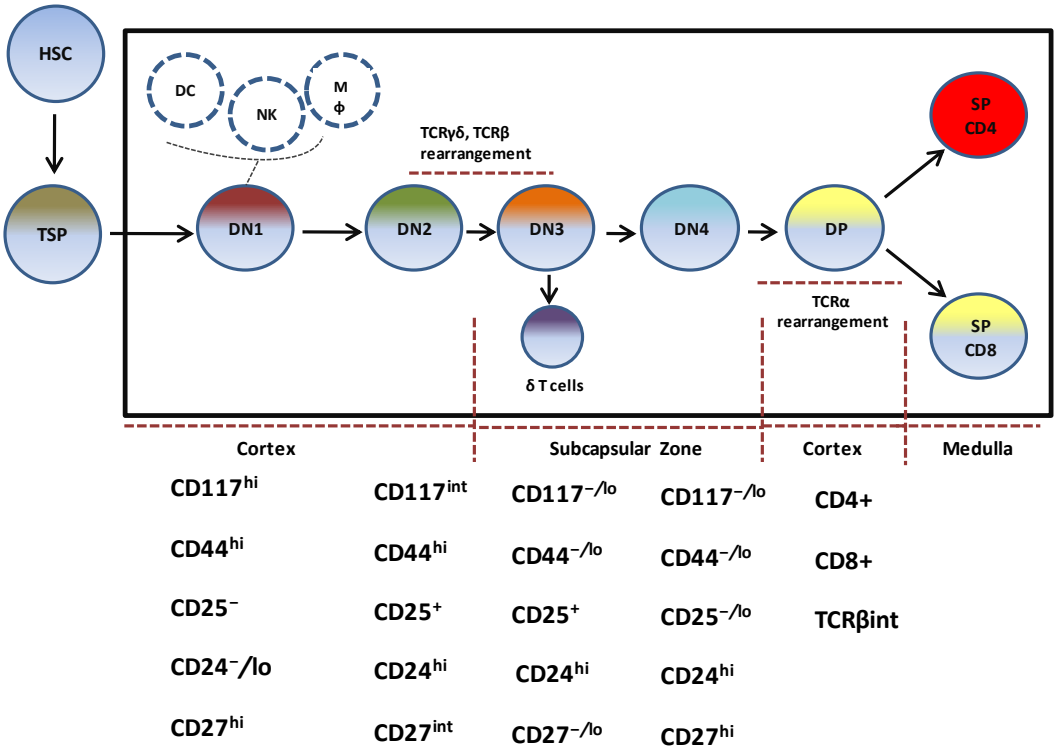

Figure 2.1: Thymic maturation of a T cell: Journey of a T cell start from the haematopoietic stem cells (HSC) in bone marrow. HSC's are quickly converted to thymic seeding progenitors (TSP) before entering in to the thymus. Subsequently these cells were passed through cortex of the thymus. At this point they have the potential to differentiate in to dendritic cells (DCs), natural killer cells (NK cells) or macrophages (Mϕ). However, tight regulation of NOTCH signalling pathways prevent these cells from entering in to that alternative differentiation and remain stick to the T cell differentiation root. TCRγδ and TCRβ rearrangement starts at the late double negative 2 (DN2) stage and ends in subcapsular zone of the cortex at the double negative 3 (DN3) stage. Two different lineages of T cells (γδ and αβ) formed at this point of late DN3. At early double negative 4 (DN4) stage, cells committed to follow the αβ lineage double positives (DP) move in to the cortex region of thymus. Most of the TCR gene rearrangements take place at this region. The cells subsequently enter into the medulla of the thymus and undergo negative and positive selection before entering into the periphery. The cellular phenotypic markers at each development stage is also given under each stages.

The ability of T cells to discriminate between 'self' (one's own proteins) and non-self (foreign antigen) has also been decided at this point of T cell development (Starr *et al*., 2003; Pardoll, 2001). This is discussed in the next sections.

2.1.2 Positive and negative selection (Thymic selection)

One hallmark of T cells is their receptor specificity towards foreign antigens and their MHC restricted recognition, combined with the ability to be tolerant of self-proteins and peptides. This ability of a T cell to discriminate between 'self' and 'non-self' is acquired during their thymic maturation processes (*figure 2.1*). In late foetal development approximately 10-100 thymic seeding progenitors enter the thymus through a guarded access point (Foss *et al.*, 2001; Shortman, 1990). These cells then undergo 20-25 divisions during their stay as double negatives (Shortman, 1990; Kyewski & Klein, 2006). Once their lineages are committed, there is an extensive rearrangement of TCRα gene locus until each T cells reaches the region of positive selection in the thymus (Klein *et al.*, 2009). Positive selection is required by the T cell in order to rescue themselves from apoptosis which is induced by the interaction of T cells with the peptide MHC complexes in the medullary region of the thymus (Kisielow & Miazek, 1995). These cells now develop the MHC restriction of their TCRs and also up regulate RAG genes for active recombination of TCR locus and Bcl- XL (Ioannidis *et al*., 2001) for anti-apoptotic characters. Several studies suggest that the journey of a T cell on its late double positive stages to reach the medulla is dependent on its optimal survival capability which is invariably determined by TCR engagement and the quality of ligand and type of presentation (Wilkinson *et al*., 1995; Yasutomo *et al*., 2000). Once the T cell reaches the medulla it has a fully developed TCR and other functional co-receptors (Anderson *et al*., 2013). There are two models widely available for describing thymic selection as "qualitative" and "quantitative". In the "qualitative" model the assumption is that the nature and type of peptide generate qualitatively different signals for the survival or death of the T cells (Jameson, 1995). The "avidity" or "quantitative" models suggest that the strength of interaction between the MHC and TCR complexes (P-MHC) determines the outcome of the T cell fate and this strength of interactions are often decided by the nature of peptide. If the strength of the interaction is too strong, the interacting T cells die off by a process referred as "clonal deletion" (death by excessive signals). Weak binding leads to death by 'neglect' (death by lack of signals) (Figure.

2.2). T cells which receive the optimum levels of binding strength and signal will proliferate and proceed to the periphery. This process of thymic selection of T cells equips T lymphocytes to recognise 'self' from 'non-self' (foreign) peptides.

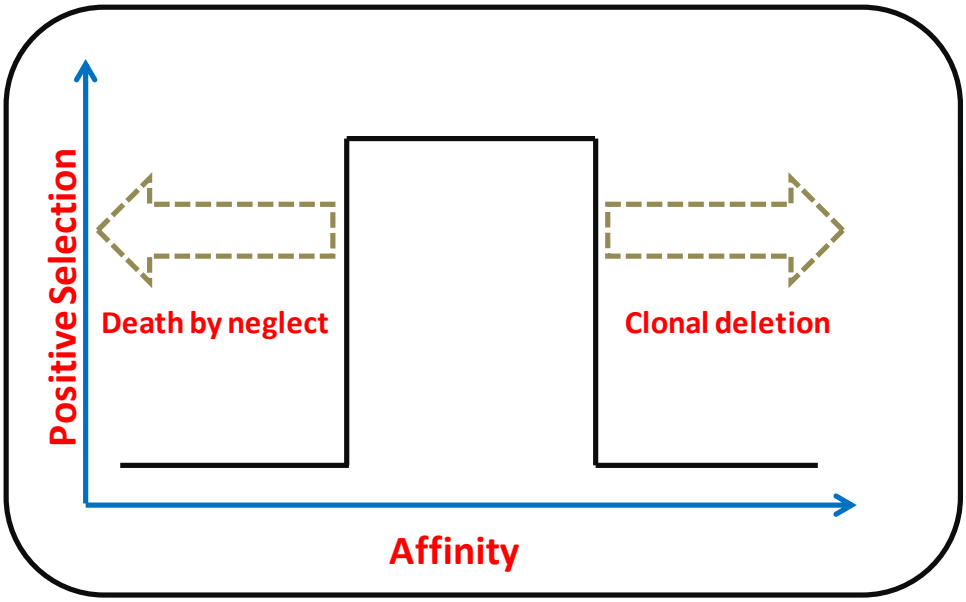

Figure 2.2: The quantitative (affinity or avidity) model of positive selection. For a T cell to be positively selected the strength of interaction (affinity) is crucial. If the affinity of P-MHC complexes is below a certain threshold, the T cells die by a process called "death by neglect". If the affinity is too high clonal deletion will occur by a process called serial triggering (super optimal signals). A T cell which receives the optimal signal by the P-MHC complexes at a suitable threshold only will survive by positive selection and enter the periphery.

2.1.3 Impact of thymic selection on cancer immunotherapy

The selective deletion of self reactive T cells in the thymus predominantly resulting in the generation of a T cell repertoire which are low avidity in their response against self antigens. During the thymic selection 95% of all T cells entering the thymic medulla perishes due to negative selection, the other 5% are released into the periphery and the majority of them do not complete maturation as a consequence of peripheral tolerance mechanisms (Huesmann *et al*., 1991). However, thymic selection is not always fool proof, T cells with specificity towards the self-antigens can also escape from thymic selection by various other mechanisms and are reviewed in the literature (Ignatowicz *et al*., 1996; van Meerwijk J.P.M, *et al*., 1996, Enouz *et al*, 2012).

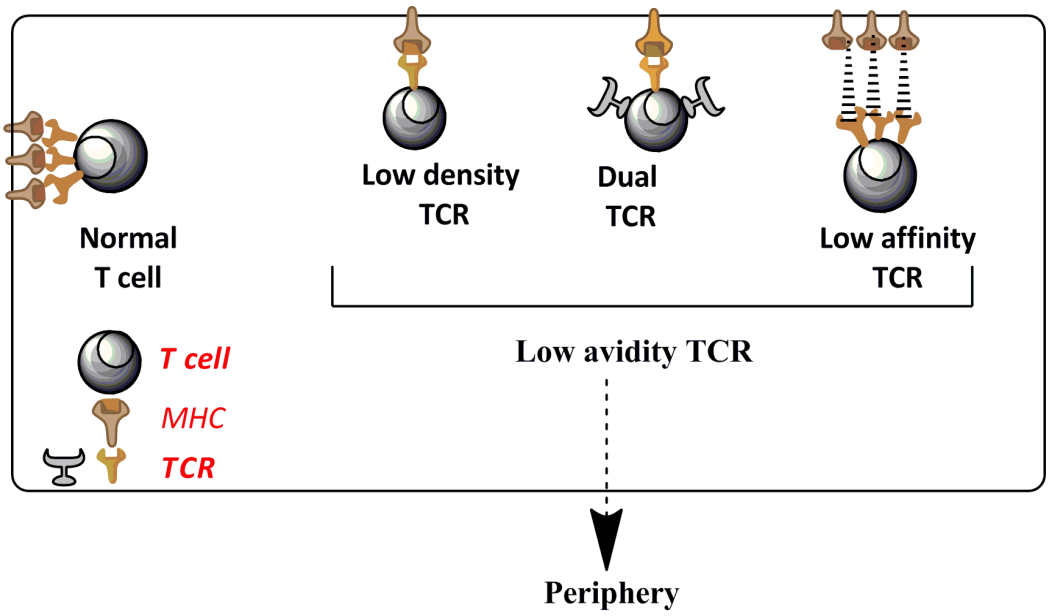

Figure 2.3: The mechanism of generation of low avidity T cells in the thymus. T cells having a moderate affinity/avidity against the self-antigens are only selected towards the periphery. They include the T cells with low number of TCR on its surface, thus having low avidity, T cells with multispecific TCR and the T cells having a certain degree of low affinity towards the self-antigens. These cells can be effectively used in cancer immunotherapy using suitable vaccination strategies against TAAs. (Adapted from Visser *et al*., 2003)

For tumour immunotherapeutic settings, the available pool of responding $CD8^+$ T cells are predominantly low avidity T cells having low to moderate affinity towards self-antigens known as naïve population. As discussed earlier the naïve population has a high degree tolerance towards the self antigens, therefore one obvious question is, "are $CD8^+$ T cells exposed to all the tissue restricted antigens in the body?" if not, the chances of highly reactive tumour specific high avidity T cells will be in the body. Studies suggested that cortical thymic epithelial cells and the medullary thymic epithelial cells express mostly all the tissue restricted antigens (TRA) in the thymus by a process referred to as, promiscuous, or ectopic gene expression controlled by autoimmune regulator genes (AIRE) (Anderson *et al.,* 2002; Gardner *et al.,* 2009). Therefore, the majority of the $CD8^+$ cells get a chance to be exposed to TRA at least once, before leaving the thymus (Derbinski *et al.,* 2001; Kyewski and Klein, 2006) and it is therefore reasonable to assume that all or most of the high avidity self reactive T cells are completely eliminated.

However, T cells having low or moderate affinity towards the self antigens escaped in the thymic selection will be still in the periphery. This has been a paradoxical situation for immunologists for many years that the cells escaped from the central tolerance live happily in the periphery without causing significant auto-immune disease (Ian & Heath, 2008). How are the cells, having a moderate affinity towards the self-antigens, tolerated in the periphery? Two main mechanisms are operating; one is the "avoidance of self-antigens" by physically separating them into the immunologically privileged regions so that T cells never see them in their life cycle (Streilein *et al*., 1997).

A second way of tolerance is by the "anergic mechanisms" in which T cells remains functionally at a nonresponsive stage even when exposed to the self-antigens (Yuuki *et al.,* 1990). Tolerogenic dendritic cells (Ma *et al.,* 2013) and regulatory T cells (Tregs) ($CD4^+CD25^{high}$ $Foxp3^+$) are also known to induce peripheral tolerance (Schmidt *et al*., 2012). This dangerous balancing act of cellular immunity could be disrupted when suitable conditions arises which break the tolerance mechanisms (Illés *et al*., 2005) and mount destructive autoimmunity such as the one observed in neonatal insulin-dependent diabetes (Radu *et al*., 1999). While this is a danger in terms of autoimmunity, it can be used as a tool in cancer immunotherapy by carefully selecting the antigens to specifically target the tumour and mobilising self reactive T cells into the tumour sites (Eggermont *et al*., 2013) for better therapeutic killing of the malignant cells providing

the tumour expresses self tumour antigens. Breaking the avidity barriers of these cells should be carefully monitored, otherwise side-effects such as vitiligo (depigmentation of the skin) can be triggered. Vitiligo mainly observed as a treatment side effect in melanoma patients, when treated with immunotherapy against the most common melanoma specific antigens such as gp100, MART1, TRP1 (tyrosinase-related protein 1) and TRP2 (Alonso-Castro *et al.*, 2013; Byrne & Turk, 2012). The reasons for this phenomenon are that targeted antigens are involved in the normal melanin synthesis and the immune-mediated loss of these melanin pigment producing cells, in extreme cases, loses of retinal pigments (Caspi, 2008). However, given the choices available for the melanoma patients, the benefits of T cell based therapies outweigh the side effects in the majority of cases. So in summary loopholes in thymic selection result in the release of low to moderate avidity T cells into the periphery which can be used to target the malignant tissues by immunotherapeutic intervention, providing the targeted antigen is not expressed by vital organs. Moreover the success of any cancer vaccination strategies depends on the naïve T cell precursor frequency against the target antigen and also vaccine's ability to recruit and expand those existing specificities (Jenkins and Moon., 2012).

2.1.4 T cell affinity, avidity and functional avidity

2.1.4.1 TCR Affinity

The terminology affinity, avidity and functional avidity are highly interconnected and often confusing in the field of immunology (Viganò *et al.,* 2012). T cell affinity is the first level of interaction where a single TCR interacts with only one MHC molecule. The strength of this monomeric interaction between a TCR and pMHC is referred to as T cell affinity (Kindt *et al.,* 2007). Unlike T cells, in B cells, the antibodies are generated and the affinity is fine-tuned by somatic hyper-mutations (SHM) in the variable region of antigen binding sites. Even though both BCR and TCR gene rearrangements shared a high degree of similarity, the lack of SHM in T cells leads to generation of TCR with fixed affinity for the rest of their life cycle. The measurement of affinity is usually carried out by measuring the dissociation constant (kD) of soluble TCR- pMHC complexes using Surface Plasmon Resonance (SPR) or by measuring the half-life ($t_{1/2}$) of TCR- pMHC interactions. (Margulies *et al.,* 1996).

2.1.4.2 TCR Avidity

Avidity or structural avidity is the cumulative strength creating from the overall interactions happening at the binding site, which is the collective strength of all the TCRs and other co-receptor molecules involved in the synaptic complex formation (van den Boorn *et al.,* 2006). The best way to measure the TCR structural avidity is using multimer staining (MHC tetramers, pentamers or dextramers) and the use of multimer dissociation assay (measuring the dissociation rate of TCR bound multimers) using flow cytometry analysis. These analysis has been usually performed using multimer decay assays (Wooldridge *et al.,* 2009), in which the multimers bound to its cognate TCR in the sample and then measure the dissociation of these bound multimers over the time. If the binding strength is high (higher avidity), then the dissociation over the time is slow and vice versa for low binding strength interactions.

2.1.4.3 T cell Functional Avidity (TFA)

While two of the above discussed factors are related to the structural property of TCR and other contributing molecules during the T cell antigen presenting cell interaction, the term "functional avidity" refers to the measure of functional sensitivity of T cells towards its cognate antigen (Viganò *et al.*, 2012; von Essen *et al*., 2012; Amoah *et al.*, 2012), in other words it is the biological readout of T cell functionality. The detailed review of functional avidity is discussed in the following section.

Affinity and structural avidity are two important parameters contributing towards the functionality of T cells, however in many cases high frequency high affinity T cell response might not indicate a successful immune reaction because of its poor correlation between T cell responses and clinical outcome (Jonuleit *et al.*, 2001; Anichini *et al.,* 1999; Lee *et al.,* 1999). Apart from structural affinity and avidity, a more reliable way to assess the quality of the immune response is the T cell functional avidity (TFA). TFA is defined as the measure of the functional response of T cell to activation by an antigenic peptide presented on a MHC molecule, usually measured by T cell activation by the target cell using techniques such as cytokine release assays (peptide titration ELISpot assays, intracellular cytokine staining) or cytolytic (chromium release) (Pudney *et al*., 2010; McKee *et al*., 2005) target cell killing assays using a titrated concentration of peptide pulsed on a TAP (cells having defective antigen processing machinery) deficient antigen presenting cell (Luft *et al*., 2001). The effector biological

function is measured as the concentration required for 50% maximum effector function (EC_{50}) of T cells by plotting a sigmoidal graph with logM concentration of the peptides (Figure 2.4).

High T cell avidity is the ability of T cell to recognise and respond to low levels of the antigen concentration (hence having low EC_{50}) and the opposite is for the low avidity (High EC_{50}) T cells (McKee *et al.*, 2005). In many clinical settings this functional measurements of T cells have been positively correlated with tumour recognition and therapeutic efficiency making it an effective indicator for successful immunotherapy (McMahn & Slansky, 2007; McKee *et al.*, 2005).

There are many contributing factors for functional avidity of T cells other than TCR affinity and avidity such as T cell receptor (TCR) expression levels, expression of co-stimulatory molecules, cellular microenvironment (McMahn & Slansky, 2007; Viganò *et al*., 2012; von Essen *et al*., 2012) and the signal modulation in T cells during their encounter with an antigen presenting cell (Sharma & Alexander-Miller, 2011). Depending on the functional profile (antigen requirement for optimal activation) T cell population can be roughly classified in to high avidity (required < 1 nM peptide), medium (required < 1-100 nM peptide) or low avidity (required >100 nM peptide) T cells (Snyder *et al*., 2003).

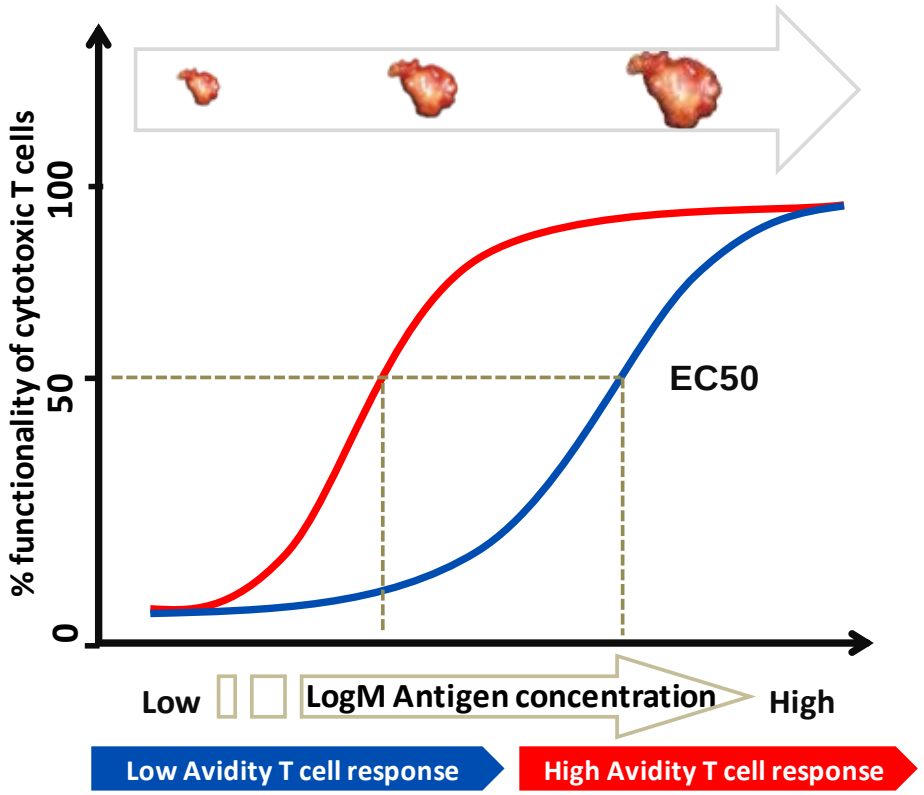

Figure 2.4: Representation of high and low avidity T cell responses, x-axis represent antigen concentration and y-axis represent percentage of T cell activity. High avidity T cell response (Red line) requires very low amounts of target antigen to generate visible populations of effector T cells. These cells recognise the typically low concentration of peptide present on the tumour surface and effectively check the tumour growth. In contrary, low avidity T cells required (blue line) very high concentration of antigenic peptides for its optimal activation; hence these cells were only activated when the tumour expressed very high level of tumour associated antigens.

2.1.5 Factors influencing T cell functional avidity

The first reported evidence of differences in functional avidity was depicted in the memory (antigen experienced) and naïve (antigen inexperienced) T cells (Byrne, 1988). This followed by subsequent studies also have demonstrated that the T cells can dramatically improve their functionality following an antigen exposure despite the fact that these cells are not capable of modifying their TCR by any type of somatic mutation in their antigen recognition motifs (Pihlgren *et al.*, 1996). Several arguments are in

place for explaining the increase in functional avidity, like existence of different TCR clonotypes having various affinities in the T cell repertoire or T cell having a fixed affinity can be converted into low and high avidity based on the stimulus they received in their clonal evolution. Most predominant support for the first argument came from the studies of a diabetes murine model (Han *et al.*, 2005), where they showed that the very low frequency existence of high avidity T cell at the beginning of the disease and when the disease progressed there is a correlated increase in the high avidity T cell population. For the support of second argument Alexander-miller and colleagues have showed that a single T cell clone can be functionally tuned into high and low avidity phenotypes by subjecting them into two extreme concentrations of peptide stimulations (Kroger & Alexander-miller, 2007). Their studies showed that the clones expanded in the presence of high concentration of antigenic peptide subsequently required high concentration of peptide for its optimal activation and vice versa for the clones expanded with the low concentration of the peptides. Both of these studies are counterintuitive in term of understanding the exact mechanism behind the shaping of functional profile of T cell repertoire.

Studies of T cell avidity in tumour antigens were first reported in murine melanoma model system (Zeh *et al.*, 1999), where they demonstrated the efficiency of high avidity T cells raised against low levels of tumour antigenic peptide TRP-2 for the elimination of lung metastases compared with low avidity T cells. The utility of high avidity response has been effectively reported in many other preclinical and clinical settings as well (Janicki *et al.*, 2008; Aranda *et al.*, 2011; Rezvani *et al.*, 2011). Adoptive transfer studies in a breast cancer model of NT2.5 tumours with high and low avidity HER2/neu $_{420\text{-}429}$ specific T cells have demonstrated that high avidity T cells were effectively capable of controlling the tumour growth after depleting the $CD25^{+}$ T regulatory cells using cyclophosphamide and antibodies (Weiss *et al.*, 2012) and pointed towards the potential problem of T cell functionality repression in the tumour environment. A recent study by Zhu *et al.*, (2013) which adoptively transferred high and low avidity TRP-2 specific T cell in a C57Bl6 mice bearing B16 melanoma tumour reported a more pronounced infiltration of high avidity T cells into the tumour in comparison to the low avidity counterparts. The high avidity T cells delayed the tumour growth initially, but were then suppressed in the tumour microenvironment. PD-1 blockade in the tumour

and the depletion of tumour associated dendritic cells quickly restored their functionality, highlighting the importance of susceptibility of these cells towards the tolerisation which is exerted by the tumour and can be reversed with targeting suppressive cells and the molecules within the tumour microenvironment.

The area of functional avidity is still controversial; the problem is mainly because all the three factors: affinity, structural avidity and functional avidity are highly interconnected and so it is very difficult to draw a clear line between them to define the functionality of T cells. Hence, it is highly important not to define functional avidity without considering the TCR peptide-MHC affinity and structural avidity. Factors that can influence avidity and thereby the property of functional avidity are discussed in the following sections.

2.1.5.1 TCR affinity is the single most influencing factor of T cell functionality?

Affinity is the first parameter to be fine-tuned in order to get a high functional avidity T cell. If pMHC complexes are naturally high in their affinity, low affinity TCRs will recognise them and mount T cell mediated immune response. Conversely, if the pMHC complexes are of very low affinity then they require very high affinity T cell receptors (Stone *et al*., 2009). There are many independent models available to explain the optimum TCR affinity (Figure.2.5), of which the two most important are the kinetic proof reading (KFR) model and serial triggering (ST) model. KFR model (McMahan & Slanskya, 2007) proposes that a TCR peptide MHC interaction should be in place right from the beginning to the end of the signalling process, whereas the ST model proposes that the pMHC complexes engaged with multiple TCRs for giving a sustained signal throughout the process of T cell activation. Studies with high affinity T cells *in vitro* (Corse, 2010; McMahan, 2006) found a good correlation between CTL activation and affinity in the laboratory. However, these correlations were not able to be reproduced in the *in vivo* models, in which the maximum response was obtained from the intermediate affinity peptide, not from the high affinity peptide. These studies suggested that even though affinity is a determining factor for the stimulation of the T cells, it is not the only a single factor which determines the functionality in the living system.

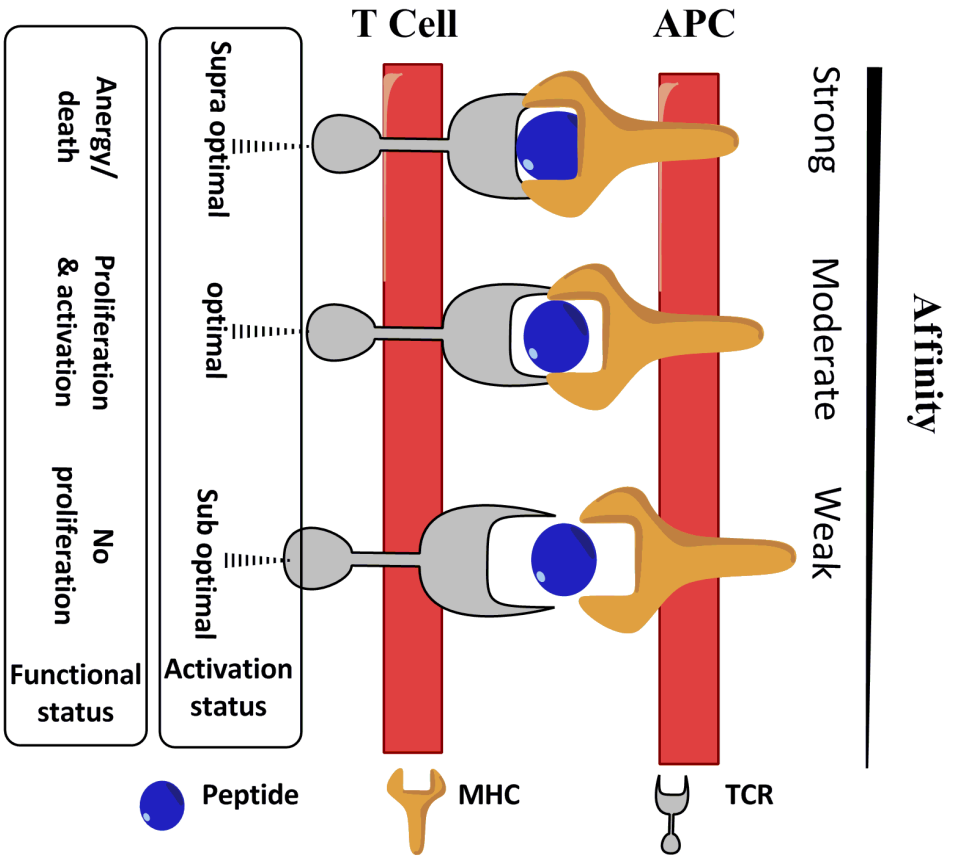

Figure 2.5: Three possible interactions of TCR and pMHC complex and their outcome as described by affinity model. Right hand side of the interaction is antigen presenting cell (APC) and the left side illustrate T cell and the possible outcome of each interactions. Despite of many existing theories about TCR Peptide-MHC, all the models are however suggesting three important aspect as illustrated in the figure. High affinity interactions will leads to overstimulation of T cells, results in functionally incapacitating the cells or ultimate deletion. Moderate affinity will be optimum for the best survival chance and the proliferation of T cells. Weak affinity leads to sub optimal stimulation and results in non-proliferation of the target cell.

Adapted from McMahan & Slansky 2007

Moreover, the affinity determinations were routinely undertaken with soluble single TCRs which might not be an accurate representation of the multimeric interactions which occur in the antigen presenting cell. Furthermore, the T cell is highly dynamic and is also in a different spatial plane (Nel, 2002). Now the question is ‘is the affinity the single most influencing factor?’, perhaps not. Studies using the viral models have

reported that T cells are capable of modifying their functional avidity several folds without selecting for high affinity receptors (Slifka & Whitton *et al.*, 2001).

2.1.5.2 Effect of co stimulatory and inhibitory molecules

The second important parameter which affects the T cell functional avidity is the co-receptor distribution and abundance on T cells. Of many co-receptors, CD8 is the most important one, which is a glycoprotein receptor present on cytotoxic T cells adjacent to the TCR-CD3 complex (Cole and Gao, 2004). (The detailed signalling mechanism from CD8 co-receptor is illustrated in Chapter 3). A slightly different role for this molecule in TCR signalling cascades has been observed in many studies that have compared naïve and primed T cells. CD8 molecules help to stabilise the interaction of TCR-pMHC at the synaptic complex formation and hence it play an important role in the stabilisation of the interactions. The critical role of CD8 in modulating the functionality in relation to TCR affinity was first demonstrated by Holler & Kranz (2003). In their study they have used engineered TCR (αβ) receptors with varying degrees of TCR affinity, as measured by the dissociation constant (kD). These cells are engineered with or without CD8 molecule to assess the role of this molecule in conjunction with TCR of varying affinity. Their study concluded that the receptors having higher affinity can stimulate with or without CD8, whereas the low affinity TCRs always required CD8 interaction. This observation is critical in terms of CTL based tumour immunology, as T cell repertoire in the periphery typically exhibits low affinity towards majority of all the tumour antigens due to their self-nature. These studies are further backed up by the data obtained from multimer staining of T cells (Choi *et al.*, 2003). Choi *et al* used an MHC molecule of mutated CD8 binding region to make the tetramers and found that the clones which are having the high affinity towards the cognate antigen will only bind to the tetramers and thereby readily identify the higher avidity T cells.

Another important co-receptor which might play an important role in functional avidity is CD28. The utility of CD28 in synaptic complex formation and TCR signal modulations has been well documented in the literature (Huang *et al.*, 2002; Yokosuka *et al.*, 2008; Acuto & Michel, 2003). Like CD8, CD28 is a glycoprotein receptor on T lymphocytes and it binds with the ligands present on the antigen presenting cells known as CD80 (B7.1) and CD86 (B7.2) to generate the second important signal (Signal 2) which is required for T cell activation. Previous studies of these molecules on naïve and

primed T cells (Manickasingham *et al*., 1998) have demonstrated that CD28 has different roles in these cell types. The involvements of other stimulatory molecules such as CD45 and CD81 in the functionality of T cells have been reported previously (Watson & Lee, 2004; Sagi *et al.*, 2012)

In contrary to stimulatory molecules, inhibitory molecules have a negative influence on the activation and proliferation of T lymphocytes. Three major inhibitory receptors of T cells are well characterised till today viz CTLA 4 (cytotoxic T lymphocyte antigen 4) PD-1 (programmed death 1 receptor) and BTLA (B and T cell attenuator). CTLA is a molecule expressed by T cell which binds to the CD80 and CD86 ligands present on the antigen presenting cells with higher affinity than their conventional receptor CD28 (activation receptor), and which results in the loss or reduced transmission of signal 2 required for the T cell activation (Korman *et al*., 2006). PD-1 is also expressed on T cells with its ligands (PDL-1 and PDL-2) expressed in many other normal organs (Riella *et al*., 2012). Expression of this ligand in normal organs was believed to be a mechanism of tolerance against self-reacting T cells (Hutchinson, 2012). In tumour immunology, PD-1 is the number one inhibitory signal which is expressed by the tumour and this will neutralise the potential cytotoxic activity of tumour infiltrating T cells (TILs) (Nirschl & Drake, 2013). The third member of inhibitory molecule is BTLA, and this is found to be upregulated in many T and B cells and its expression is used to regulate tolerance (Liu *et al*, 2009) by dampening the strength of the signals that are received by activated cells. Therefore, blocking of the expression of BTLA can resurrect the functionality of otherwise attenuated in these cells naturally (Pasero & Olive, 2013).

2.1.5.3 Other influencing factors

As has been discussed in the above sections, the temporal and spatial expression of activation and inhibitory receptors along with the affinity are not the only factors that govern the functional outcome of T cells. Physical role of cytoskeletal rearrangement and the distribution of lipid raft micro clusters are another possible area to be investigated since they are the two important parameters which determine the effective formation of a synaptic complex (Piragyte & Jun, 2012; Moran & Miceli, 1998). The involvement of these parameters are reviewed in Jenkins and Griffiths, 2010.

2.1.6 Immunologic memory, CTL phenotypic orientations implications in cancer immunotherapy

The ability of immune system to establish immunologic memory (B and T cell memory) is the hall mark of adaptive immunity. For T cell based tumour immunology it is having far more reaching implications in terms of preventing tumour reappearance after therapeutic interventions. In a broad definition, T cell memory is the ability of T lymphocytes to memorise the previous encounter of an antigen by keeping a long lasting pool of phenotypically and functionally distinct population of cell, capable of detecting subsequent repeated infection or malignancy in the future. For prophylactic vaccination settings memory is well studied and it is widely considered to be a key feature for keeping viral and bacterial pathogens in check during the rest of the life time in vaccinated individuals.

The process of establishing this immunologic memory starts at the very beginning of antigen exposure of naïve T cell population to the antigenic stimulus in the lymphoid organs. Naïve populations are expanded by receiving the appropriate antigenic and co-receptor stimulus and execute their effector functions which ultimately leads to the pathogen clearance. Ninety percent of the antigen specific effector CTLs then perishes (Gourley *et al.*, 2004) and the rest become memory T cell populations. Based on the expression of cell surface markers and secreted cytokine and other effector molecules, the memory population is further classified in to T central memory (Tcm) and effector memory (Tem) phenotypes (Zanetti *et al.*, 2010), with the former located primarily in the lymphoid organs and the latter in the peripheral tissues (Gourley *et al.*, 2004). It is widely understood that the changes in the gene expression patterns, transcription factors and epigenetic regulations control the process of converting the naïve and effector cells into memory phenotypes and keeps them in the body for longer time (Weng *et al.*, 2012). Predominantly, our understanding of memory differentiation has been derived from viral models. Studies of mouse models and chronic human viral infections have revealed the nature and establishment of the memory phenotype (Seder and Ahmed, 2003). In a basic model derived from the viral model, the memory is established in three phases. The clonal expansion of viral specific T cells takes off during the first wave of infection and reaches a peak of expansion in a few days. This population will decline

upon the viral clearance subsequently and the remaining population is converted in to the memory phenotype (Appay *et al.,* 2002).

As discussed earlier the magnitude and the nature of T cell immune response against the TAAs are slightly different due to their self-nature and hence it is crucial to understand the mechanism and the kinetics of memory establishment in major cancer vaccine trials (Seder and Ahmed, 2003). It is also noteworthy that most of the cancer vaccination strategies are in the therapeutic settings (i.e. vaccinating the patients already having the disease), hence antigen-specific T cells are presented with dual challenges of encountering a self-antigenic protein and immune suppression. However, most of the preclinical cancer trials have been conducted in mouse models and in this respect it is important to understand the phenotypic changes happening in these settings in terms of prolonged exposure of T cells to the antigenic stimulus. Cancer vaccination strategies used for the stimulation of T cell immunity are highly heterogeneous with regards to the various vaccine modalities such as whole cell, protein, peptide, DNA and dendritic cells (Ref. Chapter 1). These different vaccines are known to have different capacities to stimulate T cells at a different rate. The rate of expansion might have a consequence in the subsequent establishment of memory phenotypes (Seder and Ahmed, 2003). However, no studies were reported in the literature to study the effect of various vaccine strategies on the shaping of immunologic memory. However, many studies have agreed upon the fact that Tcm is more protective than Tem in patients undergoing a recall response with the same antigen (Goldinger *et al,* 2012; Klebanoff *et al., 2005*).

2.1.7 Aim of this study

It is widely accepted that the generation of high functional avidity T cells is an indicator of quality immune response. In the viral immunology the selection of the high avidity precursor population naturally happens because of the nature of viral antigen (foreign) and the high affinity receptors. However, in tumour immunology, because of the self-nature of the antigenic proteins combined with the tolerance mechanisms discussed above will limit the vaccine efficacy by generating inferior quality (low avidity) T cell populations. The careful vaccine designs in the recent past have demonstrated the generation of high avidity T cells in the patients are plausible. In such a scenario, if the antigen responsible for the immune response is known, it is always easy to assess the functional avidity by carrying out peptide titration cytokine assays. However, in

majority of cases this process is restricted by the limited availability of patient cells for conducting large titration assays with sufficient replicates. The situation becomes even more problematic when the responsible antigenic peptide is not known, such in whole cell and protein vaccines. In this context, we have attempted to characterise a mouse melanoma model system which is capable of generating high and low functional avidity T cells responding against its cognate antigen with different sensitivities for identifying a marker of higher functional avidity. Once, validated in the human vaccination setting, these markers will be useful for the identification of a high avidity response in the patients without undertaking cumbersome peptide titration assays. The mouse model has been previously reported by Metheringham, *et al (2009)*, which reported that immunising mice with a DNA vaccine containing a TRP-2 epitope generated a T cell response with high avidity characteristics compared to the corresponding peptide immunisations. The important advantage of this model is the ability to generate same number of peptide specific cells, but only the DNA vaccine immunisation yielded cells capable of responding to very low concentration of the antigen in the subsequent stimulations (as low as 10^{-7} M). Therefore, this is a unique vaccination model to study the behaviour of vaccine generated cytotoxic T cells and the molecular mechanisms underlying this differences in sensitivity/functional avidity. Immunological characterisations attempted in this study aimed to fully characterise and reproduce the previous observations made in this model by Metheringham, *et al., (2009)* with additional experiments and markers. The specific aims for this element of the study are given below.

- Assess the generation and frequency of peptide specific cells using multimer technology
- Complete characterisation of a high and low avidity mouse model using functional readouts such as IFN-γ
- Study the functional changes happening by *in vitro* stimulations using two peptide doses (High and Low)
- Study the immune phenotype of peptide specific cells using selected cluster of differentiation markers

2.2 Materials and Methods

2.2.1 Animal model systems

The mouse model used for the present study was C57Bl/6 (Charles River, Kent, UK) female mouse and melanoma differentiation tumour antigen tyrosinase related protein-2 (TRP-2). Immunisations were carried out with either a class 1 immunogenic TRP-2 peptide in conjunction with a class II hepatitis-B (Hep-B) helper epitope known to generate low avidity T cell responses or with a DNA based vaccine (ImmunoBody®, Scancell Ltd., Nottingham, UK) carrying the corresponding DNA fragment of the above mentioned TRP 2 epitope known to generate high avidity T cell responses in the C57Bl/6 animals after three round of immunisations. The detailed result of this model has been published elsewhere (Metheringham, *et al., 2009*). In order to cross validate the process we have used the same model workflow with a different foreign antigen derived from chicken ovalbumin. All the animal work has been undertaken with Home Office approval under the project licence number 40/3563.

2.2.2 Model characterisation

2.2.2.1 Peptide vaccine and immunisation

Synthetic HepB/TRP2 peptide TPPAYRPPNAPILAAASVYDFFVWL has been synthesised (ALTA biosciences, Birmingham, UK) with minimum purity of 90%. Upon arrival peptides were reconstituted in sterile Dimethyl sulfoxide (DMSO) at a stock concentration of 10 mg/mL and immediately frozen at $^{-}20^{0}$ C. Similar protocols were followed for the synthesis and preparation of ovalbumin epitope SIINFEKL. Prior to each vaccination peptide was prepared in phosphate buffered saline (PBS), emulsified with incomplete Freund's adjuvant (IFA) and administered subcutaneously (SC), on either side of the base of the tail at a dose concentration of 25 µg of peptide per animal. All the animals used in this study were 7-12 weeks old unless otherwise specified. Animals work was performed in a conventional animal facility or in a barrier clean environment which is specified wherever necessary in the following chapters. All the animals were received two more booster doses after the primary immunisations using the same concentration of the peptide or DNA vaccine with seven days intervals between them.

2.2.2.2 ImmunoBody® DNA Vaccine and Immunisation

ImmunoBody® DNA vaccine was kindly provided by Prof. Lindy Durrant (Scancell Ltd. Nottingham, UK), is a double expression vector (pDCorig), containing a murine variable heavy (VH) and kappa (VL) chain in one and a human kappa and heavy IgG1 constant regions in the second reading frames. Both the frames are under the control of separate CMV promoter and ends with Bovine Growth Hormone (BGH) polyadenylation signals for maximum stability of mRNAs. Complementary determining region (CDR) of the antibody sequences were removed and subsequently replaced with unique restriction enzymes containing multiple cloning sites (MCS), which is suitable for cloning different Class 1 and class II epitopes. Efficient transcription and translation of the antibody chain with inserted antigenic peptide was characterised by Metheringham, *et al.*, (2010). The detailed vector map of ImmunoBody® DNA vaccine is given in figure 2.6. Prior to immunisation, plasmid DNA (1 μg) was coated onto 1.0 μm gold particles (BioRad, Hemel Hempstead, UK) as per manufacturer's instructions. These coated DNA bullets were subsequently administered intradermally into the shaved abdomen of each mouse by a single shot using a BioRad Helios® gene gun. Primary immunised animals were subsequently boosted twice at seven days interval with the same DNA dose using the same administration protocol as above. A similar protocol was followed for ovalbumin epitope SIINFEKL, where the TRP-2 insert in the vector backbone has been replaced with SIINFEKL insert (Figure 2.6.). All the ImmunoBody® vaccines were constructed by Scancell Ltd. According to the company standard

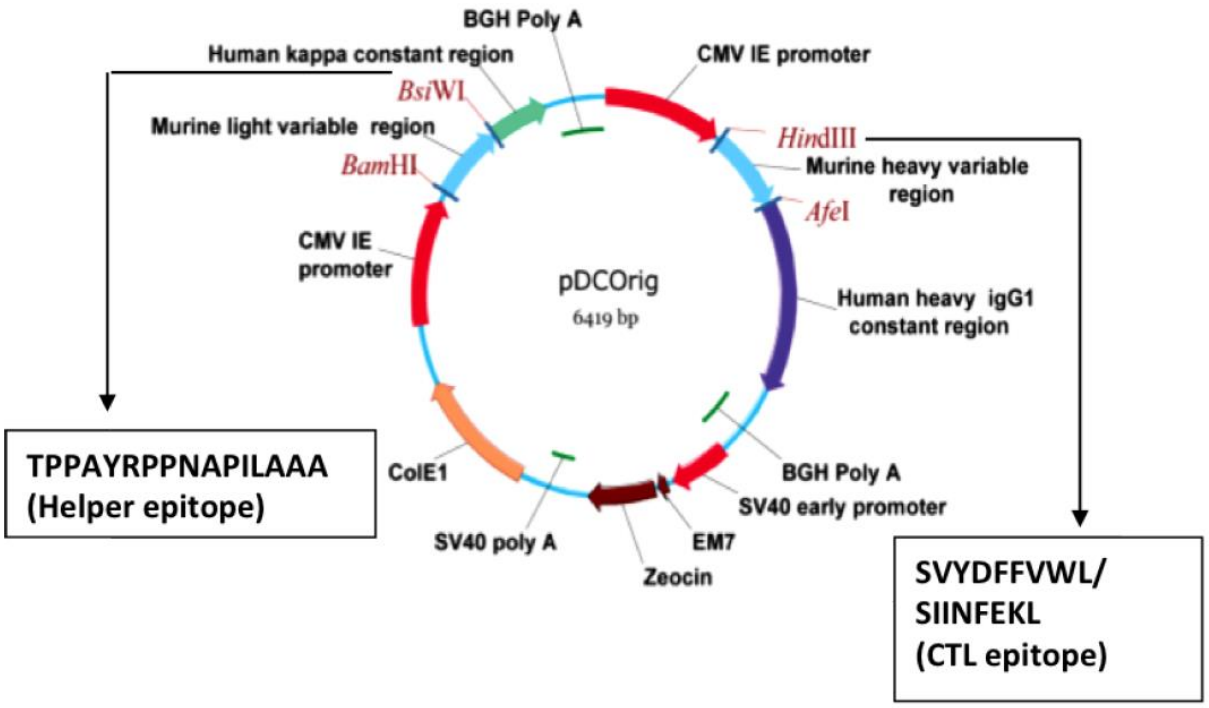

Figure 2.6: Vector map of ImmunoBody® DNA vaccine, this double expression vector carrying two open reading frames with an early CMV promoter and a BGA polyadenylation signal, the multiple cloning sites available within the two open reading frames allow the insertion of antigenic helper/CTL peptide gene sequences.

2.2.3 Isolation of splenocytes and cell counting

10 days after the last immunisation, all the immunised animals were terminated with cervical dislocation and immediately taken into a sterile laminar hood. Spleens and lymph nodes (Both axillary and Inguinal) from all the animals were harvested immediately using aseptic handling in to a pre-warmed serum free media (RPMI). Harvested spleens were placed into a sterile Petri dish containing 10 mL of serum free T cell media (RPMI 40 media containing 2mM L–Glutamine, 50 I.U./mL penicillin streptomycin and 20mM HEPES buffer). Splenocytes were carefully flushed out using a fine needle and with further 10 mL of the same media from both the sides of the spleen. The total splenocytes solution was transferred into a fresh universal tube and allowed to rest for 2 min. The solution was then carefully transferred into a fresh universal tube

leaving the large debris behind. The cells were pelleted by centrifugation in a swinging bucket rotor centrifuge at 300g for 10 min at room temperature. The supernatant was removed using a sterile aspirator and the cells were re-suspended in 10 mL of complete T cell media (10% Heat inactivated (HI) FCS (Lonza), 2mM L-glutamine(Lonza), 50 I.U./mL penicillin streptomycin (Lonza) and 20mM HEPES buffer(Lonza)) containing $7x10^{-5}$M *2*- mercaptoethanol (Sigma Aldridge). 50 µL of cell suspension was mixed with 50 µL trypan blue and counted using a disposable haemocytometer (Fast Read 102®) or a normal laboratory cytometer and the final cell number was adjusted to 5 x 10^6 T cell/mL of the media for ELISpot (MABTECH, Sweden) assays.

2.2.4 Setting up *ex vivo* ELISpot assay

TRP-2 Peptide (SVYDFFVWL) dilutions were prepared by freshly diluting the stock solution of 10 mg/mL to an initial working solution of 0.1 µg/mL ($1x10^{-1}$ M) and followed by 10 fold dilutions of up to 7 concentrations ($1x10^{-1}$ -10^{-7} M). Preparation of ELISpot plates was carried out a day before the assays by wetting the plate with 15 µL of 70% ethyl alcohol followed by washing the plates immediately with 170 µL of sterile distilled water four times. This plate was then coated with capture antibody (MABTEC INF-γ ELISpot kit) and kept in the fridge overnight. Following day the plates were washed three times with sterile PBS and blocked with complete T cell media for two hours prior to seeding the effector cells on to the plates. After two hours the medium was discarded from the plate and 100 µL ($5x10^5$ cells) of effector cells were added to each well with 100µl of the corresponding peptide dilutions in triplicates. The plates were incubated at 37^0C and 5% CO_2 (v/v) for 48 h. On the third day the plates were taken out of the incubator and cells were discarded and washed five times with sterile PBST (Phosphate Buffer Saline + 0.5% of Tween20). After the final wash100 µL of secondary antibody (1:1000 dilutions) was added to each well and incubated for 3 h at room temperature. Following the incubation the secondary antibody was discarded and washed three times with PBST. Alkaline phosphate conjugate was added to the plates and incubated for 90 min in the dark. Plates were washed again with PBST for five times and developed by adding 50 µL of development reagent (BioRad AP Substrate kit, prepared according to manufacturer's instruction) and incubated in the dark for 30 - 40 min. The development reaction was stopped by washing the plates in running tap water. The plates were dried at room temperature overnight and imaged in a plate reader

(C.T.L Technologies, USA) and the raw data was generated in an automated ELISpot counter with quality control performed for each plates.

2.2.5 Pentamer staining of peptide specific cells

For detecting the pentamer positive peptide specific cells in both ImmunoBody® and peptide immunised animals, class-I PE-2Kb/SVYDFFVWL pentamer (ProImmune) or class-I PE-2Kb/SIINFEKL pentamer (ProImmune) were used depending up on the antigens used for the immunisation. For the detection of TRP-2 specific $CD8^+$ $pentamer^+$ cells from the above immunised animals (1 x 10^6 total splenocytes prepared as in the section 2.2.3) were pelleted down by centrifugation (300 g for 10 min at room temperature). Red blood lysis was carried out by resuspending the cells in 5ml of 1X red blood lysis buffer (Miltenyi biotech) according to manufacturer's instructions and guidelines. Following red blood lysis, the cells were washed twice in the PBS with 1% heat inactivated FCS and the pellet was resuspended in residual wash solution after discarding the supernatant. The control unimmunised naïve mice also prepared in the same way and one test volume (10 µL) of class I pentamer was added to all the tubes. Tubes were incubated at room temperature for 20 min. Antimouse Fluorescein isothiocyanate [FITC] labelled CD8 (eBioscience) and antimouse Efluor 450 labelled CD3 (eBioscience) were added to each tubes and further incubated in the fridge for 15 min. Cells were washed with PBS with 1% FCS and resuspended in FACS buffer for acquiring the data in a Beckman Coulter Gallios® flow cytometry analyser. The data was further analysed using Kaluza® Flow Cytometry Analysis Software (Beckman Coulter). The analysis was performed in both TRP-2 and Ovalbumin model by the above mentioned pentamers. The specificity of pentamer binding was also confirmed by staining TRP-2 (SVYDFFVWL) high and low avidity TCR transgenic T cell clones kindly provided by Arthur Andrew Hurwitz (National Cancer Institute, Frederick, MD, USA). (Appendix 7.3)

2.2.6 Half maximal effective concentration-50 (EC_{50}) calculation

The peptide concentrations required for 50% maximum activation (Effective concentration 50% [EC_{50}]) of high and low avidity T cells was determined by plotting, a sigmoidal dose response curve and fitting this using the raw data obtained from peptide titration ELISpot assays after removing the background spot values from each

concentrations (*n=9*). A variable slope model was used for the curve fitting, top and bottom constraint were applied as '100' and '0' respectively. All the EC_{50} calculations were done in three independent occasions in triplicates.

2.2.7 Interferon-γ (IFN- γ) Intracellular cytokine (ICS) staining

To validate the results obtained from ELISpot assays, an ICS protocol was performed on cells obtained from both the immunised animals. 1.5 $x10^6$ Total splenocytes obtained from ImmunoBody® and peptide immunised animals were seeded on to each well of a 24 well culture plate with the same decreasing concentration ($1x10^{-1}$ - 10^{-7} M) of peptides used for the ELISpot assays, leaving one well as control with no peptides. Cells were rested for an hour before adding brefeldin-A (5 mg/mL stock) at final concentration of 10 µg/mL. Cells were left overnight at 37°C in a 5% (v/v) CO_2 incubator. Next day the cells were harvested in to a 15 mL conical bottom tube and washed with PBS + 1% FCS, removed the liquid completely after pelleting the cells by centrifugations as described above. Intracellular cytokine staining was performed using IntraPrep® (Beckman Coulter) intracellular antigen staining kit as per manufactures guidelines. Details on the antibodies and fluorophores that were used in this study are given in Table 2.1.

