NANOAPC DELIVER ANTIGEN, IL-2 AND CO-STIMULATORY MOLECULES TO ANTIGEN SPECIFIC T CELLS AND ACTIVATE VIRAL SPECIFIC T CELLS IN CHRONIC INFECTIONS

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DECLARATION

I declare that the work presented in this thesis is my own, and that I submit this work for my examination.

Mengya Liu

ABSTRACT

The study of the immune system has provided insight in the mechanism of protection induced by vaccination; primarily that most clinically protective vaccines are potent in generating neutralizing antibody responses. However, vaccination fails to protect against a wide range of acquired chronic infections caused by viruses, such as HIV, HBV and HCV. One of the major reasons for weak responses to therapeutic vaccine is the impaired function of effector T cells resulting from viral persistence. Although IL-2 can potently increase effect function of viral specific T cells, systemic administration of IL-2 induces organ pathology and expansion of Treg cells.

In this study, we have now developed a novel vaccine delivery system IL-2-nanoAPC delivering antigen-MHC complexes (pMHC), co-stimulatory molecules and IL-2 to antigen specific T cells. NanoAPC are derived from the endoplasmic reticulum (ER) membranes of human B cell line 721.221 engineered with selected HLA allele and IL-2 as the ER retention proteins. The IL-2-nanoAPC interacted with antigen specific T cells, induced immune synapses and expression of high affinity IL-2 receptor and enhanced effector function of antigen specific T cells, but did not affect bystander T cells and $Foxp3^+$ Treg cells. Together with pMHC, co-stimulatory molecules, the selective delivery of IL-2 not only increased the CD4 and CD8 T cell responses to viral antigens but also enhanced TCR proximal signalling and suppressed expression of PD1 molecules on IFNγ producing effector CD8 T cells. We also found that the co-induction of T helper responses by IL-2-nanoAPC in a mixed culture could increase CD8 T cell responses to viral antigen. The IL-2-nanoAPC effectively induced responses of CD4 and CD8 T cells from chronic HBV patients. The results demonstrate that selective delivery of IL-2, together with pMHC and co-stimulatory molecules, by nanoAPC to antigen specific T cells has potential to recover anti-viral immune responses in chronic HBV patients.

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIDS	Acquired Immunodeficiency Syndrome
APC	Antigen Presenting Cell
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CCR7	Chemokine (C-C motif) receptor 7
CD	Cluster of Differentiation
CPM	Count Per Minute
CPPs	Cell Penetrating Peptides
Crt	Calreticulin
CTL	Cytotoxic T cell Lymphocyte
CTLA4	Cytotoxic T Lymphocyte Antigen 4
DC	Dendritic cell
DMEM	Dulbecco's Modified Eagle's Medium
DNGR-1	DC NK C-type lectin group receptor-1
EDTA	ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunoabsorbent assay
EM	Electron microscopy
ER	Endoplasmic Reticulum
Erk	Extracellular signalregulated kinases
FBS	Foetal bovine serum
FITC	Fluorescein Isothiocyanate
FLT3L	FMS-related tyrosine kinase 3 ligand
Foxp3	Forkhead box P3
GFP	Green fluorescence protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
HBV	Hepatitis B virus

HC	Heavy chain
HCMV	Human Cytomegalovirus
HCV	Hepatitis C Virus
HEV	High Endothelial Venules
HIV	Human Immunodeficiency Virus
HLA-A2	Human leukocyte antigen with "A" serotype group
HRP	Horseradish Peroxidase
HSP	Heat Shock Proteins
HSV	Herpes Simplex Virus
ICAM-1	Inter-cellular adhesion molecule 1 or CD54
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IL	Intereleukin
ISCOMs	Immune-Stimulatory Complexes
iTreg	induced regulatory T cell
iv	intravenous (injection)
LFA-1	Lymphocyte function-associated antigen 1
LPS	Lipopolysaccharide
MARK	Mitogen-activated protein kinase
MDSC	Myeloid Derived Suppressor Cells
MHC	Major Histocompatibility Complex
min	minutes
MP-VV	Vaccinia Virus expressing Influenza A matrix protein
nanoAPC	Nano-size ER membrane mimic Antigen Presenting Cell
NBD	Nucleotide binding domain
NK	Natural Killer cell
NKT	Natural Killer T cell
nTreg	naturally occurring regulatory T cell
OVA	Chicken Ovalbumin
OVA-VV	Vaccinia virus expressing chicken Ovalbumin
P/S	Penicillin/ Streptomysin

PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PD1	Programmed cell Death 1
PE	Phycoerythrin
PFA	Paraformaldehyde
PFU	Plaque forming units
PMA	Phorbol Myristate Acetate
pMHC	Peptide/MHC complex
PMSF	Phenylmethylsulphonyl fluoride
PRR	Pattern recognition receptors
RBC	Red Blood Cell
RNA	Ribonucleic acid
RNAi	RNA interferance
rpm	rounds per minute
SD	Standard deviation
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis
SEM	Standard error mean
siRNA	small interfering RNA
SOCS1	Suppressor of cytokine signaling 1
TAP	Transporter associated with antigen processing
TB	tuberculosis
TCR	T cell receptor
TGF	Tumor Growth Factor
TH	Helping effector T cell
TipDCs	Tumor-necrosis factor (TNF)/ inducible nitric oxide synthase producing DCs
TLR	Toll-like receptor
Treg	Regulatory T cell
TTAs	tumor-associated antigens

β2m	β-2microglobulin

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INTRODUCTION

The plague of Athens in 430BCE is the earliest trail of immunity recorded by Thucydides. He noted that if the people that had recovered from a previous bout of the disease could nurse the sick without contracting the illness a second time (Figure 1.1).

Figure 1.1 Plague of Athens (430BC).

Couples of milleniums later, Edward Jenner (1749-1823) who is credited as the founder of immunology generated a vaccine against smallpox in 1798. However, the similar evidence also existed in traditional Chinese medicine. In 1000 AD the ancient Chinese custom suggest to use the powders made from the crusty skin lesions of patients recovered from smallpox inhaled by children to protect the disease. Even earlier, in AD 649, Sun Simiao, a famous Chinese doctor recorded that covers the people with the sick dog's brain (Cao, 2008) (Figure 1.2). Although they did not have any knowledge of the existence of pathogens, their experiences introduce concepts of vaccines and the foundation of immunology.

Figure 1.2 the discovery of vaccination.

Left, Dr Jenner discovered that milkmaids who came into contact with cowpox seemed to be immune from contracting smallpox. He inoculated a young boy with pus from cowpox blisters found on the hand of a milkmaid (in Latin cow = vacca, hence the term vaccination) to generate an immune response which crossreacted and offered protection against smallpox.

Right Bei Ji Qian Jin Yao Fang by Sun Simiao (581–682), published circa AD 649 and the related description of preventing the recurrence of rabies by "covering the people with the sick dog's brain" (red line) (Cao, 2008).

Then move to 19th-century the discoveries of Robert Koch showed that specific diseases were caused by specific pathogens. In the 1880s Luis Pasteur prepared a vaccine against rabies to treat a boy bitten by a rabid dog. Together with Koch and Jenner's experience, in the early of 1890s, Emil von Behring and Shibasaburo Kitasato (Figure 1.3) discovered that the serum of the animals immune to diphtheria or tetanus contained a specific antitoxic activity can protect against the toxin from the diphtheria or tetanus. This protection is due to specific antibody, which binds to the specific protein to neutralize their activity. Overall, the understanding of these mechanisms gave birth to immunology.

Figure 1.3 Emil von Behring and Shibasaburo Kitasato

1. The immune system.

The immune system, a system of biological structures and processes within an organism that protects against disease. The system consists of tissues organs, cells and molecules that work together to detect, distinguish and eliminate harmful pathogens by orchestrated mechanisms that are collectively called the Immune Response.

1.1 The cellular mediators of the immune system

All the cells in the immune system are derived from multipotent hematopoietic stem cells in the bone marrow. Before they give rise to all the blood cell types, they need to switch from highly undifferentiated progenitor cell to two different types of less differentiating plasticity stem cells, the common myeloid progenitor and the common lymphoid progenitor. The myeloid progenitor differentiates into platelets, erythrocytes (red blood cells), granulocytes, macrophages and myeloid dendritic cells (DC). The lymphoid progenitor differentiates into the T cells, B cells and Natural Killer cells (NK cells) (Figure1.4).

Granulocytes, also known as polymorphonuclear leukocytes, are a type of white blood cells which own their name by the presence of granulues in their cytoplasm. They are characterised by a short life span and are detected in increased numbers during immune responses. There are three types of granulocytes, neutrphils eosinophils and basophils, which are distinguished by the different staining properties of the granules. Granulocytes are part of the innate immune system; their role is principally phagocytic, as neutrophil is the most important cells in innate immune response, and also it provide as potent phagocytic cells, together with macrophages and dendritic cells.

T and B lymphocytes are distinguished from the other leukocytes, because of antigen receptors, and also differentiation from each other by maturation site. After T and B cells mature, they are circulate between the blood and the peripheral lymphoid

tissues to recognise the specific antigen. This characteristic makes them the principal mediators of an organised adaptive immune response launched against a specific pathogen. Unlike T and B cells, NK cells lack antigen specificity and are thought to recognise a missing self state through sets of activating and inhibitory receptors.

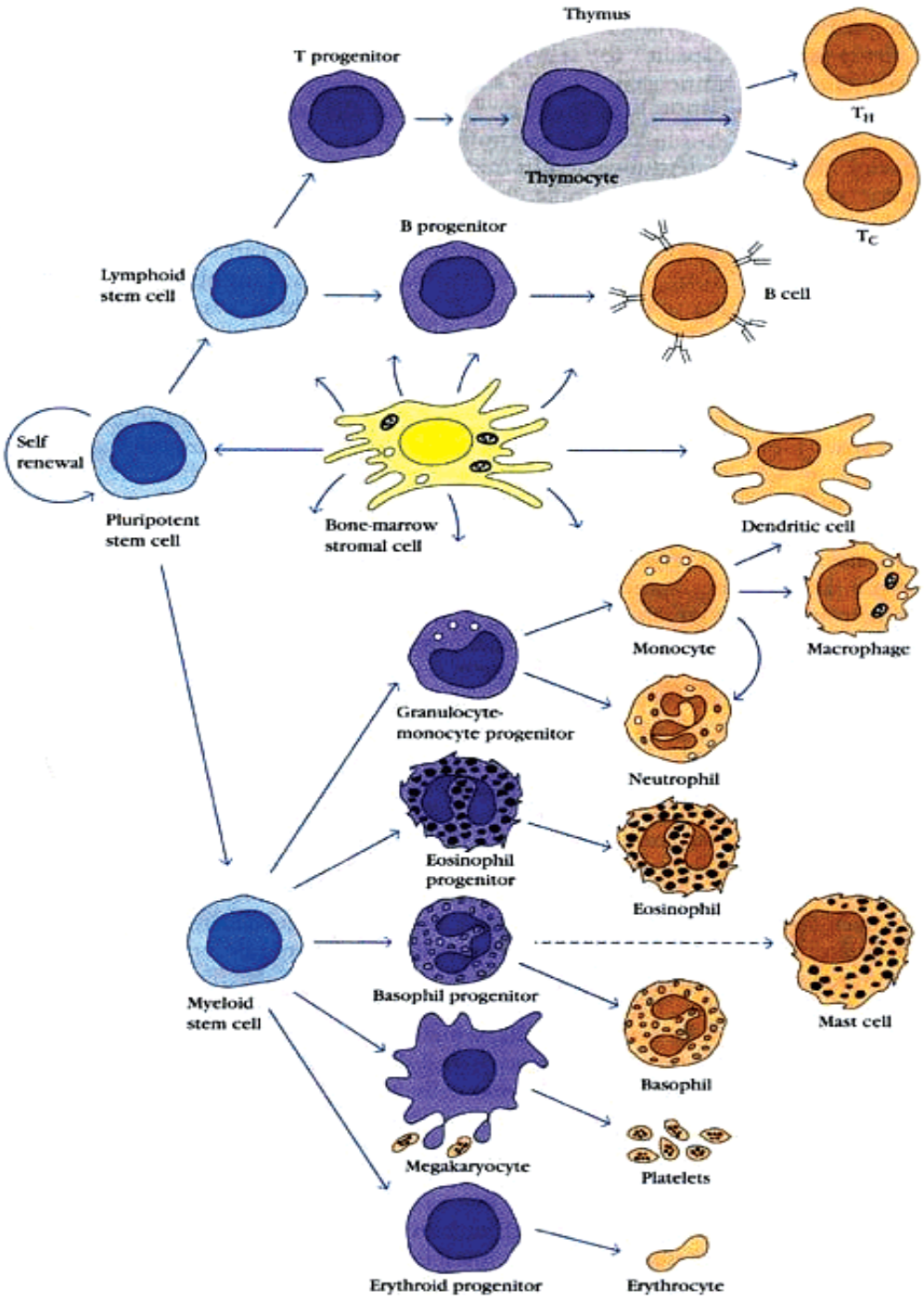

Figure 1.4 Origin of cells of the immune system.

All the cells of the immune system originate from a common pluripotent hematopoietic progenitor of the bone marrow. These highly undifferentiated cells divide to produce a common lymphoid progenitor that gives rise to NK cells, B and T lymphocytes, and a common myeloid progenitor that gives rise to erythrocytes (red blood cells), granulocytes (polymprphonuclear leukocytes), megakaryote (cells that produce platelets), macrophages and dendritic cells.

1.2 Other mediators of the immune system

Immunoglobulin also known as antibody is antigen recognition molecules of B cells. With similar structure, these proteins are produced by naïve B cells and activated plasma cell. Membrane bound immunoglobulin serves as B cell receptor (BCR). Immunoglobulin for the specific antigen is secreted as antibody by plasma cells. They have the capacity to bind specific pathogens or the toxic products.

Cytokines are secreted small proteins made by various cells in the body, which change the behaviour of cells through binding to specific receptors. Each cell type can produce only certain cytokines and in response to certain stimuli. Some cytokines have overlapping functions and some have opposite. Cytokines facilitate cell growth, chemotaxis, activation and enhance cytotoxicity. Interleukins (IL) are group of cytokines that are produced by leucocytes and act on leucocytes.

Chemokines are small chemoattractant proteins which bind to their receptors on leukocytes and induce directed chemotaxis. Normally the cytokine and chemokines released by activated macrophages initiate the process known as inflammation. And also they response to an activating stimulus which create a certain environment at the site of infection which helps the target antigen to the specific antigen presenting cells (APC), this initiates APC maturation, triggers cell migration to the peripheral lymphoid organs and regulates differentiation of T cells and antibody mediated immunity. The most important cytokines in innate immunity are TNF-α, IL-1, IL-10, IL-12, IFNα, β and γ, in adaptive immunity these are IL-2, IL-4, IL-5, TGF-β, IL-10 and IFNγ

1.3 The structure of the immune system.

Immune system is the organs, tissues, cells and molecules involved in both innate immunity and adaptive immunity. Lymphoid organs can be generally divided into central or primary lymphoid organs, where lymphocytes are generated. Peripheral or secondary lymphoid organs, is where adaptive immune response are initiated and lymphocytes are maintained. The central lymphoid organs are the bone marrow and the thymus where B cells and T cells develop respectively. The peripheral lymphoid organs are spleen, lymph nodes and lymphoid tissues associated with mucosa which are found in various locations in the body such as the gastrointestinal tract, thyroid, breast, lung, salivary glands, eye and skin. The lymphoid organs are interconnected and link with the blood via the lymphatic system. Peripheral lymphoid tissues are dynamic structures highly involved in the immune response and their appearance and function is finely coordinated.

1.4 The immune response.

The immune response consists of processes in which our body recognizes and defends itself against bacteria, viruses, and substances that appear foreign and harmful.

1.4.1 Innate immune response.

Innate immunity is the front line of host defence. It is assumed to be rapid, non-specific and identical qualitatively and quantitatively each time the same pathogen is encountered (Lanier and Sun, 2007). Similar like adaptive immune systems the innate immunity has ability to distinguish between self and non-self molecules. Innate immunity relies on receptors such as toll-like receptors (TLR) that recognize conserved patterns on different classes of pathogens to trigger an inflammatory response which limits pathogen invasion (Janeway and Medzhitov 2002) (Akira, et al. 2006) (Cooper and Alder 2006). Innate immunity largely involves three types of leukocytes, granulocytes, dendritic cells and macrophages which bind to pathogens internalise them finally to be killed. Because they use

primitive non-specific recognition systems they can bind to a variety of microbial products and induce phagocytosis (Janeway et al, 2001). And also the innate immune system provides a humeral response via cytokine such as IL-1 to activate the B cells to produce antibody. So innate immune response largely involve professional antigen presenting cells, macrophages, dendritic cells and B cells, which can trigger adaptive immunity (Figure 1.5).

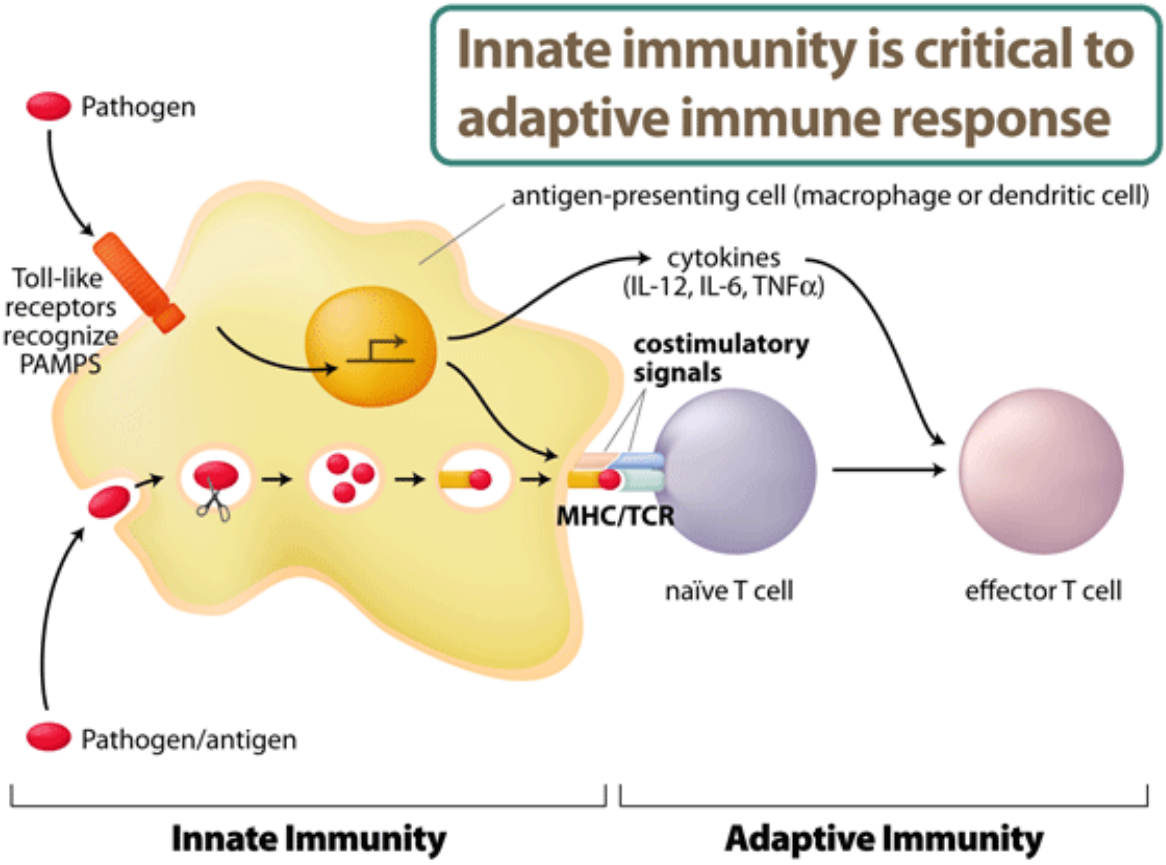

Figure 1.5 Innate immunity is critical to adaptive immune response.
Innate immunity is based on the recognition of invariable pathogen associated molecular patterns (PAMPs) by Toll-like receptors, and on phagocytosis. If the innate response fails to eliminate the pathogen, activated antigen presenting cells bearing this antigen trigger an adaptive immune response; they travel to the secondary lymphoid tissues where they present antigenic peptides and co-stimulatory signals to naïve T cells. After engaging antigen and costimulatory molecules, naïve T cells will be activated, expand and finally differentiated to effector T cells.

1.4.2 Adaptive immune response.

Adaptive immune response is the response of antigen-specific lymphocytes to antigen, including the development of immunological memory. B cells are one of the major cell types involved in adaptive immune responses. By production of antibodies, B cells mediate the humoral response. The antibodies produced by antigen specific B cells bind to pathogens and eliminate the pathogens by three main ways: a) They inhibit the toxic effects or infectivity of pathogens by binding to them (neutralisation). b) By coating the pathogen they enable phagocytic cells to recognise and kill it (opsonization). c) Antibodies can also trigger complement activation, which strongly enhances opsonization and directly lyses some bacteria (Janeway et al, 2001).

T cells are essential component of adaptive immune responses and have a wider range of activities. They are divided into two categories based on coreceptor molecules, the $CD8^+$ T cells and the $CD4^+$ T cells. These two subclasses serve different functions upon activation. $CD8^+$ T cells differentiate into cytotoxic T cells which act by direct killing of the infected cell, either by delivering cytotoxic proteins or by triggering the target cell to apoptosis (Lim et al, 2000). $CD4^+$ T cells differentiate into helper T cells which activate other cells; they either activate macrophages to kill pathogens (T helper I) or they activate B cells to produce antibody (T helper 2) (Janeway et al, 2001). T cells can only recognise antigenic peptide when in the form of complex with MHC molecules on the surface of antigen presenting cells to the two different subsets of T cells. MHC class I present peptides to $CD8^+$ T cells and MHC class II presents peptides to $CD4^+$ T cells (Moss and Khanna, 1999).

T cell activation requires T cell receptor recognition of peptide/MHC ligands on antigen presenting cells, plus co-stimulation resulting from the interaction of various molecules on T cells with complementary molecules on antigen-presenting cells (mainly dendritic cells) (Chambers and Allison, 1997). Contact with these ligands drives T cells to proliferate and differentiate into effector T cells (Figure 1.6).