2.2.8 *In vitro* stimulation of the splenocytes

This experiment was designed to investigate the ability of T cells to retain the property of functional avidity *ex vivo* under various experimental conditions, High and low avidity CTL's isolated from both ImmunoBody® and peptide groups were subjected to repeated *in vitro* stimulations using two different doses (100 µg and 10 ng) of TRP-2 peptide for 7 days. The stimulated cells were analysed by IFN-γ ELISpot assays for its functionality.

2.2.9 Preparation of syngeneic lipopolysaccharide BLAST (LPS blast)

Lipopolysaccharide is a large molecule containing lipid and carbohydrate molecules mainly present in the outer coat of gram negative bacteria is a useful tool in the laboratory for the stimulation of immune cells. LPS blasts were prepared on day one of the *in vitro* stimulation protocol as follows. One naïve seven to eight week old C57BL/6 mice was terminated by cervical dislocation and total splenocytes extracted as described earlier. The cells were centrifuged at 300 g for 10 min and resuspended in fresh

complete T cell media. Cells were counted and diluted to 1 x 10^6 cells/mL, LPS and dextran sulphate were added to a final concentration of 25 ug/mL and 7 ug/mL of the cell suspension respectively and incubated at 37°C for 48 h.

2.2.10 *In vitro* stimulation

On day three, LPS blasts were harvested into a 50 mL tube after visual inspection for activation and contamination under the microscope. The cells were irradiated for 8 min (6000 Rads) in a GamaCell® Caesium Source. After irradiation cells were washed three times with serum free RPMI complete medium and finally resuspended in the same medium at 2 x 10^7cells/mL. Two separate tubes were prepared with the above cell number and peptides were added to the tube at high (100 µg/mL) and low (10 ng/mL) concentration. Tubes were incubated at 37°C for 75 min in a 5% CO_2 incubator with cap loosened. While target cells were incubating, spleens were harvested from the ImmunoBody® and peptide immunised C57BL/6 mice. Splenocytes were extracted as described in section 2.2.3 and counted as described above. These effector cells were diluted at 5 x 10^6 cells/mL in complete CTL media. 1 mL of effecter cells were then plated in a 24 well plate from each group. After incubation, peptide pulsed irradiated target cells were taken out of the incubator and diluted to 1x10^6 cell/mL with complete CTL media. 1 mL of target cell is carefully pipette out into each well of the effector cell and incubated at 37°C for seven days in a 5% v/v CO_2 incubator.

2.2.11 ELIspot assay setting up with *in vitro* stimulated cells

On day seven of the first *in vitro* stimulation, a capture antibody coated ELISpot plate was washed four times with sterile PBS and blocked by the addition of 100 µL complete T cell media. RMAS cells (antigen processing defective cells) were used as antigen presenting cells (target cells) for all the ELISpot assays after *in vitro* stimulations, these cells were cultured in RPMI (5% (v/v) serum + L- Glutamine) at 37°C for 48 h. and harvested into a 50 mL falcon tube in a sterile tissue culture hood. The cells were counted and eight aliquots of 5x10^5 cells were prepared in a 15 mL Falcon tube, the cells were pelleted by centrifugation at 300 g for 10 min at room temperature. Six 10 fold dilutions ($^-$1M to $^-$6M) of TRP 2 (SVYDFFVWL) peptide had been prepared in serum free RPMI media. 500 µL of peptide dilutions were added to the RMAS cell pellet in a labelled 15 mL tube. To the control tube only 500 µL of serum free RPMI

was added. Cells were incubated at 37°C for 75 minutes in a CO_2 incubator with cap loosened. After the incubation cells were washed with 10 mL of sterile PBS and resuspended in complete CTL media. A further 10 fold dilution of the target cells ($5x10^4$ cells/mL) was prepared with complete CTL media. The effector cells were prepared by counting the *in vitro* stimulated cells from step 2.2.8 and diluting the cells to 5 x 10^5 cells/mL. Flick off the blocking media from ELISpot plates and 100 µL of the effector cells ($5x10^4$ cells in total) and 100 µL of target cells ($5x10^3$) were pipetted into each well of the ELISpot plate. Plates were then incubated at 37°C overnight in a CO_2 incubator. On the next day cells were discarded and washed the plates with PBS+0.5% Tween20 and the plates developed as per the protocol given in section 2.2.4. The detailed work flow is illustrated in Figure 2.7.

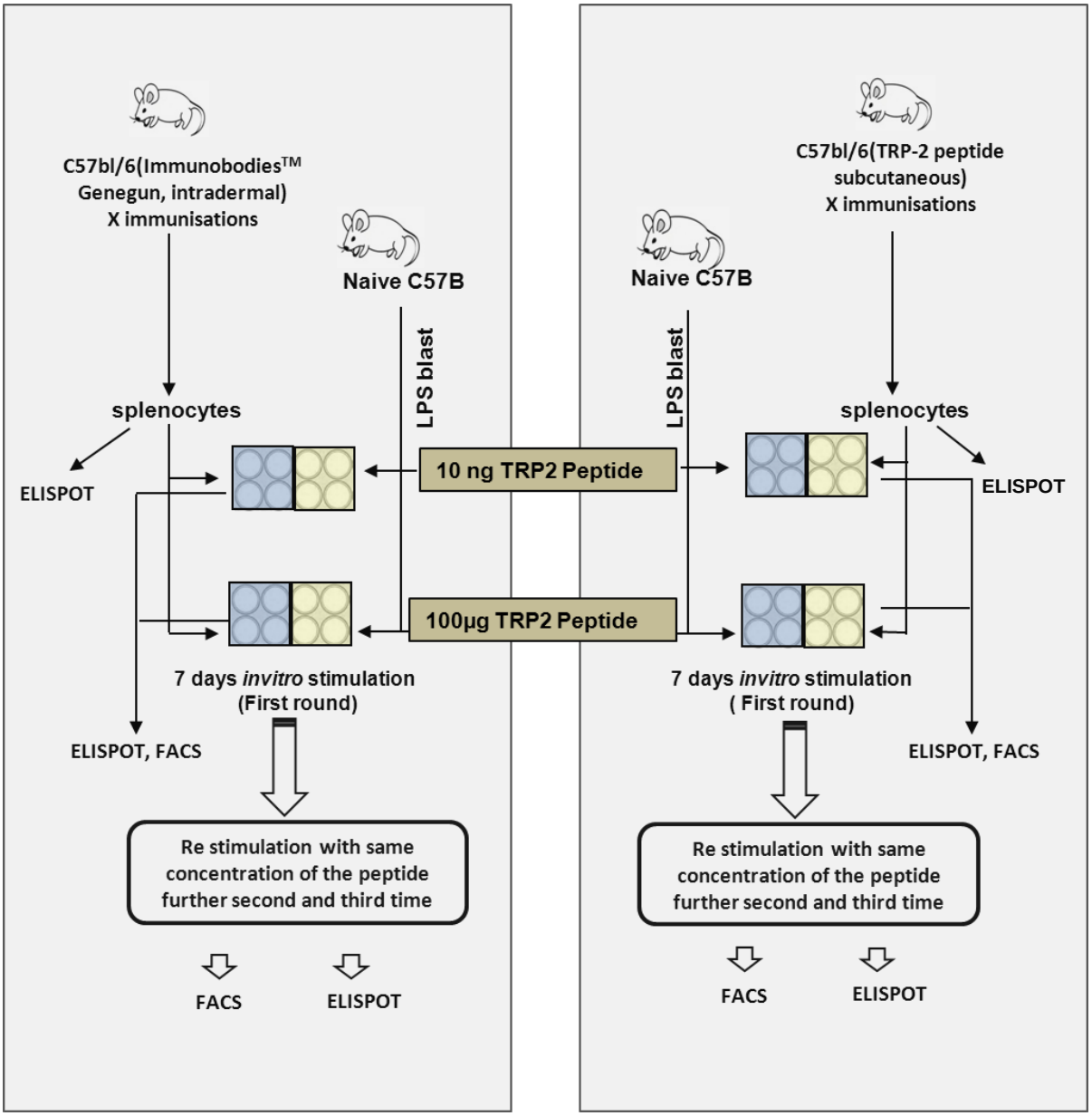

Figure 2.7: Schematic representation of *in vitro* stimulation of splenocytes using two different concentrations of peptides to generate high and low avidity CTL's.

2.2.12 Immunophenotyping of high and low avidity T cells using multicolour flow cytometry

In this study, the pentamer positive TRP-2 specific cells were immunoprofiled for their markers that were indicative of effector and memory characteristics. Markers involved in the functional activation and attenuation status were also present in the panel to obtain a comprehensive phenotypic profile of cells that had been generated by two immunisations. The markers used for the study were obtained from eBioscience and are detailed in the Table: 2.1.

Table: 2.1 Antibodies and fluorophores used for the immunophenotyping

Sl.No	Marker	Fluorophore	Made By
1	CD8	FITC	eBioscience
2	CD3	eFluor450	eBioscience
3	CD28	PerCP-Cy5.5	eBioscience
4	CD272(BTLA)	APC	eBioscience
5	CD197 (CCR7)	Alexa Fluor700	eBioscience
6	CD62L	APC-eFluor780	eBioscience
7	CD44	PE/Cy7	BioLegend
8	Mouse IFN-γ	APC	BioLegend
9	MHC Pentamer	PE	ProImmune

The animals were immunised as per the protocol mentioned in the section 2.2.1 however the immunised animals were left longer than normal (45 days) before harvesting spleen and lymph node for further investigation. The splenocytes and lymphocytes were prepared as mentioned in section 2.2.3. 1.5 $x10^6$ cells from each mouse were used for further flow cytometric analysis. Briefly, red blood cell lysis were

performed with 1X RBC lysing solution (Miltenyi Biotec GmbH) as per the manufacturer's guidelines and washed twice with PBS containing 1% v/v FCS. Subsequently cells were resuspended in 50 µL of PBS containing 1% v/v FCS buffer thoroughly. 10 µL of MHC PE conjugated TRP-2 specific pentamer was then added to each tubes and incubated at room temperature in the dark for 30 min. After 30 min, all other surface antibodies (CD8, CD3, CD28, CD272 (BTLA), CD197 (CCR7), CD62L, and CD44) were added as per the recommended concentrations of the manufacturer. Tubes were incubated at 4°C for further 15 min and washed with 5 mL of PBS containing 1% v/v FCS buffer. Cells were resuspended in flow cytometer buffer before subjected to flow cytometric analysis. Naive unimmunised animals were used as a control and the data analysis were performed in Kaluza® software (Beckman Coulter).

2.2.13 Statistical analysis

All the data in this study are presented as mean ± standard error of the mean (SEM). Pairwise comparisons were performed with non-parametric student t-tests. Multiple comparisons were performed using ANOVA and Bonferroni post test. P-value significance in the data has been represented by asterisks [$p \leq 0.05$ (*), $p \leq 0.01$ (**), and $p \leq 0.001$ (***)].

2.3 Results

2.3.1 Detection of peptide specific $CD8^+$ cells in ImmunoBody® and peptide immunised mouse models.

The rationale of this study is to understand the molecular mechanisms governing the generation of high avidity T cells, and the identification of biomarkers that would allow us to detect the presence of high avidity T cells in cancer immunotherapeutic interventions. In order to achieve the main goal, this study used two mouse models that would reproducibly generate high and low avidity T cell responses, so that any markers identified will be cross validated in separate models. which use two different antigenic epitopes. The advantage of this strategy is that any markers identified can be independent of the model or the immunogenic peptide used for the immunisations. In most of the T cell targeted vaccination settings, the generation of peptide (antigen) specific $CD8^+$ T cells is the key parameter to assess vaccine efficacy. Therefore, present study has assessed the number of peptide specific cells in both ImmunoBody® and peptide immunised animals to test the capacity of both the vaccines to generate an antigen specific response.

Splenocytes isolated from ImmunoBody® and peptide immunisations were assessed using antibody staining and flow cytometry analysis. The gating strategy used for the analysis is given in figure. 2.3.1. In TRP-2 immunised animals, $CD8^+$ pentamer positive T cells were detected in the range of 0.35 - 44% of the total $CD8^+$ population, with no significant differences noticed between the groups ($P \geq 0.05,\ n = 8$). A similar pattern was observed in the ovalbumin group, with the detection of ova specific cells in a range of 0.88 - 0.94% of total CD8 with similar number of cells between the DNA and peptide vaccine groups ($P \geq 0.05,\ n = 6$). However, ovalbumin immunisation resulted in a greater number of peptide specific cells than TRP-2 immunisation, thereby indicating that it is more immunogenic among the two model antigens studied. As a staining control, a group of un-immunised (naïve) animals were also analysed similarly (Figure 2.8. B&F). The background staining observed in the control groups was lower than in the experimental groups and is represented as a box graph in Figure 2.8.E.

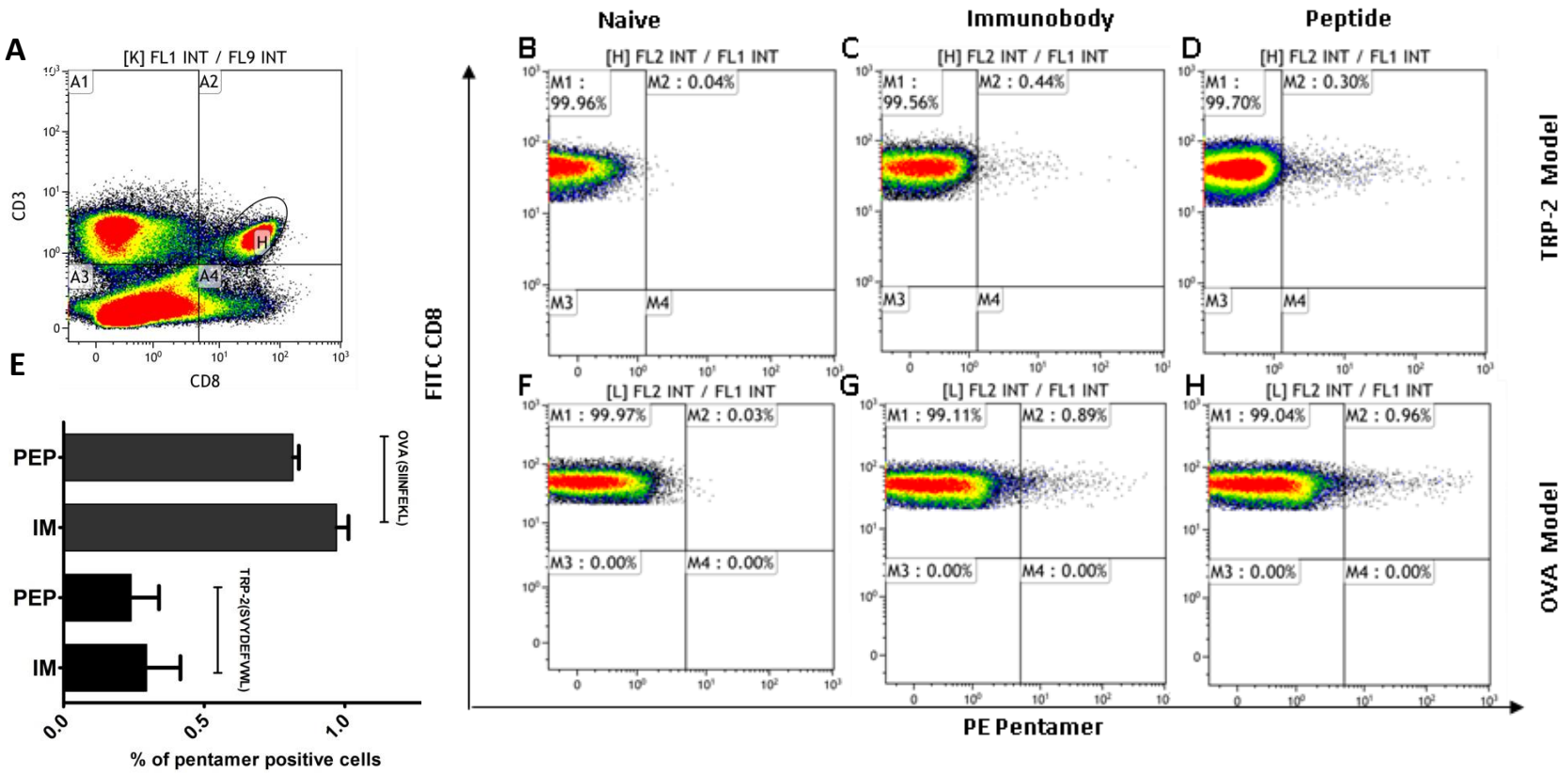

Figure.2.8: Generation of TRP-2 and Ovalbumin SIINFEKL specific cells in both ImmunoBody® and peptide immunised animals were studied after three rounds of immunisations using FACs analysis. **A:** Scatter plot showing the gating strategy used for TRP2 pentamer staining with CD3 on Y axis and CD8 on X axis. **B:** The staining of TRP-2 specific pentamers in naïve animals. **C&D:** Detection of TRP-2 specific cells in both TRP-2 (SVYDFFVWL) ImmunoBody® and peptide immunised animals **F:** The staining of OVA specific pentamers in naïve animals. **G&H:** staining of peptide specific cells in Ovalbumin (SIINFEKL) ImmunoBody® and peptide immunised animals Fig**. E**. Percentage of pentamer positive cells were calculated out of total $CD8^+$ cells and represented as mean and SEM of 8 independent experiments in TRP-2 and six in Ova models. No significant differences were noticed between two immunisations in both TRP-2 *(N=8, $P \geq 0.05$)* and OVA *(N=6, $P \geq 0.05$)* models.

2.3.2 Characterisation of functional avidity in C57Bl/6 mouse model

Although the generation of the peptide specific cells is the first step in measuring the vaccine efficacy, the functionality of vaccine generated T cells can drastically vary depending on the various factors such as vaccine framework, the mode and root of administrations (Okada *et al.*, 2001; Senovilla *et al.*, 2013, Sandoval *et al.*, 2013). So it is widely accepted that the quality of immune response is directly linked to the functionality of the vaccine generated T cells. With that view, we assessed the functionality of CTLs generated in both the mouse models (SVYDFFVWL TRP-2 melanoma differentiation self-antigen and SIINFEKL Ovalbumin foreign antigen model), using interferon-γ (IFN-γ) measurement assays.

Total splenocytes were isolated after the vaccination regime was studied for their functional ability to recognise and respond to their cognate peptides. Elispot assays of splenocytes isolated from the TRP-2 model showed differences in their ability to produce IFN-γ when challenged with TRP-2 peptides as determined by the mean spot differences between the groups (ImmunoBody® vs. peptide vaccine) in ELISpot assays (Figure 2.9 A). ImmunoBody® immunised animals were found to be better responders when compared to the peptide group (*P=0.0001, n=8*). A similar study was conducted with an identical immunisation procedure, but using a different well characterised H-2Kb restricted model antigenic peptide (SIINFEKL) from ovalbumin. ELISpot® assays were performed as described above and the mean spot differences were calculated between the peptide immunised and DNA immunised animals. Like the TRP-2 system ImmunoBody® immunised animals were more functionally superior as measured by their ability to secrete IFN - γ *(n=6, P = 0.0001)* Figure 2.9 B

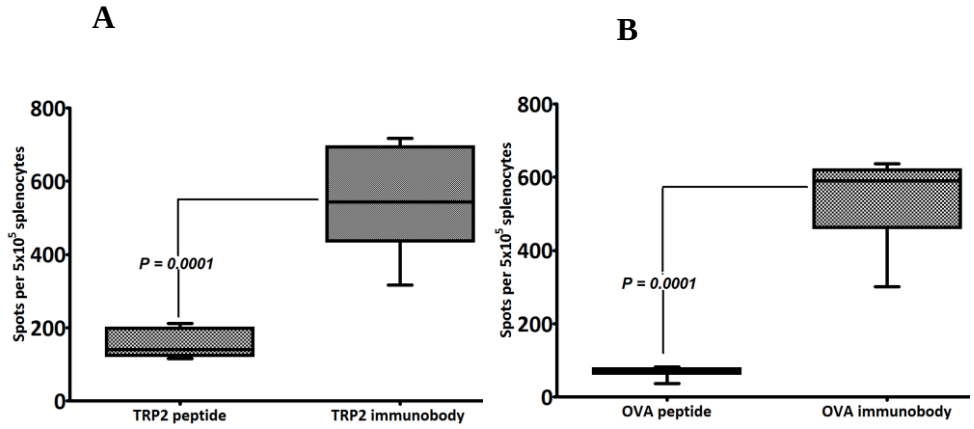

Figure.2.9. C57Bl/6 mice immunised with TRP2 (SVYDFFVWL) and Ovalbumin ('OVA' /SIINFEKL) ImmunoBody® (DNA) and peptide vaccines were assayed by IFN-γ ELISpot assays. **A**. Number of spots obtained from $5x10^5$ splenocytes of TRP-2 ImmunoBody® and peptide immunised animals were shown as box and whisker plots (*n=8*) with P-value calculated between groups. **B.** Similar results were obtained from $5x10^5$ splenocytes of 'ova' ImmunoBody® and peptide immunised animals (*n=6*).

In the next step of the study, cells generated by both the immunisations were assessed for its differences in the functional avidity (sensitivity towards its cognate antigens). This was achieved by a peptide titration IFN-γ ELISpot assay, in which the cognate peptide was (TRP-2/OVA), titrated down to seven fold dilutions from 0.1M to 0.0000001M (Fig.2.10) and splenocytes obtained from the immunisations were co-cultured in ELISpot plates with all the dilutions in triplicates. The experiment has been repeated with the same settings for at least three times. Splenocytes with no peptide was used as the control and background spots were subtracted from the test data before plotting as a graph and further statistical analysis (Figure 2.10).

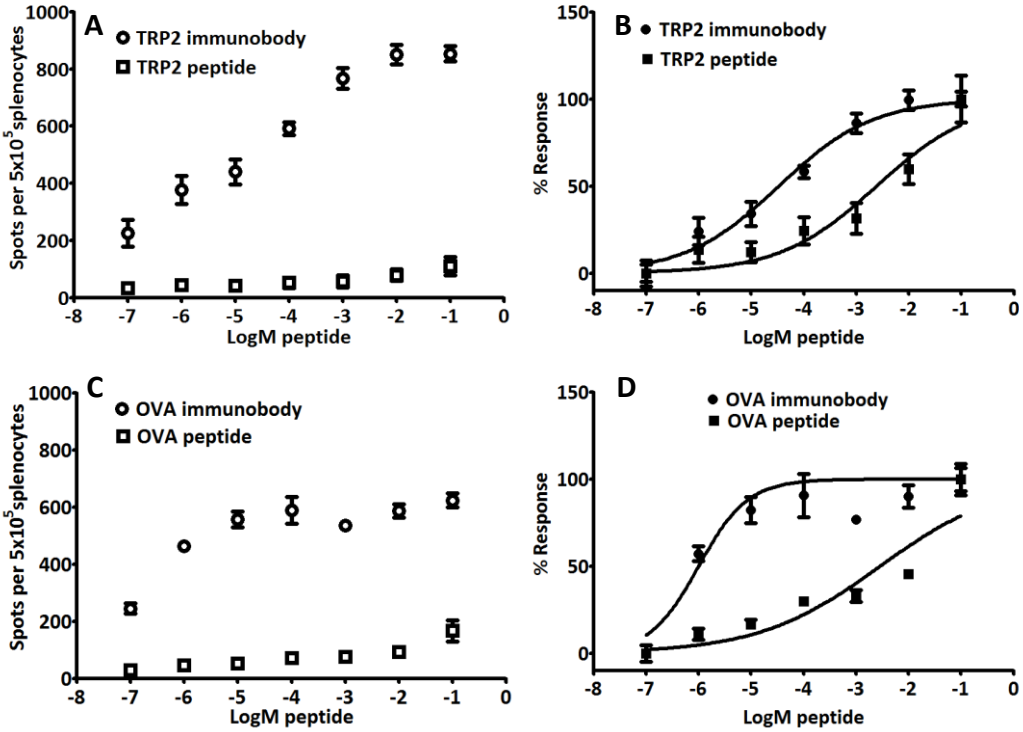

Figure.2.10. The cells obtained from the above vaccinations were assayed for their functional avidity profile by IFN-γ peptide titration ELISpot assays. **A. & C,** actual number of spots obtained from $5x10^5$ splenocytes of TRP-2 DNA and peptide immunised animals were shown with its LogM peptide concentrations on the X axis (*n=8 forTRP-2 and n=6 for OVA)* **B & D.** Figure showing the concentration requirement for 50% maximum function (EC_{50}) for ImmunoBodies and for peptides in TRP-2 **(B)** and 'OVA' **(D)** model studied.

2.3.3 Intracellular cytokine staining of high and low avidity $CD8^+$ T cells from TRP-2 ImmunoBody® and peptide Immunised animals

In order to validate the IFN-γ profiles of these two distinctly functionally different high and low avidity cells, an intracellular cytokine staining was performed. The animals were immunised as described previously and the splenocytes were isolated under sterile conditions. Cells were seeded on to a 24 well plate at a cell density of $1x10^6$ cells/well with 10^{-1} M TRP-2 peptide. IFN-γ secretion assays were then performed as described in section 2.2.7 and the IFN- γ secreting cells were determined. A similar pattern of IFN-γ profiles were observed with ImmunoBody® generated cells having higher number of IFN-γ producing $CD8^+$ cells in all the peptide concentrations studied (Figure.2.11).

The nonlinear regression of both the ImmunoBody® and peptides with decreasing titration of logM concentration of the peptides demonstrated that there is a strong differences in the concentration requirement of peptides for achieving a maximum 50% activation between ImmunoBody® and peptide derived high and low avidity T- cells [$P \leq 0.0001$] (Fig 2.10) in both the models studied. The $LogEC_{50}$ for ImmunoBody® has been calculated as $^-4.717 \pm 0.0206$ (Mean ± SEM, *n=8*) for TRP 2 immunobodies and $^-3.258 \pm 0.03223$, (Mean ± SEM, *n=8*) for the TRP-2 peptides. Similar results were obtained with the second model (OVA), however the EC_{50} calculated between these group has showed much more difference compared to TRP-2 groups with a log EC_{50} of $^-5.886 \pm 0.1562$ (*n=6*) for OVA Immunobodies and $^-2.571 \pm 0.0.1582$ (*n=6*) for OVA peptides.

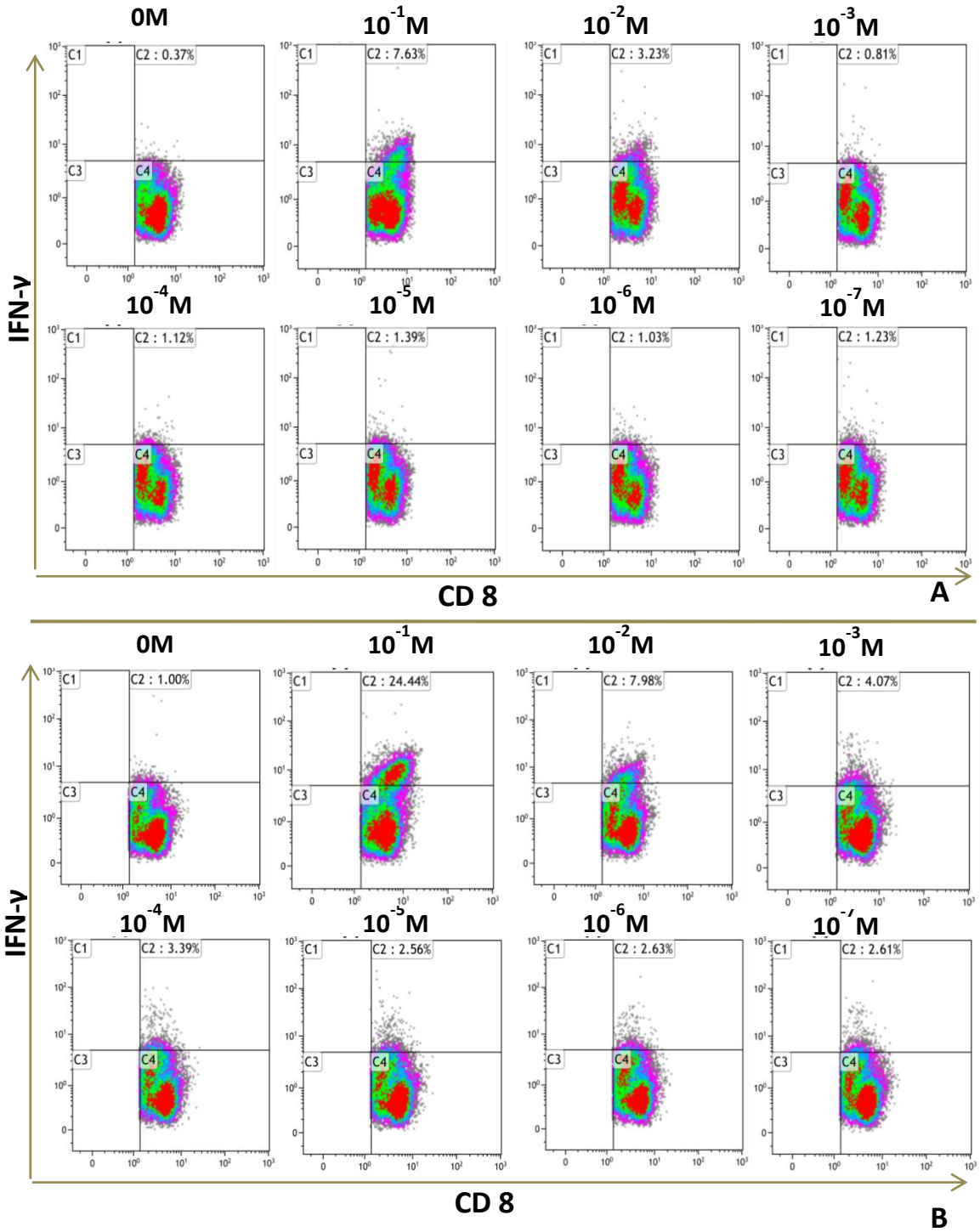

Figure 2.11: Intracellular cytokine staining of TRP-2 ImmunoBody® and peptide immunised animals showing IFN-γ production. The cells were stimulated with decreasing concentration of peptides (10^{-1}- 10^{-7} M) in the presence of brefeldin-A and stained for CD8 surface marker and IFN-γ. The upper panel **A** shows the density plot gated on $CD8^+$ cells with IFN-γ on the Y-axis. The first panel represents cells with media, brefeldinA and no peptide, following plots represent decreasing concentration of peptides (mentioned above each plot) up to the concentration of 10^{-7}. Quadrant C2 represents $CD8^+$, IFN-γ producing cells. The percentage of IFN-γ producing cells were also given in the same quadrant. The upper panel represent IFN-γ profile of a peptide titration assay of cells derived from TRP-2 peptide immunised animals and the lower panel **(B)** represent the same assay with the cells derived from ImmunoBody® immunisations.

2.3.4 *In vitro* stimulation of TRP-2 DNA and peptide vaccine generated CTLs can alter its functional properties

It has been shown by several groups that stimulation of CTLs in the lab can alter their functional ability as measured by the ability of cells to secrete IFN-γ. Total splenocytes isolated from both TRP-2 ImmunoBody® and peptide groups were stimulated by two concentrations of the peptides (100 µg/mL and 10 ng/mL). After one week of stimulation these cells were assessed for their ability to produce IFN-γ using peptide titration ELISpot assays. The *ex vivo* ELISpot assays were done with $5x10^5$ splenocytes per each well of the ELISpot plate, whereas the ELIspot assays done after *in vitro* stimulations were used only $5x10^4$ cells per well. Therefore all the cell counts were normalized to $5x10^5$ cells for a head to head comparison. Plots were generated using these normalized values and the statistical analyses were performed using two way ANOVA with Bonferroni post-test.

After a week of stimulation using high concentration (100 µg/mL) of the peptides and a low concentration (10 ng/mL) of the peptide, in ImmunoBody® group, there was an overall reduction in the spot count in comparison to the unstimulated controls in all the concentration tested in the ELIspot assays (Figure 2.12). Both 100 µg/mL and 10 ng/mL stimulated cells showed significant reduction in the spot count ($P \leq 0.001$, $n=6$). There was a complete absence of spots in the lower concentrations (10^{-4}-10^{-6} M) in both the cases indicated the disappearance of high avidity/sensitivity cells upon stimulation.

In contrary to the splenocytes derived from ImmunoBody® immunised animals, the peptide immunisation derived cells showed an opposite pattern in the interferon profile in ELISpot assays. In straight ELISpot (*ex vivo*) assays, splenocytes derived from peptide immunised animals produced a significantly lower number of spots in comparison to ImmunoBody group. However, after one week of *in vitro* stimulation of these cells with both high and low concentrations of TRP-2 peptide resulted in the production of IFN-γ in all the concentration tested in peptide titration ELISpot assays ($P \leq 0.001$, $n=3$).

One week post stimulation these cells even showed significantly higher sensitivity towards the lowest concentration of the peptides tested otherwise completely insensitive in the pre *in vitro* stimulations (Figure 2.12). Noticeably in both the studies there were no significant difference ($P \geq 0.05$) observed between high and low doses of peptides used for *in vitro* stimulations.

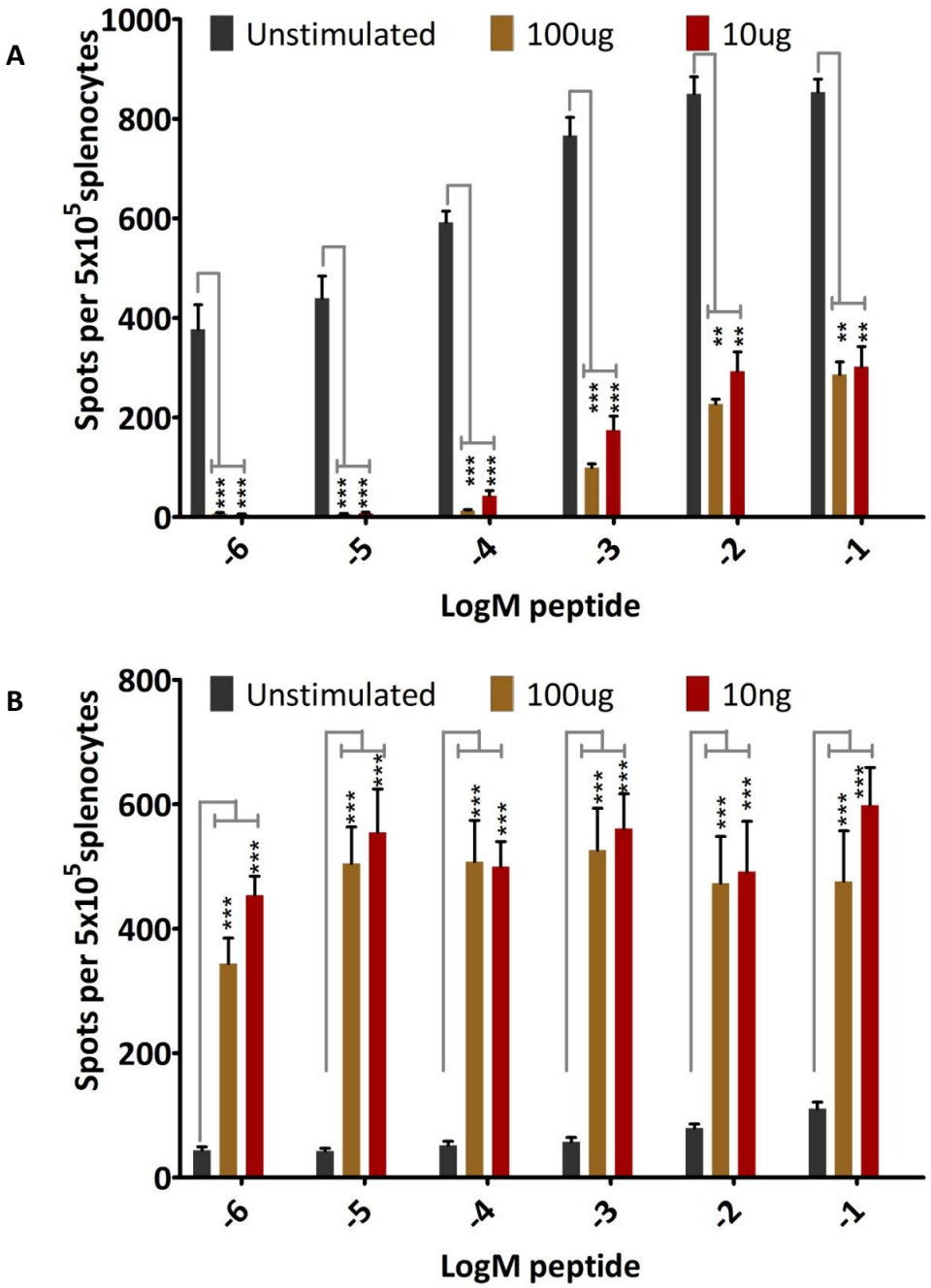

Figure 2.12. Repeated *in vitro* stimulation *in vitro* stimulations of CTLs can modulate its functional property as assessed by peptide titration IFN-γ ELISpot assays. **A.** plots showing the number of IFN-γ producing cells in unstimulated controls (*ex vivo*) and also after one week of *in vitro* stimulations using splenocytes isolated from Immunobody® Immunised animals (*n=6*) with two concentrations of TRP-2 class-I peptide. Y axis represents the normalised number of spots. **B.** Plots showing the similar data obtained using splenocytes isolated from TRP-2 peptide Immunised animals (*n=3*) with same concentrations of peptides stimulations. In both cases the P value significance is calculated for each concentration in comparison to the unstimulated controls and the asterisk represent the level of significance (** = $P \leq 0.01$, ***= $P \leq 0.001$)

2.3.5 Immunophenotyping of high and low avidity peptide specific CTLs

Phenotypic characterisations of vaccine generated T cells are a key indicator to understand the nature of response and to predict the polarisation of these cells to effector or memory phenotype. The functional fate of these cells can be decided by the nature of the vaccine and its potential to stimulate the naïve population in a short period of time. In the previous functional characterisation of the TRP-2 immunisations, we have observed two functional characteristics as assessed by interferon-γ peptide titration ELISpot assays. So we hypothesised that the functional polarisation of these cells will be different given the nature of two types of vaccines used for the epitope delivery (DNA vs. peptide). In order to test this hypothesis we have used the surface markers (section 2.2.12) which could delineate the peptide specific cells into further sub classifications. The cells were stained and subjected to flow cytometric analysis according to the optimised gating parameters. The expression of markers on TRP-2 pentamer positive cells in the spleen and lymph node of ImmunoBody® and peptide immunised animals are given in figure 2.13 and figure 2.14.

All the data from this immunophenotyping study was first analysed for the presence of TRP-2 pentamer positive cells before applying sub gating and looking for the expression of other markers. There was no significant differences in the percentage of pentamer positive cells in the spleen (1.83 ± 0.25 [mean ± SEM]) and the lymph node (1.6 ± 0.046) of ImmunoBody® immunised animals. However, the pentamer positive cells in the spleen (1.79 ± 0.18) and lymph node (0.76 ± 0.80) of peptide immunised animals were significantly ($P \leq 0.01$; $N=3$) different, with more cells detected in the spleen of all the three animals studied. These results were generated 45 days after the first immunisation, compared 21 days that was used for the previous studies (Figure 2.8). These findings demonstrate that the percentage of pentamer positive cells were increased significantly with time (Figure 2.8, 2.13) except the lymph node of peptide immunised animals.

These cells were further used for the analysis of the expression of other markers (CD44, CD62L, CCR7 and CD28) in order to investigate if any preferential phenotypic polarisation was happening over the time within the vaccine generated $CD8^+$ T cells. Expression of three memory markers (CD44, CD62L and CCR7) were first assessed on TRP-2 pentamer positive cells especially to address the question of whether these cells have more polarisation toward which memory compartment (Tcm / Tem). Majority of the cells were showed a $CD44^+$, $CD62L^+$ phenotype with a very high expression of CD44

(mean fluorescence intensity: 110.90 ± 12.1 (Mean ± SEM)) and low expression of CD62 (mean fluorescence intensity 12.20 ± 2.3 (Mean ± SEM)). Similarly, the analysis of CD62L and CCR7 on the same cells indicated positive staining but low expression of these markers were observed in the ImmunoBody® and peptide immunisations in both spleen and lymph node. CD44 expressing cells are also found to be expressing low amount of CCR7 in the spleen of both ImmunoBody® and peptide immunisations. However, a large number of CCR7 negative cells were found in the pentamer positive cells isolated from the lymph nodes of ImmunoBody® immunisations. From this analysis the predominant phenotype observed is $CD44^{hi}$, $CCR7^{lo}$, $CD62^{lo}$ in the spleen of both immunisations and the lymph node of peptide immunisations. Cells derived from the lymph node of ImmunoBody immunisations are predominantly $CD44^{hi}$, $CCR7^{-}$, $CD62^{lo}$ (Figure 14 and 15).

Expression of CD28, a costimulatory molecule, plays an important role in the T cell activation and proliferation. It is also reported to have a varied expression at different stages of antigen experience by a T cell. This study investigated the status of CD28 expression in TRP-2 pentamer positive T cell to assess their expression as a part of immunophenotyping in pentamer positive cells of both ImmunoBody® and peptide immunised animals. In the $CD28^{+}$ compartment, large proportion of antigen specific $CD8^{+}$ cells were positively stained for CD28 with low expression levels (Figure 14 and 15).

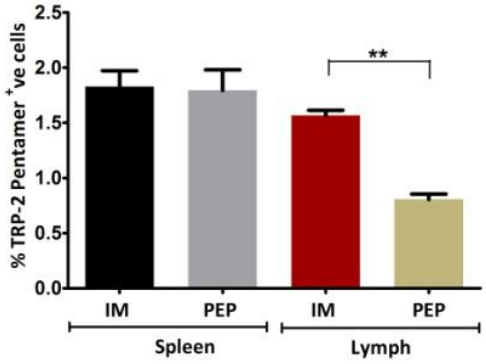

Figure 2.13: Detection of pentamer positive cells in the TRP-2 ImmunoBody® and peptide immunised animals used for the immunophenotyping study. Each bar represents an average of three animals with the error bar showing the standard error of mean. The significant difference observed were indicated as (*) above the bars.

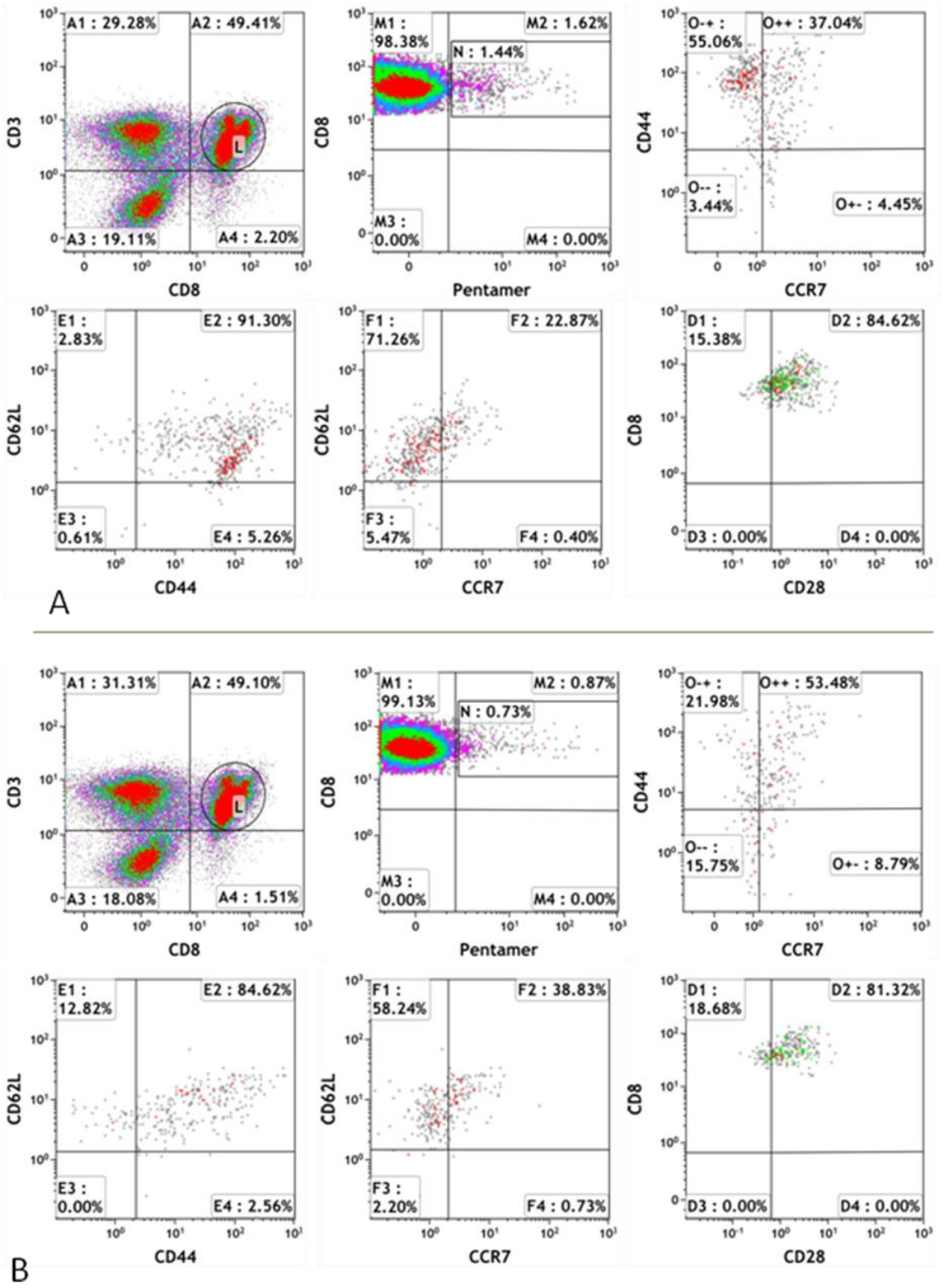

Figure 2.14: Representative dot plots showing the expression of five surface markers on the pentamer positive cells detected on the lymph node resident pentamer positive, $CD8^+$, $CD3^+$ T cells (LNCs). All the analysis was performed on TRP-2 pentamer positive T cells 45 days the first immunisation. **A.** Expression of markers in cells (LNCs) derived from TRP-2 ImmunoBody® immunised animals. **B.** Expression of markers in cells (LNCs) derived from TRP-2 peptide immunised animals.

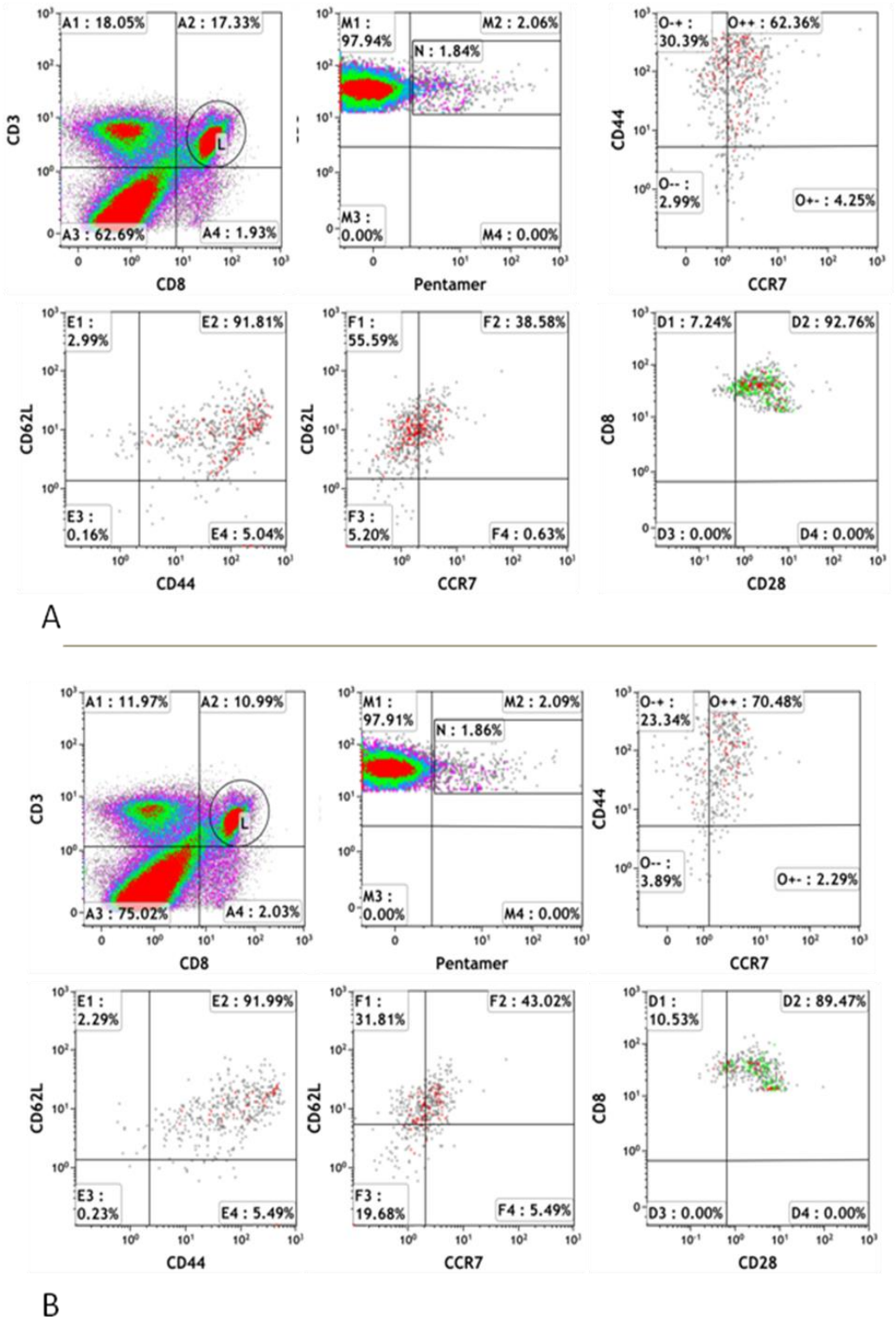

Figure 2.15: Representative dot plots showing the expression of five surface markers on the pentamer positive cells detected on the splenocyte $CD8^+$, $CD3^+$ T cells (Spleen). All the analysis was performed on TRP-2 pentamer positive T cells 45 days the first immunisation. **A.** Expression of markers in cells (spleen) derived from TRP-2 ImmunoBody® immunised animals. **B.** Expression of markers in cells (spleen) derived from TRP-2 peptide immunised animals.

2.3.6 Expression of B and T lymphocyte attenuator (BTLA) in vaccine generated CD8$^+$ T cells

BTLA (CD272) is an inhibitory molecule present in the CD8$^+$ T cells and known to play an important role in the functional suppression of vaccine generated T cells. With this view in mind this study has also assessed the expression of BTLA in TRP2 pentamer positive cells as a part of immunophenotyping.

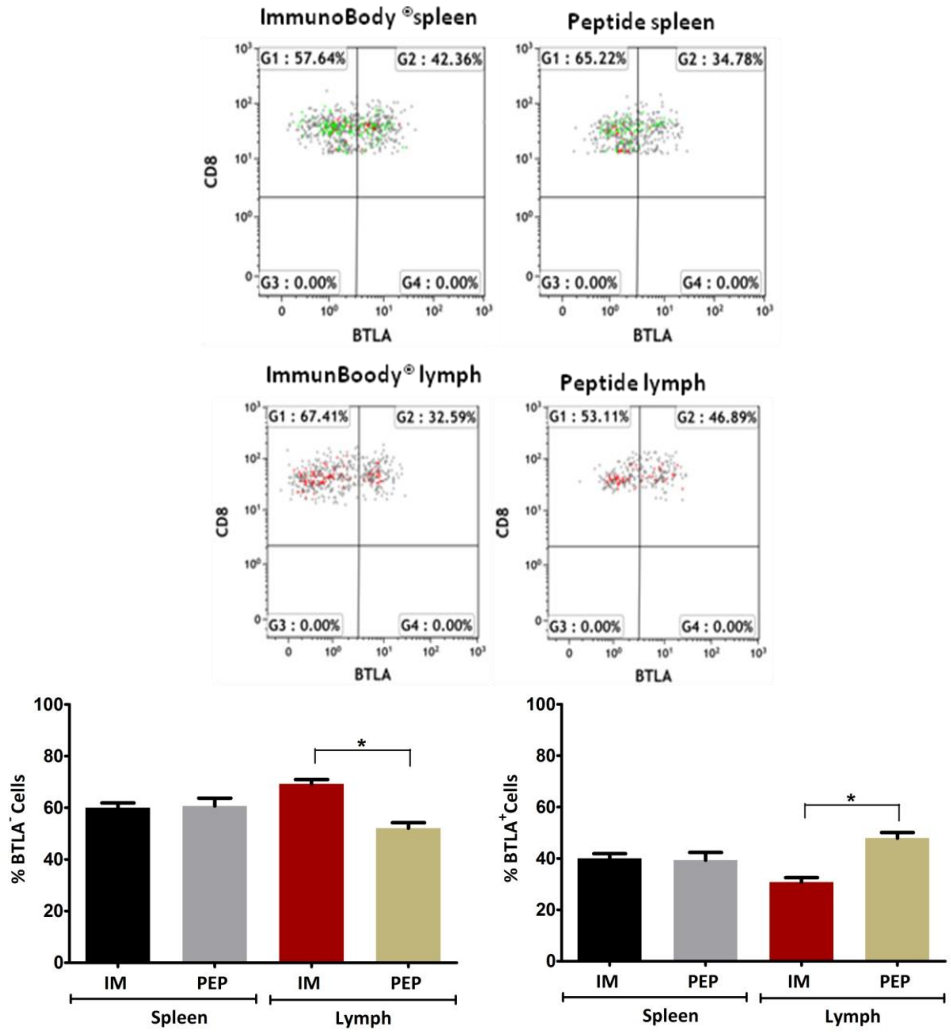

Figure 2.16: Expression of BTLA surface molecules in TRP-2 immunised animals **A**. Representative dot plot of BTLA expression on pentamer positive cells **B.** Percentage of BTLA$^+$ cells detected in the spleen and lymph node ImmunoBody® immunised (IM) and peptide immunised (PEP) animals. Significant differences observed were indicated as stars above the bars.

The pentamer positive cells in both the vaccination showed more BTLA negative cells of which a significant ($p \leq 0.01$; n=3) decrease was detected in peptide immunised lymph node (Figure 2.16).

2.4 Discussion

2.4.1. DNA vaccine (ImmunoBody®) generated CTLs with higher avidity characteristics.

Despite of our understanding of cancer initiation and immune surveillance, most of the T cell based cancer vaccine strategies remain not completely successful. One of the reasons might be attributed to the nature of the antigens used for the immune targeting. Unlike other vaccination strategies cancer vaccines are mainly developed against the self-antigens and hence the tolerance mechanisms are playing a crucial role in the attenuation of T cell responses (Pawelec & Rees., 2002; Kyewski & Klein, 2006). Even with all the failures, many of the cancer vaccination trials recently conducted were able to generate noticeable number of antigen/vaccine specific T cells but unable to translate that into clinically visible outcome due to the lack of tumour recognition and target killing. Among many other reasons identified as the limiting factors for vaccine efficacy (such as immunosubversion and immunoselection devised by tumours), the nature and functional behaviour of the cytotoxic T cells generated through each type of vaccinations are also an important factor for a positive clinical outcome. One of the efficient ways to assess this quality of vaccine generated T cell immune responses are T cell functional avidity.

Functional avidity is the measurement of T cells ability to respond against its corresponding peptide which is usually measured by peptide titration ELISpot assays or chromium release assays. However, these assays are laborious and can only be performed where the peptide responsible for the generation of this cells are known. So there is a pressing need to identify markers to measure a high avidity T cell response in cancer patients regardless of the antigen or vaccines used for the immunisations. With this in mind, this study used a mouse model system to mimic the vaccine response with varying functional avidity characteristics.

The mouse models used for this study was able to generate high (capable of recognising low dose of antigen) and low avidity (capable of recognising very high dose of antigen) responses by using two vaccine delivery systems (a DNA and the peptide vaccine). The primary model was a C57Bl/6J-TRP-2 system which uses a self-peptide (180-188, SVYDFFVWL) derived from melanoma differentiation antigen tyrosinase related protein - 2. The DNA sequence of this peptide was inserted in to the antibody heavy chain of the vaccine with a CD4 helper epitope (Hep B) in to the light chain region of the vector as

indicated in materials and method (Section 2.2.2.2). Immunisation of this vaccine to the C57Bl/6J mouse induces a high avidity T cell response compared with the corresponding peptide vaccine. The peptide titration IFN-γ ELISpot assay clearly indicated that the ImmunoBody® derived cells are capable of producing IFN-γ even at the lowest concentration of the peptide used for the assays. In contrary, peptide vaccine derived cells responded poorly in the IFN-γ assays with no or little spots were observed when the concentration of the peptide was decreased. One of the obvious questions then asked was the ability of the vaccines to generate TRP-2 specific CTLs in both the vaccinations (ImmunoBody® and Peptide). To check that $CD8^+$ cells isolated from both the immunisations were stained with a MHC class 1 TRP-2 pentamer (PE labelled) and noticed that both the immunisations were able to generate TRP-2 specific CTLs at approximately equal numbers with no significant differences. So the only difference observed in the study was their IFN-γ ELISpot profile which was an indication of their functionality.

Half maximum effective concentration 50 (EC_{50}) was determined for both the ImmunoBody® and peptides vaccines using peptide titration data. There was a 100 fold differences in the EC_{50} for peptide and ImmunoBody® derived splenocytes indicated that the ImmunoBody® derived splenocytes were more sensitive when compared to peptide groups. Similar pattern was observed with the second model also, where the TRP-2 peptide was replaced with an epitope derived from foreign protein, ovalbumin peptide sequence (SIINFEKL, $OVA_{257-264}$) in the ImmunoBody® vaccine framework. Overall the frequency of peptide specific CTLs were found to be higher in the ovalbumin model compared to the TRP-2 model which agree with the earlier findings ((Pudney, *et al.,* 2010). As with the TRP-2 system ImmunoBody® derived splenocytes showed better sensitivity towards the SIINFEKL peptides in peptide titration ELISpot assays. These experiments were repeated several times (TRP-2 model: $n= 8$ and Ovalbumin SIINFEKL model: $n=6$) and the difference in the functional properties between ImmunoBody® and peptide derived cells were highly significant $(p= 0.0001)$, hence these models were considered as an ideal tool for initial avidity molecular characterisation and the identification of avidity biomarkers.

The explanation for differences in the functional characteristics of cancer vaccine generated T cells are rooted in thymic selection, central and peripheral tolerance mechanisms. The existence of tumour reactive T cells was detected in many cancer patients. However, studies revealed that they were in a state of anergy/nonresponsive stage

partially because of keeping the low avidity-self reactive T cells under control from autoimmunity. Since most of the tumour antigens were also self-antigens or having a highest similarity to the self-proteins, it is very difficult to make the small repertoire of these low avidity T cells to break the tolerance mechanism and engage with the malignant tissues. Moreover the antigens present on the tumour surface may not be in sufficient quantities to evoke a positive response in these low avidity T cells since they need large amount of peptides to get activated.

For a successful tumour vaccination trial the available low avidity T cell pool in the immune repertoire has to be effectively primed with suitable vaccine delivery mechanisms. Different vaccine strategies are in place for this purpose such as increasing the affinity of peptide MHC interactions using various amino acid substitutions on the peptide anchor positions (Khleif, *et al.,* 1999; Loftus, *et al.,* 1998), increasing the peptide TCR binding efficiency using the same strategy (Sette & Fikes, 2003; Fong, *et al.,* 2001), genetically engineered TCRs specific to tumour antigens (Aarnoudse, *et al.,* 2002) and use of DNA vaccines (Gross, *et al.,* 1989) and anti idiotypic vaccines (Pudney, *et al.,* 2010).

Apart from the above mentioned modifications of the antigenic peptides, different vaccine delivery strategies and adjuvant settings were also effective to increase the spectrum of high avidity CTL responses in cancer patients (Stevenson *et al.,* 2004; 2010). One of the problems using the conventional peptide vaccines are their structural instability in the *in vivo* environment combined with the half-life of injected peptide, DNA vaccines such as ImmunoBody® which used in this study can efficiently overcome this problem by producing the peptide epitope with in the body and ensured the constant supply of the peptide antigens at a moderate level to the cellular immunity (Rice, *et al.,* 2008; Pudney, *et al.,* 2010).

Another possible explanation for ImmunoBody's ability to generate high avidity T cell responses might be due to its ability to perform direct and cross (indirect) presentation of the antigens to the immune system (Pudney, *et al.,* 2010; Metheringham, *et al.,* 2009). The direct presentation of the antigens was through the professional antigen presenting cells such as dendritic cells present at the site of vaccination. In this case ImmunoBody® DNA delivered intradermally transfect the DC's and translate in to peptide fragments and finally presented on a class 1 MHC complex. The indirect presentation also involves APC's, however in this case the DNA was first taken up by non APC's such as skin keratinocytes

and produce the antigens and finally presented to the APCs as a secreted protein or through apoptotic vesicles (Stevenson *et al.,* 2004; 2010). Studies suggested that ImmunoBody® vaccines were capable of generating the high avidity responses through presenting a low level of antigens to the CTLs thereby selectively expanding the population of high avidity T cells which can recognise the low doses of tumour antigens (Pudney, *et al.*, 2010). However, it is worth mentioning that this study has not detected any preferential selection of high avidity TCR clones from the total repertoire in either of these vaccines. The pentamer positive cells were uniformly distributed in the staining with our any significant difference in their staining intensity.

Though it is clear from this and other studies that using the ImmunoBody® consistently generate highly sensitive T cells, the decision point (the molecular mechanism required for high functional avidity) of this alteration of T cell functionality is largely unknown. Therefore, understanding the available pool of T cell precursors which can detect the self-antigenic epitopes and the precise mechanism to uplift their functional superiority is the key to the success of modern cancer vaccination strategies. In addition, understanding the precise molecular mechanism underlying the functional modulation of CTLs by vaccines might help in the generation of more effective vaccine framework and strategies.

2.4.2 *In vitro* stimulations might modulate the functionality of T cells

One constrain of this study was the number of peptide specific T cells available for the proteomic and genomic characterisations. So initially a separate experiment was designed for the *in vitro* generation of high and low avidity T cells by *in vitro* stimulation of the T cells with two different concentrations of the peptides. The original aim of the study was unfulfilled with little differences were observed between the groups. However an important observation was concluded from this experiment. The high avidity T cells derived from ImmunoBody® immunised animals which were responded to the TRP-2 peptides even at the lowest concentrations were disappeared after the first round of *in vitro* stimulations in both the concentrations used for the stimulation (100 µg and 10 ng). This indicates the fragility of high avidity $CD8^+$ T cells towards high peptide load. We presume that this might be due to the susceptibility of ImmunoBody generated high avidity T cell population towards excessive antigen load due to serial triggering and ultimately leading to activation induced cell death by apoptosis. In contrary to the ImmunoBody® derived T cells, peptide derived cells started to secrete IFN-γ after two rounds of stimulation in both of the above

doses tried. These results indicated that a functionally inferior pool of CTLs exists in the peptide immunised animals (low avidity) and once they received high dose of peptide stimulations they can be brought back in to the high avidity zone by breaking their avidity barrier (*inability to detect low amount of antigenic peptides*). This study partially agree with the observations made by Pudney *et al*, 2010 that the *in vitro* stimulation of TRP-2 specific T cells derived from B16- GMCSF immunised mice with low doses of peptide generated high avidity T cells whereas stimulation with high doses of peptide ended up in low avidity T cells.

2.4.3 Phenotypic changes of immunised animals after long term antigen exposure

The immunophenotypic of pentamer positive cells were carried out with the intention of studying the nature of these cells after long exposure of antigen stimulation using both ImmunoBody® and peptide immunisations. This study also aimed to understand there is any polarisation of these cells towards the central or effector memory.

The first observation noticed that the number of antigen specific cells was increased in both the cases compared to the normal immunisations used for the study (21 days vs. 45 days). There was no significant differences in the cells were observed between the spleen and the lymph node of ImmunoBody® immunised animals however a decreased homing of pentamer positive cells were detected in the lymph node of peptide immunised animals and were significantly lower compared to its corresponding spleen.

These differences might be attributed to the fundamental differences of two vaccines used for the immunisations. It is known in the literature that the stability of the immunisation and the root of administration have a knock on effect on the stimulation of naive repertoire. The DNA vaccine is known for their stability and once they have been taken up by the antigen presenting cells, there is always a constant production of the delivered antigens with in these cells. Because of that reason, the DCs, which express these antigens, will have the capacity to stimulate the T cells over a prolonged period by directly presenting the antigens. Apart from that, some of the plasmids, which have been taken up by the non-dendritic cells at the place of immunisations, also might be able to produce this antigen. Since ImmunoBody® has been engineered in such a way that the epitope is mounted on an antibody secretory chain the antigens produced by non-dendritic cells can also acts as a depot of antigens. These antigens can enter in the DCs and feed in to the class one pathway by cross presentation by the DCs. This sustained stimulation, which might be given by the

dendritic cells, could be the reason for the increased number of peptide specific cells in the ImmunoBody® immunised animals.

In contrary to the DNA vaccine peptide vaccines offers only the administered dose of the peptide as the source of antigen. The diffusions happening in the immunisation place (even with the adjuvant IFA) may add up to the availability of the antigen. The successes of peptide immunisations are also dependent on the efficiency with which the local DCs take-up the antigen and its subsequent feeding in to the class I pathway. Not only that, the DCs once taken up the antigens they have to migrate to the sentinel lymph nodes to present the antigen to the T cell repertoire for further clonal expansion of the antigen specific T cells. The maturation and the migratory capacity of the DCs can be another influencing factor, since the delivery of the antigens are in the short peptide format there is a chance of DC maturation happening fairly quickly and consequently the loss of migratory capacity to the lymph nodes. In the case of DNA vaccines, probably the vector internalisation starts up the process of DC maturation and its mobility towards the lymph nodes. However, the translation and the protein production will happen only with time compared to the readily antigen availability of peptide vaccines. This might be probably the reason by which the large number of peptide specific cells in the ImmunoBody® vaccination in both the lymph node and the spleen.

Among the markers studied in the pentamer positive cells isolated from ImmunoBody® and peptide immunised animals both in lymph node and spleen, no great differences could observed of the immunophenotype within the lymph and spleen. The predominant phenotype observed in this study was $CD44^{hi}$, $CCR7^{lo}$, $CD62^{lo}$ in the spleen of both immunisations and the lymph node of peptide immunisations. However, pentamer positive cells derived from the lymph node of ImmunoBody immunisations are predominantly $CD44^{hi}$, $CCR7^{-}$, $CD62^{lo.}$

Naive T cells are characterised with the presence of homing receptors CCR7 and very high levels of CD62L markers. The expression levels of these markers decreases up on antigen stimulation. In all the above cases pentamer positive cells showed the decreased levels of CCR7 and CD62L indicating they were subjected to antigen exposure. The presence of large number of CCR7 negative cells in the lymph node of ImmunoBody immunised animals indicating a possibility of chronic stimulation of pentamer positive cells. The predominantly observed phenotype $CD44^{hi}$, $CCR7^{lo}$, $CD62^{lo}$ is a typical effector memory

phenotype in mouse. However, these cells were obtained from the spleen and lymph nodes of immunised mice and it is a highly unlikely place to find effector memory population. Therefore, obvious observation is they are antigen experienced T cells and yet to be polarised into a memory populations with in the period of this study. High levels of CD44 and decreased expression of CCR7 and CD62L in the spleen and lymph node derived cells of peptide immunisation and also in the spleen derived cells of ImmunoBody® immunisation showed that their similarity in marker expression and therefore their antigen experience. However, significant presence of $CCR7^-$ cells in the lymph node of ImmunoBody® immunisation suggests that these cells might have exposed to the antigen more efficiently. The original hypothesis was that the DNA vaccine primed the cells more effectively and chronically so that the functional polarisations will be quicker in these groups. However, the data showed that it is not the case and both of them retained a relatively similar marker profile with subtle changes. More studies needed with longer duration with additional markers and effector cytokines will be the future direction for this study.

Chapter 3

Gene expression profiling of High and low avidity T cells

3.1 Introduction

As discussed in chapter 2, the nature and the functionality of vaccine generated $CD8^+$ T cell responses can vary significantly depending on the vaccination strategies used to deliver the antigenic peptide. These differences might be attributed to the ways in which vaccines stimulate the naïve T cell repertoire (Cao *et al.*, 2013). For that reason, the observed immune response is mainly attributed to the nature of the antigenic stimulus used, the way in which it is processed and presented to the T cell immune arm and also to some extent the precursor frequency of vaccine-specific T cells in the patient. Activation and the stimulation of T cells is not a single receptor ligand mediated process. The optimal stimulation of the naïve repertoire requires multifactorial interactions of many receptors at the initial point of contact with an antigen presenting cell (Friedl *et al.,* 2005). These interactions include the cumulative binding strength of the primary receptor responsible for the detection of the antigen (TCR), other secondary and tertiary interactions of co-receptors and all other supporting binding machinery at the synaptic complex (APC-T cell contact point) interface. If these signalling and interactions are not strong or stable, it influences the functional fate of the resulting antigen primed T cells in a more negative than positive way (Katzman *et al.,* 2010).

3.1.1 Signal requirements for T cell priming and activation

It is well known that for any optimum activation, T cells requires a minimum of two signals and three signals for maximal activation (Bretsche, 1999). The first signal is generated from the TCR- peptide MHC interactions and the second one coming from the CD28 co-receptor interactions with its ligand (B7 family ligands) on the APC (Bretsche, 1999; Baxter and Hodgkin, 2002). These two signals are thought to be essential for the optimum survival of an antigen experienced T cells. The third signal which comes from the local cytokine milieu, although not essential, is necessary for efficient clonal expansion of the antigen experienced T cells and also believed to be contributing towards the development of effector functions in these cells (Curtsinger *et al.*, 1999). Type I interferon's (∞ and β) and IL12 are believed to be key signal 3 molecules in the induction of complete signalling to the T cells during its expansion phase (Schurich et al., 2013). The

lack of signal 1 or 2 in an antigenic stimulation is known to impair the functionality of T cells by rendering them an anergic (non-functional state) phenotype (Schwartz, 2003) whereas, the lack of signal 3 leads to the reduced survival and effector function in the resulting clonal population (Sikora *et al.,* 2009). So the complete and fruitful activation of T cells requires the synergistic signalling of all the receptor molecules, which are discussed in details in the following sections.

3.1.2 T cell receptor: not a meek 'ON-OFF' switch but a dynamic signalling complex

The journey taken by a hematopoietic stem cell to become fully functional antigen specific cytotoxic T cell (CTL) in the thymus was discussed in Chapter One and Two. The antigen recognition, stimulation and expansion of T cells are also equally complex and highly organised events starting with the recognition of specific peptide-MHC among millions of other molecules present in their tissue micro niche that might only differ by few amino acids. In the lymph node where the primary antigen encounter occurs, it was observed that the T cells are in constant motion (Wei *et al.,* 2003) skimming for any danger antigenic signals. In this process it was estimated that they meet approximately 5000 dendritic cells in 1 h (Fooksman, *et al.,* 2009). Mempel *et al.,* (2004) classified the *in situ* process of antigen recognition into three different phases. In the first phase, T cells transiently interact with DCs scanning for the antigenic peptides bound on the MHC molecules. These scanning processes usually last for few seconds to minutes depending on the antigen density on the DCs they engaged. Once they have locked on to an antigenic target they stops their constant motion, believed to be by the up-regulation of adhesion molecules such as LFAI. (Balkow *et al.,* 2010). Different mathematical models are available to explain how this rarest of rare event of antigen recognition is possible within the ocean of millions of different self and few antigenic pMHC complexes, of which the most accepted is kinetic proof reading model (McKeithan, 1995). The details of this model are beyond the scope of this chapter and hence not discussed here, but have been reviewed by Goldstein (Goldstein, *et al.,* 2008). The second phase is the actual signal transduction events, which lasts up to few hours and during which the induction of necessary gene expression and cytokine production takes place (Mempel *et al.,* 2004). The third and final phase T cells gradually return to their transient phase, detach from the DCs, and start to expand clonally and finally come out of the lymphoid tissue (Mempel *et al.,* 2004). The physical nature of these interactions has been studied by fixed cell imaging and other imaging techniques (Monks,

et al., 1998 and Freiberg *et al*., 2002). These studies revealed the formation of a 'bulls eye' like structure in the region of TCR receptor complex which was named 'supra molecular activation complexes' (SMAC). Three distinct positions of the SMAC were thereby identified. The first one is known as the centre SMAC mainly composed of TCR CD3 and other main co-receptors, the second one is intracellular adhesion molecule-1 (ICAM-1) and integrins (LFA1) rich peripheral SMAC and the last one is the distal SMAC, mainly enriched with CD45 molecules. The rearrangement happening in the SMAC during APC-T cell interaction was thought to be important for the maintenance and termination of TCR signalling (Nel, 2002; Huppa & Davis 2003; Smith-Garvin *et al*., 2009).

3.1.3 The TCR signalling cascade from TCR CD3 complex

The TCR complex consists of TCR and CD3 molecules (Figure 3.1). When a ligand binds to the TCR, it transmits the signal through the immunoreceptor tyrosine based activation motif (ITAM). How the signals are transmitted from TCR to the CD3 is still a controversial area (Guy and Vignali, 2009). The receptor deformation model suggests that mechanical pull force developed at the site of immunologic synapse formation and exposes the phosphorylatable tails of CD3 molecules otherwise buried in the membrane lipids for initial phosphorylation events (Marwali, 2004). The ITAM of the CD3 chains are phosphorylated by the two Src-Kinases, LCK and FYN acts as a docking site for Syk kinase proteins (Palacios and Weiss, 2004). Syk kinase family proteins ZAP70 is the next protein tyrosine kinase (PTK) recruited onto the phosphorylated ITAM. This distal signalling phosphorylation events happening at the ITAM leads to the triggering of wider distal signalling events which ultimately leads to the complete activation of the T cell. The ITAM bound ZAP recruits and phosphorylate Linker for Activation of T cells (LAT) protein. LAT in turn bind to SH2 domain of PLCγ1 and growth factor receptor-bound protein 2 (GRB-2) and other adapter proteins GAD (Schneider and Rudd, 2008). SRC homology2 (SH2) domain containing leukocyte phosphoprotein (SLP76) is next bound to the above complex through their mutual binding partner GRB2 (Huse, 2009; Houtman *et al.*, 2005). The above signalling complex is then populated by three other molecules known as VAV1 (a proto-oncogene), NCK1 (Non-Catalytic region of tyrosine Kinase adaptor protein 1) and ITK (IL-2-induced tyrosine kinase). This binding ultimately leads to the activation of PLC-γ1 (Houtman *et al.*, 2005; Nel, 2002). The activated PLC-γ1 then hydrolyse the membrane lipids and release the second messengers IP3 (Inositolphospholipid) and DAG (Diacyl glycerol), which in turn activate two of the

important pathways involving ras and PKCθ. Ras activate a serine threonine kinase Raf-1 (MAPK kinase) which phosphorylate and activate MAPK and MAPK activates

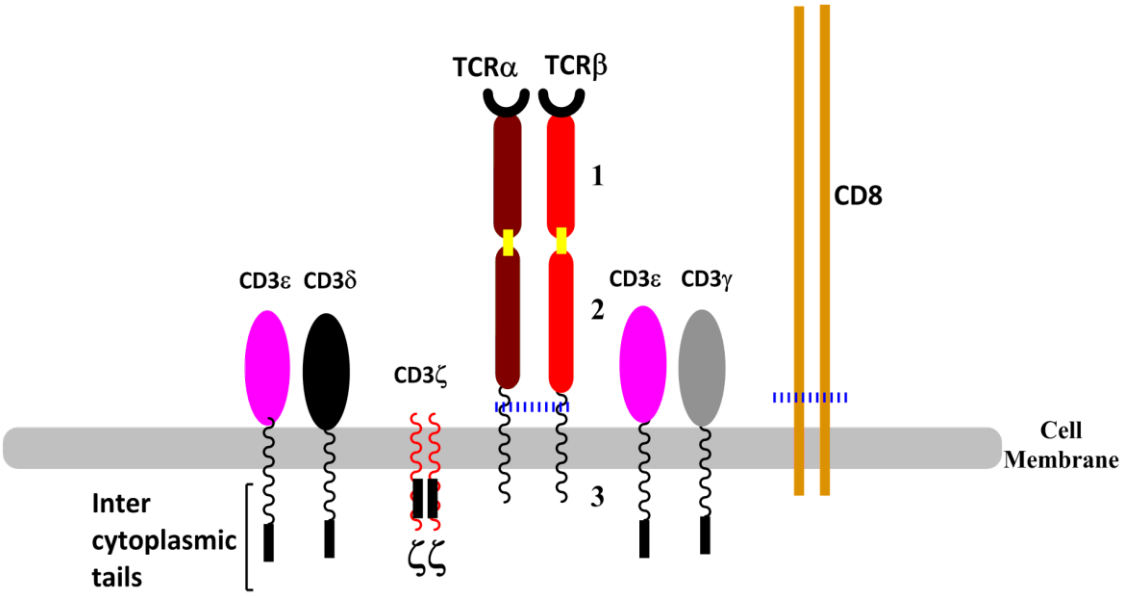

Figure 3.1: The organisation of T cell immune receptor complex, consist of the main T cell receptor (TCR), CD3 complex and the CD8 co-receptor on adjacent to it. The commonly occurring TCR is a heterodimer made up of two α (dark brown) and β (red) chain joined together by disulphide linkage (blue dotted line). It has three regions, **1.** Variable region, where the antigen recognition take place, **2.** A constant region **3.** Intra cytoplasmic tail as a continuum. CD3 complex similarly consist of two hetero (εγ, γδ) and a single homodimer (ζζ). Their intra-cytoplasmic tails carrying a immunoreceptor tyrosine based activation motif (ITAM) (indicated as black solid lines on the cytoplasmic tails) which plays an important role in the transmission of signals from TCR peptide MHC interaction in to intracellular cascades. The third receptor which is closely associated with this complex id CD8 in CTLs enables the stabilisation of interaction and also contributes towards proximal signalling pathways by acting as a docking site for many tyrosine kinases. This illustration is the TCR complex structure of a resting T cell and once it is activated an extensive reorganisation of these receptors will take place which began the TCR signalling cascade.

intracellular signal regulated kinase 1 (ERK-1) and ERK-2 (Joshi and Koretzky, 2013). The kinase activity of ERK leads to the activation of activator protein 1 (AP1) (jun/fos) transcription complex and also leads to the activation of signal transducer activator of transcription 3 (STAT-3) (Nel, 2002). Ras proteins are only active in their GTP bound stage and the gene which influences this process is guanyl nucleotide releasing protein

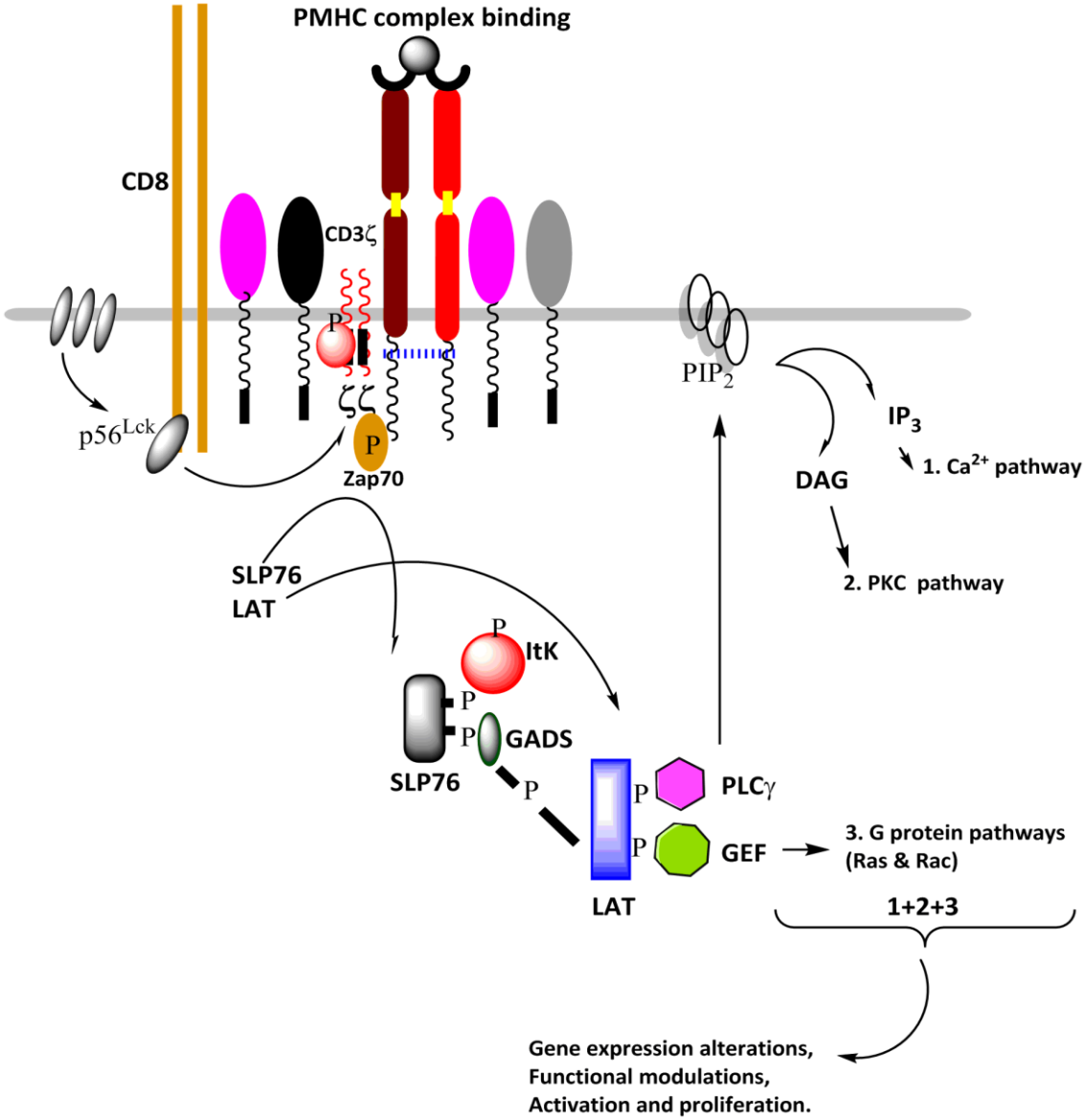

Figure 3.2: A schematic representation of TCR signalling path way. The binding of peptide MHC complexes to the TCR and the subsequent synapse formation leads to the initiation of proximal signalling pathway from TCR CD3 complex on the T cell surface. the signalling begins by the $p56^{Lck}$ in the lipid raft of activated cells and subsequent phosphorylation of ITAMS. The following signalling cascades discussed in the section 3.3.5. leads to the activation of many different patways ultimately leads to the increased Ca^{2+}, PKC pathway and Ras and Rac mediated pathways resulted in the functional changes in the activated T cells

(rasGRP) which is also up-regulate during the ras activation (Lapinski and King, 2012). Other pathways influenced by the DAG is PKCθ which contains a lipid domain a binding substrate for DAG and recruits to the proximal signalling component involving the plasma membrane. One of the key pathways regulated by PKCθ is the NF-κB pathway (Jamieson

et al., 1991). NF-κB is present in the cytoplasm bound to its inhibitor IκB in a nonactive state in resting T cells; when the T cells received the activation signals, the inhibitor IκB gets phosphorylated, ubiquitinylated and degraded (Smith-Garvin *et al*., 2009). The NF-κB is then translocated from the cytoplasm to the nucleus which leads to the activation of genes required for the activation of genes involved in T cell homeostasis. In addition to the above major signalling events, TCR signalling involves the interplay of second messenger Ca^{2+}, cytoskeletal components including actin and integrins which are also critical for the full functionality, homeostasis and development of T cells (Nel, 2002; Smith-Garvin *et al*., 2009; Fooksman *et al*., 2009) The details of these signalling events are illustrated in (Figure.3.2).

3.1.4 The decision point of avidity ?: role of signals and kinetics

In cancer vaccination settings, it is important to generate high avidity T cell responses by breaking the tolerance of the self-antigen specific T cells (Durrant *et al.,* 2010). Though much work aimed at eliciting a high avidity T cell response has been carried out using different vaccines and adjuvants, little is known about the molecular mechanisms which decide the functional fate of these cells (von Essen *et al*., 2012). Earlier studies of TCR signalling revealed that the signal requirement for naïve and primed T cells are different (Farber *et al.,* 1997; Fahmy *et al.,* 2001). The primed T cells can be sensitised with very low concentration of the antigen and less dependent on co-stimulatory signals (Yamaura *et al.,* 2010). Similar lowered activation requirements were also noticed in effector memory T cells in different studies (Watson and Lee, 2004; Tanchot *et al.,* 2004). Studies have also showed that, effector T cells can detect antigen in a CD8 independent manner, whereas a naïve T cell always require complete interaction of TCR, CD8 and peptide MHC for its optimal stimulation. This suggests a higher alertness of the proximal signalling complex due to its previous encounter (Bachmann *et al.,* 1999). These fundamental differences between naïve and antigen experienced T cell clearly show the physiological and functional changes acquired by these cells during their first antigen encounter. And these acquired functional changes might be attributed to the process of 'functional avidity maturation' a process which is discussed in detail in the previous chapter. Now the key question is, does the signals and the kinetics taking place the first contact of a naïve T cells with their antigen presenting cells have any effects on the subsequent priming of these T cell?. This is the not fully understood, but the strength and the duration of individual signal originating from the TCR and its co-receptors probably have an important role to play.

3.1.5 Contribution of co-receptor signals towards T cell activation

The formation of a mature immunologic synapse involved the recognition of TCR with its cognate antigen the initial signalling events happening in the proximal signalling complex create the protrusion towards the antigen presenting cells and several TCR-CD28 micro clusters will then aggregate towards this region. This mature immune synapse formation is the first and the fundamental prerequisite for the activation of a naive T cell. Studies conducted with different groups observed that the formation of a mature synapse is lot quicker in the primed T cells and it is less dependent on the primary co-receptor molecule CD28 (Watson and Lee, 2004). The same study also have showed that co-distribution of CD45 molecules and the TCR embedded in the lipid rafts in the antigen experienced memory T cells compared to the naive T cells indicating their membrane molecular organisational difference between these two cells (Watson and Lee, 2004). Studies conducted by O'Keefe *et al* (2004) observed that the synaptic formation of naive and effector T cells is different, in that the effector T cells the synaptic formation is characterised by the presence of central supramolecular activation cluster with the accumulation of PKC, phospho-PKC, and phospho-ZAP70 in the effector T cells.

The role of TCR signalling between the naive and the effector cells have been studied by several groups (Farber *et al.,* 1997; Fahmy, *et al.,* 2001; Pihlgren *et al.,* 1996). All the studies have concluded that there is a difference in the TCR triggering existing between them with effector cells transmits the signal much efficiently than the naive population. An important study which highlighted the difference in the TCR signalling pathways between high and low avidity T cells were published (Sharma and Alexander-Miller, 2009). They have used an ovalbumin OT-I mice derived $CD8^+$ cells for stimulation with different concentration of the peptides. A marked difference in the TCR signalling pathways were observed between these two functionally different cells. In high avidity T cells, an increased amount of phosphorylated MAPK ERK-1/2 and decreased levels of dephosphorylated NFAT were observed with increased Ca^{2+} mobilisation. They have also observed an efficient phosphorylation of CD3ζ phosphorylation. Interestingly, the high and low avidity T cells are derived from a clonal population of $CD8^+$ cells having a similar TCR receptor characteristics makes the argument that the single T cell can either modulated into high or low avidity functional state depending up on the signals they have received.

3.1.6 Transcriptomic tools to investigate gene expression changes

The post genomic era has witnessed a surge in techniques to study the expression of genes, so that the much needed functional annotations can be achieved. Among these techniques high density microarrays were the first to offer an affordable and global platform to study all the predicted and identified genes in a single experiment in different physiological and functional conditions. Since then the global transcriptomic platforms have evolved and at the latest sequencing based transcript quantification and splicing characterisations. This discovery platforms offer the changes happening in a larger scale and the real-time quantitative PCR will help the researcher to validate these changes in multiple and independent samples.

3.1.6.1 Microarrays and Affymetrix gene chips

DNA microarrays are one of the most widely used tool for parallel gene expression studies using a group of small predesigned DNA probes immobilised on a solid surface such as glass and it works based on the principles of nucleic acid hybridisation properties. The use of microarrays was first reported by Schena *et al,* (1998) since then, it has been the most widely accepted method for gene expression studies (Auer *et al.,* 2009). Success of this technique is mainly attributed to its high throughput nature and also fuelled by the technological evolution of surface chemistry and novel spotting techniques of DNA materials. Two types of arrays are available depending up on the probe material spotted on the array surface: cDNA and oligonucleotide microarrays. cDNA arrays are prepared by robotic spotting of cDNA fragments amplified from cDNA libraries onto either nylon membrane or glass slide, whereas oligonucleotide arrays are prepared by either *in situ* synthesis the predesigned gene probes onto a silicon chip using photo lithography (Pease *et al.*, 1994) or ink-jet deposition (Hughes *et al.,* 2001), or the spotting of pre-synthesised oligonucleotide probes onto a suitable surface.

The basic protocol for microarray experiments involves extraction of RNA from controls and test samples, labelling of the isolated RNA with dyes, and hybridisation to the microarray chip under suitable hybridisation parameters. Un-hybridised RNA molecules are then washed away and results are obtained using a laser scanner. Two types of detection systems are available: two-channel (Schena *et al.,* 1998; Shalon *et al.,* 1996) and single-channel detection systems (Churchil 2002). In the two-channel systems RNA

isolated from controls and the test samples are labelled separately by two fluorescent dyes such as Cy3 and Cy5.

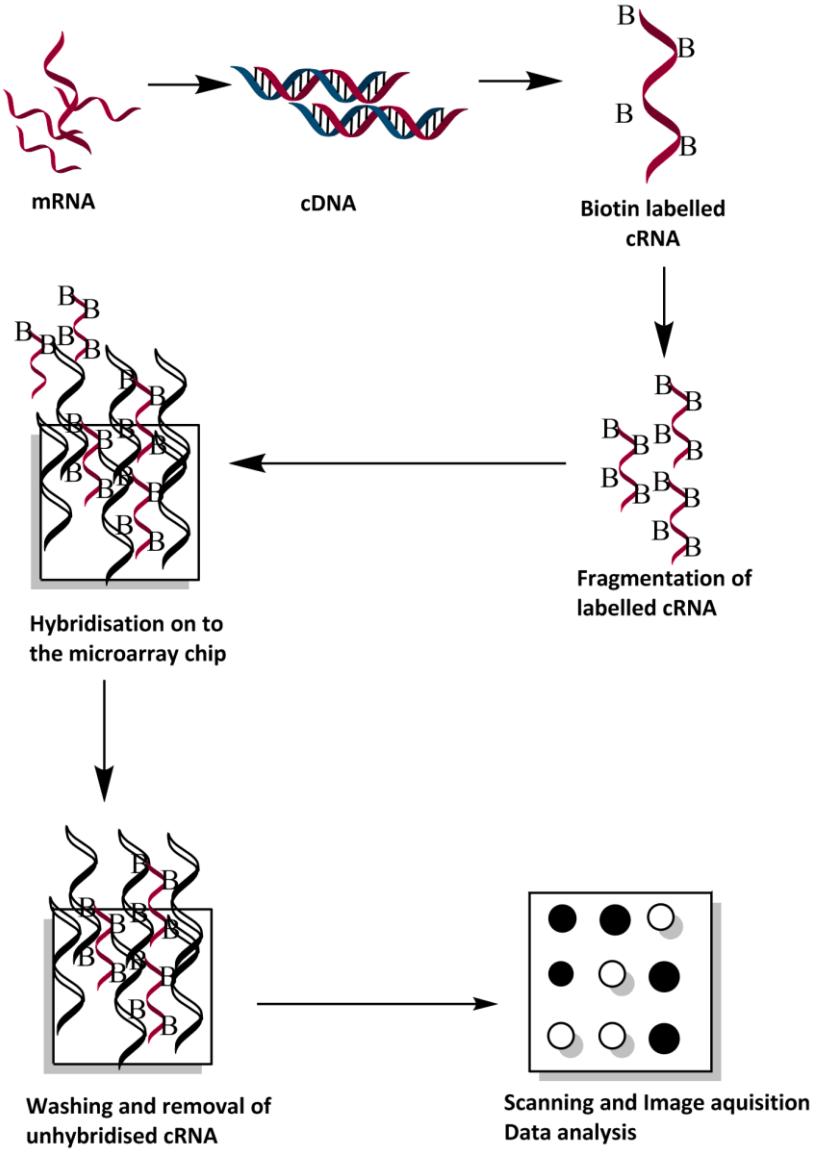

Figure 3.3: Schematic representation of microarray hybridisation work flow. The isolated mRNA is first reverse transcribed in to the cDNA and labelled using biotin. This labelled mRNA has been fragmented and then hybridised on to a chip which hold the immobilised probe sequences. The unbound fragments were then washed away and the slides can be scanned for acquiring the images. The pixel intensity of the each probe location will be interpreted as the hybridisation intensity and therefore the transcript abundance in any given RNA preparation.

The labelled RNA molecules are then mixed together and hybridised on to a single chip, whereas in a single-channel systems such as Affymetrix gene chips (Affymetrix ®, Santa Clara) or CodeLink® (GE Health Care) control and experimental RNA molecules are labelled separately with only one dye and hybridised onto two separate chips (Schuize and Downward, 2001).

One of the most widely accepted high density oligonucleotide array platforms is Affymetrix® (Santa Clara, CA), which offers several array formats such as Exon and gene arrays, 3' expression arrays, micro RNA expression arrays and tiling arrays in various organisms. The design of the probe set, which represents each of the genes present in Affymetrix slides, allows the researcher to perform highly stringent hybridisation protocols. Each probe pair consists of a perfect match (PM) and a mismatch (MM), spotted next to each other. The MM probe is exactly similar to PM probe except that it carries a single base pair change in the middle of the probe. This PM-MM way of probe design acts as an internal control during the hybridisation procedure (Lockhart *et al.,* 1996). The raw data is the scanned image of the slide which is normalized for overall brightness then analysed for the spot intensity (Illustrated workflow in Figure 3.3). Commercially available software such as GeneSpring™ (Agilent) can then be used for various statistical analyses. Use of microarrays is well established in tumour cell expression studies (Patsialou *et al.*, 2012; Buehler *et al*., 2013) and in micro genomics (Kurimoto *et al,* 2006; Hartmann and Klein, 2006).

3.1.6.2 Whole transcriptome sequencing

Recent studies indicate that the complexity of the human transcriptome is higher than expected (Kapranove *et al.*, 2007; Sulthan *et al.*, 2007). Along with the mRNA and splice variants, large numbers of non coding RNA's were also identified, with their functions largely unknown (Parkhomchuk *et al.,* 2009). Advent of next generation sequencing platforms such as Illumina/Solexa®, Roche 454® and Applied Biosystems SOLiDsystems® can generate billions of base pairs of genomic and transcriptomic sequence information in days opening up a new way to understand this transcriptional complexity. These sequencing platforms have already started to show their utility in cancer and stem cell science (Sugarbaker *et al.,* 2008, Cloonan *et al.,* 2008). Sequencing of the whole transcriptome from a single cell made possible recently with the integration of RNA

amplification technique (Kurimoto *et al.,* 2007) and SOLiD system sequencing platform (Tang *et al.,* 2009), which holds the promise of a new dimension for microgenomics.

3.1.6.3 Quantitative reverse transcription real time PCR

Unlike conventional PCR which depends on gel based techniques for product detection, real time PCR combines the target amplification and product detection into a single step (Higuchi *et al*, 1993; Heid *et al.*, 1996; Morrison *et al.*, 1998). This real time detection is achieved mainly because of the development of different fluorescent dyes (SYBR Green®, BEBO®), which can be used to relate the increase in product accumulation and fluorescent intensity quantitatively. Quantification is mainly done by the measurement of 'Ct values', which is the number of cycles required for the fluorescence values of each sample to rise above the background fluorescence (Wong and Medrano, 2005). The relationship between the copy number and Ct value is inversely proportional, i.e. higher the initial template copies, the more products accumulation and therefore more fluorescence resulting in a low Ct values.

Over the recent years, quantitative real time PCR (qRT-PCR) has become the gold standard for gene expression studies as a method to screen the expression of a given gene across samples or validate microarray result. This is mainly because of its sensitivity, reproducibility and wide dynamic range (7 order of magnitude) of quantification in qRT-PCR (Skrzypski 2007, Stahlberg *et al.,* 2005). There are two ways of quantify the gene expression in qRT-PCR: absolute quantification and relative quantification. Absolute quantification is done by plotting a linear curve based on the Ct values obtained from a serially diluted known quantity of cDNA. Absolute quantity of the unknown samples can then be deduced from the standard curve obtained. This method simplifies calculations and avoids practical and theoretical problems currently associated with PCR efficiency assessment. (Skrzypski, 2007; Bernard and Wittewer, 2002).The comparative threshold method (Relative quantification) give the gene expression values as fold change in comparison with reference samples or calibrators by assuming the PCR efficiencies of all the samples under investigation are the same. (Wong and Medrano, 2005; Skrzypski, 2007)

Uses of qRT-PCR in cancer gene expression studies are well established. Lossos *et al.,* (2004) used qRT-PCR to validate and identify six predictor marker genes in lymphoma. Similar studies in non-small cell lung cancer (Sher *et al.,* 2005) oral cancer (Li *et al.,* 2004)

and breast cancers (Bosma *et al.,* 2002) indicate that the future diagnostic value of qRT-PCR in cancer biology.

3.1.7 Aim of the study

This chapter is dedicated to studying the signals and kinetics of TRP-2 and OVA vaccine generated pentamer positive T cells using various genomic approaches and also attempts to elucidate the transcriptional landscape of these cells using global mRNA profiling. All the primary work has been conducted in the TRP-2 model and ovalbumin model has been used as an alternative model for the validation of the gene expression signatures identified in the first model. The specific T cells against the vaccinated antigens will be isolated using multimer technology for improving the quality of starting materials. The main hypotheses are addressing in this chapter are.

1. Is there an inherent difference in basal level of TCR signals which can be detected in the *ex vivo* pentamer sorted cells from ImmunoBody® and peptide immunised animals.
2. ELISpot® plate stimulated cells can be rescued to investigate the TCR signal competency between these two groups of cells using qRT-PCR.
3. A TCR independent signal such as CD3 CD28 stimulations will be used to measure the signal competency between these two cell types using real-time quantitative PCR
4. Affymetrix global transcriptomics profiling of high and low avidity T cell will be used for the identification of genes differentially expressed between the high and low avidity T cells.
5. If the markers were identified, confirm them with qRT PCR and flow cytometry.

3.2 Materials and methods

3.2.1 Immunisations and generation of high and low avidity T cell response

All the immunisations were carried out as per the protocol given in section 2.2.2. The spleens were harvested from the euthanised animals and splenocytes were prepared as described in the section 2.2.3. The cells were counted and diluted at $5x10^6$ cells per mL. The cells from each animal were tested for IFN- γ production with peptide titration ELIspot assays as described in section 2.2.4. The remaining cells (~2.0 x 10^8 total splenocytes) pooled together from three animals were pelleted by centrifugation at 300g at room temperature and proceeded immediately into CD8 negative isolations using magnetic sorting of untouched $CD8^+$ T cells.

3.2.2 Isolation of $CD8^+$ cells

Prior to the isolation of peptide specific cytotoxic T lymphocytes, $CD8^+$ cells were separated from the total splenocytes to increase the purity of the peptide specific cells. Approximately 2 x 10^8 splenocytes from three pooled immunised mice were used for the isolation using $CD8^+$ T cell isolation kit (Miltenyi Biotec GmbH, Germany) which is specially designed for the isolation of $CD8^+$ cells by the depletion of non $CD8^+$ cells. The total splenocytes were treated with 1 mL of 1x RBC lysing solution (Sigma) and incubated at room temperature for 2 min followed by passing through a 30 µm mesh to remove the clumps and cell aggregates. The cells were then washed with 20 mL of sterile PBS by centrifugation at 300 g for 10 min. The cells were resuspended in 800 µL of MACS buffer (PBS + 0.5% (v/v) of bovine serum albumin and 2 mM of EDTA). 160 µL of non $CD8^+$ antibody cocktail was added to each pool and incubated in the fridge for 15 min. After the incubation a further 600 µL of MAC buffer was added to each tubes followed by 300 µL of microbeads. Cells were incubated in the fridge for 15 min and washed with 20ml of ice cold MACS buffer (300g for 10 min at 4°C). While tubes were spinning, LS columns (Miltenyi Biotec GmbH, Germany) were prepared by assembling them into a magnet and added 3 mL of ice cold MACS buffer. The cells were resuspended in 10 mL of cold MACS buffer and applied to the above preconditioned columns. The drip through was collected which contains untouched $CD8^+$ T cell, the column has been washed further three times with 5mL of ice cold buffer for complete recovery. Cells were counted at this stage and pelleted by centrifugation at 300 g for 10 min at 4°C. The enrichment purity of magnetic sorted cells were assessed using flow cytometry by running a small volume of purified

cells with FITC stained $CD8^+$ antibodies (eBioscience). All the purifications were carried out at 4° C to keep the gene expression unchanged.

3.2.3 Isolation of peptide specific (pentamer positive) cells

The cells isolated in section 3.2.2 represent the total repertoire of $CD8^+$ cells with multiple specificities. Therefore, it was necessary to specifically isolate TRP-2 specific T cell from the whole $CD8^+$ T cell population prior to any downstream molecular characterisations. For this, a PE (PhycoErythrin) conjugated TRP-2 pentamer (ProImmune) was used for the detection and isolation of TRP-2 specific T cells were used. The isolation of pentamer specific cells is facilitated by the binding of specific TCR to the corresponding peptide present on the MHC molecule. This binding might trigger partial or complete activation of TCR signalling pathways. Since the current study mainly focused on identification of a well defined phenotypic marker and not an activation marker for high avidity T cells, most of the isolation steps were again carried out in cold conditions in the view of minimising the activation of TCR signalling pathways and thereby preserving the transcriptional and proteomic signatures undisturbed in both high and low avidity T cells. The $CD8^+$ fraction was resuspended in 100 µL of ice cold MACS buffer in a 15 mL falcon tube, 20 µL (2 test volumes as per the manufacturer's guidelines) of MHC class-1 pentamer was added and mixed thoroughly by gentle up and down pipetting. The tubes were incubated in the fridge for an hour and washed the cells with 1 mL of ice cold PBS by centrifugation at 300 g at 4°C for 10 min. The cells were resuspended in 100 µL of cold MACS buffer, a fraction (15 µL) of this cells were removed and kept aside for flow cytometric analysis. Into the remaining cell suspension, 20 µL of anti PE microbead (Miltenyi biotech) was added and incubated for further 15 min in the fridge. Following the incubation, cells were washed with 2 mL of ice cold MACS buffer and 300 g for 10 min at 4°C. While centrifuging, MS columns were prepared by adding 500 µL of cold MACS buffer. Once centrifugation was finished the cells were resuspended in 500 µL of MACS buffer and applied to the column mounted on a magnetic separator. The flow through containing non TRP-2 specific T cells were collected, columns were washed further three times with 500 µL of ice cold buffer for the complete removal of non specific cells. Finally the columns were removed from the magnet and placed on a new labelled 15 mL Falcon tube, magnetically labelled pentamer positive cells were flushed out using the plunger and 1mL of cold MACS buffer, the flushing was repeated three times with 500 µL of cold buffer and counted the cells. Cells were washed three times with ice cold PBS in a 1.5 mL micro-centrifuge tube and snap

frozen in liquid nitrogen for further downstream analysis. The overall protocol of isolation of $CD8^+$ cells and pentamer positive cells were given in figure 3.4.