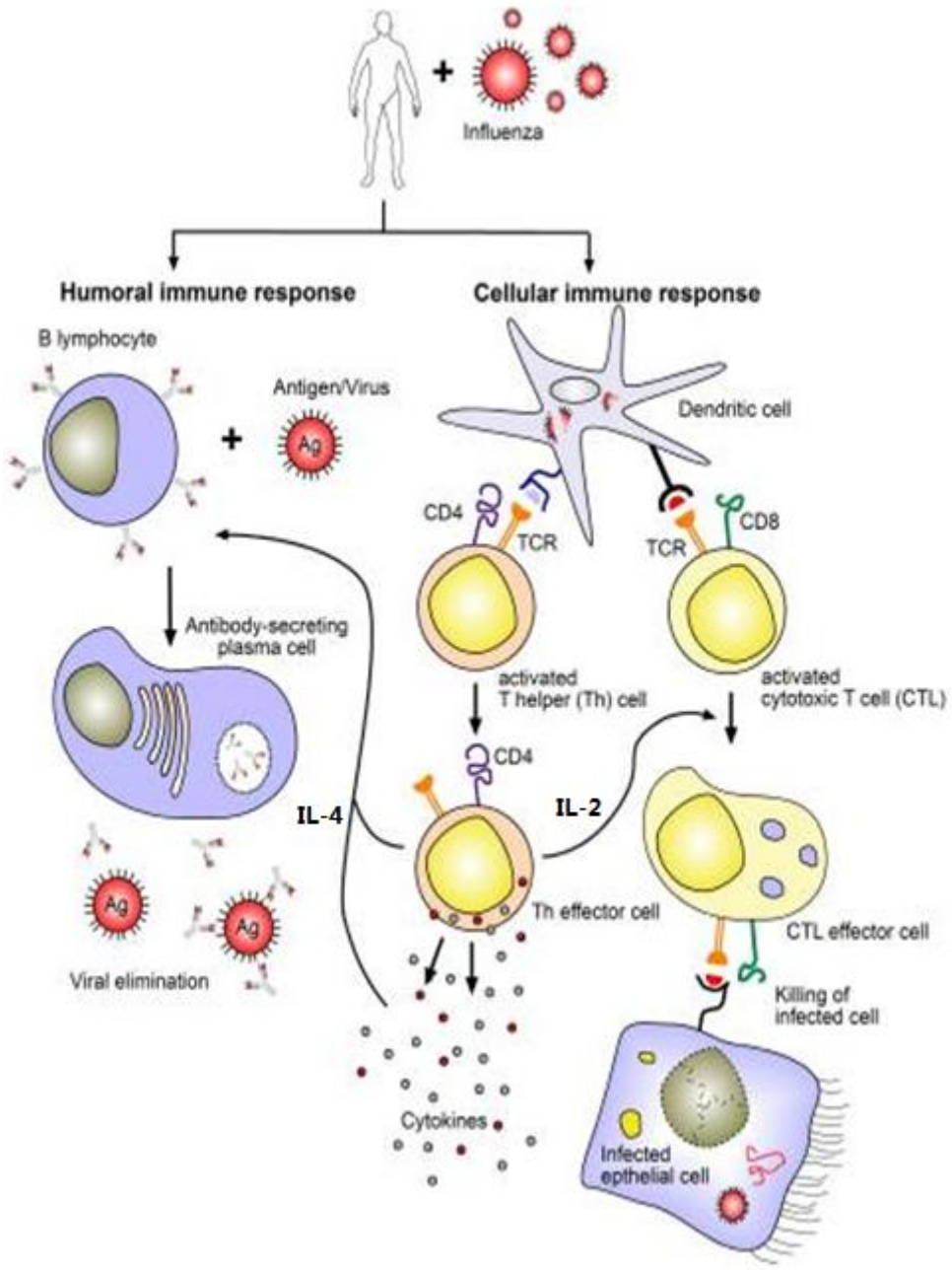

Figure 1.6 Adaptive immune response.

The humoral branch of the immune system comprises B-lymphocytes (left), which after interaction with virus differentiate into antibody-secreting plasma cells. The cellular response (right) starts with antigen presentation via MHC I (black) and II (blue) molecules by dendritic cells, which then leads to activation, proliferation and differentiation of antigen-specific T cells (CD4 or CD8). These cells gain effector cell function to help directly, release cytokines, or mediate cytotoxicity following recognition of antigen.

1.4.3 Thymocytes differentiation and central tolerance

1.4.3.1 Thymocytes differentiation

T cell maturation in thymus is strictly controlled process. T cell arise from hematopoietic stem cells, migrate to the thymus (Starr et al., 2003). The thymus is an organ that supports the differentiation and selection of T cells (Takahama, 2006). Figure 1.7 shows the overall scheme of the T cell development within the thymus.

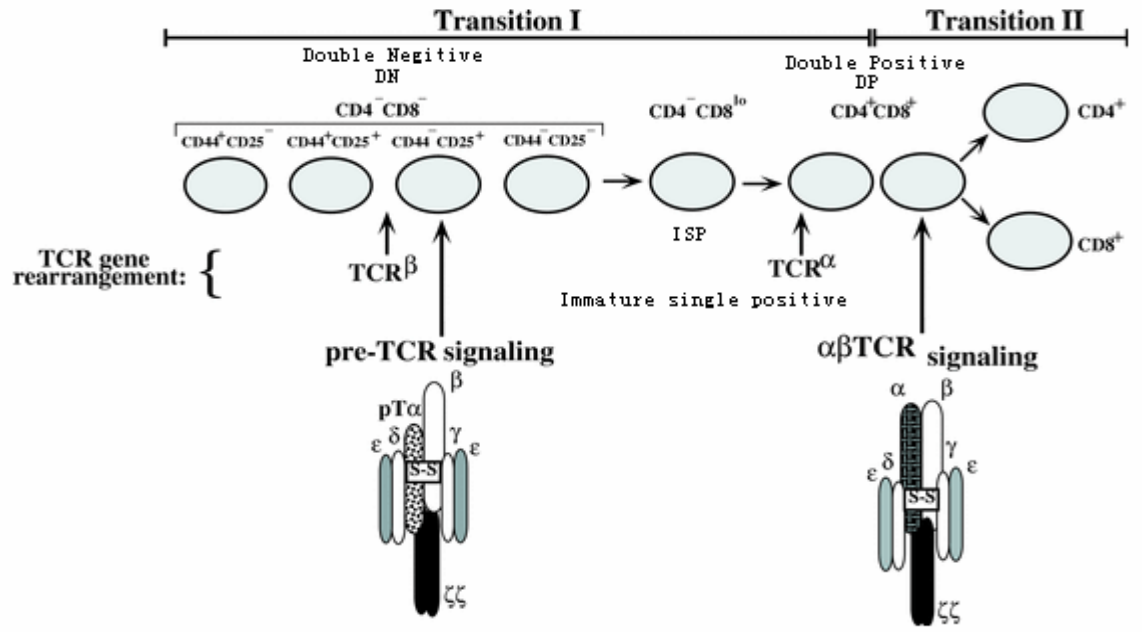

Figure 1.7 Overall summary of T cell development in the Thymus

Lymphoid Progenitors arise in the bone marrow and migrate to the thymus. In the thymus these cells lose the potential for B cell and natural killer cell development (Michie et al., 2000). The progenitor upon entry, lack expression of CD4 and CD8 and are called double negative (DN). DN thymocytes can be subdivided further into four sequential stages of differentiation, which are identified by their surface expression of CD44 and CD25: DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4;$CD44^{-}CD25^{-}$ (Godfrey et al., 1993). As the cells progress from DN2 to DN4 stages, they express the pre-T cell receptors (pre-TCR), which is composed of the non-rearranging pre-Tα chain and a rearranged TCR β-chain, forming a pre-TCR complex (von Boehmer and Fehling, 1997). Only the cells that

succeed in in-frame rearrangement of the gene encoding the TCR β-chain are selected for further differentiation beyond this DN3 stage.

In the thymic cortex, the successful expression of the pre-TCR complex on the thymocytes along with the interaction of Notch molecules with their legends such as delta-like (DII) 1 and delta-like 4 (DII 4) initiate the signals for further development to double positive (DP) thymocytes that express the TCRαβ antigen receptors. This is the first checkpoint of T cell development at the DN3 stage (Takahama, 2006). During the transition of DN4 to DP the pre-TCR expression leads to substantial cell proliferation. From the large number of DP thymocytes, the DP thymocytes that are best suited to function in the host environment is permitted to mature and migrate to peripheral lymphoid tissues. This selection is characterised by four processes; death by neglect, negative selection, positive selection and lineage-specific development (Figure 1.8)

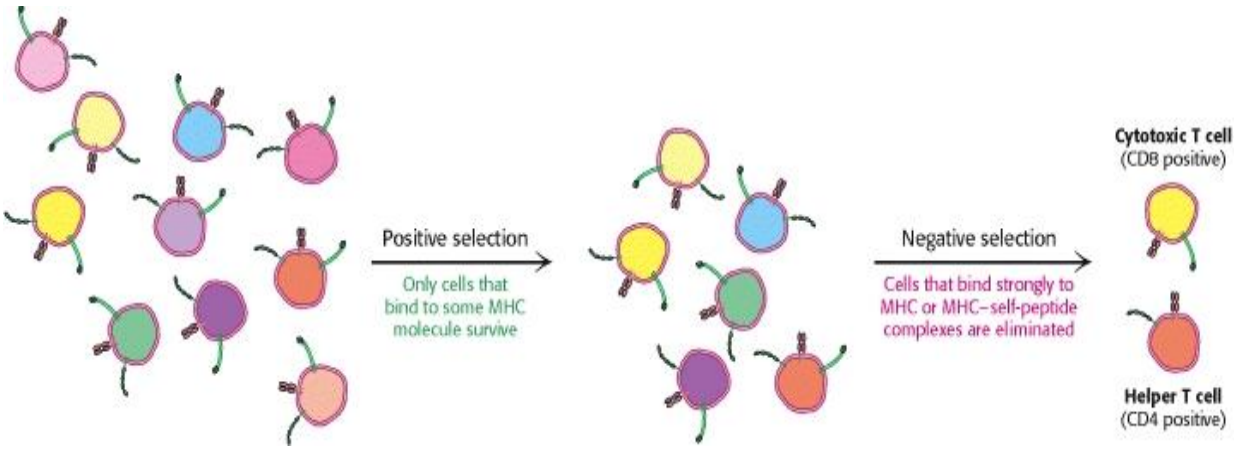

Figure 1.8 Positive selection processes, negative selection process

Death by neglect is initiated when there is too little signalling between the TCR and the self-peptide-MHC ligands, which results in delayed apoptosis. Too much signalling can promote acute apoptosis, which is caused in negative selection. Positive selection occurs when the signalling is at an intermediate level, where TCR signalling initiates effective maturation (Germain, 2002).

Positively selected DP thymocytes are induced to differentiate into single positive thymocytes, which are $CD4^{+}CD8^{-}$ or $CD4^{-}CD8^{+}$ (Hollander and Peterson, 2009). These thymocytes spend approximately 12 days in the medulla before travelling to the cortex. During this period the SP thymocytes go through a maturation process. This is accompanied by further deletion of self-reactive thymocytes that have escaped negative selection in the cortex. This process is particularly important in central tolerance to tissue specific antigens (Kyewski and Derbinski, 2004).

As well as the deletion mechanism that ensures self-tolerance, it is also thought that the medulla is the place for the production of regulatory T cells (Takahama, 2006). Treg cells have emerged to play a critical role in suppressing the response of the immune system to self-antigen (Kuhn et al., 2009).

1.4.3.2 Central tolerance

Central tolerance is tolerance to self antigens that is established during lymphoid cell development in central lymphoid organs. It is associated with the deletion of autoreactive clones. For T cells, this occurs in the thymus. Central tolerance mechanisms eliminate newly formed strongly autoreactive lymphocytes before they migrate to peripheral lymphoid organs. However, some self-reactive T lymphocytes escape central tolerance because some of the cognate self-antigens are not expressed in thymus. These autoreactive cells are inactivated in the periphery. It is unlikely that mechanisms of tolerance would be sufficient to compensate for the failure to remove self-reactive lymphocytes during their primary development. It is important that there is some mechanism to prevent auto reactivity of the T cells after they have emigrated from the thymus. This is achieved through the T cell tolerance process. Although most of the self-reactive T cells are eliminated in the thymus, negative selection has limitations and additional mechanisms of tolerance are required to limit autoimmunity (Bandyopadhyay et al, 2007). Therefore the self-reactive T cells that escape the negative selection must be kept under control in the periphery. To simplify the tolerance process the peripheral tolerance can be divided

into those that act directly on the responding T cell and those that evoke additional subsets of cells, including dendritic cells and regulatory cells (Walker and Abbas, 2002) (Figure 1.9).