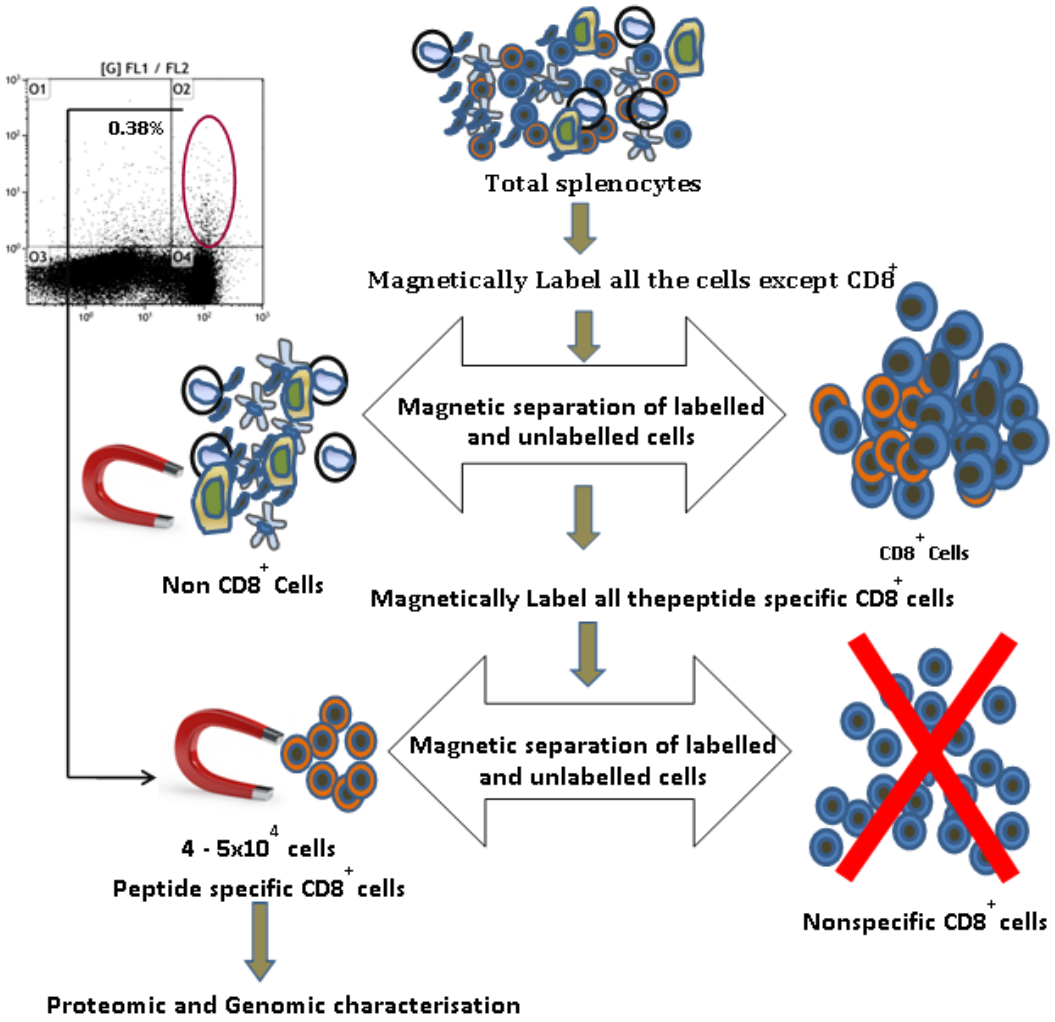

Figure 3.4: Schematic representation of isolation of pentamer (multimer) positive TRP-2 (antigen) specific cells from the total repertoire of $CD8^+$ cells

3.2.4 RNA isolation from pentamer sorted cells and cDNA synthesis

RNA isolation of pentamer positive cells were carried out initially using the cells isolated from single immunised mice. However the low number of cells recovered (~40,000 T cells from each animal), resulted in low yield of the total RNA and poor quality. To circumvent this problem all the RNA extractions were subsequently carried out by pooling three immunised animals together from each group as described above, which resulted in a threefold increase of pentamer positive cells. Cells were washed once with sterile PBS and immediately lysed the cells in lysis buffer containing chaotropic agent guanidinium

thiocyanate. The kit used for the isolation of RNA is RNAqueous® micro kit, which specialised in the isolation of RNA from very low number of cells (10 – 500,000 cells). The rest of the protocol was followed as per the manufacturer's instructions with minor modifications and the final RNA was eluted in 15 µL of elution volume. Three pools from each group were named as Immunobody pool-1, Immunobody pool-2, Immunobody pool-3, Peptide pool-1, Peptide pool-2, Peptide pool-3 were prepared for further studies. The RNA has been isolated from both the models (TRP-2 and ovalbumin) for microarray analysis. From the total RNA isolated, a fraction of RNA was used for quantitative real-time analysis (qRT) to investigate the TCR signalling status in pentamer sorted cells. DNAse treatment was performed to completely remove the residual DNA contamination prior to any cDNA synthesis. Initial quantity and quality of the RNA was assessed in NanoDrop 8000™ (Thermo).

cDNA synthesis was carried out using Superscript III (Invitrogen Life Technologies) reverse transcriptase as follows, into a nuclease free microcentrifuge tube 1 µL (200 ng) of oligo $(dT)_{15}$, 2 µL of RNA (250 ng), 1 µL of dNTP's (10 mM each) was added and adjusted to a final volume of 13µL with sterile nuclease free water. The mixture was heated in a thermal block at 65°C for 5 min and immediately chilled on ice for 2 min. Tubes were briefly centrifuged at 1000 g for 15 sec and 7 µL of RT enzyme master mix [4 µL of first strand buffer (5X, Invitrogen) 1 µL of DTT (0.1 M, Invitrogen), 1 µL of RNAsin (Promega) and 1 µL of Superscript III (200 U/µL, Invitrogen)] was added. The reaction was mixed thoroughly by gentle up and down pipetting and incubated at 50°C for 1 h in a water bath; the reaction was finally inactivated by incubating at 70°C for 15 min and stored at -20 until further use.

3.2.5 Primer designing, synthesis and optimisation

Genes involved in the TCR signalling pathway and the coding regions of all the transcript variants were downloaded from NCBI nucleic acid database. All the transcript variants were aligned and the region common to all the variants was selected as a target for primer synthesis. Primer design was carried out with primer 3.0 software (v.4.0, Whitehead Institute for Biomedical Research, Massachusetts, USA) (Untergrasser *et al.,* 2011; Koressaar & Remm, 2007). All primers were synthesised to amplify a target region of up to 200 bp at the maximum in the view of using them in real time quantitative PCR (qRT PCR). Once suitable primers were designed they were tested for their ability to form self

and hetero dimer and hairpin formations in Integrated DNA technology OligoAnalyzer 3.1. Primers which formed the above structures were avoided and the specificity of each primer was further verified by NCBI primer BLAST. All the primers were synthesised from Eurofins MWG Operon (Germany) at HPSF grade purity, the list of primers used for the present study is given in the table 3.1.

3.2.6 Optimisation of PCR primer parameters

All the primers were dissolved at a stock concentration of 100 pmol/µL in nuclease free water. A 10 pmol/µL working concentration was used for all the PCR reactions. Prior to conduct any real time PCR reactions, all the primers were optimised in a conventional gradient PCR (Thermo Scientifics) using cDNA synthesised from the RNA isolated from total splenocytes. The specificity of each primer was decided by running the above PCR products on 1.7% w/v agarose gel electrophoresis and ensured that only single specific bands was present from each reaction or picked the annealing temperature which yielded single specific band at the desired size.

3.2.7 Primer efficiency calculations

Efficiency of all PCR primers were assessed by running two fold dilution series of cDNA template synthesised from total splenocytes RNA. The efficiency of each reaction was calculated with manufacturer supplied software (RotorGene-6000). Comparable efficiencies of each primer sets were calculated in Microsoft excel by plotting the difference in C_t values against the logarithm of the template amount. The amplification efficiencies were considered as comparable when the slop of resulting straight line was < 0.1.

3.2.8 Gene expression profiling of TRP-2 peptide specific $CD8^+$ T cells using qRT PCR

Real time quantitative PCR was used for studying the gene expression patterns of TCR signalling genes in high and low avidity T cells with the template cDNA synthesised from pentamer sorted cells. PCR master mix was prepared by mixing 6.7 µL of cyber green, 0.5 µL of forward primer, 0.5 µL of reverse primer, 4.25 µL of nuclease free water and 0.5 µL of cDNA template. Thermal cycling was carried out in a Qiagen Rotor-Gene Q real time PCR cycler. Quality of amplification was monitored by plotting the melt curve after each primer amplification with the user supplied software. C_t values were determined by setting

the threshold values empirically by visual inspection. Data analysis of these genes were done with Livak method (Livak and Schmittgen, 2001; Schmittgen and Livak, 2007) and independently verified with Pfaffl method (M W Pfaffl *et al*., 2002) using Relative Expression Software Tool (REST 2009, QIAGEN) with efficiencies calculated in Rotor-Gene software.

3.2.9 RNA isolation from $CD8^+$ cells from ELISpot derived cells

This study hypothesised that the gene expression changes in the TCR signalling can be measured with the cells after 48 h of stimulation in an ELISpot plate. In routine experiment the cells after 48 h of stimulation are discarded prior to developing the plates for respective cytokine detection. Instead, these cells were saved and $CD8^+$ cells were isolated for RNA extraction. The detailed protocol is for harvesting the cells and further purification and isolation of $CD8^{+\ T}$ cells and the RNA is given below.

All the immunisations were carried out as per the section 2.3.1 in chapter 2 and an IFN- γ ELISpot assay was set up as per the protocol given in the section of the same chapter. After 48 h of incubation at 37° C at 5% v/v CO_2 the cells were harvested from the triplicate wells of the top three concentrations for both the groups by carefully mixing and pipetting out the cells (200 µL) without damaging the membrane. All the cells from top three concentrations were pooled in to a 15 mL centrifuge. Tube and pelleted down by centrifugation at 300 g for 10 min at room temperature. The cells were further resuspended in cold MACS buffer and $CD8^+$ T cells were purified with negative isolation kit as per the protocol given in the section 3.2.2 and snap frozen the cells in liquid nitrogen. RNA isolations were performed in the next day from the frozen pellets as per the protocol given in section 3.2.4 and eluted in 10 µL nuclease free water. Quantification of the isolated RNA and cDNA was carried out as per section 3.2.4. The quantitative real-time PCR has been carried out as per the protocol given in section 3.2.8.

Table 3.1. PCR primer sequences used for qRT PCR Studies

Sl. No	Primer name	Sequences 5'-3'	TM ^{0}C
1	m-LCK-F	ATGGAGAACGGGAGCCTAGT	58.0
	m-LCK-R	GGCCGTGTACTCATTGTCCT	58.0
2	m-FYN-F	TGCAAGATTGCTGACTTTGG	58.0
	m-FYN-R	TTGGTGACCAGCTCTGTGAG	58.0
3	m-ZAP70-F	CTGGTATCACAGCAGCCTGA	60.0
	m-ZAP70-R	TTCCCATAGACCAGGGACAG	60.0
4	m-LAT-F	CCCTGTTGTCTCCTCTGCTC	61.0
	m-LAT-R	CTCTGCGCTCTCCTCACTCT	61.0
5	m-MAPK1-F	TCTCCCGCACAAAAATAAGG	58.0
	m-MAPK1-R	GCCAGAGCCTGTTCAACTTC	58.0
6	m-NFkB-F	TCAGACACCTCTGCACTTGG	58.0
	m-NFkB-R	GCAGGCTATTGCTCATCACA	58.0
7	m-AKT1-F	CCCTTCTACAACCAGGACCA	58.0
	m-AKT1-R	ATACACATCCTGCCACACGA	58.0
8	m-HPRT-F	TGCTCGAGATGTCATGAAGG	58.0
	m-HPRT-R	TATGTCCCCCGTTGACTGAT	58.0
9	m-GAPDH-F	AACTTTGGCATTGTGGAAGG	58.0
	m-GAPDH-R	ACACATTGGGGGTAGGAACA	58.0
10	m-ACTB-F	AGCCATGTACGTAGCCATCC	58.0
	m-ACTB-R	CTCTCAGCTGTGGTGGTGAA	58.0
11	m-TBP-F	TGACTCCTGGAATTCCCATC	58.0
	m-TBP-R	TGTGTGGGTTGCTGAGATGT	58.0
12	m- HRAS-F	ATGGCATCCCCTACATTGAA	61.0
	m-HRAS-R	ACAGCACACATTTGCAGCTC	61.0
13	m-KRAS-F	TGCAATGAGGGACCAGTACA	61.0
	m-KRAS-R	TGCTGAGGTCTCAATGAACG	61.0
14	m-NRAS-F	TGGACACAGCTGGACAAGAG	59.0
	m-NRAS-R	CTGTCCTTGTTGGCAAGTCA	59.0
15	m-RASGRP-1-F	TGTCACAGCTCCATCTCCAG	61.0
	m-RASGRP-1-R	TTCACCTTCCCATCTTCCAG	61.0
16	m- RAF1-F	CTACACCCCATGCCTTCACT	61.0
	m- RAF1-R	GCTGAAGGTGAGGCTGATTC	61.0
17	m- JNK2 -F	CGCCACCACCTCAAATTTAT	61.0
	m- JNK2 -R	TGTGCTCAGTGGACATGGAT	61.0
18	m- VAV1-F	CTACGGGATCTGCTGATGGT	59.0
	m- VAV1-R	CTGCCGTAGGGTTTCATTGT	59.0
19	m-NFAT1-F	TCTGCTGTTCTCATGGATGC	59.0
	m-NFAT1-R	TCCTCTCCTCCTGCTCAGAT	59.0

3.2.10 CD3, CD28 stimulation of pentamer positive cells for studying signal competency

This experiment was designed to identify the differences between high and low avidity T cells in response to a uniform peptide independent signal given to them. Antibodies against CD3 of TCR-CD3 complex and the co-receptor CD28 were known to be used for the stimulation and culturing of T cells by giving them pseudo-stimulation. The hypothesis for this experiment was that if there is an inherent difference between these two functionally different cell types, they transmit the signals differentially if given a uniform signals.

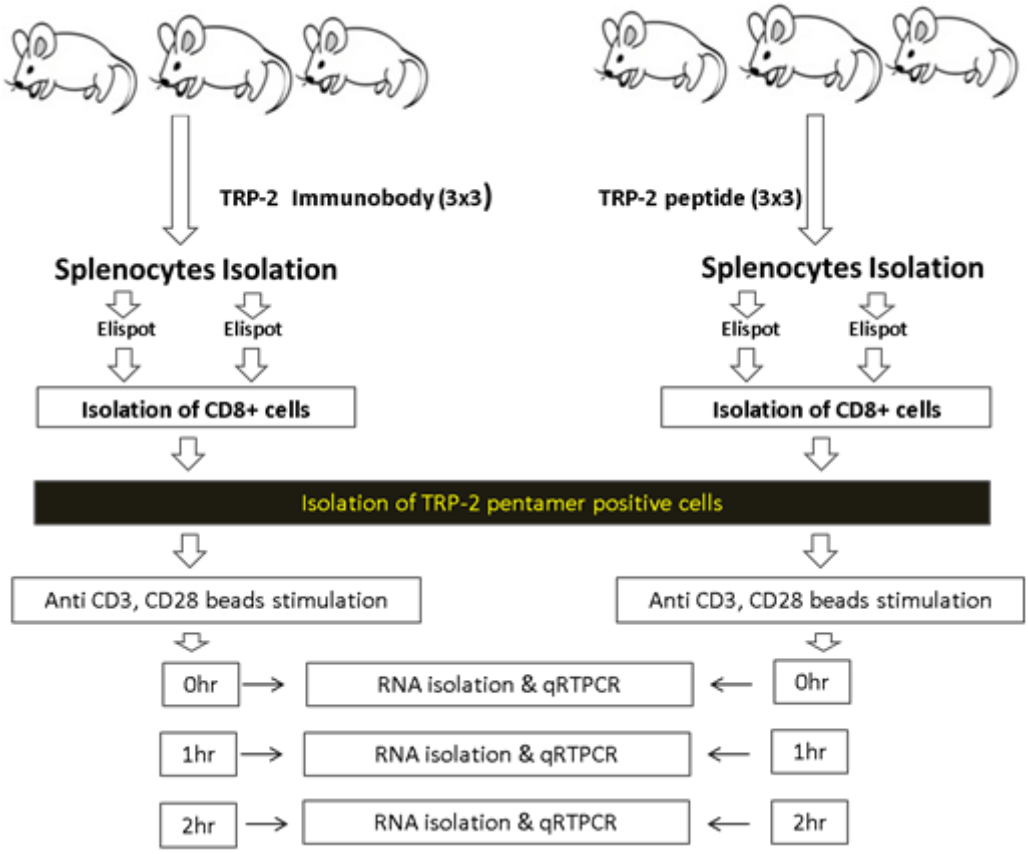

Figure: 3.5. Schematic representation of experimental procedure followed for anti-CD3, anti-CD28 stimulation studies

All the immunisations were carried out as normal; the details of immunisations were given in figure 3.5. A total of nine animals were immunised in each group. Three animals were pooled together creating three pools of immunised animals and three for peptide immunised animals. The pooling strategy has been employed to obtain enough number of

pentamer positive TRP-2 specific cells. Total $CD8^+$ cells were sorted using magnetic sorting using Miltenyi negative isolation kit from each pool using a large format column (LS columns) as per the protocol given in the section 3.2.2. Once the $CD8^+$ cells were sorted they were stained with TRP-2 pentamer (20 µL per pool) and were sorted as per the protocol given in the section 3.2.2. The sorted cells were used for the stimulation kinetic studies with CD3, CD28 coated micro beads (Miltenyi Biotec, GmbH) in 1:1 ratio. The cells were harvested three time points of stimulation at 0, 1 and 2 h in to a micro centrifuge tube (Fig 3.5). The beads were removed immediately following the harvest using a magnet and centrifuged at 1500g at 4°C. The supernatant was removed from the centrifuge tubes immediately and snap frozen using the liquid nitrogen for RNA isolation. Once all the time points were harvested as mentioned, RNA isolations were carried out using Ambion RNAaqueous micro kit. The isolated RNA was quantified, equalised by diluting everything to the lowest concentration using nuclease free water and reverse transcribed as per the protocol given in the section 3.2.4. qPCR has been carried out as per the section and the fold change has been calculated as per ΔΔCT method.

3.2.11 Gene expression studies using Affymetrix cDNA micro arrays of TRP-2 and OVA pentamer sorted cells

mRNA isolated in step 3 was used for large scale gene expression studies using Affymetrix array platform. The experiment was carried out externally at NASC arrays (The University of Nottingham, UK). Briefly, the protocol followed for the array data generation is as follows. Quality checking had been carried out using Agilent Bioanalyzer and RNA6000 ® nano kits. The biotin labelling of this products was performed using Whole Transcript Sense Target Labelling from Affymetrix® (Santa Clara, CA) with 16 h incubation. The labelled RNAs were checked for their quality using Agilent Bioanalyzer®, fragmented and hybridised to Affymetrix Gene Chip Mouse Gene 1.0 ST Arrays (Figure 3.6). The arrays were scanned after hybridisation and washing and the .CEL files were generated. These files were used for further analysis.

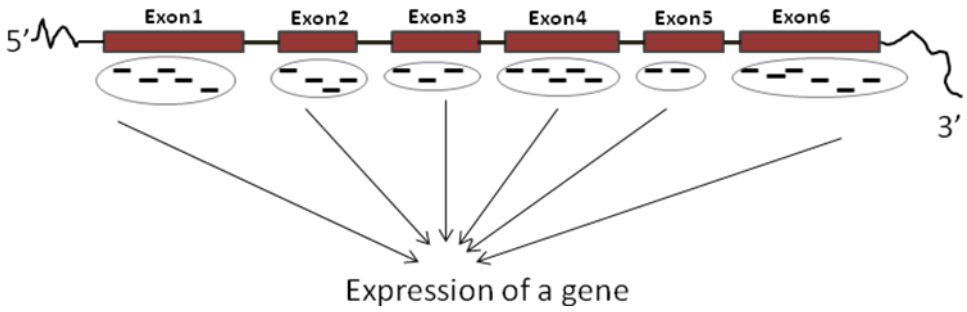

Figure. 3.6. Schematic representation of probe design of GeneChip® Mouse Gene 1.0 ST Array used for this study. A total of 28,853 genes are present on the array with each gene represented by unique 25 base pair probes spreading across the full length of the gene. Red boxes represent Exons of single gene with introgenic regions represented by solid lines. Short solid lines under each red boxes represented individual probes (the number of probes are arbitrary in the diagram for illustration purpose and it may vary between the genes). The expression of single gene is the cumulative signal values obtained from all the probe sets of a gene after hybridisation.

3.2.12 Microarray data analysis

The Affymetrix CEL file contained intensity values derived from the raw image (.dat) file calculated for each probe on the chip after hybridisation. It was necessary to summarise and transform all the probe set IDs in to genes and perform normalisation using suitable algorithms available. Two type approaches were used for data analysis, first CEL files were imported in to GeneSpring-GX11 microarray data analysis software and the samples were normalised with RMA (Robust multichip analysis) and Baseline Transformation to Median of all Samples. An experimental group was created with Immunobody as one and peptide as a second group. Fold change was calculated with a cut-off value ≥ 2 and p value ≤ 0.05. In the second approach analysis were performed with regression based method, which calculated the residuals and the standard residuals and the genes were ranked based on their expression values. *P* values for each gene were calculated with multiple testing correction using Boneferroni correction method to minimise the false discovery rate (FDR). All the array data were assessed for their quality before analysis using Affymetrix Expression console software®. All the spiking controls were tested and the correlations of arrays across the experiments were compared with Pearson correlation for its linear

dependence. The reported differential expression in this study was from the regression based approach since the differences between the two cells types were minimum regression allowed to look for the most and least differentially expressed genes.

3.2.13 Proteomic confirmation of two markers identified by transcriptome profiling using antibodies and flow cytometry

Two of the markers identified by gene expression profiling and confirmed with qRT PCR are granzyme A and B. These genes were upregulated in the ImmunoBody® high avidity group significantly. The proteomics confirmation of these markers was carried out with flow cytometry and intracellular staining. The workflow followed for the confirmation is given in figure 3.7. Briefly, 6-8 weeks old C57BL6 female mouse were immunised with TRP-2 ImmunoBody® and peptide for the generation of high and low avidity T cell response. Three animals were used in each group and immunisation regime followed was as described earlier in the second chapter (section 2.2.2).

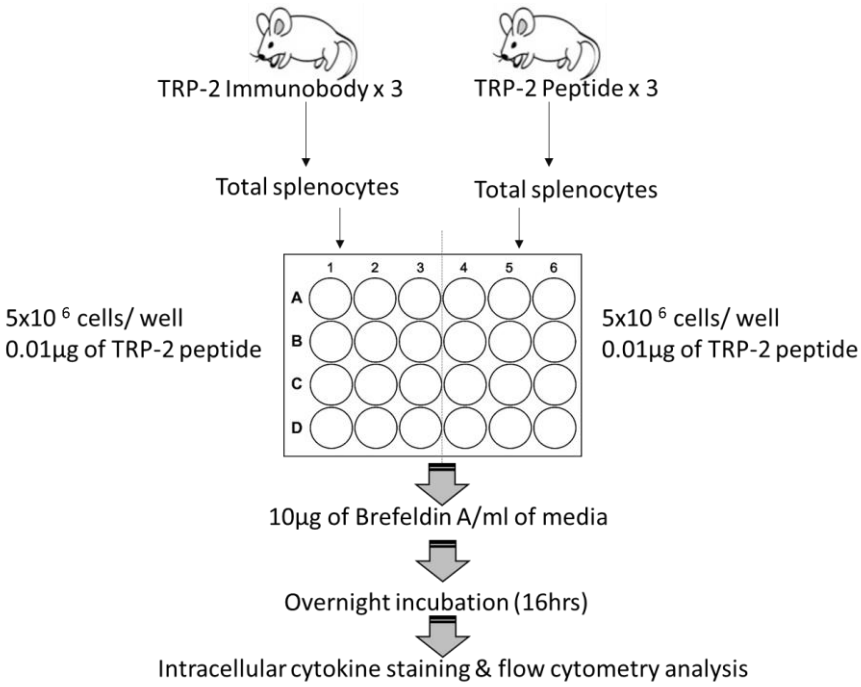

Figure 3.7: Workflow showing the protocol followed for the Proteomic confirmation of two markers identified by transcriptome profiling using antibodies and flow cytometry.

Animals were terminated by cervical dislocation and splenocytes were harvested from each animal separately. 5 X 10^6 total splenocytes were seeded on to the wells of a 24 well plate in triplicate for each animal. Cells were incubated with 0.01 μg of TRP2 peptide per well of the plate (at a concentration of 0.005 μg/mL of the media) for 3 h at 37°C at 5% v/v CO_2. After 3 h protein transport is blocked by adding 10 μg/mL of brefeldin A (Sigma), and mixed each well by gentle up and down pipetting. Cells were further incubated overnight (~ 16 h). The next day the plate was taken into a sterile hood and the cells were harvested using a 1000 mL pipette tip with gentle pipetting procedures. Intracellular cytokine staining was carried out with IntraPrep® Permeabilisation Reagent (Beckman Coulter) according to manufactures instruction. The stained cells were analysed immediately in a Gallios multicolour flow cytometer. Percp (Peridinin chlorophyll) conjugated Granzyme A (Santha Cruz, Biotechnology, Germany) and Alexa Fluor conjugated Granzyme B (Biolegand, UK) antibodies were used for the staining of the cells with anti CD3 and CD8 antibodies. Granzyme B has been used at 5 μL/10^6 cells and Granzyme A has been used at 1 μg/10^6 cells.

Results

3.3.1 RNA Isolation from pentamer positive cells

Initial RNA isolations were carried out using the pentamer positive cells isolated from single mouse. The number of cells obtained from one immunised mice were in the region of 30-40K. The initial quality assessment (QC) was carried out using NanoDrop 8000® and found to be good quality, however subsequent analysis using the Agilent Bioanalyser showed considerable degradation of the isolated mRNA. Increasing the cell number from 30K to 100K by pooling three immunised mice yielded good results both in NanoDrop and Agilent Bioanalyser®. The representative results are given in Figure 3.8.

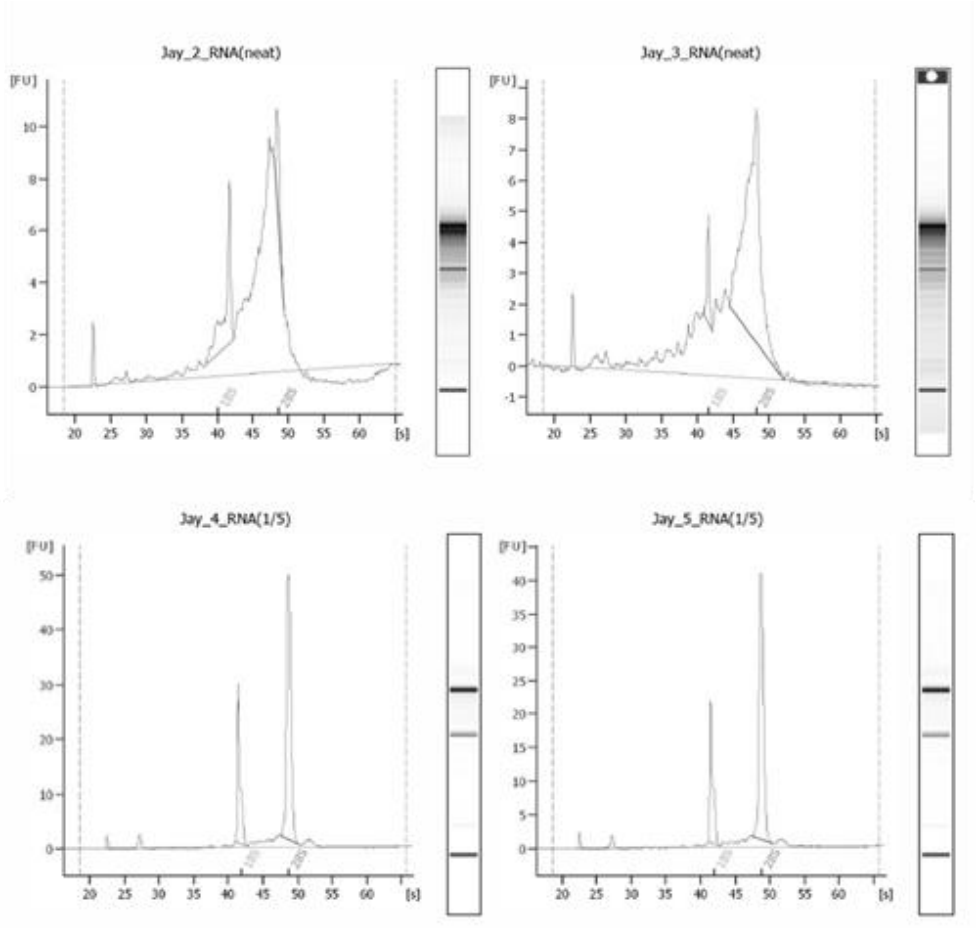

Figure 3.8: Agilent bioanalyzer results of RNA isolated from the pentamer sorted cells. A. RNA extracted from cells sorted from single mice (~ $3x10^4$cells) showing the degradation of RNA with no distinct 18S and 28S RNA peaks. B. RNA isolated from pentamer positive cells purified from three animals pooled together (≥ $10x10^4$cells). Both 18S and 28S RNA peaks are clearly visible and no sign of degradation is visible.

3.3.2 Gene expression studies of TCR signalling pathways in pentamer sorted peptide specific cells using quantitative real time PCR

RNA was isolated from TRP-2 model system and was used for the gene expression studies Initial studies were conducted with the genes involved in the TCR signalling pathway to interrogate any possible alterations during the cell sorting using TRP-2 class 1 pentamer which bind to the TCR. Replicate Ct values generated with each primer pairs with an arbitrary allocated threshold by visual inspection. The expression values were normalised to the expression of housekeeping genes. Relative expression is calculated using Livak method and no significant differences in TCR signalling pathways were detected between high avidity (ImmunoBody®) and low avidity group (*n=3, P ≥ 0.05)* Figure 3.9.

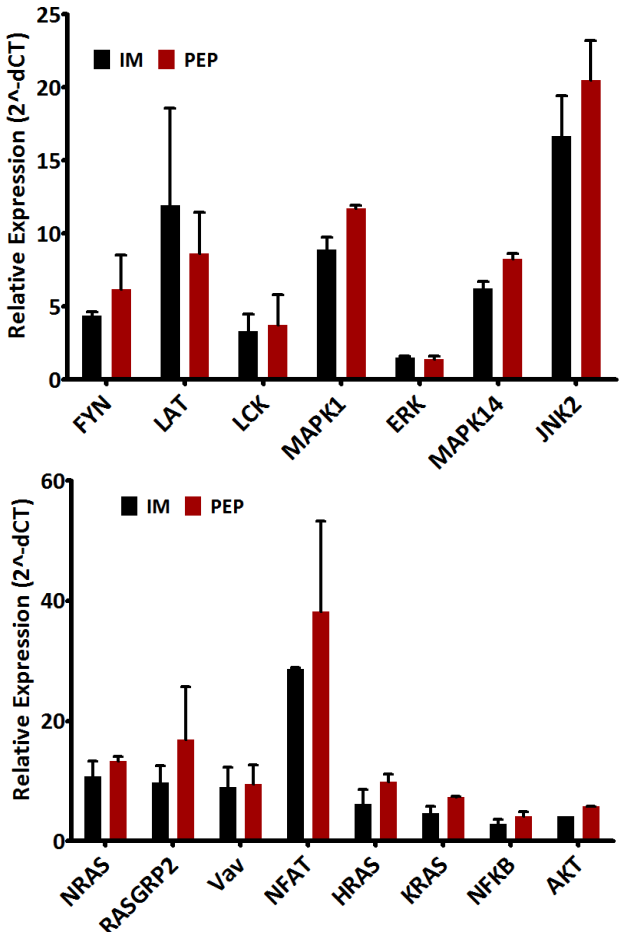

Figure 3.9: Expression of genes involved in the TCR signalling pathways in pentamer positive cells isolated from Immunobody and peptide immunised animals. The result is the representation of qRT PCR data of three independently TRP-2 ImmunoBody® (IM) and peptide (PEP) immunised animals and each bar represents the mean ± SEM.

3.3.3 Gene expression studies of TCR signalling pathways in ELISpot plate derived CD8$^+$ cells using quantitative real time PCR

Gene expression profiling of ELIspot plate stimulated cells using selected genes involved in the TCR signalling pathway did not reveal any significant differences between the stimulated groups studied ($n=3,\ P \geq 0.05$). However a significant up-regulation of VAV has been noticed in stimulated peptide immunisations in comparison to its unstimulated control (Figure 3.10).

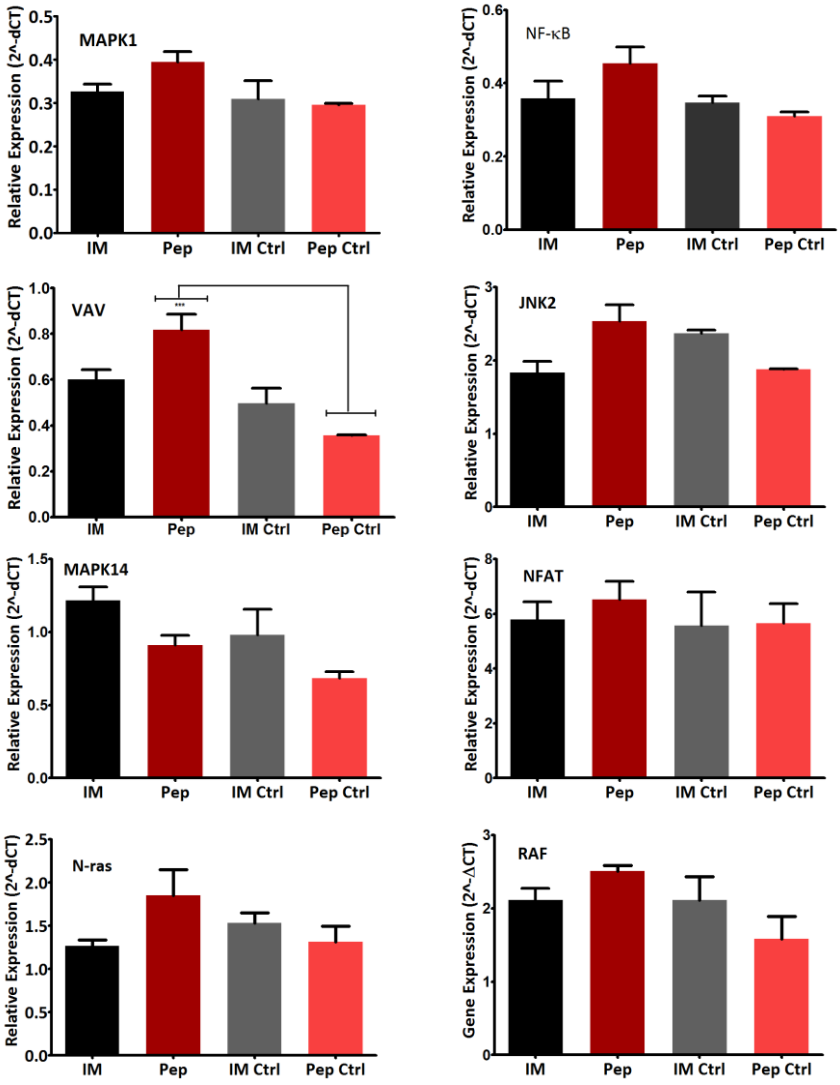

Figure 3.10: qRT PCR results showing the relative expression of selected genes in CD8+ cells obtained from ELISpot plate after 48 h of stimulation. The groups were the cells obtained from ImmunoBody® (IM) and peptide immunisation (Pep) of which IM and Pep are cells derived after the peptide stimulation and IM Ctrl and Pep Ctrl are the cells without peptide stimulations. Significance were calculated with one way ANOVA with Bonferroni post-test. The results are the representation of three independently immunised mice in each group and the bars represent Mean ± SEM.

3.3.4 Gene expression kinetic studies of TCR signalling pathway genes on TRP2 pentamer positive peptide specific cells

To investigate the signal competency between the high and low avidity T cells, this study used isolated TRP-2 specific pentamer positive $CD8^+$ T cells from the Immunobody and peptide immunised animals. These cells were subsequently stimulated with anti CD28, CD3 coated microbeads as described in the section 3.2.9. A total of 12 genes (VAV, RGRP, RAF, NRAS NF-κB, FYN, NFAT, MAPK14, MAPK1, LAT, JNK and ERK) were studied for its expression after three time point of stimulation in triplicates. Among all the above genes studied there is no significant differences in the expression were noticed with FYN (*F (5, 12) =0.7727, p=0.5874*) and LAT (*F (5, 12) = 1.197, p=0.3673*) in both high and low avidity groups. However the rest of the genes were significantly differentially regulated between the groups studied: the result of one way ANOVA are, NFAT (*F (5,12) = 18.21, p ≤ 0.0001*), MAPK1 (*F (5,12) = 29.45, p ≤ 0.001*), MAPK14 (*F (5,12) = 63.37, p ≤ 0.001*) ERK (*F (5,12) = 14.40, p ≤ 0.0001*), RAF (*F (5,12) = 17.74, p ≤ 0.0001*) RGRP (*F (5,12) = 33.16, p ≤ 0.001*), RAS (*F (5,12) = 8.357, p ≤ 0.0013*), NF-κB (*F (5,12) = 11.74, p =0.0047*), VAV (*F (5,12) = 16.08, p=0.002*), *JNK2 (F(5,12)=15.86, p=0.001) [Gene name(F (df between the group, df within the group)=F value, ANOVA p value] (n=3).*

The overall results indicated that there was a considerable increase in the expression of 10 out of nine genes studied in ImmunoBody® group compared to the peptide counterpart (Figure 3.11, 3.12, 3.13) after 2hours of stimulation. Even though there was an increasing trend in these genes in the peptide group they were not found to be statistically significant with the analysis performed. The results of all pair wise comparisons are given in table. 3.2.

	NFKB	NFAT	MAPK	MAPK	RAS	ERK	LAT	RGRP2	FYN	RAF	JNK2	VAV
IM1hr.	NS	*	**	*	*	**	NS	NS	NS	**	NS	NS
IM2hr.	*	***	***	**	*	***	NS	**	NS	***	**	*
Pep1hr.	NS	NS	NS	*	NS	NS	NS	NS	NS	NS	NS	NS
Pep2hr.	NS	NS	NS	**	NS	NS	NS	NS	NS	NS	NS	NS

NS- Not Significant, **** = P ≤ 0.05, ** = P ≤ 0.01, *** = P ≤ 0.001***

Table 3.2. Summary of significant differences observed within each group. The significance were calculated by comparing 1h and 2h stimulations to its control 0h

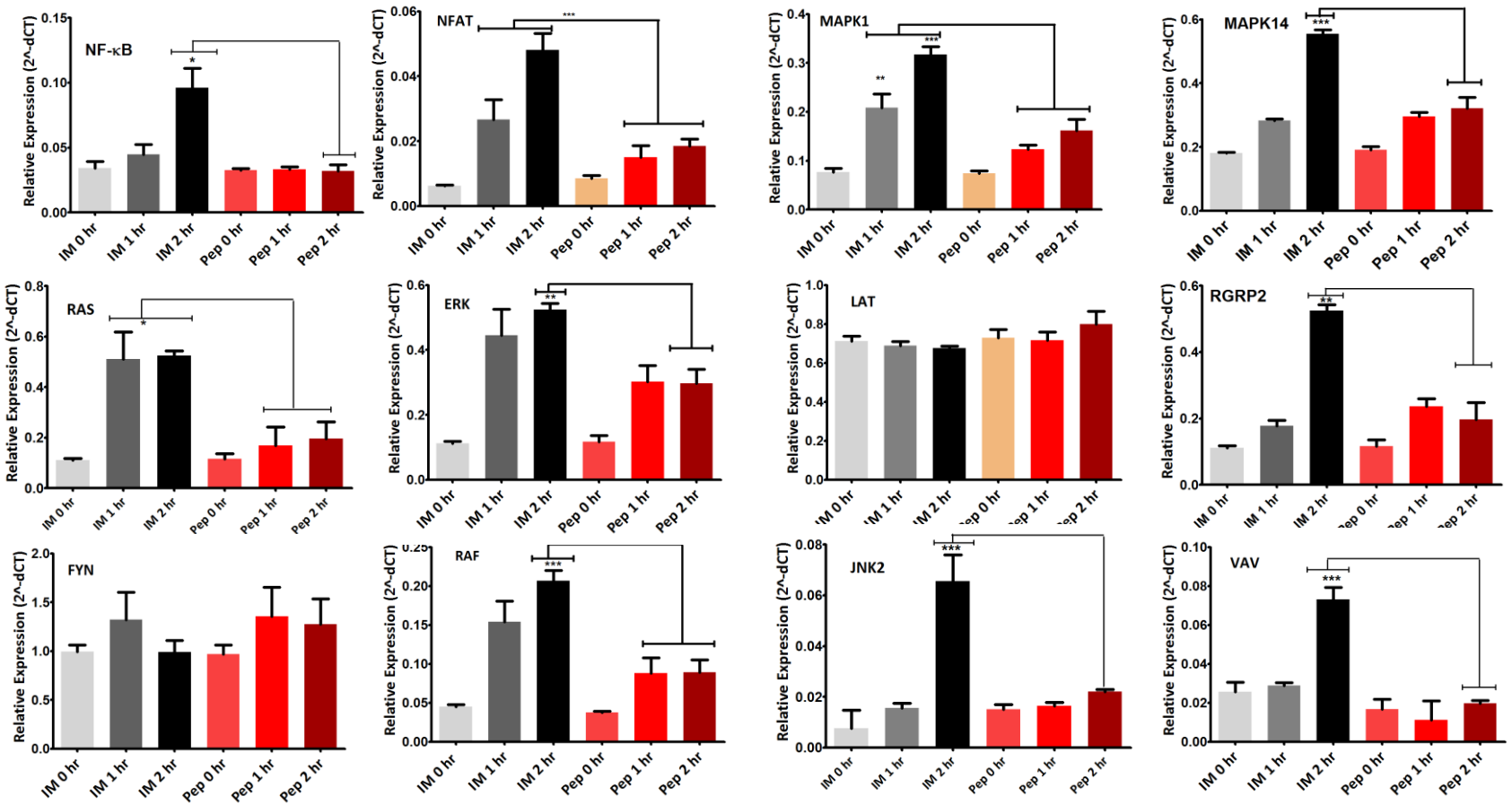

Figure 3.11: qRT PCR results showing the relative expression of selected genes in TRP-2pentamer +ve cells isolated from both ImmunoBody® and peptide immunisations. The cells isolated from each group subjected to a brief time course stimulation measuring their signal competency. The most relevant differences are marked with in each graph. The results are the representation of three independently immunised mice in each group and the bars represent Mean ± SEM.

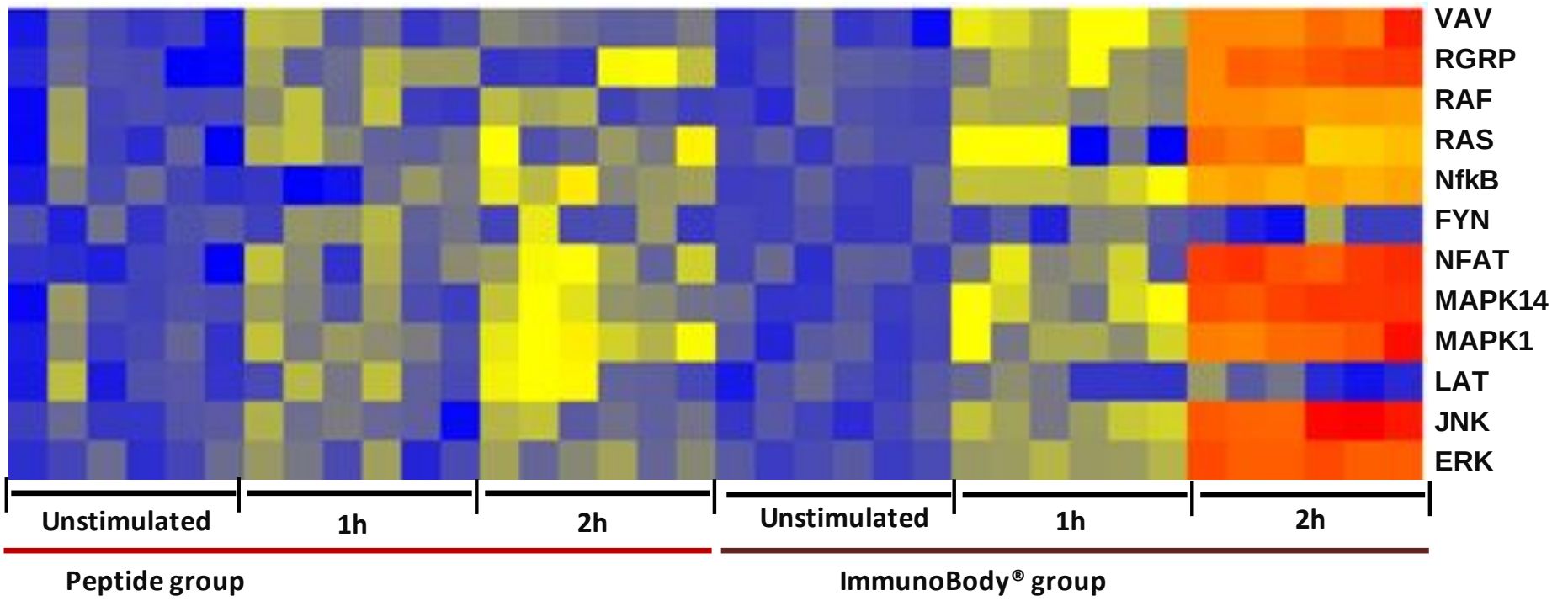

Figure 3.12: Heat map showing the expression pattern of the genes studied using qRT PCR. The colour gradient is between blue to red with yellow in the middle. The name of the genes are given on the right hand side of the heat map in the rows and the samples are given on the top.

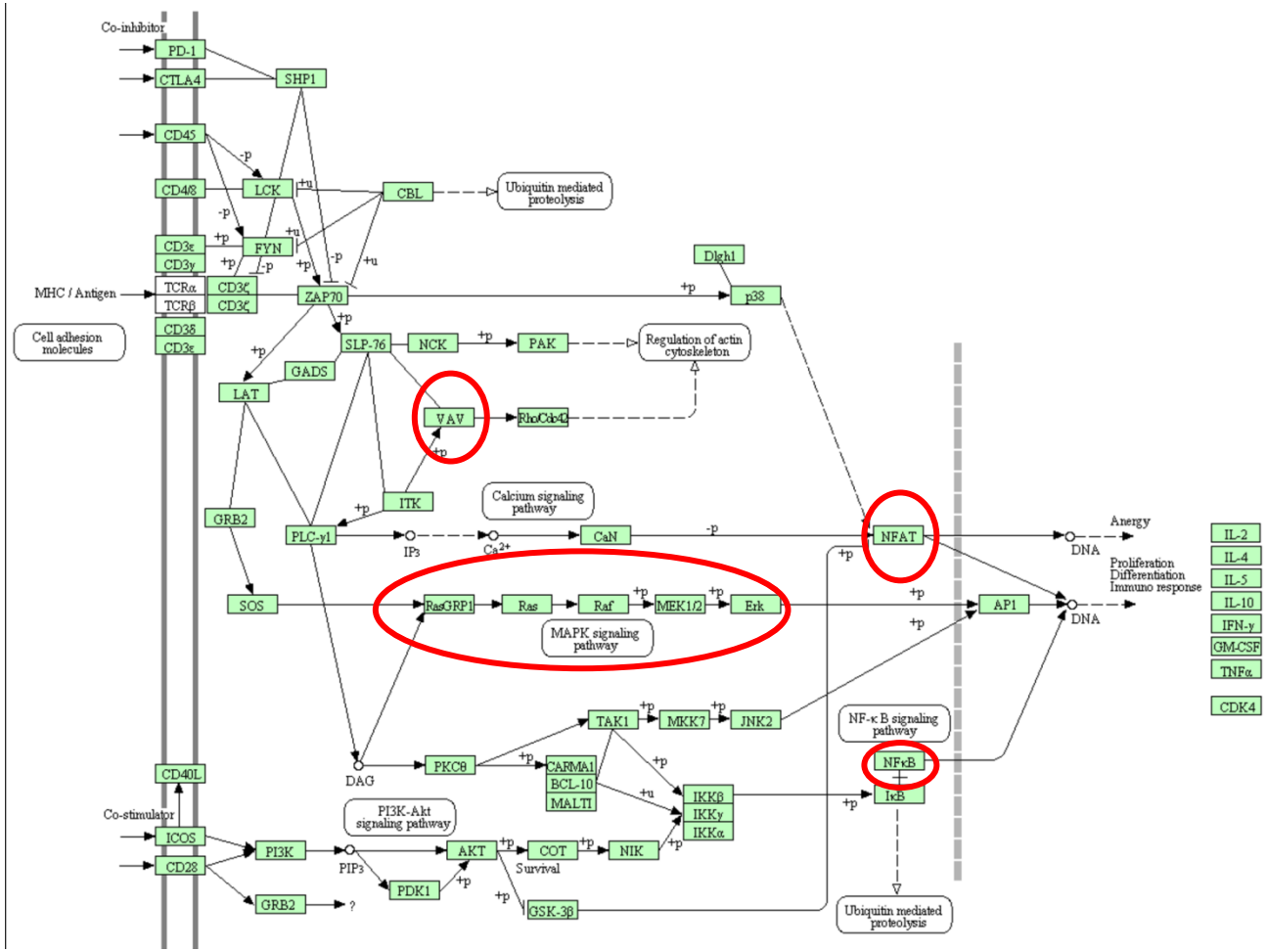

Figure 3.13: Major up regulated pathways in the kinetic gene expression study of murine TCR signalling. Signals involving the highlighted regions are highly up-regulated in the ImmunoBody® group after 2 h of stimulation using anti CD3, CD28 beads. Pathway courtesy: KEGG pathways.

3.3.5 Gene expression profiling of high and low avidity peptide specific $CD8^+$ T cells with Affymetrix® gene arrays

Global gene expression profiling was carried out on pentamer sorted peptide specific $CD8^+$ T cells using Affymetrix® high density oligonucleotide microarrays to identify the most differentially expressed genes between high and low avidity T cells. The cells were first CD8 sorted using the negative isolation kits followed by the isolation of peptide specific cells using magnetic beads. The protocol, used for the isolations were given in section 3.2.2 and carried out at 4° C to minimise the change in expression while the isolation was taking place. The first isolations were carried out using the TRP-2 model and the RNA has been subjected to both NanoDrop and Agilent Bioanalyser quality checking. The RNA quantity determined with NanoDrop 8000®, was in the range of 2.2 – 3.5 µg from each pool and 1µg of total RNA has been used for the array labelling and hybridisations. The Agilent Bioanalyser results were similar and the RNA integrity number was found to be above 8.0 with good 18s to 28s ratios in all the samples and therefore suitable for a reliable array experiment.

Two model systems were used in the gene expression profiling of high and low avidity T cells. The first model is the well characterised TRP-2 model to identify the differential gene expression between the groups and a second model, the ova (SIINFEKL) was used for validating the differentially expressed genes identified in the TRP-2 model. The array quality controls were performed in Affymetrix Expression console software.

Good correlations were observed between the arrays studied suggested by good Pearson correlations between all pair-wise comparisons (between 0.97-0.99). These correlations were calculated with the raw signal values of 28,000 genes and other transcripts and therefore justify a regression based analysis for identifying differentially expressed genes. The Pearson correlations of individual chips compared in a pairwise manner are given in Figure 3.14.

The data analysis was performed in the Microsoft excel with linear regression. Individual TRP-2 Immunobody RNA hybridised arrays were regressed on the Individual TRP-2 peptide RNA hybridised arrays with residuals and standard residuals (SR) generated subsequently. The average of SR of independent residuals was taken and reordered in the entire matrix in a descending order and thereby generated the most differentially expressed genes between the two groups studied. Similar procedures were followed with the ova

model and analysed independently. The genes which have a differential expression and the Bonferroni multiple testing correction *p* value 0.01 or less were considered as significantly differentially expressed. The complete list of the genes was given in appendix.

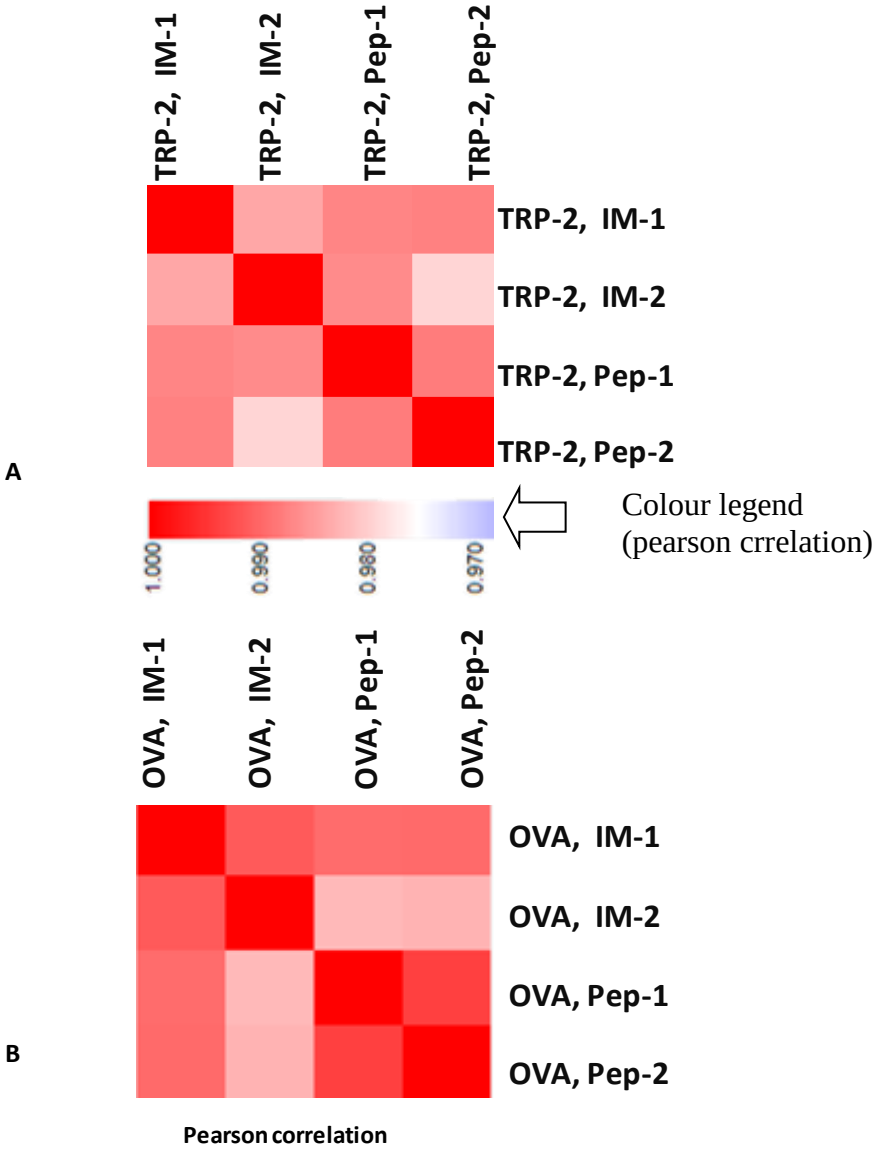

Figure 3.14: Reproducibility of arrays as showed by the correlation of 28,000 genes studied between high and low avidity T cells. A. Pair wise comparison of the expression value of these genes are expressed as Pearson correlation (PC) of the raw signal values between the array data. A. PC of TRP-2 array data. B. Correlation of OVA array data. The colour legend (marked by arrows) indicating the degree of PC calculated between each of the pair wise comparisons. The colour indicate extend of correlations between the samples (Refer to the colour legend for further information).

The genes were ranked based on their differential expression and the ones which fell above the multiple testing *p* value criteria ($P \leq 0.01$) were separated out from the main matrix in both the models. 338 genes were fulfilled these criteria in TRP-2 model after removing the noncoding transcripts and other internal control probes from the top rank ordered probes. Similarly, 239 genes were shortlisted in the ovalbumin model which showed significant up or down regulation between the groups.

3.3.6 Differential gene expression in TRP-2 specific high and low avidity T cells

The first array data for this study were generated with TRP-2 specific pentamer positive cells isolated from ImmunoBody® and peptide immunised animals. The complete list of the genes based on the rank order is given in the appendix. The major candidate genes up regulated in the ImmunoBody® group was identified with this study were Granzyme A (7.8), CD5 antigen-like (fold change: 6.02), Spi-C transcription factor (5.39), AXL receptor tyrosine kinase (5.1), EGF-like module containing, mucin-like, hormone receptor-like sequence 1 (fold change: 5.2), Fas apoptotic inhibitory molecule 3 (4.6), CD38 antigen (3.4), Granzyme B (3.4), Telomerase RNA component (3.58). Major down regulated genes in ImmunoBody group includes CD28 receptor (-3.07) (Regression standard residual distances are given in the bracket). The major differentially expressed genes which were ontologically relevant to the study are given in Figure. 3.15.

3.3.7 Differential gene expression in OVA specific high and low avidity T cells

Among the differentially expressed genes in ova model, the major up-regulated genes in high avidity group were cytotoxic effector molecules granzyme A (2.5), granzyme B (3.3), a GDP exchange factor Dennd2d (3.07), a cell cycle regulatory protein Cdk5rap1 (2.94), the down regulated ones included CD38 (- 2.2), LY6A (- 4.4), Cyclin A2 (-4.2), FOSB (-3.8) and Derlin 3 (-3.3). Although the data indicated considerable difference between the groups the magnitude of differences were not as high as the differences seen in the TRP-2 model. For the complete list of the genes refer the appendix. Genes were further hierarchical clustered in gene pattern software and the heat maps were generated using the visualisation module in the same software is given in Figure 3.16.

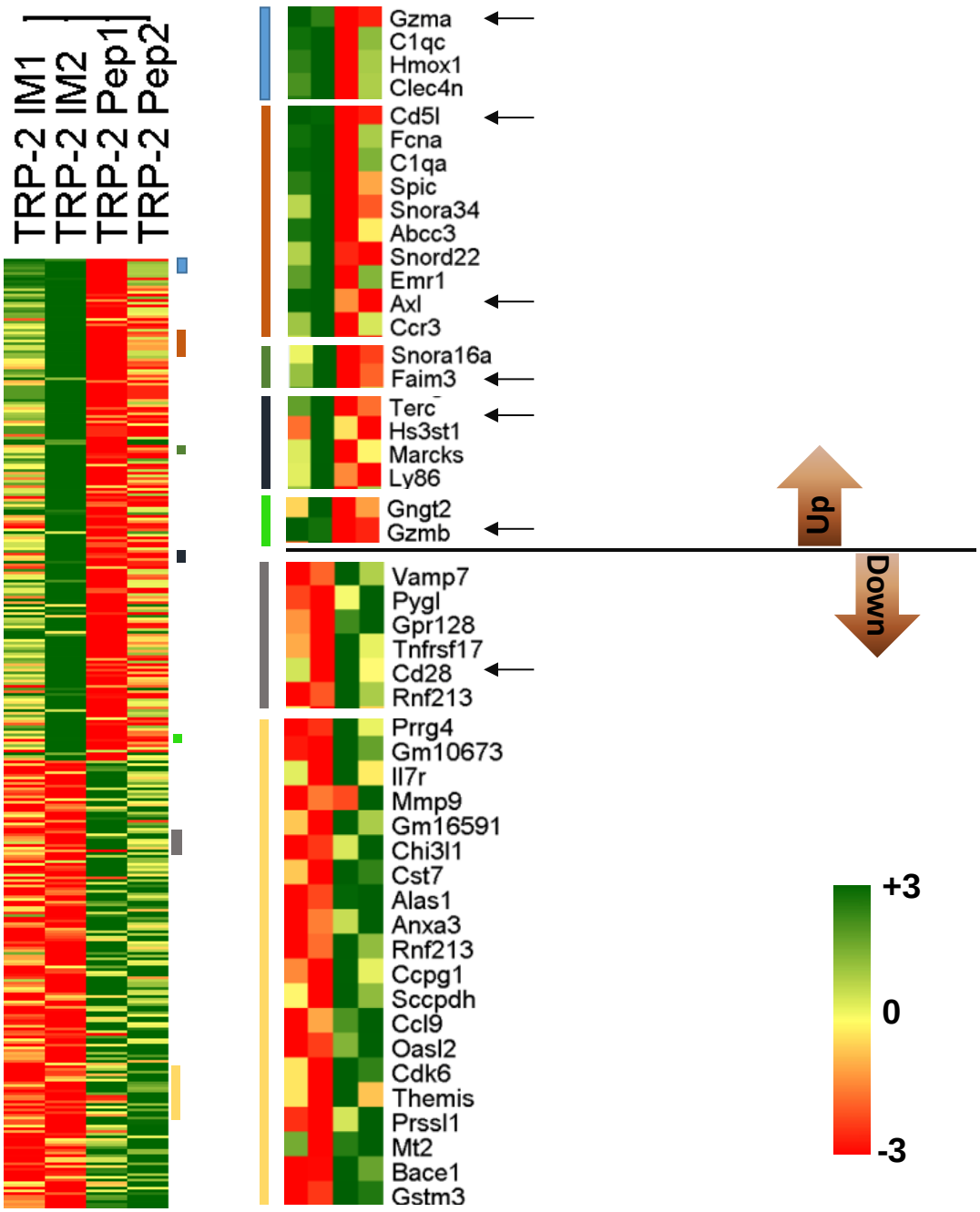

Figure 3.15: Heat map showing differentially expressed genes between TRP-2 high and low avidity peptide specific cells. The functionally important genes are highlighted. The colour code for the expression is given in the figure. Top panel represent the up-regulated in ImmunoBody® group and the bottom panel represent down regulated genes.

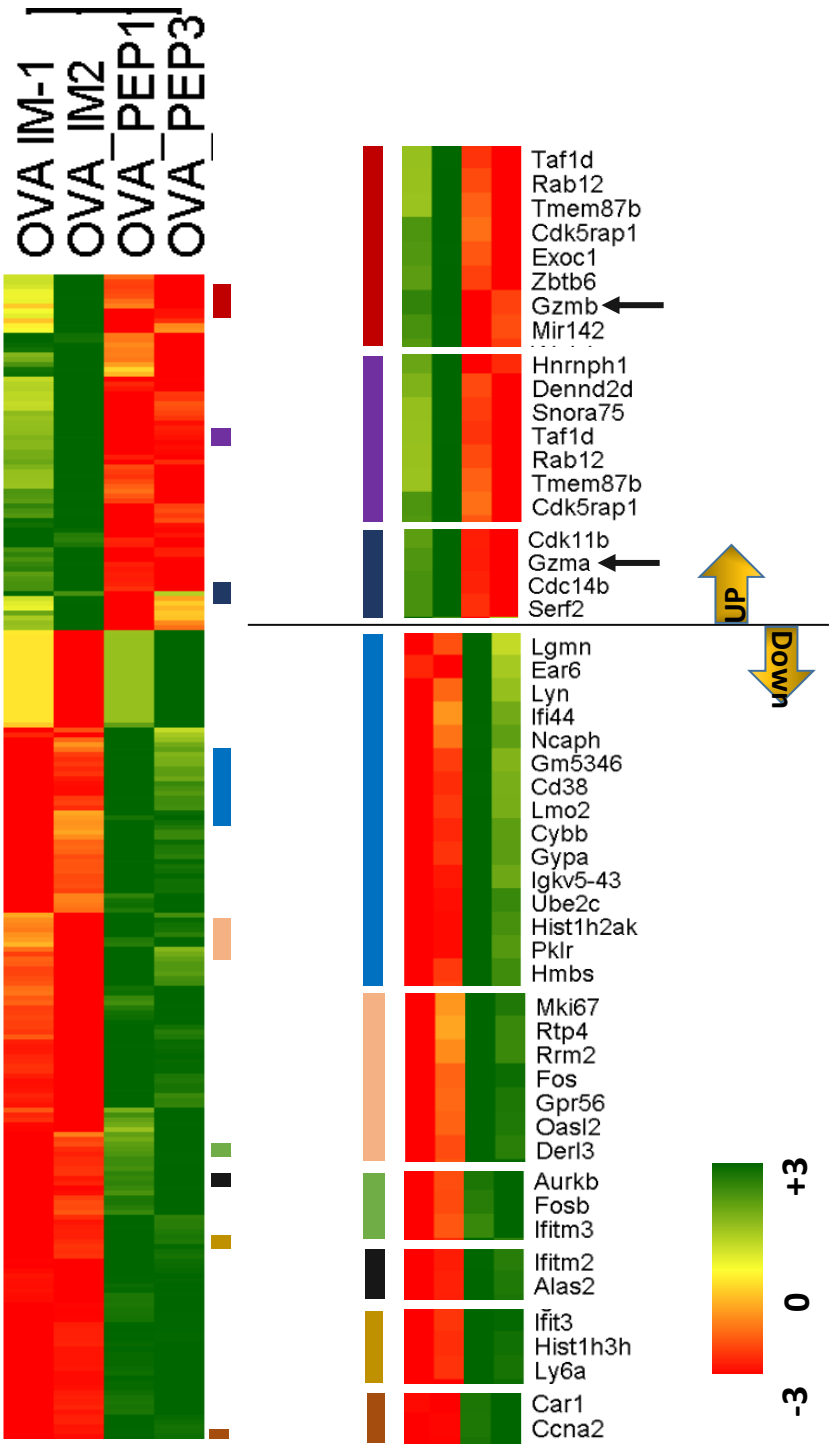

Figure 3.16: Heat map of genes differentially expressed between high and low avidity groups of OVA model. Functionally important and highly different genes are highlighted with its relative position in the main picture. The legend for colour code is also given with the picture (red the minimum value and green is the maximum expression). The top panel indicates the up regulated genes in the ova ImmunoBody® high avidity group and the bottom panel is the down regulated genes in the same group.

3.3.8 Common genes showed differential expression between TRP and OVA model

The next question was whether any of these differentially expressed genes were shared between the two model systems (TRP-2 and OVA. A Venn diagram was created to highlight the genes which had a similar expression pattern between the 2 models and also the genes uniquely expressed in each model. 305 genes were uniquely associated with the TRP-2 model and 205 for the OVA model. However 33 genes were shared between the two models studied and these genes were taken for further characterisations (Figure 3.16).

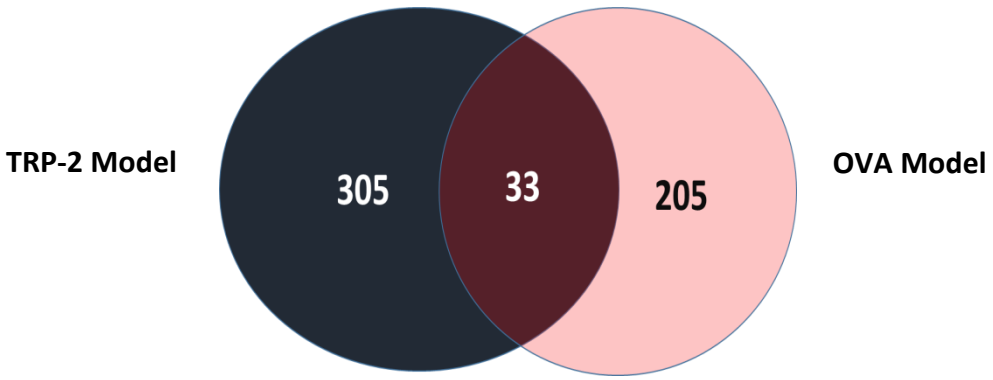

Figure 3.17: Venn diagram showing the number of genes which showed unique and shared expression patterns between the two models studied. The black area represents the number of genes having a unique expression changes in the TRP-2 model and the pink shaded area is the same for the OVA model system. The region overlapping with the brown shade representing total number of genes uniquely having an expression changes in both the models.

The expression of these genes were then further visualised in Gene Patterns (Broad institute) and the expression values were plotted as a heat map in the visualisation module of the same software. The expression pattern of these genes was given in Figure.3.18.

3.3.9 Real-time qPCR confirmation of shortlisted candidate genes

Even though the two model systems (TRP-2 and OVA specific high and low avidity T cells) differentiated the high and low avidity T cells only 33 genes were commonly found between the two models. Further literature curation of these along with the most differentially expressed genes but only present in one of the model (TRP-2 model) has also been short listed for the validation. Along with statistical relevance and rank order of these genes functional associations were also looked into before shortlisting the final list for PCR confirmation. The gene expression analysis result of these genes in the RNA of TRP2 pentamer sorted T cells are given in Figure. 3.19.

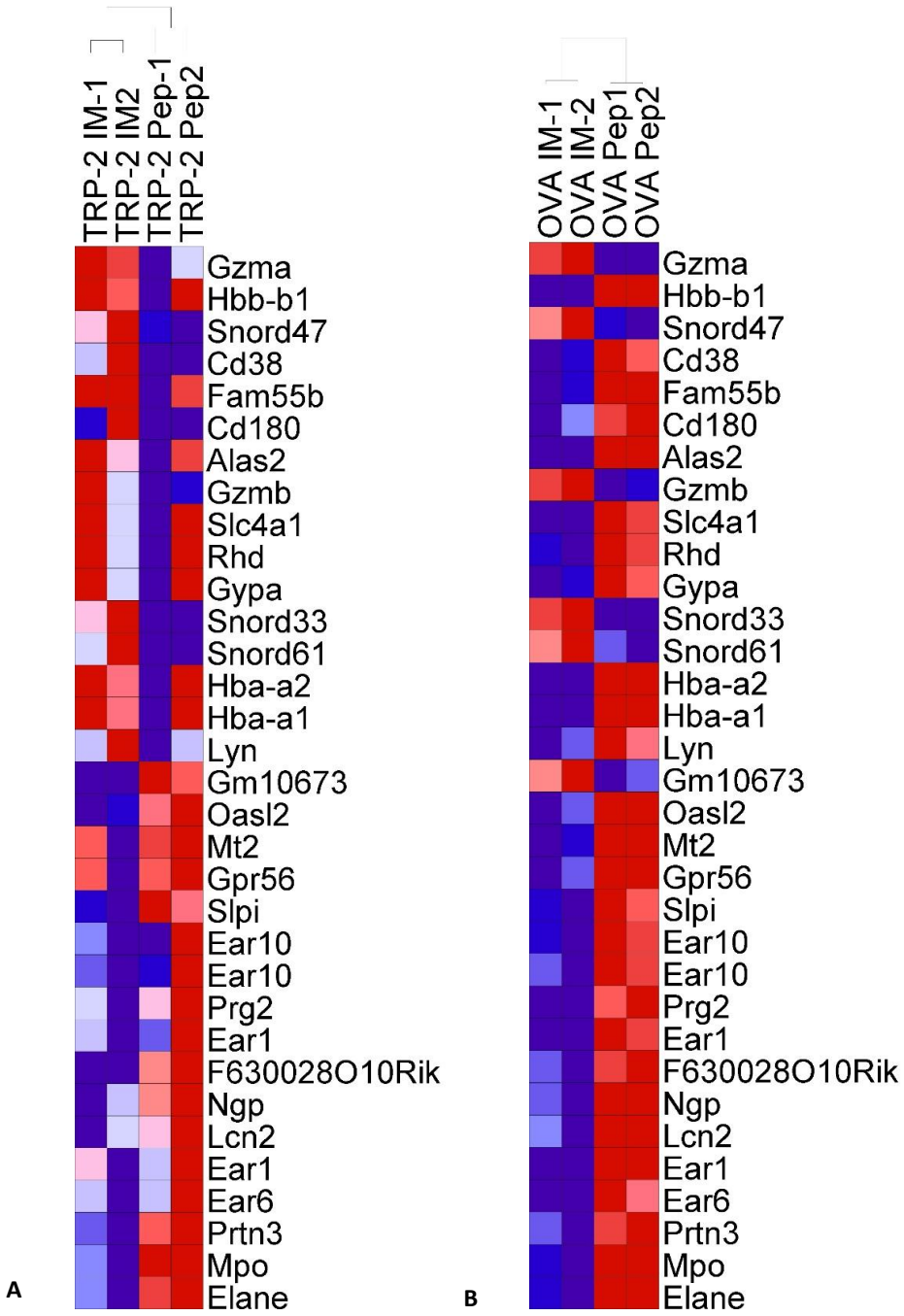

Figure 3.18: The expression pattern of 33 differentially expressed genes found common in both the models. A. TRP-2 model, B. Ovalbumin model. The red colour indicates over expression and the blue colour expressed the under expression.

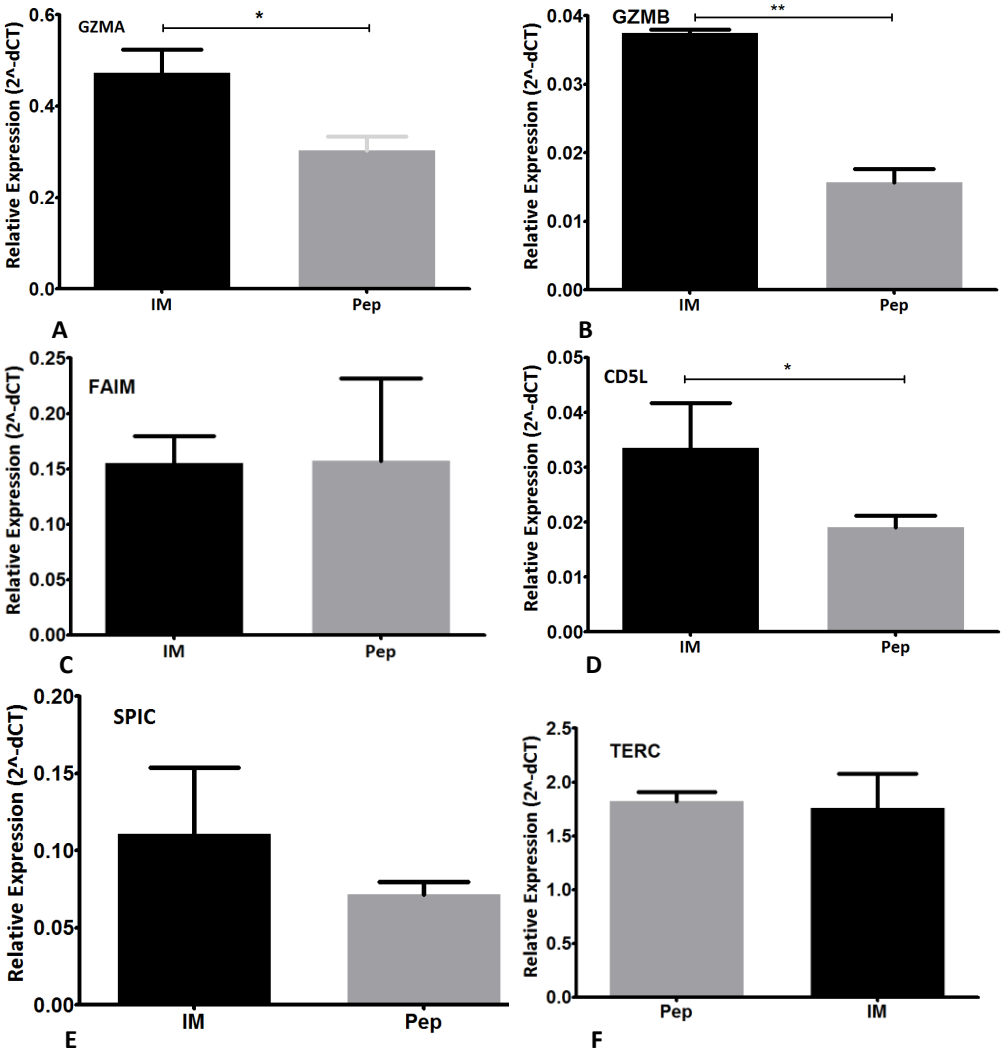

Figure 3.19: qRT PCR confirmation of the shortlisted candidate genes. The dark box represent the expression of Each genes in ImmunoBody® derived (High avidity) pentamer positive cells and the light box represent the expression of each gene in peptide derived (Low avidity) pentamer sorted cells. The data is a representation of three immunised animals in each group, the y-axis represent the normalised CT values with house keeping gene (HPRT). The relative expression is represented as 2- ΔΔCT with the mean and SEM. Significance were calculated between the group using student t-test and indicated wherever is significant as asterisk. (* = $p \leq 0.05$, ** = $p \leq 0.01$)

A. GranzymeA(GZMA), B. GranzymeA(GZMB), C. FAS apoptotic inhibitory molecule (FAIM), D. CD5 antigen like (CD5L), E. SPI-C transcription factor (SPIC), F . Telomerase RNA component (TERC).

3.3.10 Proteomic confirmation of two markers identified using gene expression profiling using flow cytometry

The gene expression studies using Affymetrix oligonucleotide arrays in the previous chapter have shortlisted genes which were differentially expressed between high and low avidity T cell responses in two models and further confirmed by real time quantitative PCR. Among all the genes two genes- granzyme A and B (Gzma & Gzmb) showed a significant up regulation in the ImmunoBody® group. These observations were further confirmed using flow cytometry in TRP2 model systems with ImmunoBody® group giving a significantly higher number of $Gzma^{+}$ $Gzmb^{+}$ cells (Figures 3.20, 21 and 22) in low CD8 expressing cells (presumably antigen experienced cells). The expression of these cells were completely absent or low in $CD8^{high}$ cells (antigen in experienced cells) (Figure 3.21 and 22). The gating strategy and the complete data is given in Figure 3.21 and 3.22.

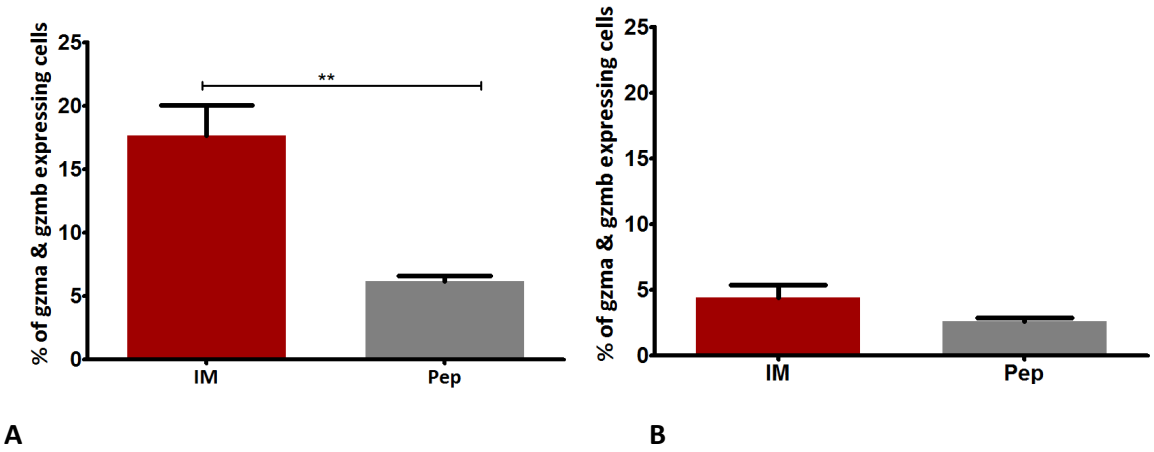

Figure 3.20: Expression of granzyme A and B on TRP-2 ImmunoBody® (IM) and peptide (Pep) immunised animals. All the immunisations were done as standard protocol given in chapter 2. The cells were given a brief stimulation with the cognate peptide and the expressions of both the proteins were measured using intracellular cytokine staining. A. % of Gzma+ Gzmb+ positive cells on CD8 low gate. B. % of Gzma+ Gzmb+ positive cells on CD8 high gate. Each bar represents the mean of three mice independently immunised and analysed. The error bar represents SEM and the significance were calculated between the group using a student t test *(n=3)*.

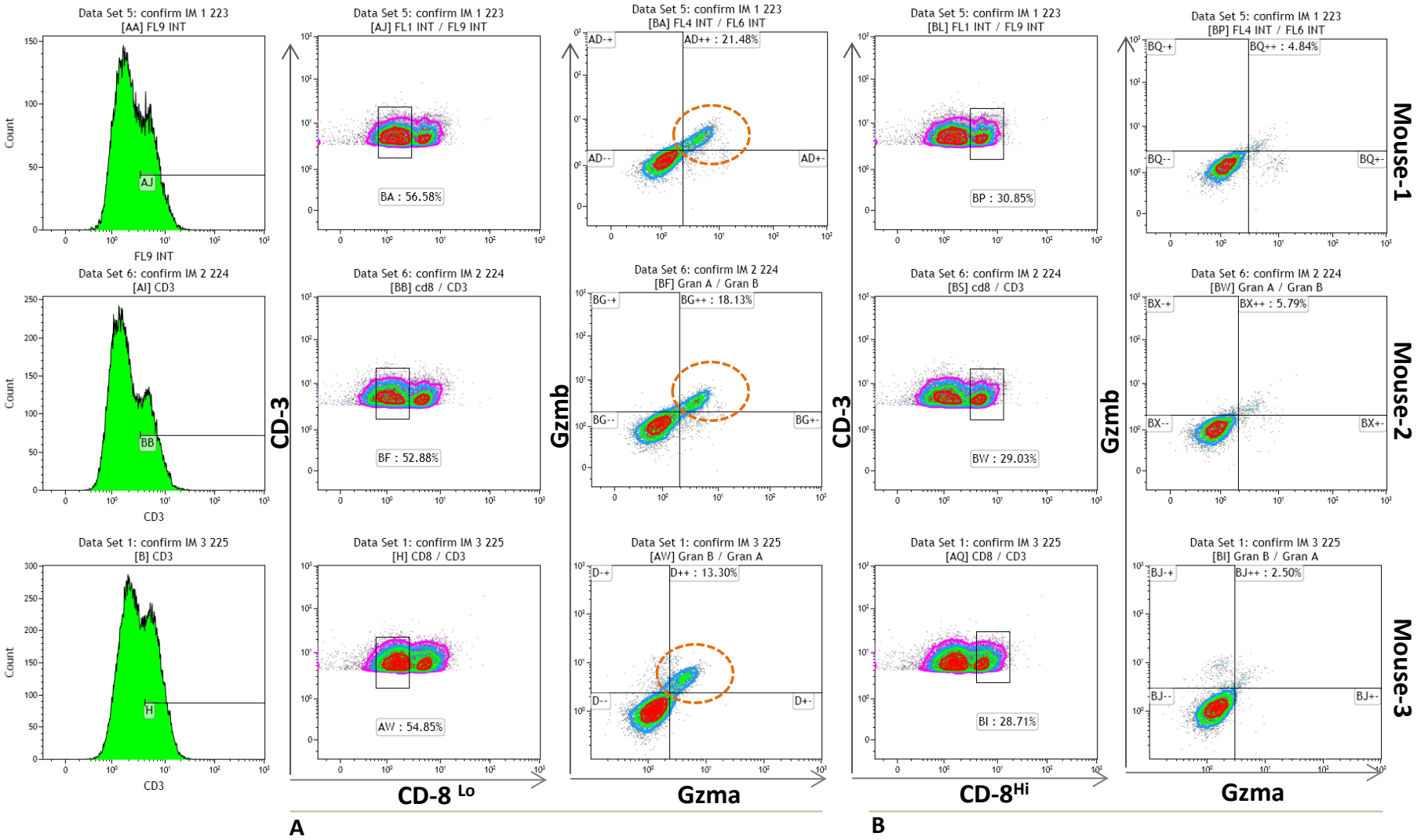

Figure 3.21: Figure showing the staining of Gzmb and Gzma gated on CD3+, CD8 ^{low} (A) and CD3^{+}CD8 ^{hi} cells isolated from ImmunoBody® immunised animals.

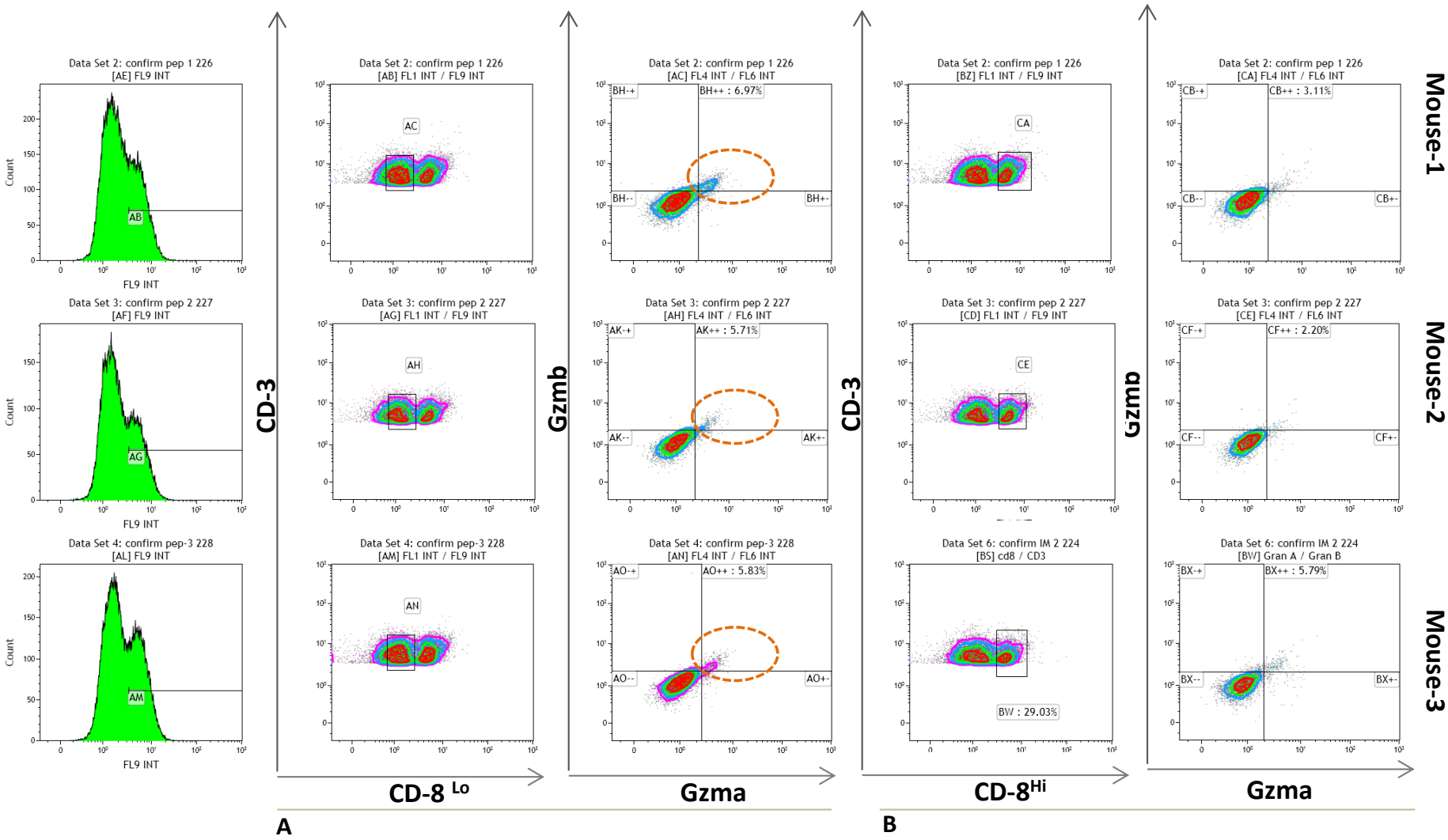

Figure 3.22: Figure showing the staining of Gzmb and Gzma gated on CD3+, CD8 low (A) and CD3+CD8 Hi cells isolated from peptide immunised animals.

3.4 Discussion

3.4.1 Pooling strategies are necessary for obtaining sufficient number of pentamer positive cells for quality RNA isolations

Isolation of specific CTLs generated using the vaccination is important in their characterisation using any downstream approaches. The early observations of vaccine generated T cells were carried out either with the pool of cells either derived from PBMC in clinical samples or from the total splenocytes for the preclinical models. This approaches mainly draws the conclusions from comparison of the stimulated and non-stimulated cells with their cognate peptides using the whole pool of cells. However, the picture often gets clear if the CTLs are purified from the total pool either by magnetic separation or by flow cytometry based cell sorting methods.

The development of multimer technology which enabled many of the immunologists to specifically detect the CTLs with desired specificity has allowed to go further and isolate the specific subpopulation of T cells from the total CD8 compartment. However, in majority of cases the frequency of specific CTLs remains small in comparison to the whole immune compartment of all the innate and adaptive immune cells. So detecting and purifying these cells from such a large and heterogeneous population is still a challenge.

To circumvent the problem of purifying specific CTLs this study made use of a two-step strategy using the total splenocyte of immunised animals. First, $CD8^+$ populations were separated out from the total splenocytes population of a single mouse using magnetic beads using a separation kit which can separate everything but the $CD8^+$ T cells. This procedure helped in such a way that no antibody was bound to the CTLs during the separation time. Therefore the CTLs were untouched and minimum changes were imposed on their gene expression patterns. Once the $CD8^+$ cells were isolated, the pentamer molecules were used to bind the specific cells and hence specifically tag the peptide specific CTLs from the total enriched population of $CD8^+$ cells. These cells were subsequently isolated using magnetic separation and lysed immediately for further RNA isolations.

Initially the isolation of peptide specific cells were carried out using the splenocytes obtained from a single mouse. A total of 25-35K cells were isolated in this way and immediately preceded for the RNA isolation using a kit specialized for the handling small number of cells. However our experience found that the quantity and the quality of RNA was inferior at every occasion when only single animal was used for CTL purification. The

outcome of this result lead to the decision of pooling multiple animals together for isolating the $CD8^+$ cells first. The pooling strategy has been done with pooling the total splenocytes from three independently immunised animals together creating three pools of three animals in each group (ImmunoBody® and peptide immunised). Total RNA was isolated from pentamer positive cells and this strategy increased the number of pentamer positive cells to more than 100K and yielded good quality RNA. One of the potential problems in the pooling strategy is the uncertainty of functional property information of CTLs. So all the animals were treated separately and an *ex vivo* IFN-γ ELISpot was performed simultaneously. This helped for the confirmation of the animals used for the pooling were high or low avidity.

Any animal showed unanticipated ELISpot results was discounted from the final study and the pool in which the respective animal belongs was also not considered for further molecular characterisation. Similar strategy has been followed for the isolation of OVA specific CTLs and the quality of RNA was analysed with the Agilent Bioanalyser®.

The low number of peptide specific $CD8^+$ T cells added difficulties to the project. One possible alternative is to isolate $CD8^+$ T cells from TCR transgenic mice where all T cells expresses same peptide specific TCR. These cells can be adoptively transferred into a wild type C57Bl6/J mice and the vaccine response can be studied with more number of vaccine specific precursor population of cells. However this fixed TCR responses may not represent the *in vivo* response happening in a natural setting where the vaccine specific repertoire selection and further functional fine-tuning are happening within a highly diverse CD8 repertoire. So this study aimed to keep to the natural setting as close as possible to draw the conclusions on a vaccine generated T cells and therefore pooling strategy has been considered as the best method to increase the cell number for further downstream processing.

3.4.2 TCR signalling gene expression studies did not detected any differences between high and low avidity *ex vivo* pentamer sorted T cells or ELISpot plate stimulated cells

Delayed response against DNA vaccines compared to the protein or peptide vaccine have been reported (Hassett, *et al.,* 2000) in viral models. This might be due to the time taken by the APCs to take up the naked DNA and subsequent migration to the lymph nodes before they start producing the antigen for processing and presentation to the T cells. The delivered antigen unlike peptide, once incorporated in to APCs can act as a continuous

production unit of target antigen (Corr *et al.,* 1996). Whereas the peptide vaccines are limited by their half-life at the site of injection due to various other mechanisms as discussed in the introduction.

If the DNA vaccines can persist in the system for longer period of time than the peptide vaccines and act as a vaccine depot we hypothesised that the TCR signalling differences would be detected in *ex vivo* isolated CTLs and therefore the ImmunoBody® immunised cells will be in a more activated state than their peptide counterpart and hence the residual TCR signalling differences would also be detected. We tested this hypothesis by *ex vivo* RNA profiling of pentamer sorted high and low avidity TRP-2 specific cells for their TCR signal status. The profiling using 15 genes involved in this pathway did not showed any signal differences. This indicated that majority of vaccine generated cells were in a resting stage at the time of their isolation. This study was conducted with the cells isolated from ImmunoBody® and peptide immunised animals with a primary and two booster immunisations a week apart with the cells isolated seven days after the final immunisation.

Mempel *et al.,* (2004), have demonstrated that the activation and the proliferation of T cells are a three step process using adoptively transferred T cells and multiphoton imaging. They have calculated the dendritic cells and T cell dwelling time for the initial recognition, homing, activation and clonal proliferation. Within the eight hour of adoptive transfer the T cells homed onto the antigen bearing dendritic cells and subsequently established a mature synapse. At 20hrs much of the T cells started to produce IFN-γ and IL-2, 1 day after the initial encounter these cells were detached from the DCs and clonally proliferated many times. This experiment approximated the kinetics of T cell activation and kinetics in an antigen dependent manner.

If the kinetics of activation and proliferation nearly follow the above path in this study most of the cells were assessed for their residual TCR signal differences might be either in the clonal proliferation or resting stage after antigen encounter. This might be the probable reason why no differences could detected in the TCR signalling pathways of *ex vivo* isolated cells. However, this expression values were used as a baseline for further activation kinetic studies which will be discussed in the later sections.

In vitro activation with the cognate peptide is an efficient way to study the signal competency between the high and low avidity T cells. With this in mind we have harvested the stimulated cells from ELISpot plate after 48 h of stimulation with two concentrations of

the peptides. $CD8^+$ cells were subsequently sorted from these stimulated cells and TCR status was assessed between the high and low avidity T cells. Again no gross differential regulation of any genes were detected except VAV, which was upregulated significantly in the control group. The window used for this experiment did not capture any of the signal differences, it is possibly the cells are exhausted with long stimulation (48 h) in the culture plate and attained a transcriptional homeostasis. These two studies indicated the importance of studying the gene expression kinetics in a time course manner with a shorter antigen exposure time prior to mRNA extraction and expression profiling.

3.4.3 Gene expression kinetics studies using anti CD3, CD28 magnetic beads revealed different signal competency between high and low avidity T cells

It is widely accepted that the way in which a naive T cells received their first stimulus has an impact on their subsequent activation by antigen encounter. This study used two radically different vaccination strategies (DNA and soluble peptide) for the delivery of a melanoma differentiation antigenic peptide (TRP-2) into the mouse. Presumably these two vaccine frameworks encountered the naive T cell repertoire in a different way and therefore they might be primed differently. If so, we hypothesised that the signal competency of these cells also going to be different if a TCR independent signal was delivered. Otherwise the signalling components of high avidity T cells are in a better equipped state compared to the low avidity one.

To test this hypothesis, all the immunisations were carried out normally and pentamer positive cells were isolated as per the pooling strategy discussed above. The cells were then stimulated with CD3, CD28 coated micro-beads in a ratio of 1:1. The cells were harvested post stimulation at 0hr, 1hr and 2hrs of stimulation and TCR signal status has been studied between these cells. Results showed that ImmunoBody® derived T cells had increased expression of genes associated with TCR signalling compared to the peptide counterpart after two hours of stimulation. Except for two of the genes (FYN and LAT) studied all other genes have showed significant upregulation in high avidity group indicating all the pathways involved in the TCR signalling responded to the CD3, CD28 stimulation quicker in ImmunoBody® derived cells than in the peptide counterpart.

The major genes up-regulated in the high avidity groups were from MAP-MAPK pathways and cytoskeletal remodelling with many of them previously known to influence the functionality of T cells during their activation and proliferation. VAV is a major SH2

containing proto-oncogene (Gouy *et al.,* 1995) which is involved in the cytoskeletal remodelling and also modulate the Ras/MEK/ERK pathways in T cells (Villalba *et al.,* 2000). The upregulation of VAV has been reported in the IL2 gene activation (Wu *et al.,* 1996) and later confirmed by RNA interference (Zakaria *et al.,* 2004).

Activation of RAS is pivotal in the T cell stimulation and signal processing (Genot *et al.,* 2000; Katz and McCormick, 1997) by positively promoting the MAP kinase pathways. The activation of RAS requires the conversion of its GDP bound state to the GTP bound stage and mainly mediated by the expression of Ras guanyl nucleotide-releasing protein (RasGRP). So the upregulation of both of these transcripts in high avidity T cells suggests the existence of an active RAS signalling pathway in high avidity T cells.

The upregulation of mitogen activated protein kinase pathway (MAPK) is one of the characteristic of T cell activation and is the direct consequences of RAS activation (Franklin, *et al.,* 1994). Activation of RAS leads to the Raf-1 activation which, in turn, leads to the upregulation of MEK pathways (MAPK or ERK kinase) (Genot *et al.,* 2000). All the classic molecules involved in this cascade were also up regulated in the ImmunoBody® group in comparison to the peptide group, including RAF, ERK1(MAPK3), ERK2 (MAPK1), p38α (MAPK14) and JNK. The importance of this pathway in the T cell activation, clonal proliferation and survival has been demonstrated previously (D'Souza *et al.,* 2008)

The fundamental difference in the RAS/MEK pathways in functionally different T cells is mainly down to the activation status. A recent study by Adachi and Davis (2011) have demonstrated that naive T cells depend more on ERK pathway than the p38 pathway which is more important for the primed T cells. Studies conducted on a single TCR transgenic mouse model derived high and low avidity T cells observed an increased phosphorylation level of the MAPK/ERK pathways in the high avidity T cells. However no transcriptomic studies were performed to study the differences in signalling pathway in the context of high and low functional avidity and to our knowledge this is the first study to report this difference in these two functional types.

3.4.4 Affymetrix gene expression studies revealed differentially expressed genes between the two groups (high and low avidity) both in TRP-2 and OVA model systems

Among the upregulated genes the most important genes upregulated were Granzyme A&B, CD-5 and Fas apoptotic inhibitory molecule-3. Granzyme A & B are the two main enzymes expressed in cells involved in the Th1 responses and have close similarity to the family of serine proteases. The main function identified with these enzymes are their cytolytic function on infected or tumour cells. Granzyme A is only detected in most mature $CD8^+$ $CD4^-$ cells and its non cytolytic functions include lymphocyte survival and immune tolerances (Chowdhary and Liberman, 2008).

CD5 is a transmembrane protein mainly present on the T cells, having a cytoplasmic domain carrying potential sites for threonine, serine and tyrosine phosphorylation. It was reported that CD5 rapidly phosphorylate after successful TCR-CD3 engagement (Perez-Villar, *et al.*, 1999). This event allows 'lck' another tyrosine kinase to bind more efficiently through its SH2 domain (Raab, et al., 1994), which might result in increased catalytic activity. The differential regulation of TCR-CD3 signalling through CD5 was demonstrated in Jurkat T cells (Carmo *et al*, 1999). Therefore the upregulation of CD5L molecules in high avidity T cells might be an important role in their functionality.

It was reported in the literature that apoptosis plays an major role in T cell homeostasis by eliminating excess antigen-specific T cells after an immune response in the peripheral tissues. A recent study by Huo, *et al* (2010) showed that Fas apoptotic inhibitory molecule (FAIM) is up regulated in T cells upon TCR engagement and Faim _/_ T cells were highly susceptible to apoptosis mediated cell death by increased levels of capsase-8 and 9. So the role of FAIM in the regulation of high and low avidity T cells need to be investigated further.

Another observation made from the array data was the consistent up regulation of small nucleolar RNAs (SNOR) and small nuclear RNA (Rnu) molecules in the ImmunoBody groups. Studies with skin and bone marrow failure syndrome dyskeratosis congenita (DC) patients showed that the significant reduction in the pseudouridylation and telomerase pathways correlated with the accumulation of a particular class (H/ACA) of SNOR (Mochizohi, *et al.,* 2003). The commonality of telomerase RNA and the SNOR is the presence of a highly similar domain called H/ACA in both the cases, however no

conclusive evidences were available for the direct relation of SNORA and the telomerase activity in humans except the above study. It is interesting to observe that telomerase RNA component (TERC) is also upregulated in ImmunoBody groups along with SNORs along with small nuclear RNA (Rnu), which is consistently up regulated in the ImmunoBody group. The main function of these small RNA molecules includes RNA splicing, regulation of transcription factors and also maintenances of telomerase. So it is worth investigating the cellular senescence pathways in high and low avidity T cells with special emphasis to small nuclear RNA (Rnu) and small nucleolar RNAs (SNORs) and telomerase RNA components (TERC).

The qRT-PCR confirmation of the above genes further narrowed down the six genes to three with GZMA, GZMB and CD5L showing significant difference between high and low avidity groups. These markers were shortlisted for further protein confirmation.

3.4.5 Expression of granzymeA and B (GzmA & B) could be used as a marker for high avidity in the current model studied

The global Affymetrix studies of high (ImmunoBody®) and low (peptide) avidity peptide specific cells have identified granzyme A and B upregulated in the high avidity group. Further qRT PCR confirmed this at the gene level. Intracellular cytokine staining (ICS) of high and low avidity T cells using antibodies against both granzyme A & B were carried out for further confirmation of these observation at the protein level. ICS were performed with a brief stimulation of these cells with one concentration (0.01 µg/mL) of peptides with the assumption that if the cells were differentially expressing the above markers then it will be clearly visible by stimulating them. As expected more granzyme A and B production was observed in the high avidity T cells. This confirmed the observations gained from the gene expression studies that these proteins could be used as a marker for differentiating high and low avidity T cells in the current model system used.

Granzymes are serine proteases secreted by cytotoxic T cells and NK cells capable of performing cell mediated cytotoxicity (Chowdhury and Lieberman, 2008). Several classes of granzymes were identified (GzmA, B, C, D, E, F, G, L, M & N**)**, of which GzmA & B are the ones well studied for their effector functions (Joeckel and Bird, 2014). Other Gzm's are known as orphan granzymes since their precise role yet to be elucidated (de Koning *et al.,* 2009; Bovenschen and Kummer, 2010). Up-regulation of GzmB was reported in many vaccine settings (Ranasinghe *et al.,* 2007; Wang *et al.,* 2013; Liu *et al.,* 2013). Up

regulation of GzmB was detected in earlier studies using microarray profiling of flow cytometry sorted $CD8^+$ cells in a vaccine trial involving Ipilimumab in metastatic melanoma (Wang *et al.,* 2013). In another study, employed cDNA microarrays to identify differentially expressed genes in a T cell *in vitro* stimulation study using a naive and agonist peptide had shortlisted granzymeB as one of the most differentially up regulated transcript in agonist group (Palena *et al.,* 2003). Unlike GzmB, GzmA is not studied extensively. However few studies indicated their role in $CD8^+$ T cell mediated cytotoxicity (Shacklett *et al.,* 2004; Kelso *et al.,* 2002).

3.4.6 Conclusions and future directions

The isolation and purification of pentamer positive cells were carried out from the enriched population of $CD8^+$ cells to minimise the total splenocyte contamination. Gene expression kinetic data revealed differences in the signalling competency between high and low functional avidity cells indicating these differences probably acquired during the priming with two radically different vaccine delivery systems. However, more studies are necessary to see the phosphorylation status of these two primed cells before reaching final conclusions. From these data it was hypothesised that the physiological and molecular changes happening during the naïve T cell priming leave the molecular machinery for better activation in high avidity cells. Whether these molecular machinery is diluted out during the clonal proliferation is another intriguing question.

Gene expression micro array data has successfully carried out with low number of peptide specific cells with out any stimulations. The data indicated there is a transcriptional differences between these two cell types. However the differences were subtle and probably the reason might be the studies were not conducted on the activated peptide specific T cells. And this will remain a future priority for this section.

Confirmation of two transcriptomically identified candidate genes (GzmA and GzmB) using antibody mediated method could be easily be the highlight of this study. Though one of them (GzmB) was well known cytotoxic molecule the second one is not reported in the literature in a greater extend. So it is ideal to confirm these markers in human vaccine trial settings to derive clinically relevant correlations.

Chapter 4.

Proteomic characterisation of high and low avidity T lymphocytes

4.1. Introduction

4.1.1. The Proteome – the functional end of the genome

Proteins are the ultimate cellular machines, the "working class" of cellular molecular framework. Proteome is the name coined by Wilkins *et al.,* in 1996 by combining the two terms PROTEin and genOME to collectively denote the protein compartment of a cell or living system. The emergence of this new field of molecular sciences coincides with our ability to study genes in detail. Until the discovery of DNA by Watson and Crick, genes, for biologist, were an abstract metaphoric entity, having no defined physical location. The invention of PCR by Karry Mullis (1990) and the subsequent large scale identification of the genes and their mapping of their physical location in the genome made a huge stride in the understanding of the genetic underpinning of many diseases and traits. The decoding of sequence information holding in the rather static DNA in humans and many other organisms left the biologist with a new set of problems, which is the functional annotation of each gene in many disease and physiological functions (Venter, 2001). Many of the answers for this functionality of the genetic information lies the protein information and therefore post genomic era exclusively demand the understanding of proteome in a wider scale. Unlike the genome, which is static, the proteome is dynamic and changes over time in response to the external stimuli. Adding to this paradigm RNA splicing (Modrek and Lee, 2002; Xing and Lee, 2007) and post translational modifications (Cerný *et al.*, 2013; Jensen, 2004; Mann, and Jensen, 2004) further diversify the genetic information therefore the complexity of proteome is several order higher than its genomic counterpart.

With this diversification happening at the mRNA and protein level, the nearly 20-25,000 genes (Collins *et al*., 2001, International Human Genome Sequencing Consortium) present in the human genome much less than the previously predicted number of genes, finally ended up in the production of over 100,000 proteins which is the functional end product of the genetic information (Gstaiger and Aebersold, 2013; Hochstrasser *et al.,* 2002). The

disparity between the number of genes and the proteins are unimaginable (Collins *et al.*, 2001) and the process involved in this complexity is illustrated in figure 4.1.