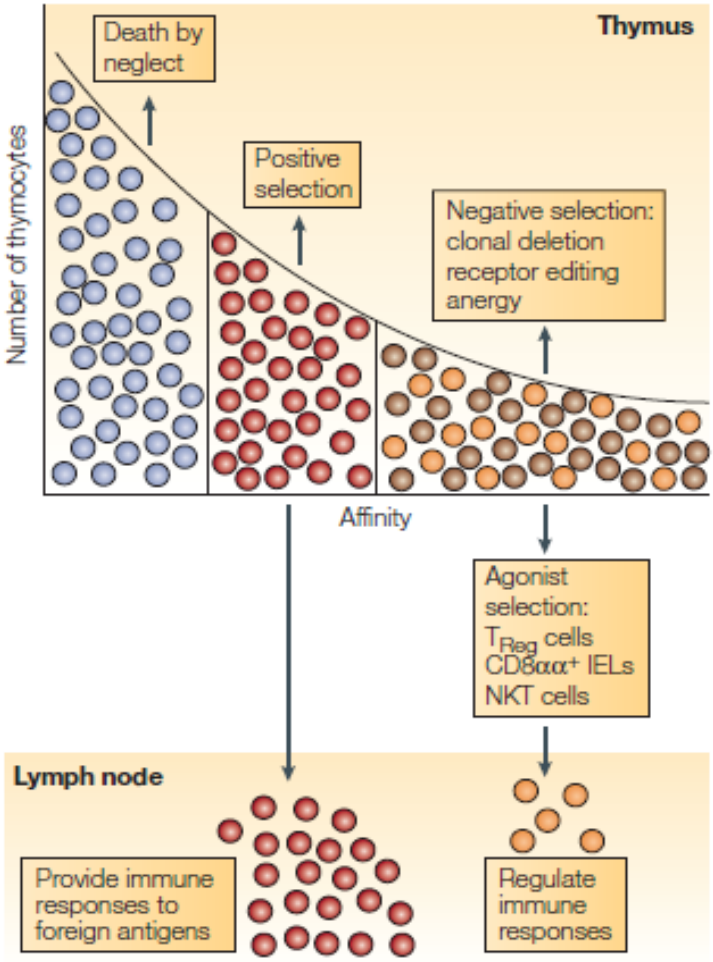

Figure 1.9 Central-tolerance mechanisms

The affinity of the TCR for self-peptide–MHC ligands is the crucial parameter that drives developmental outcome in the thymus. Progenitors that have no affinity or very low affinity die by neglect. This is thought to be the fate of most thymocytes. If the TCR has a low affinity for self-peptide–MHC, then the progenitor survives and differentiates, a process that is known as positive selection. If the progenitor has a high affinity for self-peptide–MHC, then several outcomes are possible. (Kristin et al, 2005)

2. The Major Histocompatibility complex (MHC)

2.1 MHC genes and molecule.

The protective function of T cells depends on their ability to recognize cells that are harboring pathogens or the internalized pathogens or their products. They are glycoproteins present on the cell surface of host cells. The MHC molecules which are proteins involved in antigen presentation to T cells by delivering and displaying antigenic peptide on the surface of cell membranes.

The MHC is a cluster of genes on human chromosome 6 or mouse chromosome 17. The Human Leukocyte Antigen (HLA) is the genetic designation for the human MHC. It extends over 4 centimorgans of DNA and contains over 200 genes, more than 40 of which encode leukocyte antigens. The rest are an assortment of genes that are not evolutionary related to the HLA genes themselves, although some are involved with them functionally (Klein and Sato, 2000). Some of them which have nothing to do with immune recognition which has been suggested that these phenotypes are due to accumulated mutations or genetic divergence. The MHC genes that are involved in the immune response are the MHC class I and class II genes.

In the genetic organisation of the MHC in human and mouse, there are three classes I α chain genes in humans, called HLA-A, -B and –C (the gene for β2-microglobulin is located in a different chromosome). There are also three pairs of MHC class II α- and β-chain genes, called HLA-DR, -DP and –DQ. However in many cases the HLA-DR cluster contains an extra β-chain gene whose product can pair with DRα chain. This means that the three sets of genes can give rise to four types of MHC class II molecule (Beck and Trowsdale, 1999). The expression of MHC alleles is codominant. The class II region also include the TAP, LMP, DM, and DO genes.

All the MHC class I and II molecules can present peptides to T cells (Figure 2.1), MHC class I molecules are expressed on all nucleated cells. By contrast, MHC class II expression is restricted in the professional antigen presenting cells, namely B cells, macrophages and dendritic cells. To compete with rapidly evolving pathogens the MHC is highly polygenic and polymorphic. These are important features as they allow the immune system the ability to present a wide array of peptides, preventing one disease wiping out the entire species. Much is known about MHC-peptide complexes, but each molecule binds a different range of peptides. Thus the presence of several different genes of each MHC class means that any individual is equipped to present a much broader range of peptides than if only one MHC molecule of each class was expressed at the cell surface (Janeway et al, 2001). In this way the host has a much greater chance to recognise and eliminate pathogens.

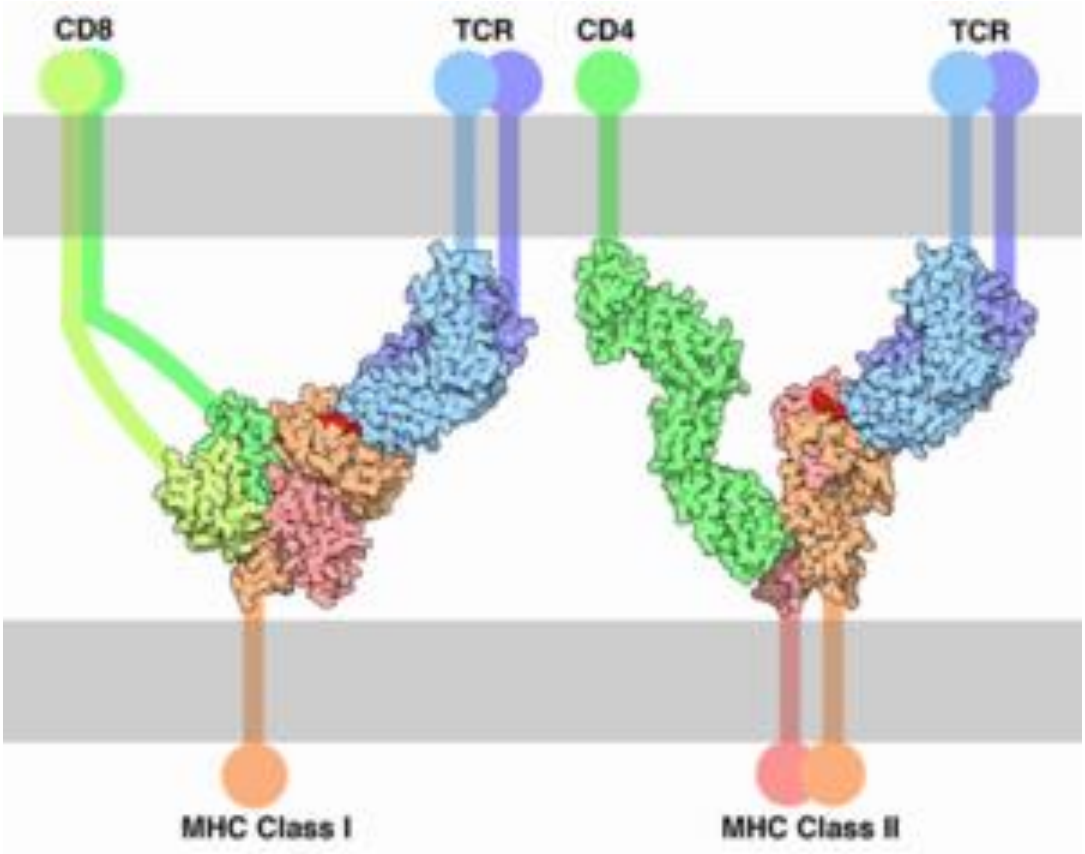

Figure 2.1 The MHC molecule bind to the T cell receptor

The MHC class I molecule (left) on most cells binds to the T-cell receptor (TCR) and CD8 receptor. The MHC Class II molecule (right) on immune cells binds to the TCR and CD4 receptor on other immune cells.

2.2. MHC polymorphism

An outstanding feature of the MHC molecules is their extensive polymorphism. There are multiple variants of each gene within a population as a whole; the MHC genes are in fact the most polymorphic genes known. There is unwarranted satisfaction with the view that MHC polymorphism evolved because there was a selective advantage in having a variety of MHC proteins to bind a variety of peptide subsets for presentation to T cells (Forsdyke, 1991).

There are more that 200 alleles of some human MHC class I and II genes, each allele being present at a relatively high frequency in the population. So there is only a small chance that an individual will be homozygous at a specific MHC locus. Thus the extensive polymorphism at each locus has the potential to double the number of different MHC molecules expressed in an individual and thereby increases the diversity already available through polygeny.

Allelic variation occurs at specific sites within the MHC molecules (Figure 2.2). The polymorphic residues are found at the peptide-binding groove. More specifically, diversity occurs at the α1 and α2 domains of class I molecules and β1 domain of class II molecules. Among the polymorphic residues there are several conserved residues within the antigen-binding sites. It has been demonstrated by Geluk et al (1993) that these reside is crucial for peptide binding and recognition by the T cell receptor, and has been conserved for over 30 million years!

MHC polymorphism appears to have been strongly selected by evolutionary pressures. Because selectively neutral polymorphisms are not expected to be maintained for long periods of time, the long persistence of MHC polymorphisms is evidence that they are selectively maintained (Hughes and Yeager, 1998). Evidence from the house mouse (Mus) suggests that the extreme diversity of genes of the

MHC results from three different forms of selection: i) pathogen driven selection (hypotheses of heterozygote advantage and rare allele advantage), ii) sexual selection (hypotheses of mating preferences, infertility and foetal loss and scarcity of homozygotes) and iii) inbreeding depression, the most important being the first one (Potts and Wakeland 1993) (Jeffery and Bangham, 2000) (Grosberg and Hart,2000) (Gruen and Weissman, 1997).

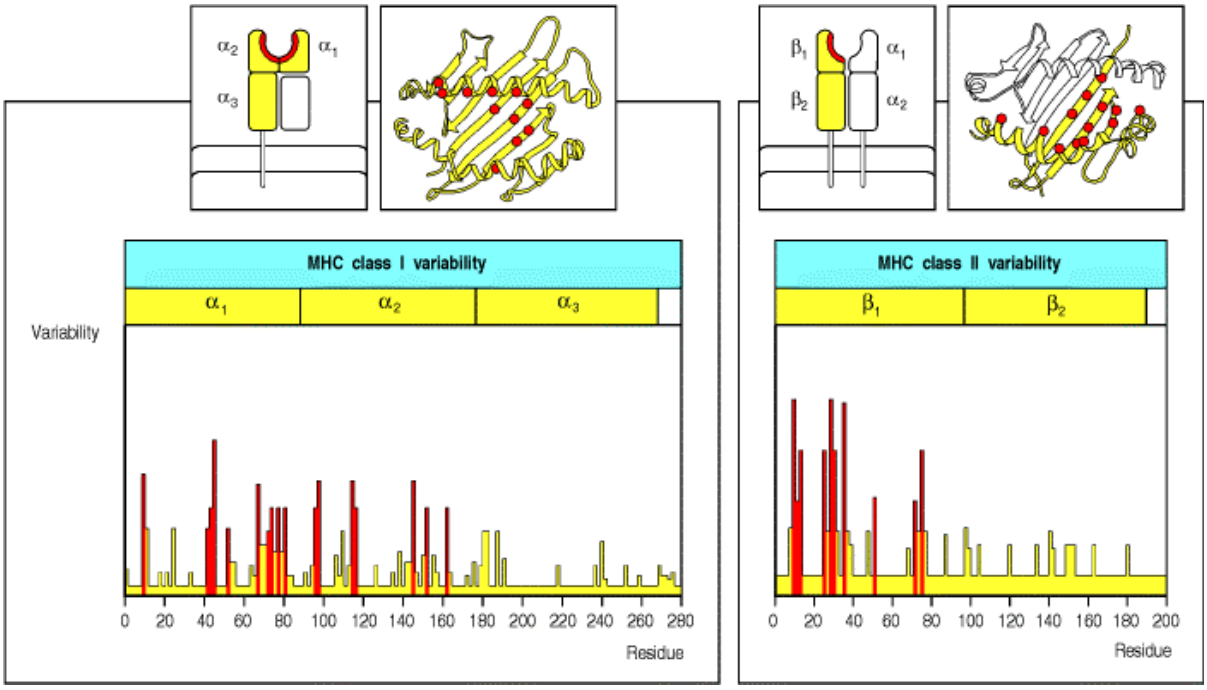

Figure 2.2. Allelic variation occurs at specific sites within MHC molecules.