4.1.2. Proteomics

Proteomics is defined as "the study of the proteome of a living entity" (Wilkins *et al.*, 1996). It has emerged as a systematic discipline in the last few decades along with our increased ability to study large numbers of proteins in parallel.

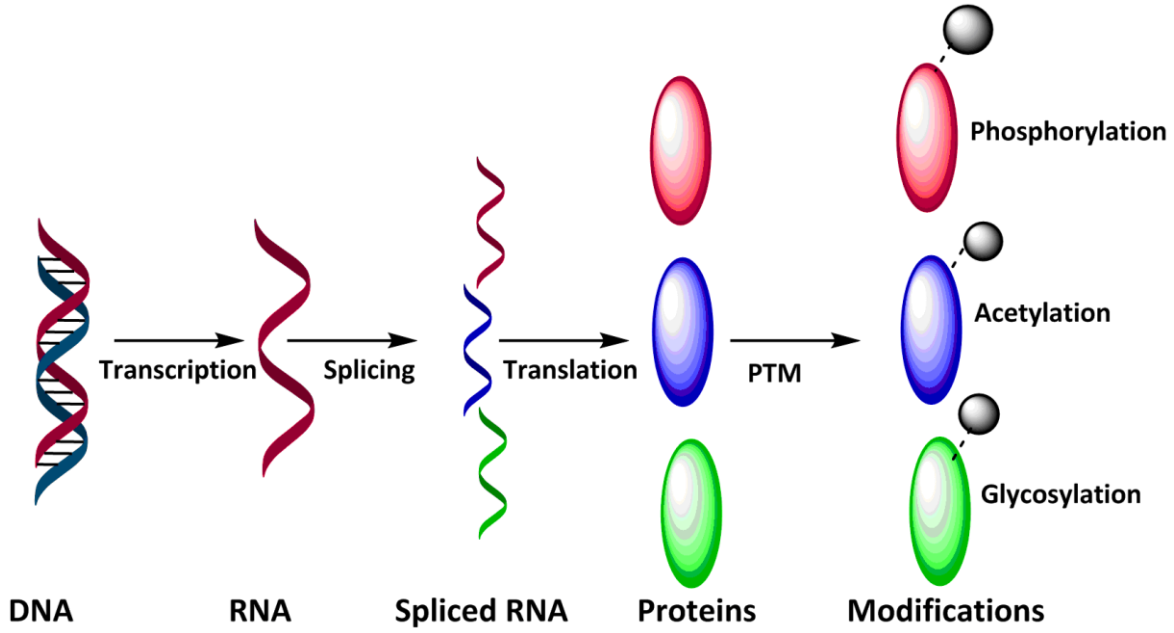

Figure 4.1: Increasing complexity and diversification of genetic information from DNA to proteins. Each stages of this central dogma of molecular biology is adding a higher level of diversification of the gene by RNA splicing mechanism and post translational modification.

Proteomics can be divided into many categories depending on the type and nature of the samples and methodologies used for any particular study. Two main categories are "functional proteomics" (Monti *et al.*, 2005; Strosberg, 2001) and "structural proteomics" (Jhoti, 2001). The former is the systematic study of function of the proteins and the latter exclusively elucidates the structural basis of the proteins and thereby determines the structure and the binding characteristics of a given protein. Depending upon the nature of starting material used for the study, proteomics can either be *targeted* (Boja and Rodriguez, 2012) or *global* (Tomlinson *et al.*, 2002); targeted proteomics looks for the particular compartments of proteins whereas global proteomics study the whole proteome complexity and changes. Subcellular fractionation (Huber et *al.*, 2003), antibody mediated immune

precipitation (Le Guezennec *et al.*, 2005), affinity purification (Mechref *et al.*, 2008) are some of the tools to enrich particular fraction of the global proteome for targeted evaluation. The main advantage of global proteomics is the ability to detect novel pathways and proteins in disease versus non disease conditions, normal versus drug treated cell line and various other biologic scenarios (Vaudel *et al.*, 2011). No prior knowledge of protein expression and/or modification are necessary for global profiling. A drawback to this type of analysis is the availability of a suitable analytical platform for analysing such large numbers of proteins (≥ 100K) in one go (Tomlinson *et al.*, 2002; Vaudel *et al.*, 2011). Traditionally, both targeted and global proteomics were studied with both one- and two-dimensional gel based approaches (O'Farrell, 1975; Molloy, 2000; Vanrobaeys *et al.*, 2005) to identify differentially expressed proteins between different conditions and subsequent identification of the protein by mass spectrometry (Gomes, *et al.*, 2013).

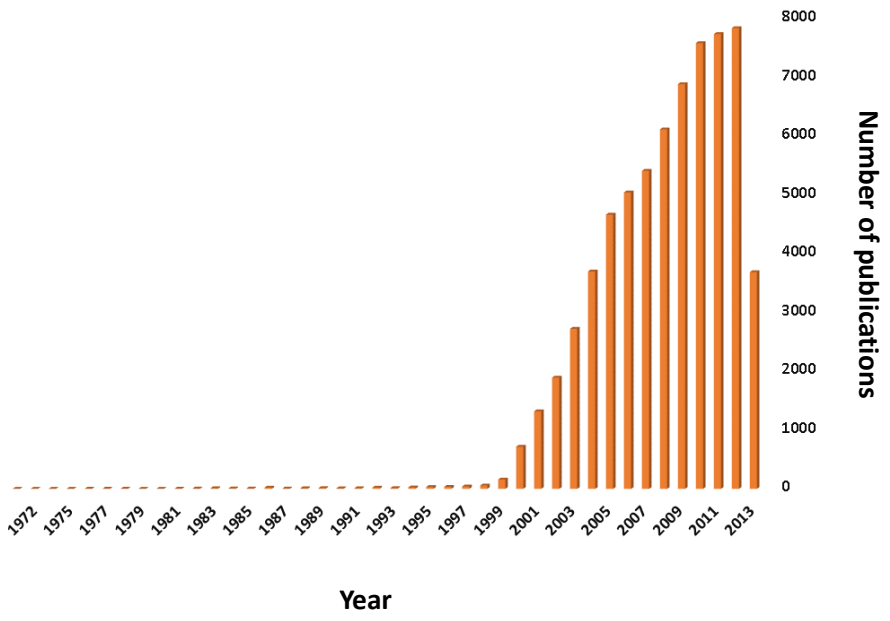

Figure 4.2: Graph showing the number of journal articles returned from a Pubmed search using the keyword 'protein profiling'. The data shows an exponential increase in the publication during the last 10 years.

Though 1 and 2-D gel based proteomics have been the mainstay of the proteomics over the years, it is limited with its detection ability and the proteome coverage. This limitation leads to the quest for gel free proteomics and finally leads to the introduction of a new tool set known as mass spectrometry (MS) for the quantitative and qualitative detection of proteins. During the past decade huge interest has been generated in the area of proteomic

profiling (figure 4.2) and mainly fuelled by the generation of novel mass spectrometric based platforms to study large number of proteins in one experiment. This study used mass spectrometry based global proteomics; therefore the subsequent literature will be restricted to mass spectrometry and shotgun proteomics.

4.1.3. Mass Spectrometry (MS) based proteomics

4.1.3.1. Origin and development of the mass spectrometer

A mass spectrometer is an instrument capable of measuring the mass to charge (*m/z*) ratio of a substance in its ionised state. Usually these measurements are done in the vacuum, free of any other materials, so that the ions can travel from one place to another without any obstruction. The developmental history of modern mass spectrometry (MS) has been long. At the beginning, substances with high vapour pressure only are analysed in the spectrometers due to their existence in the gaseous phase and hence easy to move from one place to another for the measurement of their mass. Later the development of disruption ionisation techniques in 1970's allowed the ionisation of substances in the solid/condensed state, so that they can also be analysed in the MS. The early ionisations were "hard", using chemical and electron ionisations methodologies. While these ionisations are good for studying small chemical molecules they were not very useful for the analysis of biological macromolecules. Most of the biological molecules are heat sensitive and easily fragmented upon any hard ionisation methods. The real alternative known as "soft" ionisation was first invented by Fenn (1989) in the form of electrospray ionisation (EI) and followed by Matrix-assisted laser desorption/ionisation (MALDI) (Hillenkamp F *et al.*, 1991; Hillenkamp, F. and Peter-katalinic J, 2007). This breakthrough in soft ionisation techniques lead to the development of mass spectrometers exclusively used for studying large biological molecules without the problem of unintended molecular fragmentation.

4.1.3.2. Basic Components of a mass spectrometer

The basic structure of a modern spectrometer contains 3 parts - an ion source, a mass analyser and a detector (Hillenkamp, F. and Peter-katalinic J, 2007) as seen in figure 4.3. The ion source is the place where the ionisation of the biological samples takes place, the mass analyser is where the resolution of the ionised biological fragments happening and finally the detector complete the story by recording the *m/z* of the fragment.

4.1.3.2.1 The Ion source

In MALDI, the ionisation of biological samples is achieved by mixing biological samples with a solid matrix compound. This matrix helps in the protonation of the biological samples when a low energy UV is fired on to them and lifted in to a strong electric field and migrate towards the detector and finally hit the detector. The rate of migration of the ions is represented as the *m/z* ratio (Hillenkamp *et al.,* 1991; Hillenkamp, F. and Peter-Katalinic, 2007). ESI is probably the most commonly used method used for the ionisation of biological samples in proteomic mass spectrometry. Samples are subjected to a high voltage electric field to create charged droplets (Lin *et al.,* 2003). These charged droplets

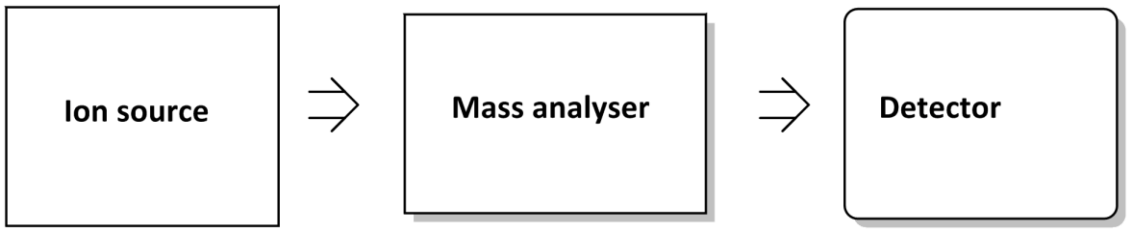

Fig.4.3. Schematic representation of the basic design of a mass spectrometer. The instrumentation starts with an ion source which is capable of ionising fragile biological samples by a process known as soft ionisation in MALDI or an electrospray ionisation. The ionised fragments then enter the next section of the instrument which is a mass analyser capable of resolving the fragment based on mass to charge ratio (*m/z*). Finally the resolved ions hit the photomultiplier detector; the signal is amplified and recorded as a peak or 'mass spectrum'.

can enter in to the inlet of mass spectrum for mass characterisation in the mass analyser. The charging can either be applied as positive or negative, however most peptide/proteins are ionised with positive charge due to their molecular structure. The sample is introduced, usually following separation by high pressure liquid chromatography (HPLC) or nano-flow chromatographic separation (nLC) (Postle, *et al.*, 2007). The volume of the flow also can be varied from few nL/min to mL/min for generation of the droplets.

4.1.3.2.2 The Mass analyser: The resolution of a mass spectrometer is the ability of its mass analyser to separate the charged biomolecules produced in the gaseous/ vapour phase carrying different m/z values effectively. The primitive type of mass analyser is the magnetic sector mass analyser (Hart-Smith and Raftery, 2012) used widely in the early

stages of MS. This had been soon replaced by different mass analysers developed by different vendors in the pursuit of enhancing the resolution of the mass spectrum such as quadruple, ion traps, orbitraps, Time-of-Flight, Fourier Transform Ion Cyclotron Resonance (FTICR) Mass Analysers are most commonly used in present day MS and extensively reviewed (Zhang *et al* ., 2013; Hart-Smith G and Blanksby S J, 2012).

4.1.3.2.3 The Detector

Once the ions pass through the mass analysers, it hit the detectors and each of the ions record electronically to generate the mass spectrum of that particular ion. The most common detector used in biological mass spectrometry is an electron multiplier (Ver Berkmoes *et al.,* 2004) which works by generating a multitude of electron or photon when an ion hit the detector surface. The initial signals are amplified a million fold and finally the conductor measure the current and amplify it before converting it in to a measurable peak or mass spectra (Rubakhin and Sweedler, 2010).

These are the basic components of a basic MS instrument; however modern MS machines employ a wide array of improvement to improve the speed, resolution and mass accuracy.

These improvements are more evident in electrospray MS machines than MALDI MS instruments (Scigelova and Makarov, 2009; Campbell and Le Blanc, 2012). This study used MALDI-TOF and liquid chromatography coupled MALDI-TOF for global profiling, therefore next few sections are dedicated to the LC-MALDI

4.1.3.3 Matrix-assisted laser desorption/ionization (MALDI) Time of Flight MS. (MALDI-TOF MS)

The working principal of MALDI –Tof MS is based on the capacity of a matrix to ionise (protonate) the biological molecules using a short wave laser power in vacuum. The ionised molecules are subsequently pushed (guided) into the mass analyser, in this case a Time of flight (TOF) analyser with the help of an electrostatic field generated by the electrode positioned opposite to the sample ionisation plane. Once they passed the acceleration electrodes they enter the drift tube where they travel towards the detector under the condition of vacuum. Depending on the mass and charge on each ion they move in slightly different velocities in the drift region and the *m/z* of each ion can be calculated from the acceleration voltage and the length of the flight tube. MALDI-Tof MS measurements can do either in linear or reflector mode. Measuring the ions in linear mode

is more often result in poor resolutions (ability to discriminate ions with similar *m/z* values), therefore a reflector has been fitted towards the end of drift tube which deflect the incoming ions into a different direction effectively increasing the flight time and path.

The normal MS in a MALDI-TOF instrument can only yield the information of the m/z value of any particular peptide fragment, but unable to give any structural information about the sequence of that peptide. Studies conducted by Kauffmann et al in observed an important property of this ionised biological molecule in the MALDI MS instrument known as Post Source Decay (PSD). This is a phenomenon by which the parental ions (original peptide fragment) decompose in to its fragment ions in the flight tube and this decomposition has been influenced by the initial kinetic energy holding within the parental ions. The latest MALDI-TOF instruments have exploited this PSD and used it for effectively deriving the sequence information of the peptides in the mass spectrometer. There are patented technologies available (LIFT in case of Bruker Daltonics) which selectively filter the ion of interest in to the drift tube and also increase the kinetic energy of the parental ions to obtain the complete sequence. The working principle of the MALDI machine and LIFT technology is given in Figure 4.4.

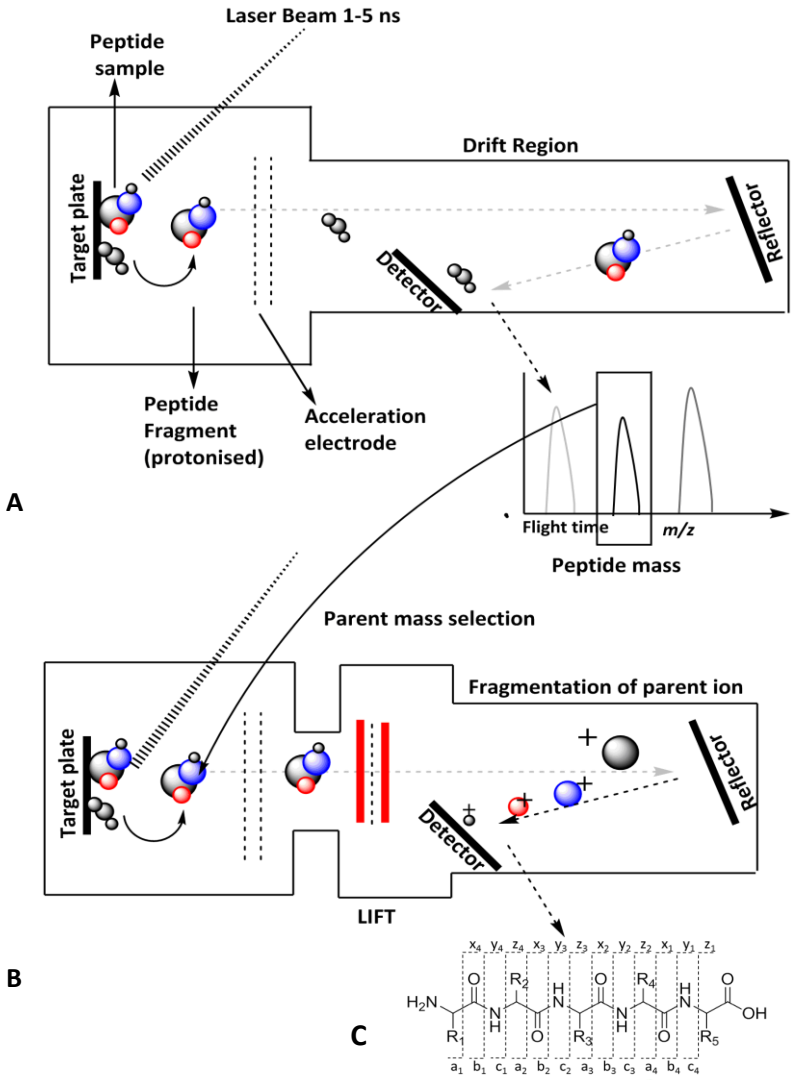

Fig.4.4. Figure representing the working of a MALDI-TOF MS. **A**. a MALDI MS identifying mass spectra for a peptide ion. The trpsinised peptide fragment obtained from single protein/ protein complexes/ global proteins were spotted on a ground steel plate with appropriate matrix. A short lasure impulse protonate the peptide fragment by impart them with a positive charge. An acceleration electrode positioned above the ionisation plane guide the protonated peptide fragment towards the drift tube (Tof tube). Modern machine fitted with the reflector deflect the ion path and thereby increase the resolution of the spectra **B.** Figure showing the extraction of sequence information of a selected (interested) parent peptide mass to identify its associated proteins. Once the peptide ion differentially expressed between the test control samples were identified and assigned for obtaining sequence information, the parent ions were selected and further energy is added to the ion before let the ion in to the drift tube for sequencing. Due to the increased kinetic energy attained after the parent ion selection (which happened in the LIFT cell of Bruker Daltonk Machines) the parental ions start to decompose by a process called post source decay (PSD**). C.** Extraction of sequence information from the mass spectra. PSD induced MS MS spectra contain 'b' and 'y' ions depending on the representative terminal from which the ions are represented. B ions originate from the amino terminus and the y ions from the carboxyl terminus. This allow the experimenter to determine the peptide structure in both the directions

4.1.3.4 MS approaches to study the proteome

Three main approaches are used in the general MALDI Tof MS and other MS platforms. They are bottom up or shot gun proteomics, top down proteomics and middle down approach. Bottom up proteomics referred to the characterisation of the proteome by fragmentation of the proteome by specific enzymes such as trypsin. This is also known as shot gun proteomics in analogy to shotgun DNA sequencing (Zhang et *al.,* 2013). The digested peptide fragments are fractionated with liquid chromatography followed by the identification of mass spectrometry (Koutroukides *et al.,* 2013). The tandem mass spectrum generated after each mass spectrum analysis are then compared to the theoretical mass spectra generated *in silico* databases such as MASCOT (Nesvizhskii, 2007). The bioinformatics analysis of mass spectrum derived peptide sequences are then assigned either uniquely or shared with specific or many proteins depending on the sequence. The overall work flow of bottom up proteomics are given in the figure 4.5.

There is no enzymatic fragmentation and the proteins used as intact as possible. The main advantage of this approach is the ability to detect post translational modifications (PTM) and protein isoform (Lanucara and Eyers, 2013; Peng *et al.,* 2013). Another advantage is the ability to give more proteome coverage and therefore eliminate the ambiguity in the sequence assignment to a specific protein. The major disadvantages of top down approaches are the difficulty of fragmenting and ionising large proteins in the ion source (Zhang *et al*., 2013). These two approaches are illustrated in figure 4.5.

4.1.3.5 Reducing sample complexity for increased proteome coverage by MS

One of the bottleneck for studying global proteome using mass spectrometry is their complexity. Several pre fractionation techniques are therefore employed to reduce this complexity. They are either electrophoresis or liquid chromatographic separation of proteins and peptides based on their physical and chemical attributes. Two methods are widely employed for reducing the overall complexity in the sample are the selective depletion method (Fang and Zhang, 2008) and combinatorial ligand libraries (Santucci *et al.,* 2012). This enrichment is mainly used in the profiling of serum proteomics where the complexity and the dynamic range of the proteome is enormous (Ly and Wasinger, 2011).

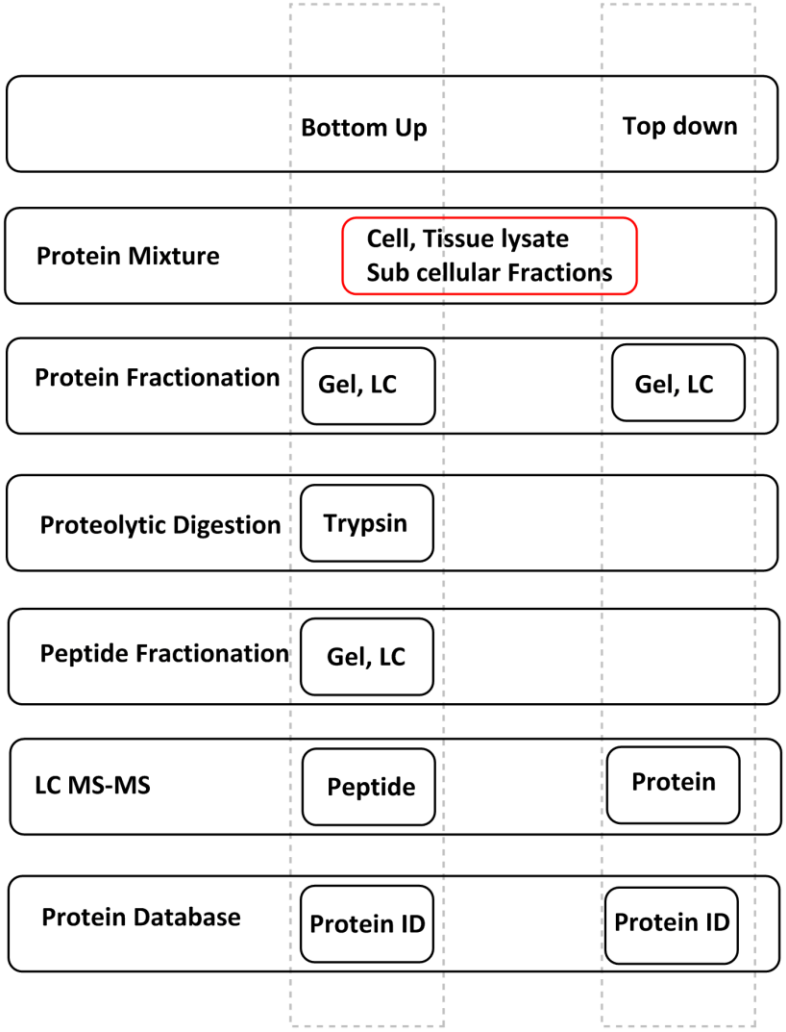

Fig 4.5. Two approaches used for mass spectrometry based proteomics are given. Bottom up proteomics is most widely used approach because of its simplicity. It is also known as 'shot gun' proteomics (analogy to DNA shot gun sequencing), involved several steps. The protein obtained from different sources can be fractionated (optional) prior to trypsinisation and the whole proteins are cleaved in to manageable chunks before subjecting to another round of fractionation. Each fractions coming out of the LC, can either constantly sprayed in to an electro spray ionisation source for identifying the peptide sequences and associated proteins using a tandem mass spectrometer or spotted on to an anchor chip for using in a MALDI mass spectrometer. In contrary to bottom up approach top down proteomics involved no trypsinisation but an optional LC or Gel fractionation can be carried out before subjecting to mass spectrometric analysis.

Advances in analytical chromatographic technologies and improvement in column chemistries enabled the separation and elution of digested peptide mixtures before subjecting to ESI or MALDI MS profiling. These pre fractionation techniques can further enhance the proteome coverage several folds (Van Riper *et al.*, 2013).

The most widely used chromatographic separations for pre fractionations are size exclusion (separations based on size), affinity chromatography (separations based on affinity tags) ion exchange chromatography (separations based on charge) and reverse-phase chromatography (separations based on hydrophobicity) (VerBerkmoes *et al.,* 2004). Most of the above separations can be performed to reduce the complexity either independently or in combination. Liquid chromatography (LC) using reverse phase columns are favoured for normal ESI and MALDI MS workflow because of its advantage for using straight away for MS analysis. LC instrumentation such as nanoLC which can handle nano litre volume flow rate can either be used in line with ESI by continuous injection of the eluted peptides in to the MS instrument or offline with MALDI MS by spotting on to a ground steel target plate for analysing later. Among this the most favoured one over the conventional LC which is limited by its inability to maintain a lower flow rate and poor separation (Gaspari and Cuda, 2011). None of this techniques are isolated and in many studies they have been used in different combination to improve the depth of fractionation (Zhang et *al.*, 2013)

4.1.3.6 MS based quantitative proteomics

Traditional quantitative proteomics has been done with gel based techniques where the proteins are separated on a one or two dimensional gel followed by transfer them on to a nitrocellulose membrane before probing with the antibodies against the interested protein. The quantity of the interested proteins can be deduced by comparing the densitometry of the normal and the test sample after normalising with the internal standard housekeeping proteins known as western blotting (Burnette 1981). One of the inherent problem with this technique is its low throughput nature. The emergence of MS based quantitative proteomics enabled the experimenter to study thousands of proteins in parallel with the quantitative information (Liang *et al.,* 2012). There are two main areas of MS based quantitative proteomics have emerged recently known as labelled and label free quantitative proteomics. Labelling approaches can either be used for the relative quantification of the proteins or can be used for the absolute quantitation (Coombs, 2011). Several libelling approaches are used routinely for quantitation such as *in vivo* labelling (SILAC) (Ong, 2012), isotopic (Mirgorodskaya *et al.,* 2000) or isobaric (Sturm *et al.,* 2012; Liang *et al.,* 2012) tags or spike in controls (Kuhn *et al.,* 2004). Once labelling has been done with any of the above approach the peak intensity differences between the control and test samples are used to compute the relative abundance of the peptide fragments (Coombs,

2011). Label free quantitative proteomics mainly carried out by ion peak intensities or spectral counting (Lundgren *et al.,* 2010; Old *et al.,* 2005). Ion peak intensity based quantitation is entirely dependent on the quality of the LC MS data generated by the LC separation and spectrometer and simply measuring the peak intensity at a given retention time of the test samples against the control samples for a given ion peak (Old *et al.,* 2005). Spectral counting on the other hand counting the number of single MS MS spectra associated with a single protein between the test and control runs. The number of MS MS spectra generated for a given protein is highly correlated with the abundance of that protein in the solution (Liu *et al.,* 2004).

4.1.4 Aim of the study

T cell development and differentiation is a tightly controlled process. Many of these processes are controlled at the protein level rather than the genetic level. Even though genetic analysis is useful in analysing the general trend of a process, it is the proteome that gives a comprehensive idea of the functional status of the cell. With this view in mind this chapter intends to carry out a global profiling of TRP-2 specific pentamer sorted cells using shot gun bottom up proteomics using liquid chromatography-matrix assisted laser ionisation desorption time of flight mass spectrometry (LC-MALDI-TOFTOF). The resulting data will be used to study the differences between high and low avidity T cells qualitatively and semi quantitatively based on ion peak intensity.

- The hypothesis is global proteome analysis can distinguish high and low avidity T cells based on the proteome profiles. Once it is achieved the identified markers can be used for the rapid detection of high avidity T cells (measure of a quality immune response) in the vaccine generated T cells.
- Group uniquely identified proteins through LC-MALDI-TOFTOF as functional categories and investigate their pathway association using database searching.

4.2 Materials and method

4.2.1 Isolation of peptide specific cells for proteomic characterisation

Isolation of peptide specific cells was carried out using the same way described in section 3.2.2 and 3.2.3 and counted using 10 µL of cell suspension and 10 µL of trypan blue. Cells were washed three times with cold PBS 3 times to remove any residual BSA from MACS buffer used for the magnetic sorting of these cells. After the final wash cells were snap frozen in liquid nitrogen and immediately transferred into -80° C until cell lysis.

4.2.2 Cell lysis and protein quantitation

Frozen cells were taken out of the -80° C freezer and immediately added 15 µL of cell lysis buffer (9.5 M Urea + 2% Dithiothreitol + 1% octyl b-D-glucopyranoside) tubes were subjected to repeat sonication (5 min) and chilling on ice (5 min) three times. 15 µL of 0.1% TFA is added to the cell lysate and sonicated again for 5 min centrifuged the whole mixture at 7000 g and transferred the lysate into a new tube and left the pellet undisturbed and stored at -80°C. Protein quantity determination of cell lysate was carried out using Bio-Rad Bradford protein assay kits with modifications. A twofold standard dilution series starting from 200 µg/mL – 6.125 µg/mL of BSA was prepared by diluting the stock solution of 2 mg/mL in lysis buffer and used for the generation of linear curve in NanoDrop 8000 ® prior to unknown sample measurement. 5 µL of each sample and dye reagent was used for setting up the calorimetric reaction in a PCR plates; blank reaction was prepared by replacing the cell lysate with 5 µL of lysis buffer. The reaction was mixed thorough and incubated briefly for colour development. NanoDrop 8000® was prepared by blank measurement and a linear curve had been established using the serially diluted BSA samples by pipetting 2.5 µL of each standard to the sample pedestals. Samples were prepared the same way as that of standards and quantity is estimated with the previously established standard curve. All the samples were equalised to the lowest concentration prior to subjecting any proteomic analytic techniques**.**

4.2.3 Trypsinisation and sample clean-up

Prior to any liquid chromatography and mass spectrometric experiments all the samples were digested with proteolytic enzyme Trypsin (Trypsin Gold, Promega) which cleave the proteins specifically by hydrolysing the peptide bond connecting any lysine and arginine residues. Trypsinisation reactions were set up by adding 16.6 µL of ammonium

bicarbonate, 7.6 µL of water, 8 µL (0.7 µg) of protein lysate and 0.7 µL of trypsin. The tubes were mixed by brief vortexing and incubated at 37°C for 16 h (overnight) in a temperature controlled hot air incubator. On the next day the reaction was stopped by adding 1 µL of 1% TFA and thorough mixing by vortexing. After trypsinisation the digested samples were subjected to clean up and concentration using ZipTip^{TM} (A C_{18} immobilised chromatography pipette tip, Millipore). The ZipTip clean-up protocol was carried out by wetting the ziptips in 80% acetonitrile by up and down pipetting three times using a 10 µL pipette, followed by equilibration of the tips in 0.1% TFA three times. In the next step sample clean-up was carried out by gentle up and down pipetting of the sample using the equilibrated tip 15 times without introducing air bubbles, Unbound peptides and salts were washed out by pipetting the ZipTips in 0.1% TFA 15 times. The bound peptides were finally eluted into 5 µL of 80% acetonitrile and diluted with 0.1% TFA prior to LC separation.

4.2.4 Nano LC fractionation of the peptides and robotic spotting on MALDI target

The optimised work flow of John van Geest proteomics laboratory has been followed for peptide mass profiling with LC-MALDI Tof (Liquid chromatography – Matrix Assisted Laser Desorption/Ionisation Time of Flight) spectrometry. This is a powerful technique in proteomic analysis by combining the physical separation capability of liquid chromatography and the analysis capability of mass spectrometry. A nanoflow HPLC system was used for this study (Proxeon EASY-nLC system, Bruker Daltonics, UK) and the separated sample fractions were spotted on to a 384 anchorchip plate (Bruker Daltonics GmbH) using a robotic spotting system (PROTEINEER fcII, Bruker Daltonics, UK) with a matrix solution 748µl of TA95 (95% acetonitrile, 5% water, 0.1% Trifluoroacetic acid), 36 µL of saturated CHCA matrix (α-Cyano-4-hydroxycinnamic acid) solution in TA 90 (8 µL of 10% TFA in water, 8 µL of 100 mM $NH_4H_2PO_4$ in water). The LC gradient used has a total run time of 86 min with 30 nL/ min, flow starts with 98% of solvent A (0.1% TFA in H_2O) and 2% of solvent B (0.2% TFA in acetonitrile) with an initial time delay of 15 min followed by a 10 s time slice segment of 64 min. At least three blanks were run between the sample runs to avoid cross contaminations. A commercially available peptide standard digest was used with each plate for the calibration of mass accuracy. Care was taken to minimise the anchor chip plate contamination by following dedicated washing and handling protocols of the plates between the runs.

4.2.5 Generation of MS and MS/MS profiles in MALDI-TOF

After spotting the peptide fractions using the robotic systems the calibration standards were spotted to the designated place of the anchor plate. After drying the plate was taken into MALDI-Tof (Bruker UltrafleXtreme). A new run was created in user supplied software WARP LC (Workflow Administration by Result-driven Processing) for each sample. Laser power was optimised by manually firing on a single spot for each samples and calibrants. Mass accuracy of each calibrants were manually checked and automatically assigned. MS profiling of the samples were carried out by automated running with set parameters followed by the MS/MS on top 10 peaks. MS/MS data of each automated runs were used for searching in in-house protein database MASCOT server through Biotools^{TM} and finally displayed the matching proteins with its Mascot score and number of peptides in ProteinBrowser^{TM}.

4.2.6 Data analysis and generation of differentiating peaks between the sample groups

Two data analysis strategies were followed for analysing the proteomic data. First one is more conventional differential analysis in which the peptide which was uniquely identified with each group was shortlisted by manually comparing the protein identities obtained from each run. Second strategy involves a statistical modelling and the identification of the MS peaks which shows maximum differences between the samples were carried out in Profile Analysis^{TM} software. These peaks were then used as precursor ions for MS/MS and the generation of protein identities. For the T test model generation, MS data from two groups, High (DNA vaccine generated) and low (peptide vaccine generated) avidity with three samples in each groups were analysed in the same software. T test modelling was carried out with following bucker generation parameters *m/z* 800 – 40000 with advance bucketing and get parameters and time alignment selected, normalisation followed is the sum of bucket values in analysis ≥ 6. With these parameters a T test had been performed and the most significant ions were organised with *P value* ≤ 0.05 and fold change ≥ 4. A separate list was created with this ions and this list was used as a shortlisted precursor list for MS/MS peptide and protein identification in tandem mass spectrometry. The distribution of these top ions in the population was then assessed manually from the retention time and *m/z* value arranged matrix table. The complete list of the shortlisted peptides were given in the appendix

In a separate approach the protein identities generated from each MS/MS experiments were grouped together in ImmunoBody™ group and in the peptide group separately using Venn diagrams (*http://bioinfogp.cnb.csic.es/tools/venny/index.html.)*. The protein identities common to all the biological replicates were then selected from each group. These common proteins from within each group (ImmunoBody™ and peptide) groups were used for the generation of a separate d Venn diagram which differentiate the unique proteins identified with immunology and peptide groups separately. These unique proteins were then used for further functional characterisation using Protein ANalysis THrough Evolutionary Relationships (PANTHER) functional classification tools (www.pantherdb.org/).

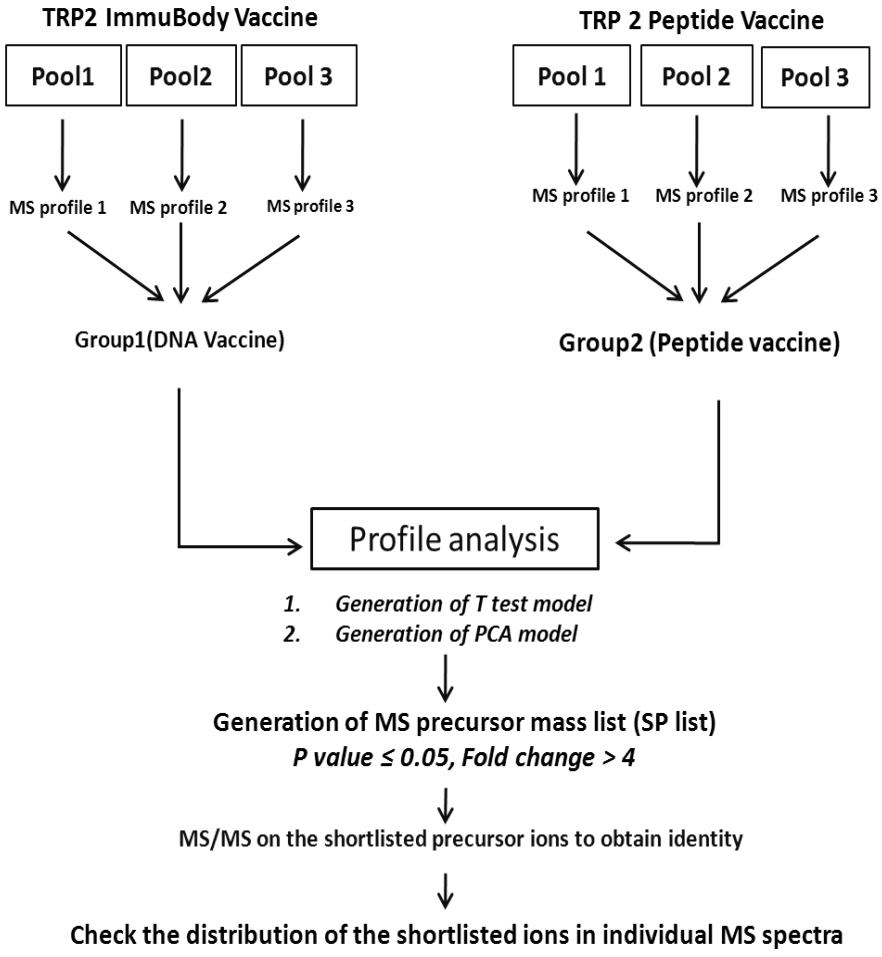

Fig 4.6. Schematic representation of proteomic workflow for the characterisation of peptide specific cells

4. 3 Results

4.3.1 Isolation of pentamer positive cells, Protein quantitation in Nanodrop 8000®

TRP-2 specific pentamer positive cells were isolated from the TRP-2 model after pooling three animals were lysed for global proteomic profiling in LC MALDI platform. The number of cells obtained and the protein quantity obtained are given in the table 4.8. The quantity determination has been done in Nanodrop 8000® with the linear graph fitted with diluted BSA. The result of Nanodrop quantitation of protein is given in appendix.

Table 4.3.1. Protein quantitation obtained from pentamer positive cells

Name	Number of Cells	Proteins Quantity (ng/µl)
IM Pool 1	1.2×10^4	128
IM Pool 2	0.9×10^4	113
IM Pool 3	0.8×10^4	85
Pep Pool 1	1.13×10^4	117
Pep Pool 2	1.06×10^4	155
Pep Pool 3	1.3×10^4	128

The number of cells used from three animals was within a range of $0.8 \times 10^4 - 1.3 \times 10^4$ cells from each of the pool. The normal protein quantitation kits were not ideal for this study due to its larger sample volume requirement and sensitivity. The normal assay works with a 1 in 10 ratio of the Bradford dye and sample, however the Nanodrop protein quantification protocol was modified with a ratio of sample to dye as 1:1 to increase the sensitivity. All the proteins were normalised to the lowest concentration (i.e. IM pool 3, 85 ng/µL) and 8 µL of this protein equalised solutions from each samples were used for trypsinisation.

4.3.2 LC separation and MALDI-TOF profiling of high and low avidity pentamer sorted cells

8 – 10 µg of total proteins were obtained from each animal pool and pentamer sorted cells the gradient used for the LC separations created a good spread of the peptides and spotted almost uniformly on to an anchor chip plate (figure 4.7). The MS data generated from the LC MALDI run was used for principle component analysis and the ImmunoBody and peptide groups were well separated from each other in the first and second component (PC1& PC2) (Fig 4.7).

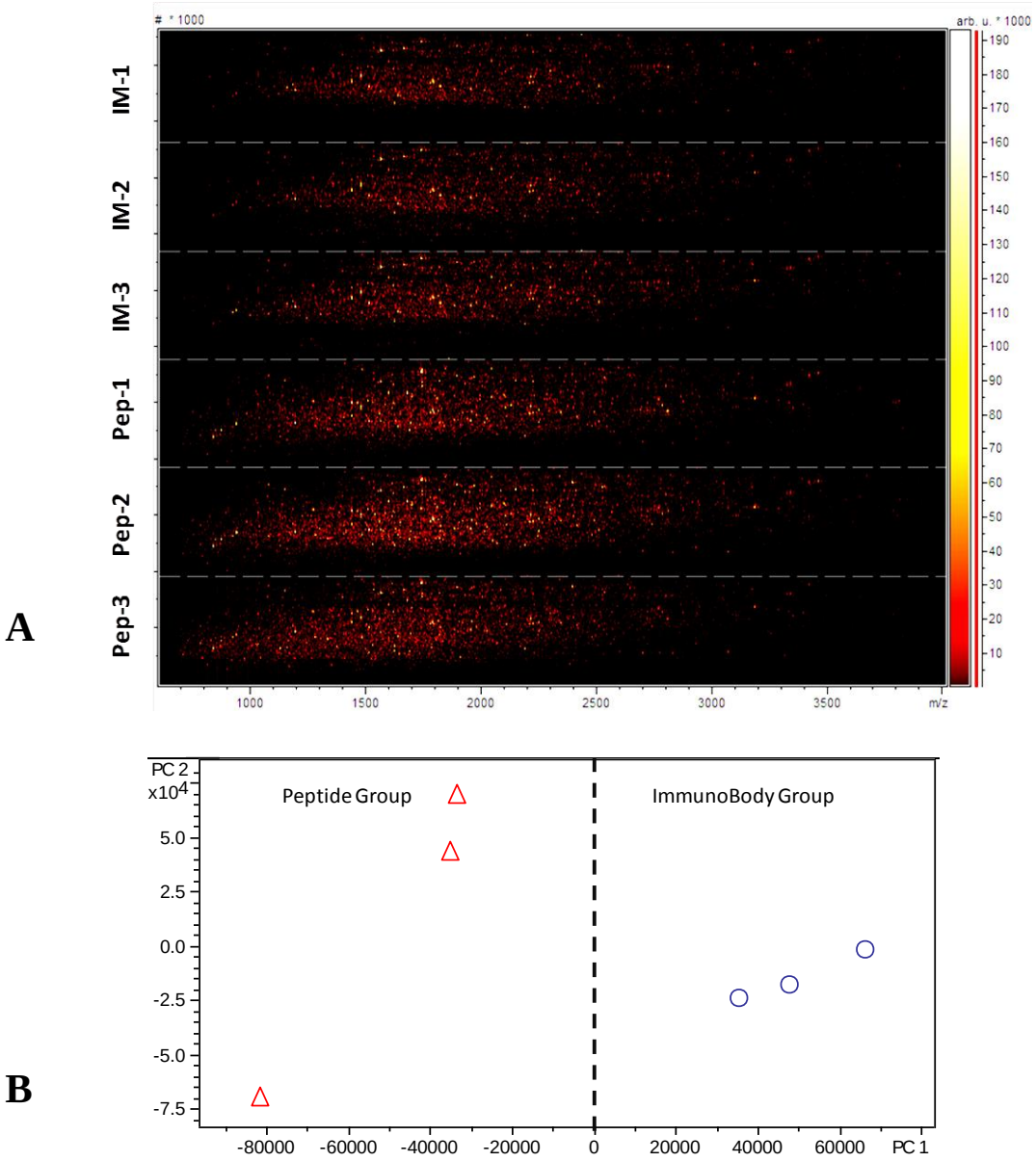

Figure 4.7: A. Gel view showing the LC separation of the peptide fragments of six samples, (picture generated from the MS data) B. Principle component (PC) analysis of ImmunoBody® and peptide group based on two component PC1 and 2 showing separation of two groups.

4.3.3 Pre-selection of ions prior to MS/MS using profile analysis identified nine candidates

Highly up regulated ions were subjected to further fragmentation and the amino acid sequences were searched against highly curated protein database and the unique proteins associated with the that peptide fragment was identified. The *m/z* values which showed consistent expression within the groups (figure 4.9), the proteins which showed any previous functional association with the $CD8^+$ T cells were shortlisted for further analysis. Table 4.2.

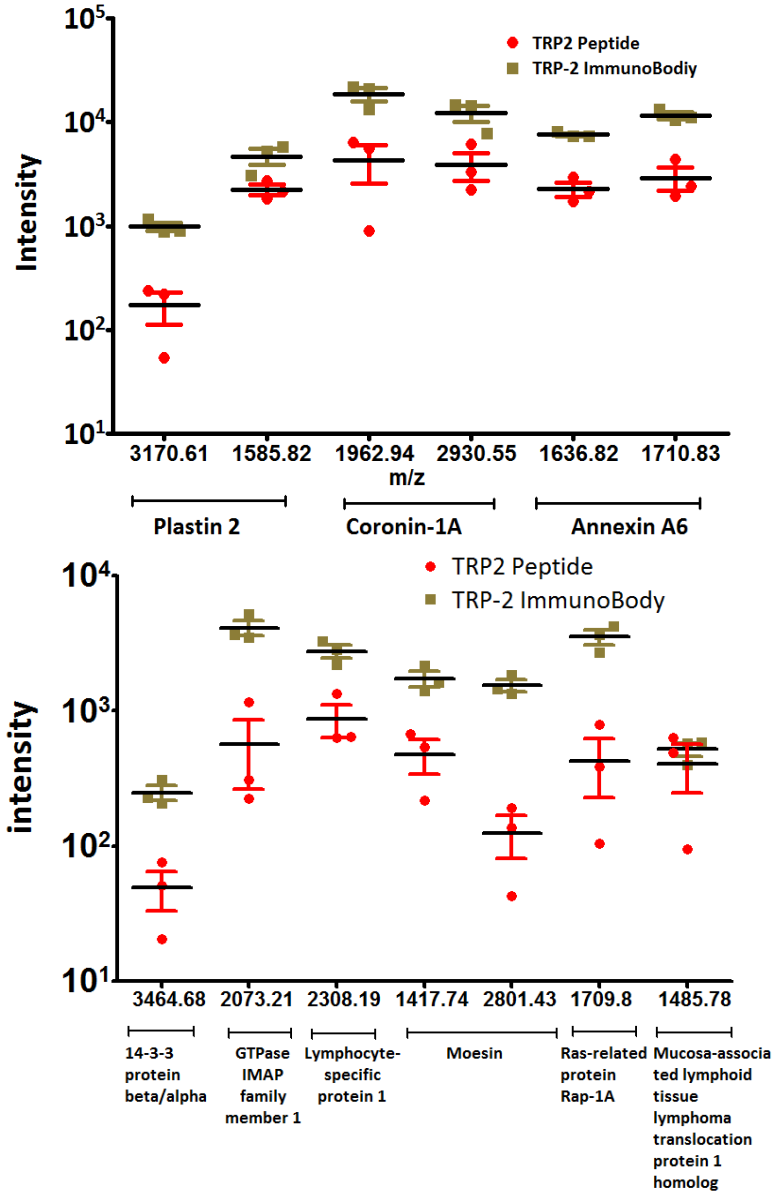

Figure 4.9: Candidate ions identified with LC MALDI profiling and its intensity distribution between the high and low avidity TRP-2 specific CD8+ cells. The proteins identified are given under each ion and the intensity is given as log scale in the Y axis. Error bars showing the mean and SD.

Table 4.2: Candidate protein identified for future studies using LC MALDI profiling

Protein	Protein function	Peptide sequence	m/z
Plastin 2	Crucial role in the formation of immunological synapse, Involves in TCR/CD3 and CD2 or CD28 mediated stimulation, cytoskeleton remodelling up on serine phosphorylation.	VYALPEDLVEVNPK	1585.84
		AYYHLLEQVAPKGDEEGIPAVVIDMSGLR	3170.61
Coronin-1A	T cell survival and Ca^{2+} mobilization capabilities. When T cell is activated, helps in the generation of inositol-1,4,5-trisphosphate from phosphatidylinositol-4,5-bisphosphate	HVFGQPAKADQCYEDVR	1962.91
		KGTVVAEKDRPHEGTRPVHAVFVSEGK	2930.55
Annexin A6	Membrane-binding protein, Interacts with GTPase-activating protein p120GAP	SELDMLDIREIFR	1636.83
		GIGTDEATIIDIVTHR	1710.90
14-3-3 protein beta/alpha	An adapter protein known to binding to phospahatases and kinases, influence the expression of RAF-1 and AKT	YLSEVASGENKQTTVSNSQQAYQEAFEISKK	3464.68
GTPase IMAP family member 1	Trans membrane protein. Plays a critical role in the T cell differentiation	YVALTSPGPHALLLVVPLGR	2073.22
Lymphocyte-specific protein 1	Phosphoprotein bind to F-actin. Main reported functions are cell - cell interactions and mobility.	SSELDEDEGFGDWSQKPEPR	2308.00
Moesin	Actin cytoskeleton organisation, role in T cell activation by helping in cell polarity during synaptic complex formation	SGYLAGDKLLPQR	1417.77
		GSELWLGVDALGLNIYEQNDRLTPK	2801.44
Ras-related protein Rap-1A	A Ras-like GTPases, play an important role in the cytoskeleton reorganisation during T cell activation	SKINVNEIFYDLVR	1709.92
Mucosa-associated lymphoid tissue lymphoma translocation protein 1 homolog	Helps NF-kappa-B in a BCL10 mediated manner also helps in integrin adhesion in T cells	RLSESLDRAPEGR	1485.77

4.3.4 Differential analysis identified 42 unique proteins in high avidity group

MALDI shotgun approach using the tryptic digests obtained from TRP-2 specific pentamer positive cells have identified large number of proteins in both the groups through LC

MALDI spectrometry. The LC MS run conducted using three independently isolated TRP-2 pentamer positive cells in each group have identified 408 ± 15 proteins in the ImmunoBody group and 498 ± 8 proteins in peptide group. Three of the runs in each data set were combined together using a Venn diagram to identify the common proteins between all the three runs. 294 proteins were identified as common between all the three samples in the peptide group and 312 proteins were identified as common between the ImmunoBody® runs.

Table 4. 2: List of Unique proteins identified in Immunobody® Pentamer positive cell

ID	NAME	Pathway, cellular function/Proceses/Component	# peptides
Bax	Apoptosis regulator BAX	Apoptosis signaling pathway	4
Creb1	cAMP response element-binding protein	p38 MAPK pathway	2
Ptprc	Leukocyte common antigen	JAK/STAT signaling pathway, T & B cell activation	6
Grap2	GRB2-related adaptor protein 2	PI3 kinase & Ras pathway, T & B cell activation	6
Rac2	Ras-related C3 botulinum toxin substrate 2	p38 MAPK, Integrin & Ras pathway, T & B cell activation	4
Cdc42	Cell division control protein 42	p38 MAPK pathway, T cell activation, Integrin signalling	3
Stmn1	Stathmin	Cytoskeletal regulation by Rho GTPase	1
Ywhah	14-3-3 protein eta	P53 & PI3 kinase pathway	3
Rap1a	Ras-related protein Rap-1A	Heterotrimeric G-protein signalling, MAPKKK pathway	2
Cltc	Clathrin heavy chain 1	Heterotrimeric G-protein signalling	18
Gnai2	Guanine nucleotide-binding protein	PI3 kinase pathway,	3
Fkbp2	FK506-binding protein 2	TGF-beta signaling pathway	4
Cox6b1	Cytochrome c oxidase subunit VIb isoform 1	oxidative phosphorylation	5
Bub3	Mitotic checkpoint protein BUB3	chromosome segregation	2
Pdcd6ip	Programmed cell death 6-interacting protein	induction of apoptosis	2
Psmb1	Proteasome subunit beta type-1	proteolysis	3
Samhd1	SAM domain and HD domain-containing protein	hydrolase activity	11
Tardbp	TAR DNA-binding protein 43	regulation of transcription	4
Thrap3	Thyroid hormone receptor-associated protein 3	receptor activity, transcription factor activity	6
Eef1g	Elongation factor 1-gamma	immune system process, Response to toxin	4
Nedd8	Nedd8	proteolysis	2
Ubtf	Nucleolar transcription factor 1	transcription factor activity	6
Cpne1	Copine-1	intracellular protein transport	5
Lgals1	Galectin-1	receptor binding	4
Il16	Interleukin-16	Immune response	11
Cct2	T-complex protein 1 subunit beta	chaperonin	8
Otub1	Ubiquitin thioesterase OTUB1	hydrolase activity	2
Gimap4	GTPase IMAP family member 4	immune system process	2
Fkbp2	FK506-binding protein 2	immune system process	4
Gdi1	Rab GDP dissociation inhibitor alpha		5
Cct7	T-complex protein 1 subunit eta	chaperonin	7
Got1	Aspartate aminotransferase, cytoplasmic	transaminase activity	3
S100a10	Protein S100-A10	calcium ion binding	2
Sub1	transcriptional coactivator p15	transcription factor activity	4
Kpnb1	Importin subunit beta-1	transmembrane transporter activity	4
Psma2	Proteasome subunit alpha type-2	Proteolysis	4
Cct4	T-complex protein 1 subunit delta	chaperonin	4
Pml	Probable transcription factor PML	ubiquitin-protein ligase activity	5
S100a11	Protein S100-A11	calcium ion binding	1
Sept1	Septin-1	small GTPase	6
Ran	GTP-binding nuclear protein Ran	GTPase activity	5
Ranbp1	Ran-specific GTPase-activating protein	G-protein modulator	3

These commonly identified proteins in each group were further used for shortlisting uniquely identified protein in each group. 183 proteins were common to both the group

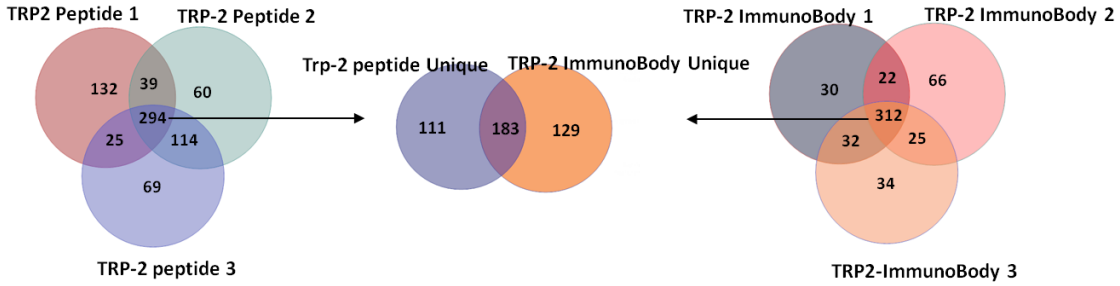

Figure 4.10: The shared identities obtained from each LC MALDI runs in ImmunoBody® and peptide groups. The common identities obtained from each groups were combined in a separate Venn diagram to obtain unique identities for each groups (129 identities for ImmunoBody® groups and 111 for peptide group).