2.3 The structure of MHC molecules.

As mentioned before, the overall structure of the two classes of MHC molecules is very similar. However they bear some critical differences that allow them to serve their distinct functions in antigen presentation.

2.4 MHC class I molecule.

There are three components consist of the structure of a mature MHC class I molecules which is a heterodimer of the polymorphic heavy chain (HC) noncovalently associated with β-2microglobulin (β2m), where does not span the

membrane and then loaded with the antigenic peptide, all of which are essential for the formation and stability of a functional MHC class I complex. The heavy chain (43kDa) α chain, a transmembrane glycoprotein, folds into three different structural domains (α-1, α-2, α-3), a transmembrane segment and a cytoplasmic tail. The folding of α1 and α2 domains creates a long cleft or groove, which accommodates the peptide antigens (Klein and Sato, 2000). The peptides that can bind to a given MHC molecule have the same or very similar amino acid residues (‘anchor’ residues) at two or three particular positions alone the peptides in the MHC molecule (Bouvier and Wiley, 1994). MHC ckass I molecules bind short peptides if 8-10 amino acids by both ends.

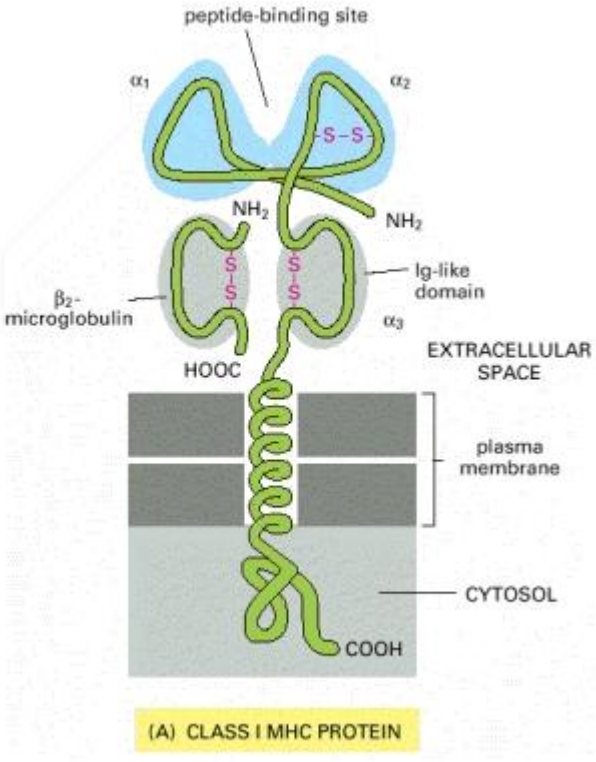

Figure 2.3 MHC class I.

Scematic representation shows the MHC class I molecule is a heterodimer of a transmembrane α chain bound non-covalently to β2-microglobulin, which does not span the membrane. The α1 and α2 segments of the heavy chain fold together to create the peptide-binding site.

2.5 MHC class II molecule.

The MHC class II molecule (Figure2.4) is composed of two transmembrane glycoprotein chain, α and β, which are both encoded within the MHC region of the genome. Each chain has two domains and the two chains together form a compact four-domain compact structure, similar to that of MHC class I molecule (Fremont et al, 1996). The two domains forming the peptide-binding cleft are contributed by different chains and are therefore not joined by a covalent bond (Klein and Sato, 2000). The major difference between the two molecules is that for MHC class II the membrane distal domains are not joined by covalent bonds, thus forming a peptide-binding groove that is open at both ends. As a result, the peptide ends are not bound into pockets, but instead the peptide lies in an extended conformation between the two chains and binds by interactions along the length of the binding groove. Because the peptide is bound by its backbone and allowed to emerge from both ends of the binding groove, the length of the peptides that bind to MHC class II molecules are at least 13 amino acids long and can be much longer than of those that bind to MHC class I molecules, in most cases between 13-17 amino acids long (Rammensee, 1995) (Sofra, 2009). Similarly to MHC class I molecules, the sites of major polymorphism in the MHC class II molecule that determine antigen binding are located in the peptide binding cleft; different allelic variants of MHC class II molecules bind different peptides. However, the more open structure of the MHC class II peptide-binding site and the greater length of the peptides bound in it allow greater flexibility in peptide binding (Sofra, 2009).

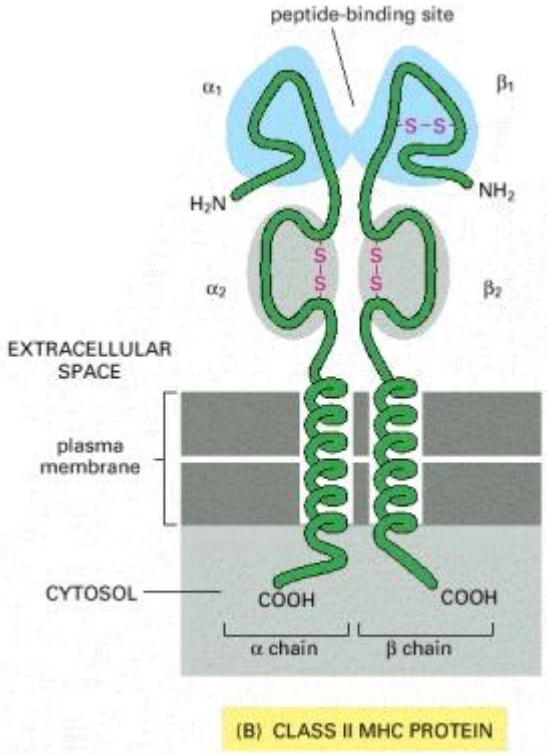

Figure 2.4 MHC class II.

Schematic representation shows MHC class II is formed by two transmembrane glycoprotein chains, which are not covalently bound. Thus, the peptide binding site that is formed is open at both ends**.**

2.6 Peptide-MHC complex.

MHC molecules assemble with short antigenic peptides to form antigenic peptide MHC complexes. TCR interacts both the antigenic peptides and MHC to respond effectively to antigen under MHC restriction. As mentioned before the mature MHC molecules consist of three components: the polymorphic heavy chain (HC), (light chain) and the antigenic peptide which is a necessary and integral part of a stable MHC molecule structure; this serves to prevent random peptide exchanges at the cell surface, thus making the peptide/MHC complex a reliable indicator of infection or of uptake of a specific antigen.

MHC class I molecules are translocated during synthesis into the lumen of Endoplasmic reticuculum (ER). Here the chains must fold correctly and assemble with each other if necessary, before the complete protein can be transported to the cell surface. Thus the peptide-binding site of the MHC class I molecule is formed in the lumen of ER and is never exposed to the cytosol. Newly synthesized MHC class I molecules are held in the ER until they form stable peptide/MHC class I complexes (Townsend et al, 1989), so antigenic peptides are transported into the lumen of ER. The antigenic fragments derive from proteins found in the cytosol and they are formed by degradation of larger proteins by the proteasome, which is protein degradation machines located in the nucleus and the cytoplasm, before they enter the ER through the transporters associated with antigen processing (TAP). The TAP transporter is responsible for antigenic peptide translocation. This is a heterodimer of TAP1 and TAP2 proteins, which are members of the ATP-binding cassette (ABC) superfamily and are characterised by the ABC unit. ABC proteins mediate ATP-dependent transport of ions, sugars, amino acids, and peptides across membranes in many types of cells, including bacteria (Klein et al, 1999).

In the ER, every step of the assembly and forming of the MHC class I molecule undergoes extensive quality control by a wide array of chaperones and specific proteins. Thus there is a recognition point on class I heavy chain molecules single N-linked glycan for the ER membrane chaperone calnexin and soluble chaperone calreticulin, both of which aid in the proper folding and retention of the class I molecules (Amy et al, 2002). Initially, partly folded MHC class I α chains bind to a chaperone protein, calnexin, and form a complex that binds to β2m. The MHC class I α: β2m dimmer is then released from calnexin to become associated with the loading complex, which consists of the chaperone molecules calreticulin and Erp57, the transporters associated with antigen processing (TAP1 and TAP2) and tapasin as the specific chaperone only located at the membrane of the ER. Tapasin bridges TAP and MHC class I to facilitate the delivery of suitable TAP-associated peptides and their loading on the MHC class I. A stable peptide/MHC class I complex is then allowed to travel to the cell surface (Figure 2.5).

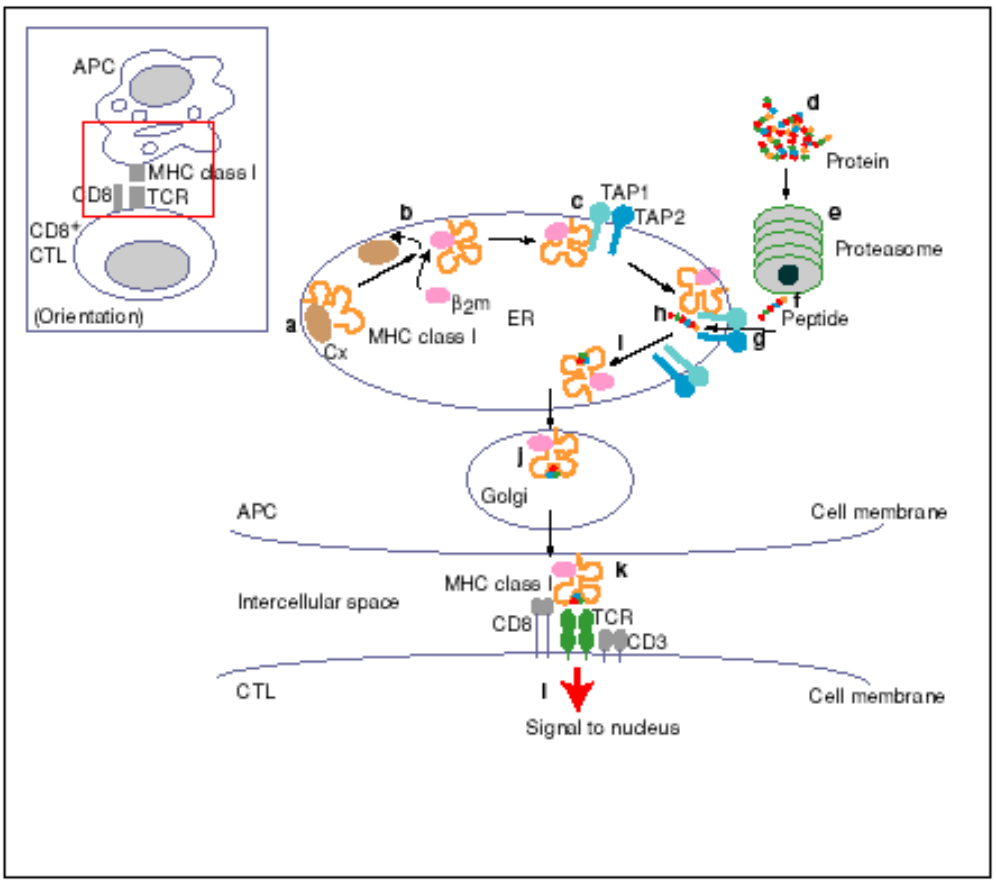

Figure 2.5. Degradation and Transport of antigens that bind to MHC class I molecules
Schematic representation of peptide loading mechanism on MHC class I molecules. Peptides generated by the immunoproteasome are transported through the ER membrane by TAP. Tapasin links TAP to MHC class I heavy-chain/β2- microglobulin dimers to facilitate peptide loading onto MHC class I. Other components of the loading complex, such as calreticulin (Crt) and ERp57, ensure correct folding and assembly of the MHC class I with the peptide.

Conversely, MHC class II antigen presentation pathway is geared towards the presentation of extracellulaely derived antigens or antigens generated within the endolysosomal pathway. MHC class II expression is confined only in a small subset of antigen presenting cells grouped as professional APC, which include macrophage, B cells and dendritic cell. Like MHC class I, MHC class II molecule is also translated into ER, but class II molecules has to transport to the lysosome associated endosomal compartment with a third transmemebrane glycoprotein invariant chain (Ii), which protects the peptide binding site and targets delivery of MHC class II

molecules to the acidic endosomal compartment. As a molecular chaperone for class II this invariant chain also provide targeting information in its cytoplasmic N terminus to direct the next step for class II /Ii complex (Amy et al, 2002). Once they arrive in the endosomal compartment, the MHC class II will form stable complexes with antigenic peptides derived from extracellular proteins before it is allowed to reach the cell surface. After transport into the acidified vesicle, acid proteases such as cathepsin S sequentially cleave the invariant chain leaving only the short fragment, called CLIP that blocks the MHC class II α: β peptide binding groove. The class II-associated invariant chain peptide (CLIP) remains bound to the MHC class II molecule until it encounters suitable peptides; upon peptide competition it is dissociated or displaced to allow peptide binding to MHC class II. There are other proteins also playing an important role called HLA-DM (DM) which form a similar structure of that class II molecule. It like the tapasin with class I molecules, DM stable class II molecule and confer upon the class II molecule with high affinity bind with peptide into the peptide-binding groove. Finally stable peptide/MHC class II complexes travel to the cell membrane where they are presented to T cells. This process is slower than class I; new synthesized peptide/MHC class II molecules present to cell surface approximate 2-4 hours (Figure 2.6).

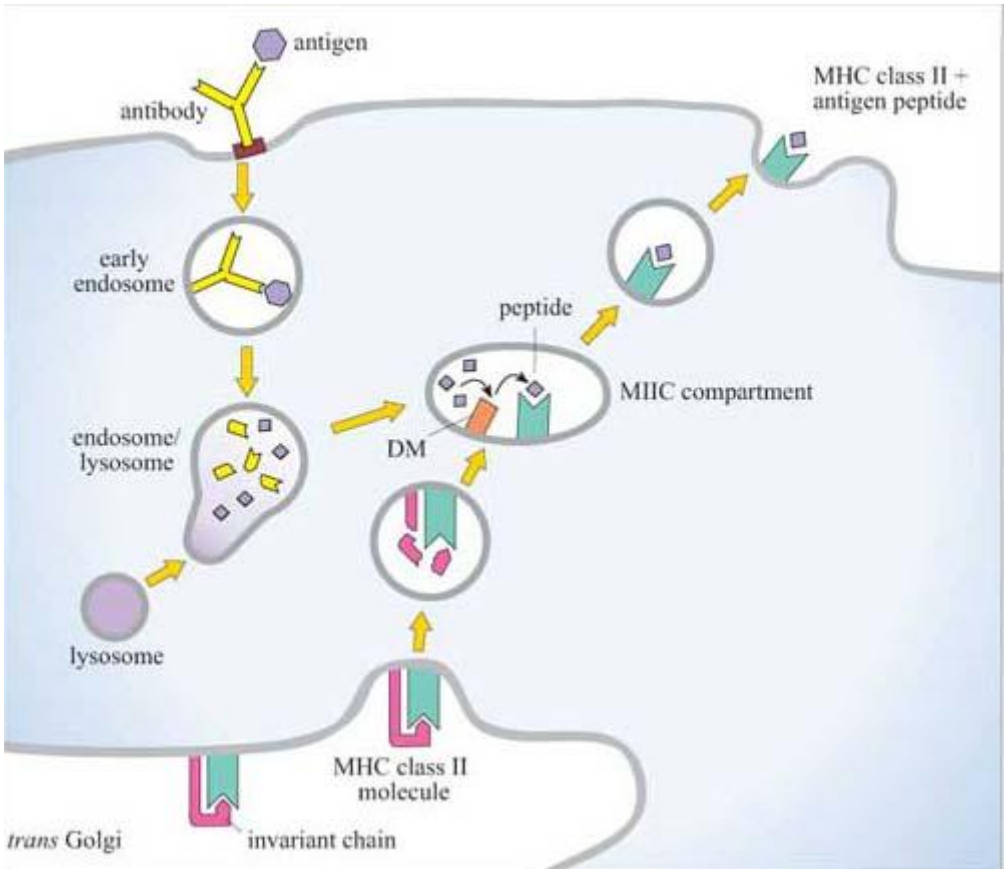

Figure 2.6. Antigen **degradation, transport and binding to MHC class II molecules**

3. T cell activation

T cell activation is initiated by interaction of the TCR-CD3 complex with a processed antigenic peptide bound to either a class I ($CD8^+$ cells) or class II ($CD4^+$ cells) MHC molecule on the surface of an antigen presenting cell. $CD8^+$ and $CD4^+$ cells also require a co-stimulating boost called B7, and then following activation from the APC cells there are a number of substances called cytokines trigger the rapid growth and proliferation of more T cells and are thought to increase the cytotoxic effects of the $CD8^+$ cells. So three signals are required for T cell activation, which are peptide- MHC complex binding with TCR, co-stimulate molecule and cytokines (Figure 3.1).

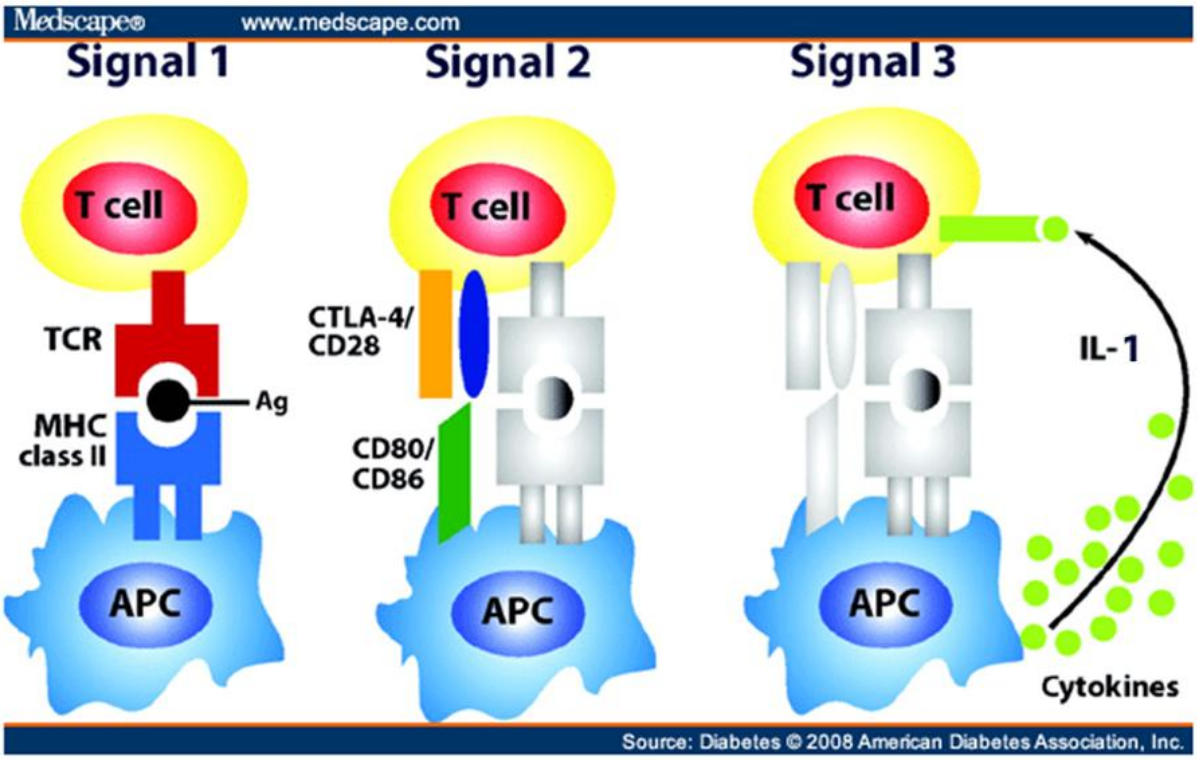

Figure 3.1 three signals induce T cell activation

The outcome of specific antigen recognition by T cells (signal 1) is determined by co-stimulation (signal 2) delivered by fully activated APCs and the presence of inflammatory cytokines (signal 3). Activation of naïve T cell in the presence of all three signals leads to full effector function.

3.1 T cell receptor (TCR).

The antigen recognition molecule of T cells is the T cell receptor (TCR) located on the plasma membrane of T cell that recognises peptide/MHC combinations on antigen presentation cells. TCR is a disulphide-linked heterodimer which is formed by two transmembrane glycoprotein chains, α and β, which contain highly variable antigen recognition sites (Figure 3.2) A minority of T cells bear an alternative but structurally similar receptor made up of a different pair of chain designated γ and δ; the function of γ; δ T cells in immune responses is not yet entirely clear.

The TCR associated with CD3, forming a TCR-CD3 membrane complex. CD3 expression in required for membrane expression of α: β and γ: δ TCRs. The rearrangement of antigen-recognition segments in TCR gene results in highly diversified TCR pool sufficient to recognise a plethora of antigens potentially encountered through life time. The α: β chains form extracellular disulfide-linked heterodimers responsible for MHC/antigen recognition. The TCR complex is formed with various other components that are required for initiating signalling when the TCR complex binds to peptide/MHC. These components include various CD3 molecules which are five invariant polypeptide chains γ, δ, ε, ζ and η chain. These chains associate to form three dimmers, γ: ε, δ: ε and a ζ: ζ or a ζ: η.

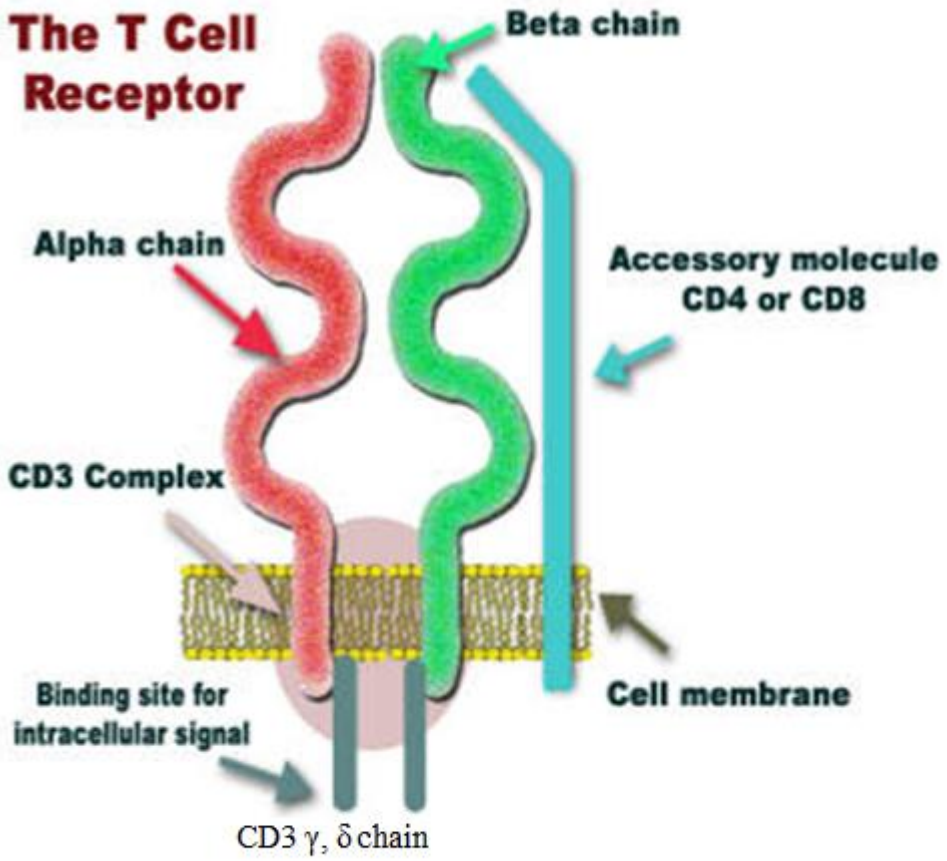

Figure 3.2. T cell receptor

Schematic representation shows that the T cell receptor is formed by two transmembrane glycoprotein chains, the α and β. The extracellular portion of the chains consists of a constant region and a viariable region, which is the site of peptide/MHC recognition.