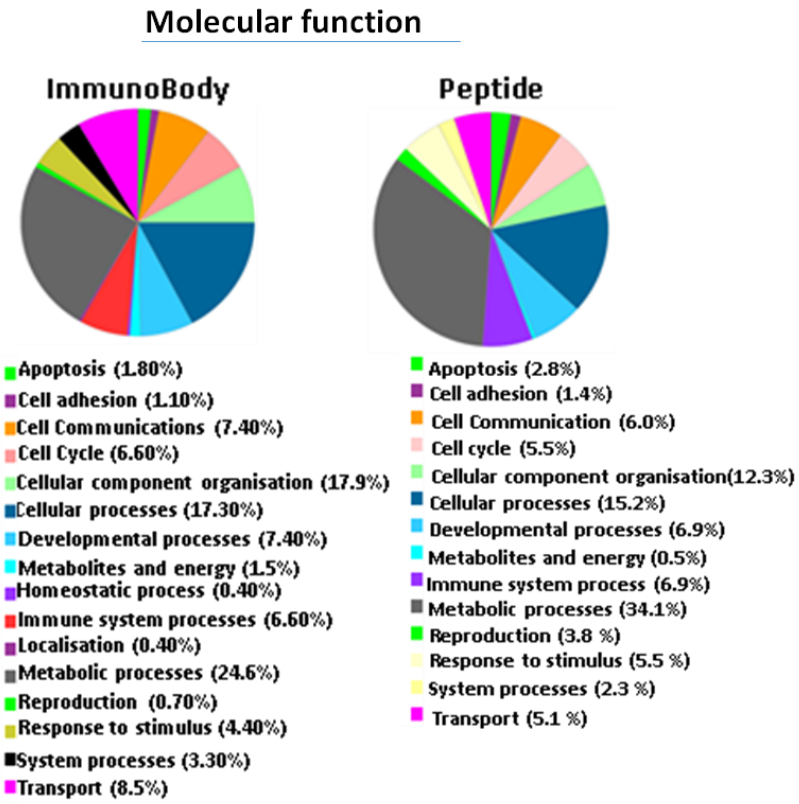

Figure 4.11: Panel showing their functional classification of unique proteins based on their enrichment in two major ontological classes (Biological processes and Molecular function).

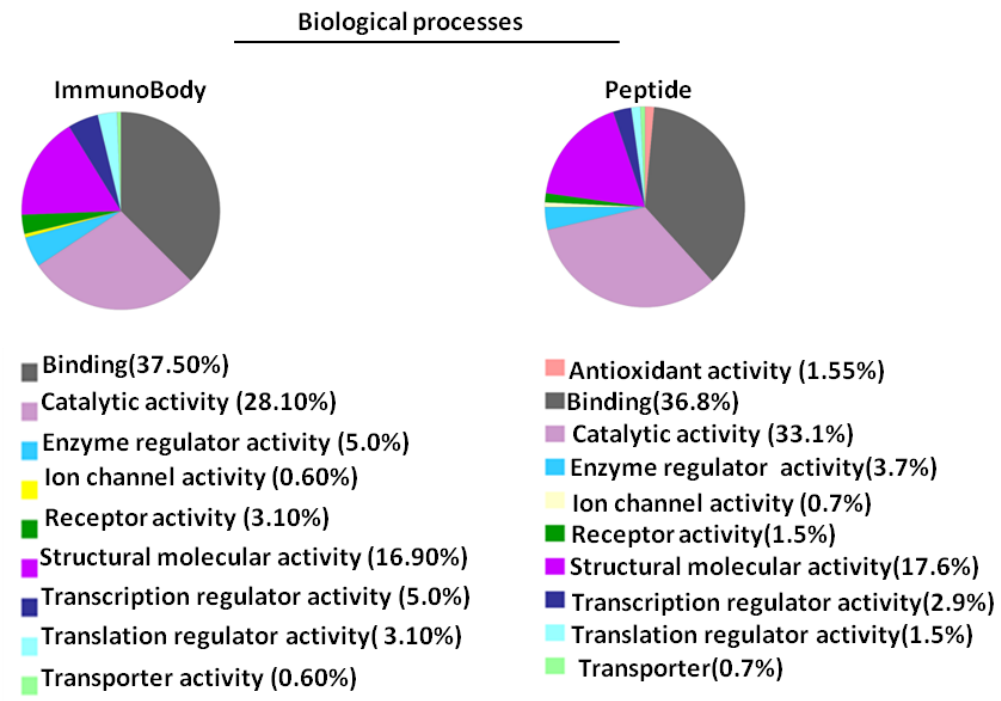

Figure 4.12: Panel showing the classification of these unique proteins based on their cellular component.

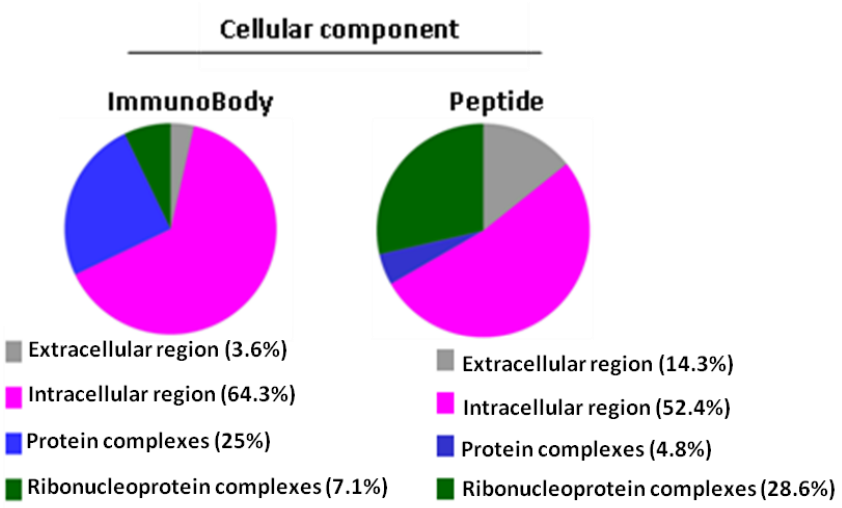

Figure 4.13: Panel showing the classification of these unique proteins based on their biological process.

with 111 unique proteins identified only in peptide group and 129 identified in the ImmunoBody® high avidity group. The proteins identified in the high avidity groups were further filtered by removing any of the protein identities having only single peptide fragment associated and also by literature driven functional classification. This qualitative analysis shortlisted 42 protein identities associated with TRP-2 derived pentamer positive high avidity T cells. The detailed list of the proteins along with its functional association and number of peptide fragment identified in each of the protein are given in the table 4.2.

4.4 Discussion

4.4.1 Proteomic characterisation and the identification of candidate markers for high avidity responses

Proteomic characterisations of the peptide specific cells were initially conducted with peptide specific cells isolated from single mouse. One of the bottlenecks of this study was the quantity of the proteins obtained from these cells were very low typically within the range of 5-10µg. Most of the proteomic techniques which involved any gel separations or fractionations need much higher quantity of proteins for a reproducible experiment. The only available technique to study this limited amount of proteins was separation in nano-HPLC and analysis with MALDI-TOF. The initial studies done with LC-MALDI experiments yielded low number of identities (100-190 identities) combined with poor reproducibility between the runs indicated the protein quantity problems. To overcome this problem three animals were pooled together in to one and repeated the study which resulted in three times higher peptide specific cells and protein quantity. The experiments were repeated with good reproducibility and higher number protein identities (420-550 identities) were obtained from these studies. The candidate peaks identified as differentially expressed between the groups were shortlisted and the identities for those peaks were obtained. Nine proteins were identified by these experiments as potential candidates. The detailed functional roles and their possible significance in avidity are given in the following section.

Plastin-2 also known as L-plastin or lymphocyte cytosolic protein is having a known functional association with the immune synaptic complex formation. This is an actin binding protein which relocalises to the immune synapse upon activation by TCR-CD3 complex (Janji *et al.,* 2006). The phosphorylation of plastin also facilitates the surface transport of the T cell activation molecules CD69 and CD25 (Wabinitz, *et al.,* 2007).

Coronin 1A plays a crucial role in the cytoskeletal reorganisation of highly active and motile cell by forming invaginations of large plasma membranes (Ferrari, *et al.,* 1999). Recent study showed the importance of coronin 1A in αβ T cells. The αβ T cells produced by coronin 1A deficient mice leads to the development of poor quality T cells with impaired signalling properties such as decreased TCR-induced Ca^{2+} influx and phosphorylation and poor IFN-γ production (Mugnier, *et al.,* 2008).

Annexin A6 also known as Calphobindin-II is a calcium-dependent membrane-binding protein. This protein interacts with signalling proteins such as GTPase-activating protein p120GAP which is considered to be one of the most important inactivators of Ras (Grewal, *et al.,* 2005). They are Ca^{2+} dependant phospholipid binding proteins which act as membrane-membrane or membrane cytoskeleton linkers. So it may play an important role in the APC CTL interaction.

14-3-3 protein beta/alpha protein is an adapter protein mainly involved in the modulation of signalling processes by binding to different binding partners usually involving phosphoserine and phosphothreonine motifs. The role in T cells yet to be addressed.

GTPase IMAP family member-1 is the member of a new family of cell survival regulators (through apoptosis regulation) especially expressed on T lymphocytes. Studies have shown that these proteins played a crucial role in the survival of T cells during its development.

Lymphocyte-specific protein 1 also known as 52 KDa phosphoprotein mainly reported to be present in the neutrophils involved in the phosphorylation events. Not many reports were available about its function in T lymphocytes. Since it is involved in the phosphorylation events heavily, therefore, it is worth investigating its role in T cell functionality further.

Moesin with its partner ezrin plays an important role in the cytoskeletal organisation of activated T cells and the formation of synaptic complexes. Main function of these proteins was linking proteins to the actin cytoskeleton. Studies demonstrated that the deficit in ezrin and moesin in T cells leads to the decreased production of IL-2 and also low level production of phospholipase C-γ-1 phosphorylation and calcium flux (Shaffer, *et al.,* 2009). So upregulation of this protein may play a crucial role in the T cell functional avidity.

Ras related protein RAP 1 was first reported that it operates as an antagonist of Ras, competing c-Raf-1 (Dhillon, *et al.,* 2005). More recently it was reported that Rap1 plays

additional roles such as activation of the ERK/MAP kinase pathway via B-Raf, promoting cell-matrix adhesion through the activation of integrins, and regulation of cell-cell junctions (Dhillon, *et al.,* 2005). So it is an ideal candidate to investigate its role in high avidity T cells.

The above candidate proteins identified were broadly involved in the functional category of cytoskeletal signalling and Ca^{2+} mobilisations, hence it is necessary to investigate the role of above identified proteins in the T cell activation events using time lapse confocal microscopy and FACS analysis in detail.

The differences in functional avidity might attribute to the difference in the cell to form rapid cytoskeletal reorganisation (the ability to form quick synaptic complexes between high and low avidity T cells. It is also worth noting from the previous studies done else where highlighted the importance of membrane rafts and Ca^{2+} mobilisations in determining the functionality of T cells (Oh-hora and Rao, 2009), however, no one has ever investigated the role of these components in functional avidity maturation of antigen specific T cells. Apart from the above broad objectives all these protein identities will be studied further specifically in high and low avidity T cells with an objective of translate this data in to a clinically valid tool.

4.4.2 Identification of uniquely expressed proteins in ImmunoBody® and peptide group using qualitative approach

One of the potential limitations of LC MALDI based proteomics is that it's not inherently good for quantitative studies. Often qualitative analysis is a useful tool for the identification of the proteins uniquely associated with one or other group. This qualitative segregation of the proteins were based on the assumption that if a protein is expressed high abundant, then the chances of identifying that protein is higher in shotgun bottom up proteomics given the fact that the control and test group were prepared in the same manner. This study has identified 111 unique proteins only in peptide low avidity group and 129 identified in the ImmunoBody® high avidity group.

The functional classifications of uniquely identified proteins in ImmunoBody® and peptide groups were checked for its functional (ontogenic) properties using PANTHER ontology classification programme (Thomas *et al*., 2003). This classification allowed the grouping of the proteins in to three basic functional terms known as molecular function, biological

processes and cellular components. This will allow us to detect the enrichment in each class by looking in to the whole identities qualitatively.

In the functional class of molecular function major differences were identified in the number of proteins identified with cellular component organisations (17.9% vs. 12.3%), metabolic process (24.6% vs. 34.1%) and transport associated proteins (8.5% vs. 5.1%) (ImmunoBody® Vs. Peptide). In the biological processes class the most differences were observed are in the receptor activity (3.10% vs. 1.5%), Transcription regulator activity and translation (5.0 % vs. 2.9 %)) regulator activity (3.10% vs. 1.5%). Based on the location at which the identified proteins predominantly employ the function, the cellular component classification showed that the identified proteins were highly different between the high and low avidity group (ImmunoBody® and Peptide). ImmunoBody® group has identified mostly protein complexes (25% vs. 4.8%) whereas the most predominant class identified in cellular component ontology class in peptide group was ribonucleoprotein complexes (7.1% vs. 28.6%) .

The regulation of T cell activation and function by ribonucleoproteins were studied and found their significant regulatory role in those processes (Mukherjee *et al.,* 2009). However, how these complexes affect the positive or negative regulation of T cell functionality is not yet known. The enrichment of protein complexes in ImmunoBody® (high avidity) group might be due to the better priming of these cells using the DNA vaccine. It is evident from the literature that T cell activation and priming leads to the assembly of many protein complexes to complete the extracellular signals in to intracellular processes (Hartgroves *et al.,* 2003)

By removing high abundant cellular proteins from the list 42 proteins were uniquely shortlisted which also showed previous T cell functional association. The protein identities and their unique pathway association studies using literature survey suggested that some of these proteins have unique association to the pathways involved in T cell activation and functionality (Table: 4.2). Protein with most peptide fragment identified were clathrin heavy chain (18 peptides), IL16 (11 peptides), T complex protein 1 subunit beta and eta (8 & 7 peptides respectively), ptprc (6 peptides), and Grap 2 (6 peptides).

Clathrin heavy chain is involved in the receptor trafficking and endocytosis with in the cells (Brodsky, 2012) is a key molecule in T cell activation. This molecule is involved in the receptor internalisation of TCR after its activation by antigen (Crotzer *et al.,* 2004).

IL16 is widely known as lymphocyte chemoattractant factor has been secreted by many immune cells including CD8 and CD4 T cells (Laberge *et al.,* 1996). They present as an inactive form and required caspase mediation to get the active protein (Cruikshank *et al.,* 2000). The role of this cytokine in T cell functional modulations was not well studied. Ptprc (CD45) is a trans-membrane tyrosine phosphatase known to regulate various functions of T cell activation (Turka *et al.,* 1992). Early studies showed its importance in preventing programmed cell death of T cells (Poggi *et al.,* 1996), and also have the ability to modulate the TCR-CD3 mediated stimulation (Turka *et al.,* 1992). The quantitative differences of CD45 has been also reported to have an influence on B cell development, survival and functionality (Zikherman *et al.,* 2012), and in general a key controller of signal requirement (threshold) for the activation of many immune cells (Hermiston *et al.,* 2003).

Grap2 also known as GADS is a SH2 & 3 containing adaptor protein which acts as a scaffold for many phosphorylation events during T cell activation. This is also involved in the activation of NFAT pathway (Law *et al.,* 1999) and also plays an important role in the calcium mobilisation within the T cells (Yankee *et al*., 2004). So the identification of these proteins uniquely with high avidity group might be associated with the better signal competency of those cells.

In general the enrichment of large number of proteins associated with T cell functionality such as small adaptor proteins and GTPases suggesting a better active and sensitive machinery of T cell are operating in high avidity TRP-2 ImmunoBody® derived pentamer positive cells. This might be probably due to the way these cells are primed using a DNA vaccine in an antigen specific manner. However further studies are required for the conformation of these observations using supporting experiments.

5.4.3 Conclusions and future directions

Global proteomic profiling of high and low avidity T cells using purified TRP-2 specific T cells were achieved successfully using LC MALDI platform. Good number of proteins was identified with this limited number of cells showing the utility of high throughput platform to study the limited but more meaningful biological samples in large scale. Looking into the immune cell profiling only few studies have looked the protein signatures in such a detail with very pure vaccine induced T cells. Many studies on T cells usually conducted with transgenic TCR bearing $CD8^+$ cells such as OT1 (Ovalbumin TCR transgenic), or

Jurkat cell lines mainly because of the availability of large number of cells for sample demanding proteomic studies.

Though the platform was able to identify large number of meaningful information from the high and low avidity samples, the inherent inability of LC MALDI to precisely quantify the proteins make the analysis difficult. Therefore, this studies employed a 'pseudo-quantitation' approach to quantify the proteins by aligning the retention time across the samples and then looking for peak intensity differences between the samples. Even though it is a crude way of doing quantitation, it is the only label free method available for the machine setup used for the study. The qualitative differential analysis revealed more biologically relevant information associated with high avidity T cell, by identifying multitude of proteins associated with functionally important associations in many T cell pathway. These proteins were completely absent in the peptide group indicating these proteins were less abundant in the low avidity (Peptide) group.

Chapter 5

Summary of Discussion

5.1 Functional avidity (sensitivity) can be different in peptide specific cells having single epitope specificity.

The goal of cancer immunotherapy is to help one's own immune system to detect and destroy the malignant transformations happening within the body. However the hope for a successful therapeutic intervention still remains marginal, with only one successful FDA approved dendritic cell vaccine Sipuleucel-T (developed by Dendreon) providing a modest increase in life span of 4.1 months (median survival) with a huge therapy cost ($90,000/treatment) in asymptomatic or minimally symptomatic metastatic castrate resistant (hormone refractory) prostate cancer.

Over the past few decades, identification of tumour antigens as potential vaccine targets has become routine in the field of tumour immunology. Despite this, most T cell based cancer vaccine strategies remain unsuccessful (Jonuleit *et al*., 2001; Anichini *et al*., 1999; Lee *et al*., 1999). Some of these failures can be attributed to the nature of the antigens and the immune avoidance mechanisms operating in patients. Unlike prophylactic vaccinations using foreign antigens, tolerance mechanisms plays a key role in the attenuation of T cell responses in many therapeutic vaccine trials (Pawelec & Rees., 2002; Kyewski & Klein, 2006). Trials conducted with many of these antigens generated sufficient numbers of antigen specific T cells, however, in the majority of cases the success was hampered by poor clinical efficacy. Antigens that performed well in preclinical models unfortunately did not translate into clinical outcome and some were even found to generate T cells which did not recognise and kill targets (Kudrin and Hanna, 2012).

Detailed studies looking for the cause of these failures attributed mainly to immunosubversion and selection strategies devised by the tumour and its micro-environment (Zitvogel *et al*., 2006). Despite these immune suppression mechanisms, the type and functionality of the cytotoxic T cells generated through each vaccination are also important for mounting an efficient clinical outcome (McKee *et al*., 2005). There are many ways to assess the quality of T cell responses in vaccine trials. Among them affinity and structural avidity are two important indicators of T cell binding capacity at two different levels with the former indicate TCR binding strength and the latter being the overall

interaction strength between T cell and antigen presenting cell. Though in many cases the above two are good indicators of a good T cell functional prediction, it is functional avidity (biological readout of a T cell up on antigen stimulation) which is widely considered as the efficient way to assess the quality of T cell immune responses. Functional avidity, also known as "functional sensitivity", is the measurement of a T cell's ability to respond to its cognate antigen in a dose dependent manner (Viganò *et al*., 2012; von Essen *et al*., 2012; Amoah *et al*., 2012). The measurement of this functional property is usually carried out by T cell functional assays such as ELISpot, and chromium release killing assays in a dose dependent (titrating down target antigenic peptide concentration) manner (Pudney *et al*., 2010; McKee *et al*., 2005). Depending on the antigenic stimulus requirement for half maximal activation (EC_{50}), the T cell population can be generally classified into high avidity (requiring < 1 nM peptide), medium (requiring < 1-100 nM peptide) or low avidity (requiring >100 nM peptide) T cells (Snyder *et al*., 2003). In more generalised terms high avidity T cells requires very low concentration of antigens for their activation and vice versa for low avidity T cells.

Success of an immunotherapy could be measured by the generation of high avidity T cells (Viganò *et al*., 2012; McKee *et al*., 2005). However, the current assays to measure these responses are laborious and need millions of PBMCs to generate the necessary titration curves. The difficulty increases if the peptide(s) responsible for the generation of these cells are unknown, such as with whole protein or cell vaccines and anti-checkpoint therapies. Therefore, there is a need to identify surrogate markers which can quickly and reliably identify high avidity T cell response in cancer vaccination settings. This study precisely focused on the identification of markers for a high avidity T cell response and investigating the mechanism at the gene and protein level using high throughput genomic and proteomic platforms.

With the previously discussed need in mind, two mouse models, kindly provided by Prof. Lindy Durrant (Scancell Ltd., Department of Clinical Oncology, The University of Nottingham, UK) was used for this study. The first model was the C57Bl/6J-TRP-$2_{180\text{-}188}$ system which uses a self-peptide (SVYDFFVWL) derived from melanoma differentiation antigen tyrosinase related protein-2 (TRP-2). The DNA sequence of this peptide was inserted into a vaccine framework known as ImmunoBody®, developed by Scancell Ltd., known to generate a high avidity response in mouse compared to its peptide counterpart (Metheringham *et al*., 2009; Pudney *et al*., 2010). The second model, uses a foreign (non-

self) peptide (Ovalbumin ($OVA_{257-264}$) (SIINFEKL) antigen and was also used in this study to confirm the results.

The first question to be answered in this study was the reproducibility of the model, and it was confirmed with multiple experiment that the ImmunoBody® vaccinations are consistently higher avidity than their peptide counterpart in both self (TRP-2) and foreign (OVA) peptide as measured by IFN-γ ELISpot assays (section 2.3.2). ImmunoBody® derived cells are capable of producing IFN-γ even at the lowest concentration of the peptide used for the assays. The EC_{50} calculated for ImmunoBody® and peptide immunisations showed a two log difference for their antigen requirement for maximal 50% of activation in both TRP-2 and OVA. These sensitivity differences were further confirmed by intracellular cytokine staining and flow cytometry. These observations were in agreement by the previous observations made by Pudney *et al*., 2010 on this model.

The only functional readout used for this study was peptide titration ELISpot assays against IFN-γ. This assay to measure functional avidity has been used widely in the literature (Berger *et al*., 2011; Draenert *et al*., 2004; Chen *et al*., 2009). Killing assays with TRP-2 specific high and low avidity T cells were conducted by Prof. Lindy's group showed that ImmunoBody® generated high avidity T cells kill the antigen pulsed target cells more efficiently than the peptide counterpart (Pudney *et al*., 2010).

The differences in spot count in ELISpot might be due to the differences in number (frequency) of peptide specific cells generated by both the vaccinations. To test this hypothesis, the splenocytes obtained from ImmunoBody® and peptide immunised animals were stained with TRP-2 or OVA pentamer depending on the antigen used. In both the OVA and TRP-2 immunised animals there was no significant differences were noticed between the group. However, OVA immunised animals generated more pentamer positive cells in both the peptide and ImmunoBody® model, indicating that it is more immunogenic in this settings. The low frequency of TRP-2 specific cells due to its self nature and therefore, T cells might be more tolerogenic towards TRP-2 compared to OVA.

Although both vaccination strategies could induce the same number of TCR positive $CD8^+$ T-cells, as assessed by the number of $CD8^+$ T-cells stained for peptide-specific pentamer by flow cytometry, only the ImmunoBody®-vaccinated mice could induce T cells capable of secreting IFN-γ in response to very low concentration of peptides. If both immunisations generated the same overall number of peptide-specific T-cells then one might think that the

ImmunoBody® derived-T cells have more TCR on their surface and therefore less peptides are required for a signal to be triggered and the IFN-γ to be secreted. However, if this was the case the fluorescence intensity of the pentamer staining should be stronger with the ImmunoBody® derived-T cells but this was not observed so it appears that the difference between the T cells generated by these two very different vaccine strategies lies within the special organisations of the TCRs on the surface of the T cells with the CD3 and other molecules forming a sort of "pre-arranged/pre-functional" groups of TCR machinery which would then be extremely sensitive to the next peptide/MHC encounter or solely down to a more efficient signalling. However, this study hasn't carried out a multimer decay assay to validate this assumptions.

The presence of similar number of peptide specific cells in peptide and ImmunoBody® group and their differences in functionality towards the peptide assays suggesting that functional avidity maturation is the possible mechanism operating in ImmunoBody® high avidity group. The nature of the vaccine delivery might have played an important role in this process. Conventional peptide vaccines are known for their structural instability in the harsh *in vivo* environment full of peptidases (Slingluff, 2011). On the other hand, DNA vaccines are not susceptible to this problem since they are producing the encoded peptide epitope with in the transfected cells of the body and therefore ensure a constant supply of the peptide antigens at a moderate to low level to the cellular immunity (Rice, *et al*., 2008; Pudney, *et al*., 2010). The T cells are then constantly getting a chance to expose to this low but continuous antigenic stimulus for a longer period of time. This continuous stimulation of the repertoire is probably improving the sensitivity of T cells over the time. In contrast, peptide vaccines usually deal with periodic dosage of high concentration of the peptide and this might be selecting the low avidity T cells which required high concentration of peptides for its stimulation.

Another possible reason for the ImmunoBody® vaccine generating high avidity T cell responses may be the coverage of the antigen presentation pathway. The ImmunoBody® are capable of perform direct and cross (indirect) presentation of the antigens to the immune system (Pudney, *et al*., 2010; Metheringham, *et al*., 2009). In the direct pathway, the injected plasmid can be taken up by dendritic cells and the ImmunoBody® produced within the dendritic cell before undergoing an endogenous processing pathway. The cross presentation route is by through exogenous processing of secreted ImmunoBody® epitopes. This epitopes has an antibody FC region which binds to the FCgR1 (CD64) present on the

dendritic cells and internalised by receptor mediated endocytosis (Durrant *et al*., 2011). This leads to the exogenous antigen processing pathway of DCs and cross present to the cellular immunity (Durrant *et al*., 2011; Pudney, *et al*., 2010; Stevenson *et al*., 2004; 2010). This way the antigen visibility is greater in ImmunoBody® compared to the peptide counterpart. The quantity of ImmunoBody® produced within the cell and the number of target peptide MHC complexes on the dendritic cells has not been previously studied. It is reasonable, based on this research to suggest that ImmunoBody® vaccines are capable of generating a high avidity response through presentation of low level of antigens to the CTLs, thereby selectively expanding the population of high avidity T cells (which can recognise the low doses of tumour antigens) (Pudney, *et al*., 2010).

In vitro stimulations of both high and low avidity T cells at two different and extreme concentration (100 μg/mL and 10 ng/mL) of peptides reinforce the above argument that the high avidity repertoire in the ImmunoBody® immunised animals was shaped by the exposure of a precursor T cells to low concentration of the antigens. In both the concentrations tested in the above stimulations, high avidity T cells showed an overall reduction in the spot number indicating that they are susceptible to in vitro stimulations with relatively high concentration of peptide (section 2.3.4). This reduction in the spot number may be due to activation induced cell death (Vigano *et al*., 2012) due to serial triggering. Contrasting results in the peptide group by giving large number of spots in all the concentration tested after a week of in vitro stimulation with (100 μg/mL: and 10 ng/mL) of peptides (section 2.3.4) suggesting that antigen sensitivity of T cells can be extensively modified depending on the nature stimulations they have received.

In viral systems, it has been showed that the T cells can undergo functional maturation during the course of infection, without selecting high affinity receptors (Slifka & Whitton *et al*., 2001). Similarly, functional avidity maturation might be happening in tumour vaccination settings as well, but it is not well studied. Instead tumour vaccination strategies always looked in to the improvement of TCR affinity to achieve maximum clinical outcome (Wooldridge *et al*., 2012). Unfortunately tumour immunologist choose another path of transfecting the T cells with high affinity receptors for making a super affinity T cell which can detect and mobilise immediately to the tumour site and eradicate them (Savoldo and Dotti 2013) However, like dendritic cell based vaccines this is also expensive and requires an expert skill set and advanced laboratory conditions to perform the adoptive transfer of engineered T cells (Kodumudi *et al*., 2012).

The immunophenotype of pentamer positive cells revealed there was no noticeable phenotypic polarisation of these cells after 45 days of initial immunisation (section 2.3.5). Probably longer exposures might be required for this to be happened. One inhibitory molecule studied (BTLA) also showed only less pronounced differences between high and low avidity T cells indicating these cells are undergoing a more or less similar developmental path up to 45 days. However studies with these models by Brentville *et al*., 2012 found that after 70 days of initial immunisation with TRP-2 ImmunoBody® high avidity T cells are selected in to the memory and the recalling of memory with ImmunoBody® boost results in the resuming of the function. However, peptide boosts lead to the rapid reduction in the avidity. All these data indicating the plasticity of avidity as a functional property, now it is up to the tumour immunologist to tap in to this area to modify physiologically occurring T cells properties against the TAAs with low avidity into higher functionality.

5.2 Global and targeted transcriptomic changes could differentiate high and low avidity T cells

Normally, gene expression studies of immune cells are carried out using bulk immune cells such as PBMC's, whole blood cells or total splenocytes. Though such studies are useful at times, the cellular heterogeneity in the initial sample population is very high. This heterogeneity often leads in to misinterpretation and hence modern gene expression studies demand purified or enriched starting cell populations. (Espina *et al*., 2006; Demou & Hendrix, 2008). Therefore this study decided to enrich the population of antigen specific $CD8^+$ T cells from the total CD8 repertoire and so that any observed expression changes can be clearly attributed to the cellular phenotype.

The successful enrichment of TRP2 and OVA peptide specific cells were carried out using the magnetic separation (section 3.2.3). The enriched populations were further studied using various expression platforms to interrogate the expression differences between high and low avidity T cells (sections 3.2.8-3.2.11). At first the basal expression of TCR signalling was investigated real time quantitative PCR. Even though some differences could be observed in general, none of them were statistically significant. This may be due the resting stage of the TRP-2 specific cells or that the differences were very subtle and not to be resolved by the relative quantitation method used in this study.

If there is any differences in the signal competency between these cells, activation of these cells *ex vivo* would be an ideal mechanism to study the signal difference upon exposure to the antigenic peptide. In the lab ELISpot has been routinely used for measuring the functional avidity of the T cells. The $CD8^+$ T cells isolated from these ELISpot plates can be an ideal material for assessing TCR status was assessed between the high and low avidity T cells. No significant differences could also be detected between these cells, indicating the time used for harvesting the cells (48 h after the initial stimulation as per the normal ELISpot protocol) were not ideal for studying the gene expression differences, though they were appropriate for secreted protein studies such as IFN-γ.

In the next stage of this study, a more structured approach was followed, with the TCR signalling competency were studied at three stages of activation (0h, 1h and 2h). CD3, CD28 coated micro-beads were chosen to avoid the TCR stimulation so that cells in each group (high and low avidity) will receive a uniform signal. If there are differences in their cellular machinery, they would transmit the signals differentially. TRP-2 pentamer purified population of T cells from ImmunoBody® and peptide immunised animal were used for the study with the assumption that these the naive T cell repertoire was shaped in a different way between the two vaccine strategies (Peptide/ ImmunoBody®) because these two vaccines might have primed the T cell differently. This kinetic study yielded some surprising results with up regulation of predominantly all the genes studied in the ImmunoBody® group compared to the peptide group. The genes used for these studies can be broadly classified into three categories as 1) genes involved in the proximal signalling (beginning of TCR signalling) 2) the ones which associated with the distal signalling (further down the cascade) 3) T cells associated transcription factors (final point). The two genes which did not showed any differences were associated with the proximal signalling events in TCR signalling and this suggested that the signalling starts uniformly and when it cascaded down the molecular machinery of ImmunoBody® derived cells are better equipped and therefore transmitted the signals more efficiently leads to the up regulation of other genes. The major genes that were upregulated were associated with MAP-MAPK pathways and cytoskeletal remodelling with many of them previously known to influence the functionality of T cells (Villalba *et al*., 2000; Wu *et al*., 1996). So upregulation of these pathways can be used as a good marker for functional avidity.

Global gene expression profiling was carried out with TRP-2 and OVA specific pentamer sorted high and low avidity $CD8^+$ T cells using Affymetrix® high density oligonucleotide

microarrays to identify the most differentially expressed genes. The array hybridisation has been successful with low number of cells ($1x10^5$ cells) and the analysis performed between the high and low avidity groups have identified genes which have a differential expression (338 genes in TRP-2, 239 genes in OVA model) pattern between high and low avidity T cells. Though there are differences between the two groups they were very subtle. This might be probably due to the resting stage of these cells.

The major genes identified using these global platforms, which are also functionally important for T cell biology were Granzyme A, Granzyme B, Fas Apoptotic Inhibitory Molecule, Telomerase RNA Component, CD5 Antigen-Like, Spi-C Transcription Factor. The up-regulation of Granzyme transcripts was unanticipated. One possible explanation for that might be attributed to the constitutive expression of TRP-2 ImmunoBodies® in the mice compared to the peptide counterpart. As discussed in the previous section the constitutive expression of a DNA vaccine has the possibility of priming the naive, already antigen experienced and clonally proliferated T cells again and again.

At this point it is worth mentioning that the gene gun immunisations on the shaved abdomen of the mice resulted in vitiligo at the site of immunisation in the majority of the prophylactic strategies we have tried (Pudney *et al*., 2010). This vitiligo is the destruction of melanocytes by T cells indicating that they are in a more activated state in the Immunobody immunised mice compared to the peptide immunisation, where no indication of depigmentation was observed. Selected microarray shortlisted markers (Granzyme A, Granzyme B, Fas Apoptotic Inhibitory Molecule, Telomerase RNA Component, CD5 Antigen-Like, Spi-C Transcription Factor) were subjected to qRT PCR for confirmation. Only three out of six genes tested showed positive correlation with the array data which included granzyme A and B, CD5L. Further proteomics confirmations were done on these cells using murine granzyme A and B. Initial studies failed to detect any significant changes between high and low avidity groups without stimulation probably due to the low abundance of the protein to be detected by intracellular staining. Hence the cells were stimulated with a low concentration of TRP-2 peptide and the study carried out with the assumption that if there were any differences in granzyme A and B expression, these differences should be amplified by peptide specific stimulation. The initial data suggested that there was a difference in the protein expression of granzyme A and B between high and low avidity T cells and therefore could be used for an ideal marker for differentiation of high and low avidity T cell response. If it is successfully validated, they could be used

as a marker for monitoring avidity in patients on vaccine trials, since they are the effector molecules of cellular cytotoxicity. The expression of these molecules is also an indication of how effectively they would be at killing the target. Granzyme B is a classically studied molecule of T cell cytotoxicity; however granzyme A is not reported very well in the literature in association with CTL function. In this respect it is worth investigating the dual role of these molecules in ImmunoBody® generated T cells by selectively blocking one or other using antibodies in killing assays.

5.3 Successful global proteome profiling of pentamer sorted T cells identified uniquely expressed protein in high avidity group

Studying low number of cells using global proteomic characterisation is a huge challenge; still within these limitations, this study successfully produced a global proteomic data for antigen specific T lymphocytes using around 1x 10^5 cells. Protein concentrations were low (Table 4.1), however, LC fractionation was successful. 420-550 proteins were identified from these set of samples. A 'pseudo' quantitative method based on the peak intensity was employed to identify candidate precursor mass which was different between Immuno-Body® and peptide group, these peaks were subsequently fragmented using MS/MS and protein identities were obtained. The protein identities obtained are Plastin-2, Coronin 1A, Annexin A6, 14-3-3 protein beta/alpha protein, Lymphocyte-specific protein 1, Ras related protein RAP1 and Moesin. Plastin and coronins are involved in the cytoskeletal remodelling (Janji *et al.*, 2006; Wabinitz, *et al*., 2007) and coronin along with annexin involved in Ca^{2+} signalling and also interact with GTPase-activating protein p120 GAP an inactivators of Ras (Grewal, *et al*., 2005). GTPase IMAP family member-1 contributes towards the enhanced survival of T cell. Identification of these proteins with higher peak intensities in the ImmunoBody® vaccinated group indicated that these proteins might be highly expressed in high avidity group. These studies further looked with the unique identities identified with ImmunoBody® group (Table 4.3). Ontology classification allowed further dividing these proteins in to three functional ontologic classes (molecular processes, biological function and cellular component) to investigate any enrichment of particular functional classes of the proteins in the total differentially identified proteins. Generally large no large differences were observed, however, the ImmunoBody® vaccinated group showed significant functional enrichment in proteins involved in the intracellular regions (mainly signalling components) again indicating that these cells may be constantly exposed to a low dose of ImmunoBody® antigen within the body. This might be possible since

these vaccine has been incorporated into the cellular system of the host; hence it is reasonable to imagine that the cells will get a constitutive stimulation. This argument was again reinforced in the qualitative analysis, where 129 protein identities identified in the ImmunoBody® high avidity group. Among the proteins identified, the pathway associations of these molecules were Investigated in to literature and found that some of these proteins were associated with pathways related to T cell activation and differentiation (Table 4.3). Molecules such as IL16 (11 peptides), T complex protein 1 subunit beta and eta (8 & 7 peptides respectively), ptprc (6 peptides), and Grap 2 (6 peptides) were involved in the T cell functionality when they are in active stage. The identification of Clathrin heavy chain particularly significant since it was involved in the receptor trafficking and endocytosis within the cells (Brodsky, 2012) might be playing an important role in the TCR recycling (Crotzer *et al*., 2004) in the ImmunoBody® derived cells. Overall, an enrichment of large number of small adaptor proteins and GTPases and GADS pointing towards a better active and sensitive machinery of high avidity T cell in ImmunoBody® derived pentamer positive high avidity T cells. However, further studies are required for the conformation of these observations using further experiments such as western blots or FACS. However this work has proved that it is possible to get a glimpse of the proteome of antigen specific T cells and study more than 400 proteins in a single experiment.

5.4 Conclusions and future directions

The two different vaccination strategies generated equal number of peptide specific cells as measured by pentamer staining. However only one of them (ImmunoBody®) was capable of producing IFN-γ in peptide titration ELISpot assays, indicating that they are functionally different in their response to the cognate antigenic stimulus. The avidity measurement indicated that ImmunoBody® derived high avidity, and peptide derived low avidity, has a 2 log difference in their antigen requirement for maximal activation. However, low avidity T cells were shown to restore their functionality once exposed to the antigenic stimulus *in vitro* whereas the low avidity ones die, possibly due to activation induced cell death. This suggests that great care should be taken while designing the cancer vaccines and appropriate consideration must be given to determine the avidity of vaccine generated T cells to avoid the killing of high avidity T cells. Gene expression kinetic studies indicated that there are significant differences in the signalling competency between high and low functional avidity T cells with high avidity transmit the signals more efficiently. However more studies are necessary to examine the phosphorylation status of

these two primed cells before reaching final conclusion. A new hypothesis can be derived from this study is that the physiological and molecular changes happening during the naïve T cell priming leave the molecular machinery for better activation in high avidity cells, so that they will respond to the subsequent activation much efficiently. Gene expression microarray analysis was successfully carried out with low number of peptide specific cells with observed transcriptional changes between high and low avidity T cells types. However the differences were subtle and the reason may be that the studies were not conducted on the activated peptide specific T cells and this will be a future priority for global gene expression profiling of resting and activated high and low avidity T cells. Global proteomic profiling of high and low avidity T cells using purified TRP-2 specific T cells were successfully completed. Though several identities were obtained, the quantitation still remains a problem because of the inherent inability of LC MALDI to precisely quantify the proteins. Future work will be needed utilising more appropriate quantitative mass spectrometry instrumentation.

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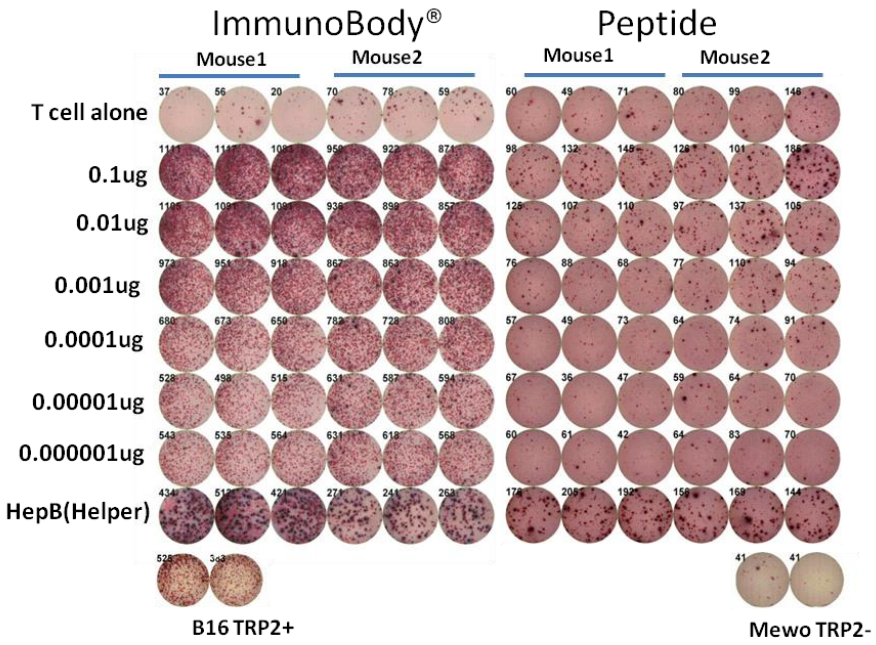
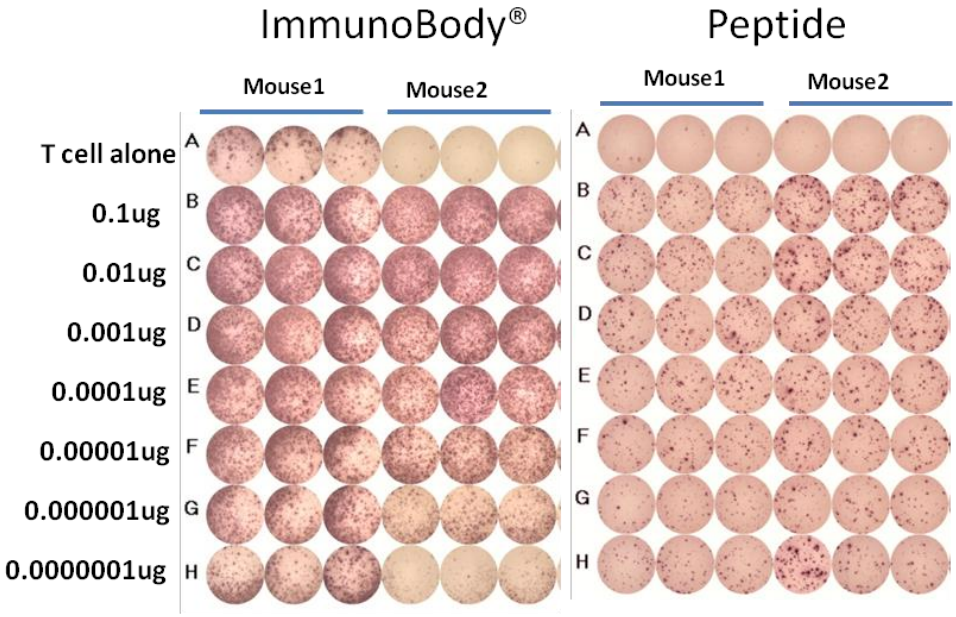
Chapter 7 Appendix

Figure 7:1 Pictures of *ex vivo* IFN-γ ELISpot Plates from TRP-2 and ova antigen immunisations



C57Bl/6 mice immunised with TRP2 (SVYDFFVWL) ImmunoBody® (DNA) and peptide vaccine were assayed by IFN-γ peptide titration ELISpot® assays. Concentrations are given in the left side . In the bottom cells showing positive control (B16 which expresses TRP-2) and negative control Mewo (which is TRP-2 negative)



C57Bl/6 mice immunised with OVA (SIINFEKL) ImmunoBody® (DNA) and peptide vaccine were assayed by IFN-γ peptide titration ELISpot® assays. Concentrations are given in the left side .

Figure 7.2: Pictures of IFN-γ ELISpot Plates from TRP-2 and ova antigen immunisations after *in vitro* stimulations

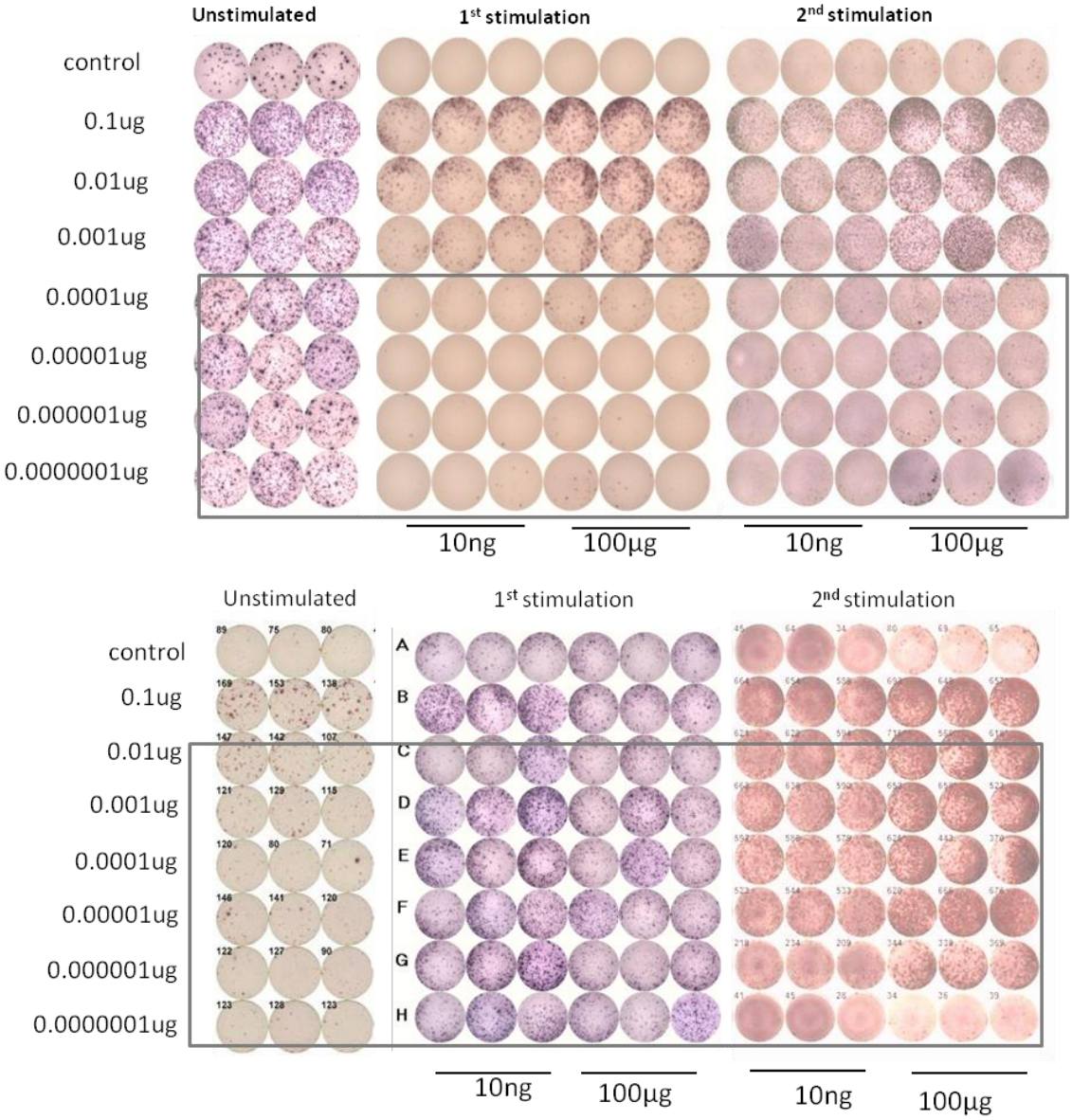


Figure 7.3: Binding specificity of MHC multimer used for this study

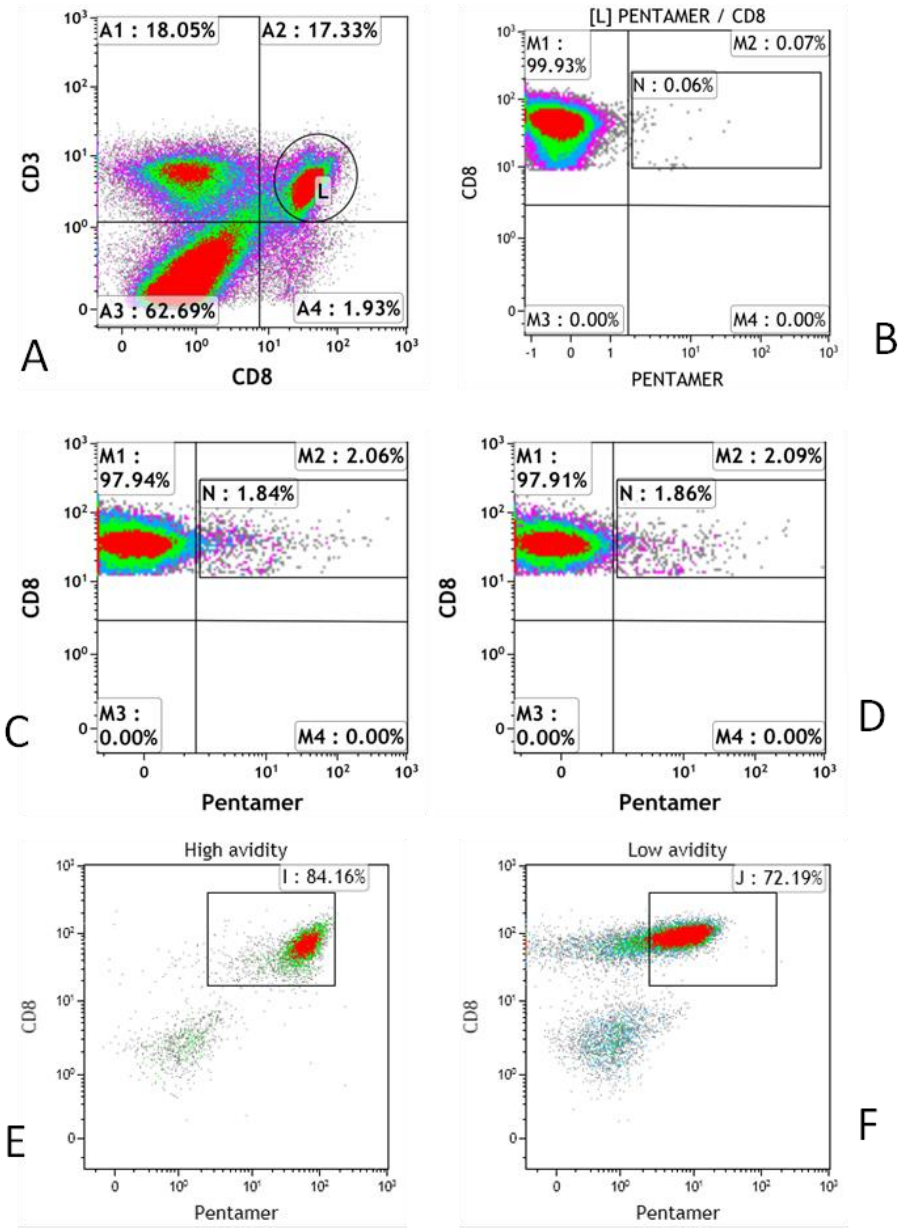


Figure:7.3. Binding specificity of the pentamer used for this study. **A**. Staining of CD3 and CD8 markers showing $CD8^+$ $CD3^+$ T cells. **B**. Staining of MHC pentamers on the cells isolated from the spleen of a naive mice (Gated on $CD8^+$ $CD3^+$ cells). **C and D** Staining of MHC pentamers on the cells (spleen) isolated from Immunobody and peptide immunised animals (Gated on $CD8^+$ $CD3^+$ cells) after 45 days of the first immunisation. **E and F** MHC pentamers tested on the TRP-2 SVYDFFVWL TCR transgenic splenocytes kindly provided By Prof. Arthur Andrew Hurwitz, National Cancer Institute, USA**. E**. Detection of high structural avidity T cell (SVYDFFVWL specificity) C. Detection of low avidity T cell (SVYDFFVWLspecificity).