3.2 Co-stimulatory molecules.

Although recognition of antigen: MHC complexes are mediated solely by the TCR- CD3 complex, co-stimulatory molecule play an important accessory role to fully active T cell. In the absence of co-stimulatory signals with disturb of specific signalling through the TCR will lead to T cell non-responsiveness or 'anergy '. Co-stimulatory signals are delivered by co-stimulatory molecules on APCs via their cognate receptors on T cells. The two major co-stimulation signals following the TCR: CD3: MHC interactions are i) the prototypical CD28 interaction with either CD80 (B7.1) or CD86 (B7.2) and ii) the binding of CD40:CD40L (Figure 3.3).

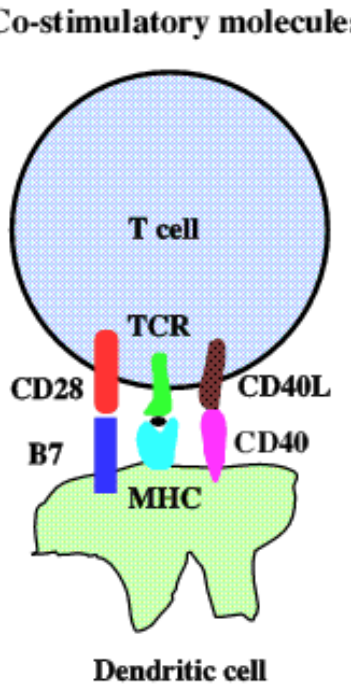

Figure 3.3. Two major co-stimulation signals

CD80 and CD86 bind CD28 with equal affinity but are present in differing densities on the various APCs (Chambers and Allison, 1997). For this reason the resultant T cell response can alter markedly depending to which ligand CD28 binds. It must also be noted that upon activation of the T cell a newly expressed CTLA-4 receptor may also bind the CD86 ligand but with higher affinity, and acts as an inhibitory signal leading to deceleration of the activation process (Chambers and Allison, 1997). The role of CD28 as a co-stimulatory entity is observed through its collaborative actions in ultimately recruiting transcription factors for interleukin-2 (IL-2) gene expression (Figure 3.4).

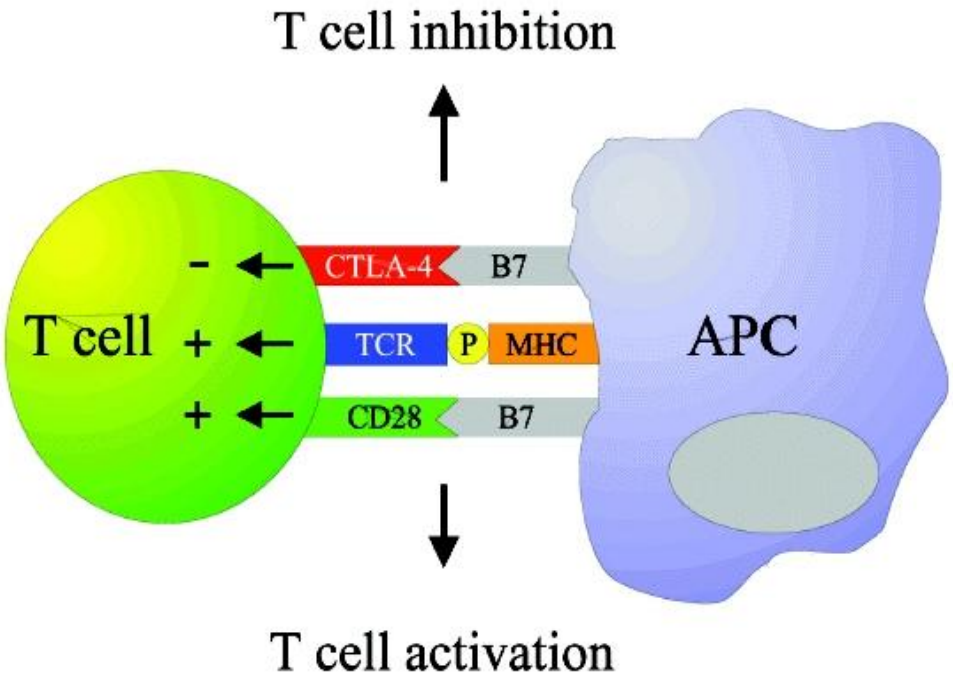

Figure 3.4. Co-stimulation signalling control T cell activation

The CD40: CD40L co-stimulation acts in two directions. It is involved in the upregulation of CD80 (B7.1) and CD86 (B7.2) surface expression on the B cell and thus in driving the T cell response. Alternatively, CD40 ligand expression by the T cell may act upon macrophages to provide the contact dependent macrophage response to interferon-γ; although it has been found that tumour-necrosis factor α or β can substitute for CD40L in macrophage activation (Janeway et al, 2001).

3.3 The immunological synapse.

The immunological synapse is the interface between an antigen presenting cell and a lymphocyte as well as other critically important accessory ligands. This structure forms around the site of contact between the T cell and APC as consequence of reorganization of T cell membrane proteins. This advance in live 2-photon-microscopy have revealed that the immunological synapse is an active and dynamic structure that allows T cells to recognise and respond to antigenic molecules on the surface of APCs. The formation of the immunological synapse is thought to take place in three general steps; first, an interaction occurs between the accessory

ligands on the T cell, e.g LFA-1, and those on the APC, e.g. ICAM-1 (Sofra, 2009). This contact provides a stable structure which allows the T cell to stop and 'sample' the available peptide/MHC complexes through its TCR. Subsequently, if the TCR recognises and binds to the peptide/MHC complex, the entire TCR/peptide/MHC structure is transported and becomes the centre of the synapse during which time signalling occurs (Figrue 3.5).

The immunological synapse requires hours of stable interaction between a T cell and an APC to result in effective T cell activation (Garcia et al. 2008). Once synapse formation is underway, the size and stability of the assembled cluster are determinants of the signal initiation which triggers T cell activation. Thus, the molecules involved in the cluster define the fate of T cells following their engagement with APC (Sofra, 2009). The function of immunological synapse is still in research step, but it is thought to have an important role in regulating signalling, and also involved in the directed secretion of cytokines and cytotoxins by effector T cells in contact with their target cells.

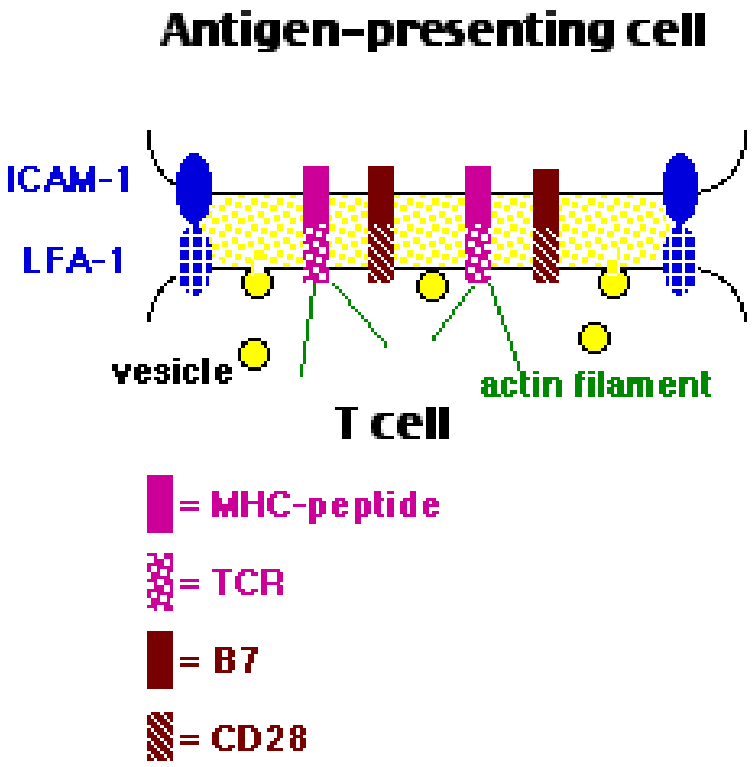

Figure 3.5. Immunological synapse

3.4 T cell activation and immune response to target infected cells

The responses of T lymphocytes to cell associated microbial antigens consist of a series of sequential steps that result in an increase in the number of antigen specific T cells and the conversion of naïve T cells to effector cells. Naïve T lymphocytes constantly circulate through peripheral lymphoid organs until recognition of specific antigenic peptide in context with MHC on APC. The recognition leads to activation and expansion of naïve T cells (Figure 3.6).

The activation of naïve T cells leads to their proliferation and the differentiation of their progeny into effector T cells. Both of proliferation and differentiation depend on the production of cytokines whose multiple functions in cell-mediated immunity. Once naïve T cells differentiated into specialized effector T cells is associated with their enhanced functional potential to orchestrate pathogen clearance. After differentiated into distinct Th cells, the major function for CD4 T cells is to produce specific effector cytokines that induce specific type of immune responses. So in other words this primary cell-mediated immune response when challenged by the same pathogen not only provides a specialized ‘army’ of effector T cells, but also generates a state of immunological readiness for protection from subsequent challenge with the same pathogen.

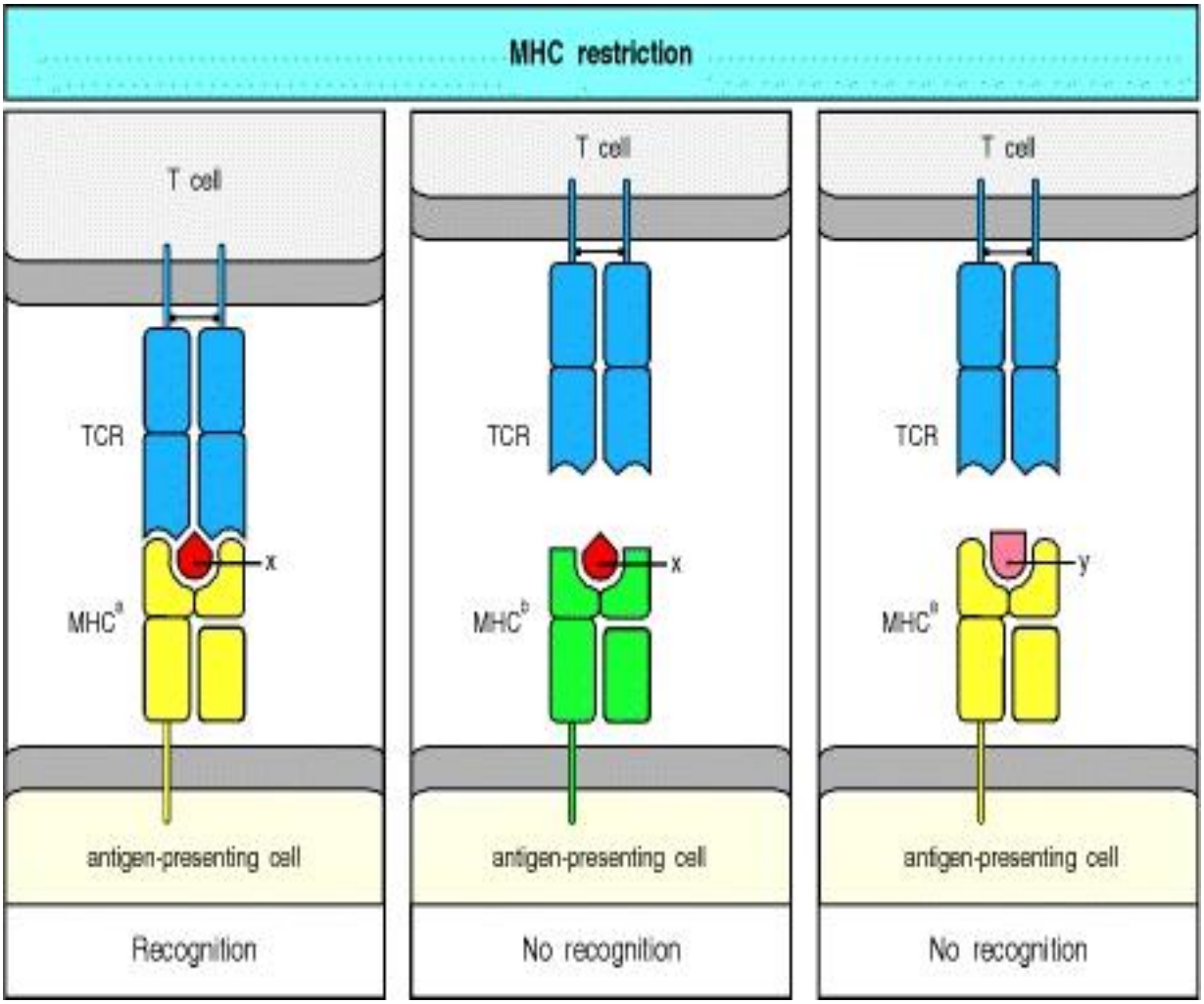

Figure 3.6. The recognition of antigens by T cells is MHC restricted. Naïve T cells recognise specific complexes of peptide/MHC. For example, a T cell that has a TCR that can recognise antigen x on MHCα would not recognise the same antigen on a different MHC (e.g. antigen x on MHCβ), or a different antigen on the same MHC (e.g. antigen y on MHCα).