Figure 7.4: Purity of CD8 enrichment

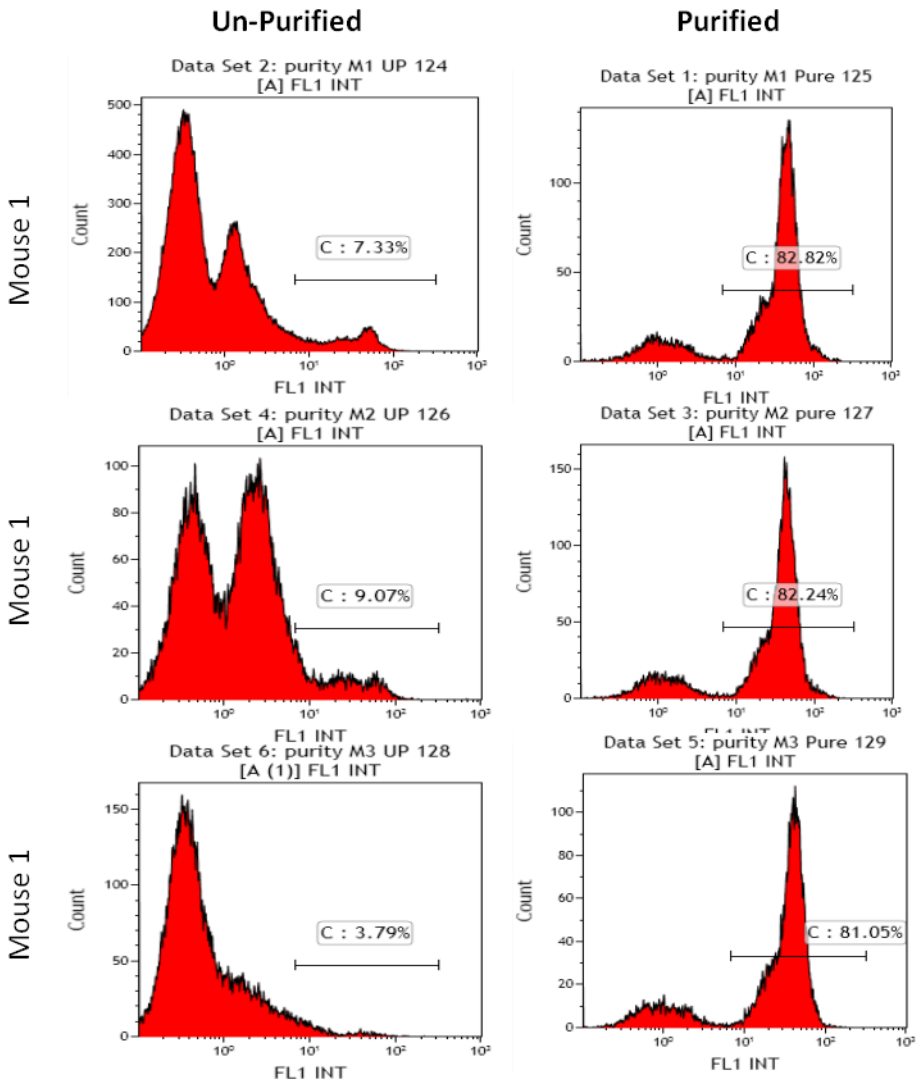


Figure showing the enrichment of CD8+ cells prior to the isolation of pentamer positive cells. The left side panel shows total splenocytes and the right side shows purified fraction

Figure 7.5: Binding specificity of antibodies used for immune phenotyping

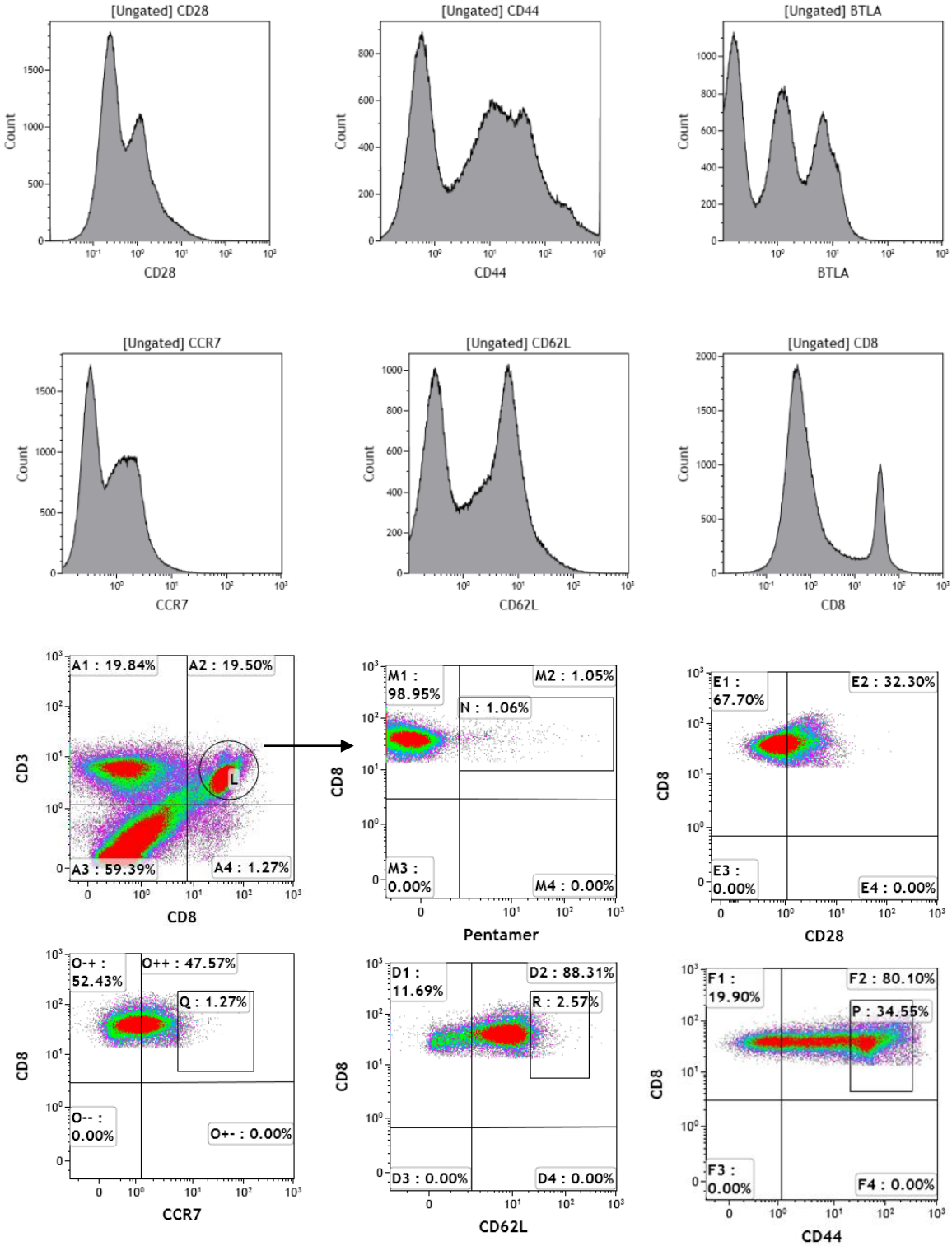


Figure: Effective binding of the antibodies used for immuno-phenotyping. Top panel showing a histogram plot with positively stained population. Bottom panel is showing the positive gating of these cells as a density plot. For the purpose of identifying positive staining the cells were gated on t. otal CD8+ CD3+ population and not on antigen specific pentamer positive cells

Appendix VI Genes up-regulated in Immunobody. Raw expression values are given in a log scale (TRP-2 model)

NAME	Descrption	TRP-2 IM1	TRP-2 IM2	TRP-2 Pep1	TRP-2 Pep2
10412211	Gzma	9.589867	9.08598	5.731719	7.910787
10517513	C1qc	9.915211	10.07822	5.80855	9.193348
10572897	Hmox1	10.87203	11.17641	7.251457	10.20731
10541605	Clec4n	7.82252	8.360159	4.335455	7.207325
10517508	C1qb	10.29355	10.54361	6.732691	9.586124
10354374	Slc40a1	9.374151	10.05637	6.020717	8.896749
10501608	Vcam1	9.940613	10.07201	6.774196	9.313292
10492964	Cd5l	7.999214	7.922586	4.941354	7.083319
10480849	Fcna	9.464057	9.581237	6.44323	8.831555
10517517	C1qa	9.426667	9.470099	6.348067	8.9394
10371662	Spic	8.086142	8.361504	5.5297	7.458381
10432176	Snora34	9.773756	10.79492	8.46547	8.787658
10389894	Abcc3	7.823934	7.958979	5.513885	6.856365
10461162	Snord22	9.405281	10.35747	8.280205	8.16065
10446282	Emr1	8.210877	8.678833	5.523114	8.043721
10561104	Axl	8.773106	8.826503	6.169617	8.179367
10590628	Ccr3	7.182745	8.04994	5.042803	6.902711
10461148	Snhg1	6.847205	8.113957	5.876047	5.804968
10487588	Il1a	6.580138	6.957129	4.286168	5.994854
10475890	Mertk	7.997388	8.232366	5.7192	7.35084
10571840	Hpgd	8.124514	8.688388	6.084416	7.617432
10516906	Snora73b	12.67959	13.12858	11.10118	11.80032
10576757	Fcer2a	7.054664	10.36507	7.722066	6.66023
10362674	Rnu3a	11.68785	12.06138	10.18383	10.71121
10604076	Snora69	9.504164	10.70633	8.840189	8.452264
10376887	Snord49a	9.310322	10.78306	8.543922	8.639715
10498367	P2ry13	7.589698	8.129551	5.499421	7.226751
10508719	Snora16a	10.19817	11.49014	9.330112	9.520072
10349593	Faim3	10.37903	11.51175	9.273946	9.784533
10445758	Treml4	9.258013	9.518743	7.164486	8.745387
10420758	Blk	8.778323	10.11146	7.749572	8.294958
10516908	Snora73a	12.71691	12.91903	11.11018	11.83317
10377429	Snord118	9.840086	10.91598	8.680268	9.278258
10399428	Snord118	9.840086	10.91598	8.680268	9.278258
10557928	Itgad	8.493612	8.792257	6.380451	8.042044
10445953	Emr4	7.24434	7.905445	5.316432	6.990599
10603860	Cfp	9.903337	10.47845	7.880783	9.784018
10572669	Fam129c	8.095046	9.628594	7.173551	7.785551
10434932	Fam43a	7.934694	9.46143	7.222821	7.437933
10444284	H2-Ob	9.369182	11.00211	8.717368	8.991334
10567863	Cd19	8.841819	10.62243	8.383888	8.433625
10347335	Slc11a1	9.182103	9.442764	7.292714	8.667005
10481101	Snora43	10.14342	10.5157	8.620813	9.429207
10368277	Rps12	8.732088	9.439003	7.665389	7.87585
10508721	Snora44	11.85772	11.34685	9.985884	10.70804
10369301	Chst3	6.309884	8.257577	6.1151	5.766916
10444223	H2-Oa	8.446677	10.14124	7.687684	8.317366
10494413	Rnu1b1	10.39444	10.66964	9.224343	9.312216
10494421	Rnu1b1	10.39444	10.66964	9.224343	9.312216
10500343	Rnu1b1	10.39444	10.66964	9.224343	9.312216
10500358	Rnu1b1	10.39444	10.66964	9.224343	9.312216
10512937	Rnu1b1	10.39444	10.66964	9.224343	9.312216
10489246	Mafb	6.983207	7.065101	5.351636	6.046548
10421128	Adamdec1	6.924807	7.544652	5.016084	6.820369
10583312	Taf1d	9.524443	10.5322	8.402685	9.176725
10592888	Cxcr5	6.944347	8.681185	6.64759	6.4082
10419674	Snord8	9.96397	11.1686	9.324019	9.38326
10501922	Snhg8	10.67548	11.28314	9.419006	10.14829
10360145	B930036N1	9.97566	10.52721	9.297438	8.802517
10491058	Rpsl2	11.79775	12.20421	10.77675	10.90046
10492971	Fcrl1	7.082126	9.05241	7.070755	6.574193
10548504	Klra8	5.414029	6.884009	4.315355	5.411291
10494411	Rnu1b1	10.39305	10.61123	9.367935	9.269728
10494417	Rnu1b1	10.39305	10.61123	9.367935	9.269728
10500356	Rnu1b1	10.39305	10.61123	9.367935	9.269728
10358399	Rgs13	4.197691	6.447623	4.083524	3.988812
10590031	Itga9	7.138973	7.679647	5.471708	6.8682
10566258	Hbb-b2	13.30799	13.08477	10.84867	13.34158
10566254	Hbb-b1	13.35606	13.08733	10.87341	13.38039
10444306	H2-Eb2	8.130914	9.729858	7.742852	7.757351
10444236	H2-DMb2	10.33709	11.63158	9.590322	10.11351
10492983	Fcrl5	7.623876	8.733533	6.881544	7.087032
10351515	Rnu1b1	10.1555	10.4423	9.176587	9.128
10351043	Snord47	11.232	12.12241	10.6244	10.51655
10562720	Siglece	7.643154	7.809888	5.537147	7.541424
10445293	Pla2g7	8.115096	8.500911	6.434896	7.878008
10544523	Rny1	10.7967	11.84141	10.6095	9.878319
10461487	Cybasc3	9.10546	10.26627	8.551228	8.60676
10579331	Gdf15	7.632402	7.790726	5.888381	7.250942
10512470	Cd72	9.874437	11.35883	9.51758	9.563251
10531952	Abcg3	7.469491	8.123319	6.006011	7.31124
10466172	Ms4a1	9.925154	11.6663	9.636942	9.8137
10461154	Snhg1	6.231544	7.05336	5.097746	5.864447
10500345	Terc	12.29068	12.83291	11.28422	11.79408
10529732	Hs3st1	6.124646	8.403053	6.528101	5.756297
10368675	Marcks	8.461849	9.336082	7.343792	8.287498
10404606	Ly86	10.18803	11.47179	9.554667	10.03653
10560624	Apoe	10.87007	11.33939	9.33447	10.81945
10375360	Ebf1	8.180679	10.34128	8.248497	8.155461
10521678	Cd38	8.964314	10.36575	8.573173	8.657997
10505071	Tmem38b	9.208268	9.514099	8.422078	8.199805
10433578	Snn	6.965196	8.216871	6.639387	6.364717
10430186	Apol7c	8.113007	8.366554	6.600251	7.734829

NAME	Descrption	TRP-2 IM1	TRP-2 IM2	TRP-2 Pep1	TRP-2 Pep2
10593198	Fam55b	8.56933	8.533388	6.607595	8.381689
10525365	Hvcn1	9.249723	10.69202	8.991926	8.90536
10528207	Cd36	8.380288	9.683527	7.571501	8.418983
10562812	Spib	8.696977	10.4688	8.364212	8.759653
10406928	Cd180	9.066186	10.93495	8.977813	9.003679
10467979	Scd1	9.226393	11.42562	9.375266	9.272377
10557399	Sbk1	8.115637	9.212299	7.525062	7.745033
10602372	Alas2	8.861356	7.982565	6.247273	8.537067
10444291	H2-Ab1	12.54194	13.16456	11.40329	12.44177
10360684	Ephx1	7.802707	9.163388	7.598841	7.335053
10531610	Rasgef1b	8.74185	9.797302	8.015669	8.530432
10382844	Snord1c	6.273273	7.03448	5.694345	5.50728
10386093	Snord1c	6.273273	7.03448	5.694345	5.50728
10439312	Cd86	7.842793	8.885528	6.795788	7.906504
10461158	Snhg1	8.462978	8.651784	7.393661	7.705073
10512669	Pax5	8.150686	10.03154	8.134851	8.056373
10432178	Snora2b	6.24492	7.502478	5.908464	5.749757
10380571	Gngt2	6.999265	8.341341	6.43324	6.854496
10420308	Gzmb	9.922928	8.828732	8.345399	8.446661
10460392	Pold4	8.111415	9.717979	7.891159	7.960004
10562132	Cd22	8.115824	10.34049	8.534281	7.964089
10416340	Gfra2	7.534735	7.674738	6.012338	7.170517
10549647	Ncr1	6.21598	8.352924	5.640357	6.890463
10404840	Cd83	10.60115	11.41025	9.939986	10.20299
10565813	Snord15a	9.744101	10.61673	9.14128	9.314711
10359754	Mpzl1	7.898453	7.716675	6.276272	7.331823
10444298	H2-Eb1	12.27614	12.92093	11.21675	12.19009
10456071	Csf1r	9.208694	9.498959	7.884416	8.899006
10556206	Snora3	10.85592	11.62804	10.4058	10.24237
10570432	Snora3	10.80808	11.59458	10.37434	10.1937
10365559	Igf1	6.34639	6.68378	4.884417	6.103601
10552380	Siglecg	7.398795	9.102331	7.078908	7.457997
10446553	Epb4.1l3	6.304914	6.314565	4.90934	5.65973
10450363	Snord52	6.313813	7.771177	5.987106	6.08076
10391649	Slc4a1	11.1644	9.719302	7.972857	11.05589
10500295	Plekho1	9.880741	10.7892	9.092258	9.722798
10489204	Tgm2	8.704157	8.638174	7.064716	8.365243
10515429	Snord55	10.50967	11.53235	10.15942	10.07807
10509002	Rhd	9.879602	7.918889	6.282712	9.61893
10358733	Rgl1	7.230938	7.912672	6.167732	7.027921
10455015	Vaultrc5	9.33708	9.856716	8.397053	8.940981
10545192	Rprl1	9.500235	9.78429	8.707607	8.723711
10353034	Snord87	10.09444	11.04696	9.651169	9.690675
10408600	Serpinb6a	6.863996	6.959473	5.373998	6.503882
10573054	Gypa	9.39338	7.996819	6.213872	9.311585
10465831	5730408K0	9.47921	9.33142	8.336817	8.689937
10563085	Fcgrt	8.543394	8.544001	7.291391	7.975061
10443506	Fgd2	8.512315	9.472265	7.746667	8.441962
10590267	Snora62	7.850598	8.264646	6.976917	7.304422
10506488	Ppap2b	5.858487	6.673142	4.997478	5.625985
10351781	Kcnj10	6.516372	6.943178	5.111654	6.487891
10600355	Snora70	8.458153	8.631198	7.441567	7.872429
10417526	Dnase1l3	9.511496	10.04841	8.234589	9.612614
10376885	Snord49b	12.65885	13.37	12.18243	12.28089
10537909	Rny3	13.53883	13.73761	13.05898	12.6803
10557895	Itgax	8.169209	8.522425	6.814348	8.104808
10515425	Snord38a	5.942158	7.205748	5.720703	5.580857
10489235	9430008C0	8.865037	9.365599	8.030067	8.472673
10563099	Snord35b	11.72145	12.65988	11.47022	11.34032
10363860	Slc16a9	6.660229	6.819273	5.583716	6.085406
10563112	Snord33	11.84336	12.51105	11.41631	11.37053
10484987	Nr1h3	6.931929	7.356722	5.92274	6.577335
10576784	Cd209a	6.929454	8.057871	6.354747	6.860675
10390505	Snora21	9.019244	9.406793	8.174484	8.560115
10502335	Bank1	8.499734	10.86738	8.937159	8.760662
10534935	Pirb1	7.546444	7.687526	5.719011	7.757407
10352000	Kmo	7.207993	8.886125	6.966573	7.39296
10470555	Gbgt1	6.179564	6.093713	5.23596	5.217745
10565811	Snord15b	12.18854	12.55946	11.61969	11.58797
10450154	H2-Aa	12.15588	12.94525	11.22451	12.35182
10477250	Hck	8.511951	9.59916	7.773345	8.657495
10469358	Mrc1	7.579521	7.964271	6.441591	7.364956
10478678	Cd40	7.321283	8.333824	6.842777	7.078229
10556297	Adm	8.249062	7.596461	6.313666	7.802503
10548422	Klri2	5.12991	6.528691	4.293539	5.547582
10596148	Trf	9.555169	9.783866	8.281093	9.416269
10556244	Snora23	8.911791	9.615429	8.844393	8.023393
10553559	Siglech	6.211782	7.411462	5.850393	6.005884
10519607	4930420K1	9.196369	10.57047	9.098394	9.053179
10533720	Niacr1	7.15239	7.385021	5.763688	7.050668
10402512	Scarna13	10.76602	10.82987	9.710416	10.32201
10415857	Fam167a	6.739068	7.718857	6.255171	6.486626
10363475	Prf1	8.123374	8.358061	7.274094	7.540363
10604743	Snord61	7.891029	8.807808	7.501167	7.543459
10551025	Cd79a	10.65185	12.17768	10.67324	10.64272
10484888	Ptprj	9.16923	10.13482	8.682972	9.031823
10454807	Snora74a	11.32218	11.6171	10.66083	10.77147
10576034	Irf8	10.01123	10.88662	9.256189	10.09975
10361292	Cr2	6.299931	9.565512	7.80933	6.418388
10531407	Cxcl9	8.429946	8.140371	6.534154	8.418097
10497481	2810416G2	7.191314	7.635533	6.195167	6.976489
10444229	H2-DMa	10.22089	11.49057	9.649928	10.56882
10593473	1810046K0	9.230952	9.145272	7.581236	8.70209

Appendix VII Genes down-regulated in Immunobody. Raw expression values are given in a log scale (TRP-2 model)

NAME	Descrption	TRP-2 IM1	TRP-2 IM2	TRP-2 Pep1	TRP-2 Pep2
10569017	Ifitm3	9.637287	9.857903	10.49467	11.11666
10403821	Tcrg-V3	8.820829	8.122258	10.04784	8.955576
10457733	B4galt6	6.308984	6.334962	7.227243	7.386437
10365749	Lta4h	7.987337	7.987864	8.896038	9.127562
10596279	Dnajc13	5.071257	5.796882	6.716968	6.087835
10396712	Fut8	8.004765	7.836905	9.405346	8.489271
10589654	Als2cl	6.149069	5.42206	7.431859	6.097953
10596543	Rad54l2	5.747794	5.739632	7.052429	6.398836
10415784	Trim13	7.051756	6.623665	7.983385	7.708501
10551815	Zfp260	6.093111	6.250867	7.638571	6.697249
10571274	Gsr	8.78372	8.293302	9.226282	9.96719
10542993	Pon3	8.473999	8.275257	9.840219	9.028057
10536505	Met	4.988557	4.896088	6.104732	5.75148
10393887	Pycr1	7.5927	7.121058	8.780922	8.018259
10363082	Lilrb4	6.263291	7.172405	7.351449	8.14273
10454632	Camk4	5.854827	5.219637	7.05974	6.028613
10547740	C1s	3.758599	3.990427	5.464801	4.224479
10560242	C5ar1	4.96135	5.309272	5.515269	6.755356
10548385	Olr1	4.154801	4.312653	5.014004	5.420711
10392183	Ern1	7.756928	7.190834	8.844728	8.221866
10495596	Frrs1	6.74356	5.809929	7.411004	7.210453
10391697	Itga2b	6.583876	6.423945	7.516446	7.573705
10565994	Art2b // A	4.788126	5.126945	6.993124	4.936018
10407327	Emb	9.682821	8.756377	10.49698	10.15176
10542857	Far2	5.293806	5.11835	6.282934	6.160043
10367843	Utrn	7.154034	7.214114	8.730898	7.770291
10607952	Vamp7	7.594597	7.840863	9.098844	8.498391
10400844	Pygl	6.875453	6.72273	7.346099	8.412729
10440002	Gpr128	4.97195	4.584596	5.73121	5.905748
10433584	Tnfrsf17	8.541339	8.018451	9.892799	8.906086
10346783	Cd28	8.862856	7.407469	9.887501	8.624049
10383204	Rnf213	7.671423	7.88681	9.187749	8.595829
10469255	Prkcq	8.517771	7.538423	9.932522	8.368923
10387855	Alox15	6.418158	6.462885	5.723978	9.333157
10402991	Gm16970	12.18146	11.22852	13.37507	12.44687
10502224	Sgms2	5.725321	5.367957	6.260623	6.971786
10385533	Tgtp1	8.293904	8.00921	9.526764	9.043917
10344817	Cspp1	5.139129	6.378856	7.078473	6.600583
10496091	Lef1	8.898957	7.721509	10.03113	8.868871
10383200	Rnf213	7.285742	7.472165	8.816395	8.18348
10485624	Prrg4	6.770383	6.87612	8.43206	7.434144
10501046	Gm10673	4.518981	4.476056	5.705172	5.407722
10427628	Il7r	8.716152	7.37142	9.900121	8.473452
10478633	Mmp9	6.577484	6.882494	6.761191	8.933296
10414953	Gm16591	4.40368	3.808361	5.46169	4.87117
10349968	Chi3l1	5.637611	5.757745	6.361109	7.234969
10476945	Cst7	8.121135	7.433799	8.999371	8.851639
10596454	Alas1	6.840498	7.01092	8.043525	8.071169
10523451	Anxa3	6.257323	6.595372	7.166331	7.92976
10383206	Rnf213	7.466496	7.758605	9.011381	8.520266
10587012	Ccpg1	8.393986	7.993329	9.873787	8.857033
10352178	Sccpdh	5.56175	4.608797	6.433246	5.949596
10389214	Ccl9	6.305702	6.817595	7.590185	7.825161
10524621	Oasl2	6.724374	6.869962	7.733228	8.163691
10519324	Cdk6	9.027987	8.105388	9.838401	9.685663
10362350	Themis	6.817914	5.97667	8.414124	6.682087
10370644	Prssl1	5.426135	5.320988	6.073639	6.931323
10574023	Mt2	11.65075	9.711828	11.86205	11.99759
10584941	Bace1	6.362609	6.375094	7.702717	7.368254
10362511	Gstm3	5.389682	5.517532	6.653011	6.548241
10538887	Gm5153	11.40671	10.81736	12.81975	11.95094
10401935	BC005685	6.77597	6.441141	8.481771	7.087939
10497646	Phc3	5.381682	5.973351	7.228276	6.445515
10508074	Csf3r	5.971431	6.308368	6.915699	7.705809
10365482	Timp3	5.165476	5.403523	6.770527	6.102465
10360338	Fcer1a	4.478628	4.645157	5.229639	6.191607
10481262	Fcnb	5.937644	6.022153	6.445356	7.882596
10571815	Gpm6a	5.385189	5.219183	7.110599	5.834912
10497451	Cpa3	6.50266	6.480574	8.112594	7.26506
10538901	BC005685	6.746758	6.487041	8.577344	7.066647
10414728	Gm7174	5.034709	4.492245	6.286873	5.569557
10574259	Gpr56	9.384345	7.361527	9.480116	9.768934
10403006	Gm7112	9.459102	8.890182	10.75812	10.14645
10466659	Gda	6.395913	5.859464	7.000996	7.674215
10467216	Cpeb3	5.943641	5.153401	7.315778	6.180797
10530516	Txk	7.479335	6.859753	8.938855	7.874419
10358421	Rgs18	6.33867	7.480301	7.962712	8.330256
10383192	Rnf213	7.121958	7.293264	8.442578	8.460281
10466606	Anxa1	7.183536	7.380604	7.927135	9.14292
10563715	Mrgpra2b	5.21449	5.174658	5.91498	6.890467
10380174	Mpo	9.121368	7.791436	11.88338	11.87076
10364535	Elane	8.433015	7.372181	11.13115	11.56763
10420261	Ctsg	7.595968	6.231912	10.48756	10.95787

NAME	Descrption	TRP-2 IM1	TRP-2 IM2	TRP-2 Pep1	TRP-2 Pep2
10386058	Sparc	6.287923	6.142701	8.378756	6.516499
10385428	Itk	9.477957	7.72432	10.30216	9.473875
10385518	Tgtp1	8.056376	7.760482	9.476921	8.885838
10379633	Slfn1	6.96968	6.542465	8.299047	7.711625
10554094	Igf1r	7.451108	6.377673	8.207121	8.135328
10421517	Cysltr2	4.871775	4.164931	6.199124	5.27795
10402981	Gm900	5.859273	6.108444	7.756779	6.749736
10597098	Camp	7.109243	7.993564	8.040306	9.68683
10488382	Cd93	6.893817	7.489754	8.906891	8.14621
10472235	Dapl1	9.577905	8.432557	10.95892	9.816936
10543120	Ica1	4.808833	4.379165	6.101148	5.659763
10363070	Gp49a	6.611547	7.164611	7.764619	8.698249
10563712	Mrgpra2a	5.13889	5.159265	5.877431	7.105302
10485622	Qser1	6.158895	6.160885	7.721083	7.337097
10504757	BC005685	6.748187	6.4626	8.704298	7.288231
10347291	Cxcr2	4.739384	4.58604	5.166677	6.857328
10487238	Hdc	7.197366	7.16186	8.034441	9.18722
10403066	Gm16710	8.5035	8.421247	10.68936	9.176223
10497548	Fndc3b	7.839392	7.009982	9.561564	8.184456
10543239	Tcfec	5.832731	6.110675	7.463833	7.326643
10547664	Clec4e	5.526101	5.288092	6.249053	7.398663
10414262	Ear2	9.2597	9.078118	8.884237	12.47751
10501229	Gstm1	7.033997	7.125841	8.391186	8.745188
10415015	Gm13949	4.708076	3.535243	6.299806	4.79687
10401900	Sel1l	9.246717	8.739494	11.21195	9.850601
10474700	Thbs1	7.638734	7.917637	8.428293	10.15216
10435288	Muc13	6.631204	5.442288	7.515738	7.508502
10574276	Gpr97	6.478508	6.084798	7.755714	7.770935
10408928	Hspb1	8.075319	7.329114	8.845095	9.586127
10429573	Ly6c2	11.1268	10.44939	12.5463	12.2063
10526410	Hspb1	7.961002	7.231628	8.78004	9.478753
10423049	Prlr	4.422682	4.108268	6.782434	4.670915
10569020	Ifitm6	5.278641	6.681057	6.242225	8.76821
10445746	Trem1	5.643428	5.676318	6.470429	7.889835
10429580	I830127L07	8.562352	8.981112	9.926787	10.80186
10496872	Eltd1	5.039892	5.210004	6.911381	6.36945
10451953	Lrg1	5.49291	5.675477	6.058494	8.177898
10523128	Ppbp	7.318691	8.105155	9.176453	9.419589
10501020	Chi3l3	4.59649	5.60985	5.792056	7.472202
10538875	AY498738	9.00958	8.722036	10.68031	10.33631
10545180	Gm10879	11.40371	10.27454	12.77111	12.28398
10489463	Slpi	8.616098	8.397497	10.49968	9.821762
10429560	Ly6i	6.304932	6.378236	8.22738	7.67772
10523134	Pf4	6.531179	6.362988	7.811449	8.328002
10419156	Ear10	8.801408	8.313081	8.437481	12.08597
10598175	Ear10	8.534323	8.082186	8.239657	11.80634
10379636	Slfn4	5.751188	6.016742	6.28997	8.821088
10487208	Atp8b4	6.754921	6.285714	8.609427	7.831455
10424683	Ly6g	5.720295	5.917487	6.561203	8.462098
10429568	Ly6c1	10.78453	10.37706	12.65546	12.10703
10367436	Cd63	5.554529	5.746743	6.450701	8.254933
10473406	Prg3	6.173438	5.845471	6.37689	9.068511
10381096	Igfbp4	7.738251	6.642073	9.162525	8.744167
10410386	E430024C0	4.019139	3.748498	5.604944	5.577785
10410388	E430024C0	4.019139	3.748498	5.604944	5.577785
10473399	Prg2	9.290556	7.693725	9.421315	11.26007
10581605	Hp	6.376187	6.360035	7.649928	8.721138
10419154	Ear1	9.014612	8.186208	8.582313	12.45986
10600852	F630028O1	5.486126	5.612344	6.811297	8.01732
10484463	Serping1	4.839833	4.817619	7.663381	5.769885
10499861	S100a9	6.437485	8.081434	7.949113	10.50551
10589535	Ngp	8.74703	10.0792	10.79197	12.17001
10445753	Trem3	5.366235	5.90491	7.087781	8.202077
10478048	Lbp	6.125412	5.983109	7.892826	8.259671
10599348	Gria3	4.810802	5.087077	7.449424	6.536249
10355403	Fn1	6.019461	6.779673	8.977273	8.020826
10442762	Prss34	6.840974	6.831545	8.682114	9.269894
10589703	Ltf	6.147509	7.546659	8.118481	9.898699
10481627	Lcn2	6.664207	8.155189	8.720603	10.52452
10352905	Cd34	6.056442	5.655152	8.450757	7.628768
10583100	Mmp8	4.34091	5.357017	5.579257	8.550432
10420254	Mcpt8	5.943045	5.805286	8.024098	8.276974
10544273	Clec5a	4.259262	4.360202	6.457104	6.802654
10452316	C3	7.817947	7.989816	10.98809	9.676615
10493831	S100a8	7.308252	8.972199	9.321878	11.8728
10563597	Saa3	4.258882	4.6221	7.45257	6.252638
10419151	Ear1	9.000506	6.769539	8.312558	12.43943
10414590	Ear6	7.547207	6.06633	7.526339	11.28144
10365974	Dcn	5.297863	5.286076	9.277538	6.628817
10389654	Epx	6.984108	5.110147	7.376402	10.12368
10408693	F13a1	5.690486	6.228181	8.870432	8.605432
10466224	Ms4a3	6.057644	5.367032	8.19968	9.057676
10364529	Prtn3	7.478862	6.691831	9.876183	10.68878

Appendix VI II: Genes up-regulated in Immunobody. Raw expression values are given in a log scale (OVA model)

Probe Set I	Gene Symb	OVA IM-1	OVA_IM2	OVA_PEP1	OVA_PEP3		Probe Set I	Gene Symb	OVA IM-1	OVA_IM2	OVA_PEP1	OVA_PEP3
10545180	Igkv4-91	9.69314	9.805981	6.91714	6.99322		10583312	Taf1d	8.645541	8.958839	8.118399	8.040622
10563112	Snord33	8.60014	8.818928	7.375708	7.449839		10389561	Dhx40	7.417555	7.959614	7.00306	6.906667
10384968	Bod1	6.485357	6.448826	5.588012	5.293087		10577910	Fnta	6.802185	7.246727	6.260534	6.345529
10604743	Snord61	5.130029	5.877207	4.605886	4.36947		10470318	Gm11362	7.8614	8.107421	7.267119	7.289405
10351039	Mir5117	5.887665	6.16469	4.764966	5.343298		10515986	Zfp69	5.503265	5.529018	4.943538	4.737007
10420308	Gzmb	7.492692	7.599083	6.534068	6.667619		10570434	Ifitm1	6.421075	6.366988	5.710132	5.735517
10365003	Snord37	7.596515	7.936535	6.990962	6.702778		10603706	Med4	5.087574	5.736595	4.511792	4.874906
10501048	Dennd2d	7.580863	7.899948	6.903404	6.759975		10398693	Snora28	9.477392	9.738741	8.903022	8.909912
10356329	Snora75	7.435209	7.814739	6.798612	6.685408		10535894	Hmgb1	9.187836	9.311234	8.710701	8.409495
10488748	Cdk5rap1	7.006035	7.166324	6.34201	6.116799		10465244	Malat1	10.97988	11.25908	10.40462	10.43884
10380135	Mir142	8.442287	8.589273	7.59488	7.737499		10400350	Cfl2	7.893459	8.103149	7.420601	7.220647
10567171	Snord14a	8.940478	9.430416	8.35562	8.290436		10419288	Gch1	6.651633	7.067834	6.13947	6.213923
10456717	Snord58b	4.430719	5.224023	4.03425	3.910317		10547469	Wnk1	8.422059	8.558064	7.780632	7.865306
10523901	Rpl5 // Gı	6.409052	6.745096	5.691262	5.808454		10493626	1700094D(	6.850286	6.860258	6.147926	6.261266
10464642	Carns1	8.628381	8.712394	7.835826	7.885286		10366407	Gm10752	6.893124	7.264099	6.423843	6.398476
10454828	Pnet-ps	7.895741	8.241735	7.241679	7.261731		10576216	Snord68	6.596814	7.368963	6.168699	6.40834
10414659	Snord58b	4.41347	5.200002	4.038794	3.900672		10470316	Gm11362	4.699151	5.611107	4.428719	4.488603
10501046	Gm10673	7.642265	8.178261	7.010998	7.166273		10452556	Rab12	7.319747	7.608991	6.861599	6.754037
10470320	Gm11362	6.363622	6.950526	5.656157	6.020112		10368486	Rnf146	6.092801	6.170103	5.514164	5.475348
10469720	Acbd5	6.151396	6.955054	5.709156	5.735511		10485633	Gm10796	6.868388	6.831445	6.239313	6.199461
10518145	Prdm2	6.914974	6.923786	6.291751	6.024115		10494369	Sf3b4	5.695186	5.719399	5.297318	4.859215
10344803	Cspp1	5.747438	6.07411	5.115907	5.16254		10511113	Cdk11b	8.409454	8.556264	7.858694	7.820117
10351043	Snord47	7.611231	8.150832	7.147291	7.034295		10488029	Zfand1	7.282542	7.256142	6.73473	6.552051
10427253	Map3k12	6.206707	6.71437	5.674802	5.678596		10409709	Mir7-1	8.531556	8.775112	7.995221	8.011959
10558454	Glrx3	5.746104	5.616892	4.496005	5.427341		10554895	Crebzf	7.848598	8.121634	7.221683	7.453065
10412211	Gzma	5.583329	5.724157	4.952904	4.914941		10564573	Chd2	8.295896	8.495142	7.772614	7.737996
10403073	Ighg	5.81477	6.847641	5.673496	5.436073		10586168	Snord16a	10.417	10.47286	9.823563	9.795281
10476104	Nop56	6.121158	6.746631	5.679972	5.705353		10491438	Ttc14	7.353605	7.640796	6.769305	6.940662
10439980	Pcnp	6.508757	6.442314	5.81006	5.761304		10522606	Exoc1	7.381692	7.503676	6.883982	6.76347
10389882	Luc7l3	8.402536	8.72374	7.799734	7.880173		10520388	Rbm33	7.720439	8.357555	7.256856	7.506729
10453555	2610044O	5.970062	6.579965	5.30556	5.769284		10442206	Zfp51	6.936976	7.504477	6.657868	6.487235
10440953	Donson	7.701056	7.71559	7.27808	6.958601		10482167	Zbtb6	6.373755	6.512095	5.873817	5.789457
10475912	Tmem87b	7.290926	7.563529	6.889805	6.755482		10363903	Hbs1l	9.807161	9.913036	9.246777	9.248672
10443459	Srsf3	7.033446	7.417471	6.561611	6.665708		10406614	Mtx3	6.137709	6.329241	5.623382	5.63205
10497300	Zfand1	7.523465	7.5157	6.933628	6.940027		10402564	Setd3	7.732155	8.054579	7.088573	7.45635
10410099	Cdc14b	8.015813	8.115256	7.49514	7.455571		10375735	Hnrnph1	9.984821	10.14665	9.426685	9.482327
10475350	Serf2	8.24432	8.342276	7.731792	7.673965							

Appendix IX: Genes down-regulated in Immunobody. Raw expression values are given in a log scale (OVA model)

Probe Set I	Gene Symb	OVA IM-1	OVA_IM2	OVA_PEP1	OVA_PEP3
10509002	Rhd	4.915914	4.825545	5.572963	5.445599
10456005	Cd74	9.415863	9.652457	10.14615	10.12623
10461798	Olfr1463	7.060061	7.260168	7.699183	7.846801
10521678	Cd38	6.692955	6.750337	7.449569	7.249082
10553299	Ifitm2	7.979477	7.740631	8.509089	8.500862
10502791	Ifi44	6.420697	6.665099	7.251917	7.068855
10408081	Hist1h1b	7.188938	7.218439	7.795668	7.874993
10500324	Hist2h2ac	11.05011	11.01361	11.67572	11.64247
10462623	Ifit1	6.924688	7.215146	7.692296	7.680799
10404069	Hist1h1a	6.912033	6.823286	7.501864	7.527173
10503098	Lyn	6.442467	6.593576	7.285135	7.015993
10403957	Hist1h4m	11.00908	11.06604	11.61906	11.70539
10408092	Hist1h4m	11.00908	11.06604	11.61906	11.70539
10474875	Casc5	4.941171	5.205425	5.691702	5.720735
10409220	Hist2h2aa	11.19541	11.17953	11.84357	11.80031
10402268	Lgmn	5.190751	5.305191	6.104516	5.683601
10564177	Snord116	9.782273	9.221884	10.03219	10.33153
10434778	Rtp4	7.693832	7.984877	8.508713	8.426792
10578413	Gm5346	5.349745	5.434859	6.164013	5.928683
10566678	Olfr485	7.772089	7.618953	8.443921	8.285086
10564163	Snord116	9.798928	9.248939	10.06616	10.36345
10564167	Snord116	9.798928	9.248939	10.06616	10.36345
10564171	Snord116	9.798928	9.248939	10.06616	10.36345
10564173	Snord116	9.798928	9.248939	10.06616	10.36345
10564175	Snord116	9.798928	9.248939	10.06616	10.36345
10564179	Snord116	9.798928	9.248939	10.06616	10.36345
10564181	Snord116	9.798928	9.248939	10.06616	10.36345
10564185	Snord116	9.798928	9.248939	10.06616	10.36345
10564187	Snord116	9.798928	9.248939	10.06616	10.36345
10564189	Snord116	9.798928	9.248939	10.06616	10.36345
10564191	Snord116	9.798928	9.248939	10.06616	10.36345
10564193	Snord116	9.798928	9.248939	10.06616	10.36345
10564195	Snord116	9.798928	9.248939	10.06616	10.36345
10564197	Snord116	9.798928	9.248939	10.06616	10.36345
10564199	Snord116	9.798928	9.248939	10.06616	10.36345
10564201	Snord116	9.798928	9.248939	10.06616	10.36345
10564205	Snord116	9.798928	9.248939	10.06616	10.36345
10564207	Snord116	9.798928	9.248939	10.06616	10.36345
10390707	Top2a	6.313098	6.405108	6.917174	7.114111
10603551	Cybb	6.514248	6.567877	7.2821	7.129576
10414590	Ear6	5.50648	5.453753	6.329952	5.988084
10445192	Rhag	5.005081	4.943321	6.297024	6.153286
10429564	Ly6a	9.74416	9.87386	11.07964	11.01913
10531126	Igj	9.144139	9.28445	10.45521	10.59454
10403009	Ighg	5.955611	6.043149	7.261371	7.407386
10545242	Igkv6-20	7.415425	7.362882	8.76242	8.699686
10487011	Gatm	5.975191	6.362548	7.527967	7.568853
10364535	Elane	6.22934	6.088137	7.642186	7.628078
10545194	Igkv4-59 /	8.37042	8.324935	9.852571	9.868026
10481627	Lcn2	5.110229	4.552481	6.525882	6.361879
10362896	Cd24a	7.189897	7.220691	8.805318	8.760952
10490923	Car2	6.623505	6.70909	8.236732	8.330407
10403060	Ighv1-72	9.271645	9.111667	10.89878	10.88278
10574023	Mt2	8.606429	8.832493	10.4396	10.38246
10545175	Igkv10-96	10.253	10.2876	11.99741	12.01908
10569017	Ifitm3	6.195415	6.515269	7.981906	8.222151
10545173	Igkv10-96	9.506121	9.578478	11.3659	11.29345
10589535	Ngp	5.084825	4.744139	6.813802	6.682652
10419151	Ear1	4.505959	4.571469	6.396954	6.52073
10403069	Ighm	7.093417	7.406801	9.221152	9.157598
10545217	Igkv5-43	5.853404	5.937531	8.208887	7.58828
10391649	Slc4a1	6.307032	6.244219	8.433184	8.182203
10403031	V165-D-J-(	7.211883	7.720778	9.491369	9.640642
10545215	Igk-V28	8.361155	8.754202	10.7611	10.69134
10402991	Igh-VX24	8.759705	8.820551	11.06357	11.08999
10545247	Igkv6-14	9.651943	9.838027	12.10501	11.94946
10375058	Hba-a2	9.126966	9.141016	11.59062	11.72808
10380174	Mpo	5.663897	5.405417	8.215822	7.973115
10375051	Hba-a1	9.120332	9.13518	11.61618	11.75747
10566254	Hbb-b1	8.313478	8.505347	11.22721	11.38285
10566258	Hbb-b1	8.199006	8.223135	11.2548	11.40889
10482030	Stom	4.995683	5.158698	6.13506	6.128513
10473384	Slc43a3	6.830793	6.898653	7.937542	7.909772
10568714	Mki67	6.503299	6.912265	7.793732	7.719363
10377405	Aurkb	6.029736	6.203056	7.153509	7.216507
10538871	Igkv1-133	10.73658	10.83158	11.93002	11.7627
10419154	Ear1	6.870685	6.795013	7.995491	7.843473
10543067	Asns	5.919641	6.253086	7.129128	7.174603
10560481	Fosb	8.434941	8.607538	9.545672	9.638711
10493382	Pklr	5.977372	5.996243	7.200623	6.968264
10524621	Oasl2	7.161689	7.40784	8.427088	8.351056

Probe Set I	Gene Symb	OVA IM-1	OVA_IM2	OVA_PEP1	OVA_PEP3
10494386	Hist2h2ab	10.58032	10.6065	11.28061	11.22537
10592816	Hmbs	7.285876	7.366234	8.041426	7.943898
10399710	Rsad2	5.646624	5.706326	6.279614	6.420312
10566626	Olfr715	6.872515	6.915068	7.503333	7.629521
10487340	Ncaph	5.965194	6.151942	6.805748	6.648603
10494395	Hist2h2aa	10.45082	10.51281	11.17758	11.11378
10408197	Hist1h2bh	8.502542	8.206079	9.029217	9.075888
10598507	Slc38a5	5.857539	5.527244	6.465425	6.347484
10478572	Ube2c	6.271914	6.289079	7.017772	6.928547
10594774	Ccnb2	7.482479	7.488847	8.14045	8.228992
10440160	Olfr192	5.097438	4.960466	5.631385	5.868066
10541307	Usp18	8.071953	7.975669	8.749675	8.723195
10458278	2010001M	6.581431	6.654547	7.265029	7.382614
10379727	Gm11428	5.428035	5.239229	6.053626	6.083939
10462796	Kif11	5.769148	5.622241	6.384153	6.477353
10366180	Gm4340	6.509685	6.038043	6.939265	7.1251
10551347	Blvrb	7.403169	7.307667	8.057964	8.114697
10403938	Hist1h2ao	11.21598	11.18911	11.91754	11.9188
10381187	Atp6v0a1	5.506072	5.306466	6.299344	6.016981
10408118	Hist1h2ag	11.03688	11.03162	11.77089	11.75583
10462618	Ifit3	7.504037	7.585826	8.283919	8.2768
10383756	Ifitm2	7.814249	7.855592	8.607857	8.544074
10404045	Hist1h2ao	11.14157	11.14158	11.88349	11.87006
10598175	Ear10	6.321431	6.164641	7.082469	6.927879
10408077	Hist1h2ak	9.870657	9.884653	10.67732	10.55715
10372648	Lyz2	5.765269	5.550415	6.416943	6.43749
10408072	Hist1h2ai	11.38985	11.34416	12.11364	12.10259
10408111	Hist1h2ah	11.33518	11.27975	12.05106	12.05029
10481304	Gfi1b	7.243656	6.982396	7.901143	7.872323
10451670	Tspo2	5.104416	5.004099	5.941382	5.705724
10404026	Hist1h2af	11.39225	11.35926	12.13057	12.11629
10403955	Hist1h2ao	11.32148	11.28478	12.05946	12.05155
10408085	Hist1h2an	11.05695	11.04336	11.81519	11.80632
10408094	Hist1h2ao	11.14209	11.11843	11.89371	11.89045
10602372	Alas2	5.782949	5.831751	6.607224	6.554678
10403941	Hist1h3h	11.35816	11.43147	12.16844	12.14394
10408246	Hist1h3a /	11.32796	11.37794	12.13447	12.12346
10593198	Fam55b	5.432575	5.578039	6.315976	6.278376
10374727	Bcl11a	6.882184	7.034981	7.751101	7.744086
10574259	Gpr56	6.226099	6.421151	7.137237	7.09022
10404028	Hist1h3g	11.34708	11.37873	12.16583	12.13317
10394978	Rrm2	6.481926	6.763412	7.461812	7.35609
10404049	Hist1h3d	11.38842	11.43767	12.20783	12.20364
10404065	Hist1h3b	11.39621	11.44188	12.22151	12.21968
10574027	Mt1	6.750691	6.684824	7.549112	7.540235
10408202	Hist1h3e	11.30255	11.35221	12.13292	12.13219
10408239	Hist1h3c	11.37181	11.40495	12.19534	12.19687
10408083	Hist1h3i	11.34135	11.37262	12.16301	12.17356
10406928	Cd180	6.49757	6.760734	7.394032	7.48318
10473399	Prg2	5.698457	5.687837	6.406479	6.65654
10494402	Hist2h3c1	10.90512	10.95603	11.75216	11.74663
10511258	Fam132a	6.515189	6.508718	7.16104	7.534299
10494405	Hist2h3b	11.33089	11.31137	12.14532	12.16168
10473367	Slc43a1	6.129727	6.107075	6.98951	6.947611
10474201	Lmo2	6.708872	6.807498	7.741296	7.470375
10404063	Hist1h2ab	4.046447	4.067121	4.84863	5.010705
10403959	Hist1h2br	9.928025	9.961787	10.80614	10.81833
10408087	Hist1h2br	9.928025	9.961787	10.80614	10.81833
10573054	Gypa	4.262294	4.35241	5.293264	5.086112
10397346	Fos	8.197109	8.396738	9.173129	9.153563
10559261	Cd81	7.72242	7.660189	8.513938	8.654209
10403018	Ighg3	10.93873	10.99162	11.8639	11.81752
10534389	Cldn13	5.042794	4.894657	5.959202	5.807876
10489463	Slpi	6.399591	6.269768	7.352597	7.145491
10402347	Ifi27l2a	7.140572	7.291275	8.004104	8.210841
10419156	Ear10	6.476151	6.305223	7.383574	7.244441
10413710	Nt5dc2	6.373025	6.49045	7.311954	7.367415
10574572	Ces2g	5.951755	5.828533	6.821517	6.823285
10404061	Hist1h2bb	6.751467	6.659152	7.523697	7.758821
10515848	Ermap	6.719698	6.216194	7.400573	7.483231
10420261	Ctsg	4.831854	4.552722	5.68084	5.637224
10364093	Derl3	6.739131	6.890574	7.788571	7.700519
10403948	Hist1h2bn	10.28543	10.30475	11.22633	11.22825
10408210	Hist1h2bf	10.2796	10.30092	11.2146	11.24415
10403943	Hist1h2bn	8.184858	8.036425	9.073481	9.100347
10438415	Iglv1	9.216059	9.370726	10.26516	10.23235
10403980	Hist1h2bj	10.60824	10.65145	11.60415	11.62265
10408070	Hist1h2bl	10.60506	10.6571	11.60753	11.63696
10545196	Igkv4-68	10.85706	10.92595	11.89001	11.9018
10538459	Aqp1	6.119696	5.881849	6.982844	7.153708
10403978	Hist1h2bk	10.42978	10.4946	11.47072	11.52333