The effector T cells act very rapidly when they encounter their specific antigen on other cells because of their requirement to recognize peptide antigens presented by MHC molecules. And all of them act on other hast cells but not the pathogen itself. Subsequently, the effector T cells secrete IL-2, which drives them to proliferate and differentiate into armed effector T cells.

3.4.1 T cell differentiate

On antigen recognition, naïve T cells differentiate into several functional classed of effector T cells that are specialized for different activities. There are two main functional categories that detect peptide antigens derived from different types of pathogen, the cytotoxic $CD8^+$ T cells and the helper $CD4^+$ T cells. Circulating effector $CD8^+$ T cells recognize antigenic peptide derived from intracellular pathogens in the context of peptide/MHC class I complexes on the APC surface and naïve $CD8^+$ T cells all differentiate into cytotoxic effector T cells that respond by initiating a cytotoxic response towards the antigen presenting cell that synthesized the antigenic peptides. Effector $CD4^+$ T cells have more flexible repertoire of effector activities. After recognition antigens derived from pathogens replicating in intracellular vesicles, as well as extracellular bacteria and toxins, in the context of peptide/MHC class II complexes on the APC; in response, they can differentiate down distinct pathways that generate effector subsets with different immunological functions.

3.4.2 $CD4^+$ effector T cell subsets

The naïve $CD4^+$ T cell is a multipotential precursor with defined antigen recognition specificity, but substantial plasticity for development. It has the ability to differentiate into several different subtypes of effector T cells in response to specific cytokine environment. The subsets that were defined first are called T_H1, T_H2; more recently, a third population has been identified and called T_H17 cells because its signature cytokine IL-17 and several regulatory T cell subsets that have inhibitory activity that limits the extent of immune activation (Figure 3.7).

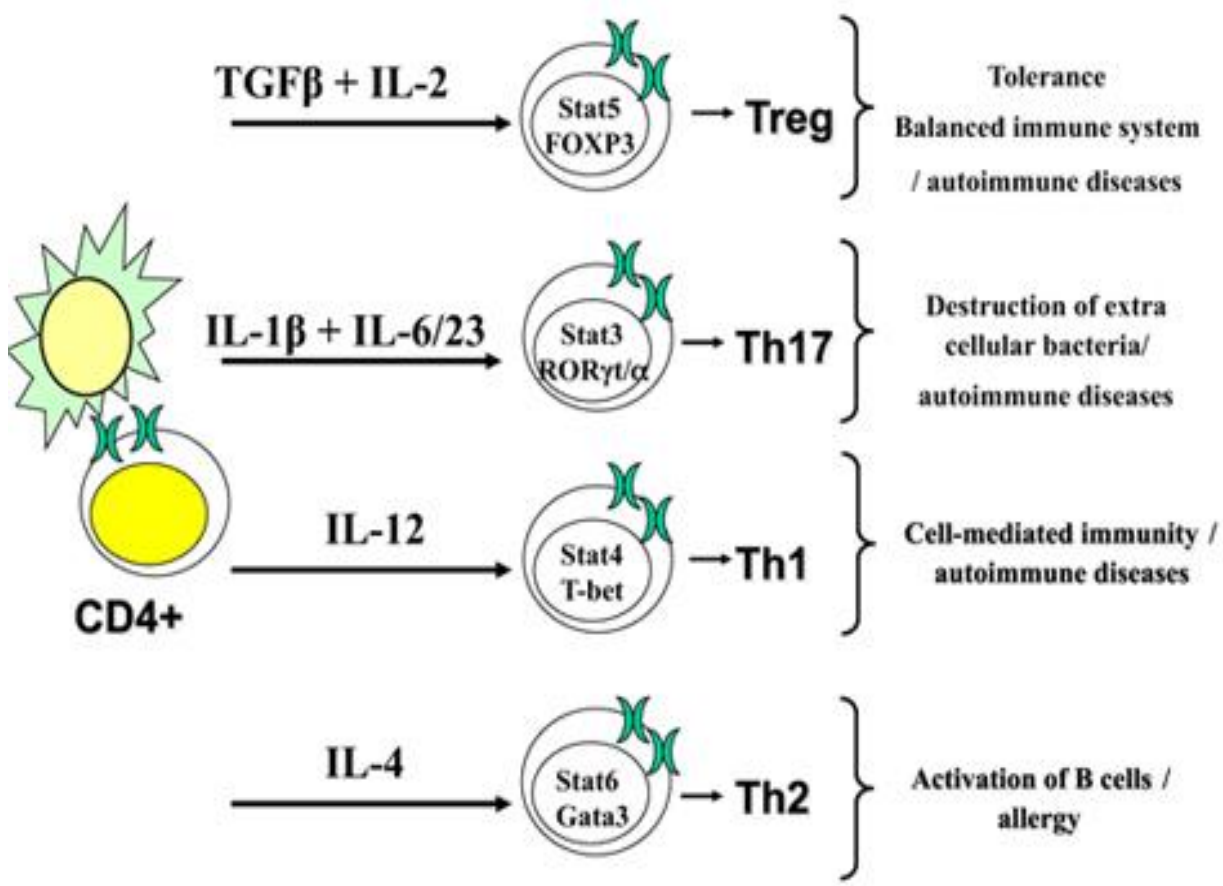

Figure 3.7. Cytokines involved in CD4 T cells differentiation, flagship cytokines produced by the differentiated CD4 T cells and their effector functions

Intracellular bacterial infections tend to stimulate the development of T_H1 cells, which differentiate in the presence of IL-12 and are defined on the basis of their production of IL-2, IFNγ and lymphotoxin (Macatonia et al. 1993; Scharton and Scott 1993). T_H1 cells have the capacity to trigger an inflammatory response and support macrophage activation and migration. These cells provide additional activating signals for macrophages that are chronically infected with certain pathogens and are unable to destroy them. This is done through producing CD40 ligand for CD40 macrophage receptor and through IFNγ the generation of cytotoxic T cells and the induction of B cells for the production of opsonizing antibodies. These effector T_H1 cells will generate copious IFNγ when they recognize antigen on a target cell, thus reinforcing the signal for the differentiation of more T_H1 cells.

Conversely, extracellular antigens tend to stimulate the differentiation of T_H2 cells (also called helper CD4 T cells), which develop in the presence of IL-4. The T_H2 cells make IL-4, IL-5, IL-13 and other cytokines that help B cell activation, production of neutralizing antibodies, control of allergic reactions and expulsion of extracellular parasites (Mohrs et al. 2002; Prout et al. 2004). T_H2 cells largely promote immune response to parasites. They secrete cytokines that promote B cell growth: IL-4, IL-5, IL-9 and IL-13. They express CD40 ligand that also induces B cell proliferation and isotype switching. Recent evidence has suggested that certain protein secreted by activated dendirtic cells can lead to the activation of the IL-4 and GATA-3 gene in cells, thus starting a cascade of positive feedback for differentiation as T_H2 cells as a result of continued IL-4 secretion. Each subset promotes its own development and inhibits the development of the other subset via their secreted cytokines (Sanders et al. 1988; Gajewski and Fitch 1988), such that in that particular environment the induction of one type of response suppresses the induction of the other (Mosmann and Coffman 1989).

Recently an IL-17-producing subset, known as T_H17 cells (Harrington, Hatton et al. 2005), has been described. T_H17 cell differentiation is driven by TGFβ in combination with the pro-inflammatory cytokines IL-6, IL-21, and IL-23 (Zhou, Lopes et al. 2008), and it is antagonised by products of the TH1 (e.g. IFNγ) and T_H2 (e.g. IL-4) lineages (McGeachy and Cua 2008), and they express the receptor for cytokine IL-23 rather than the receptor for IL-12 expressed by T_H1 cells. In addition to IL-17, T_H17 cells are characterised by their ability to produce IL-22 and IL-17F (Boniface et al. 2009). They promote the recruitment of neutrophils and moncytes, and this may be their principal role in inflammatory disorders. The commitment to the T_H17 lineage is thought to be under the control of the transcription factor RORγT, which is induced in these conditions and which drives expression of the receptor for IL-23. T_H17 cells are thought to be an evolved arm of the adaptive immune response specialized for enhanced host protection against extracellular bacteria and some fungi (Horton et al. 2000) (Stefanelli et al. 2005) (Harrington et al. 2006) particularly at mucosal surfaces (Pallone et al. 2009). They are thought to contribute to homeostatic

maintenance of mucosal tissues such as the gut (Izcue et al. 2008), and emerging information suggests that T_H17 cells may also be involved in antiviral immune responses by protecting the host against secondary infections involving gastrointestinal microbes (Brenchley and Douek 2008).

Besides armed effector cells, $CD4^+$ T cells can also differentiate into distinct regulatory subsets (Treg cells) which found in the periphery are a heterogeneous group of cells with different developmental origins. They are $CD4^+$ and also express the α chain of the IL-2 receptor (CD25) with high levels of the L-selectin receptor CD62L and represent about 10-15% of the CD4 T cells in the human circulation. They suppress the proliferation and differentiation of T_H or cytotoxic T cells; serve to limit potential immunopathology and autoimmunity that may be caused by an over-exuberant immune response (Sakaguchi. 2000). Treg cells are defined by the expression of a forkhead transcription factor, Foxp3, which is essential for programming their regulatory effector function. Regulatory T cells can be further divided in two categories, the naturally occurring $CD4^+CD25^+$ subset that develops in the thymus (nTregs) and the TGFβ-induced $CD4^+CD25^-$ subset that differentiates in the periphery (iTregs) (Curotto de Lafaille and Lafaille 2009) (Josefowicz and Rudensky 2009). Generally, both types exert regulatory function by suppressing immune responses via the secretion of specific cytokines, for example by producing IL-10 and TGFβ, they inhibit T cell proliferation. Inter-linking mechanisms for $CD4^+$ T cell effector and regulatory lineage specification have been described (Veldhoen and Stockinger 2006); for example T_H17 differentiation depends on the pleiotropic cytokine TGFβ, which is also linked to regulatory T cell development and function. Low concentrations of TGFβ drive T_H17 cell differentiation, while high concentrations of TGFβ inhibit T_H17 cell development and induce differentiation of regulatory T cells. Even after differentiation a helper T cell of a specific lineage can convert to another helper phenotype, within a certain cytokine environment. For example, iTregs can become IL-17 producing cells in the presence of IL-6 and IL-21, T_H17 cells can switch to IFNγ producing T_H1 cells in the presence of IL-12 or, in the presence of IL-4, to IL-4 producing T_H1 cells. Treg is involved in controlling

adaptive immune responses, so failure of natural Treg function is known to be involved in several autoimmune syndormes (Figure 3.8).

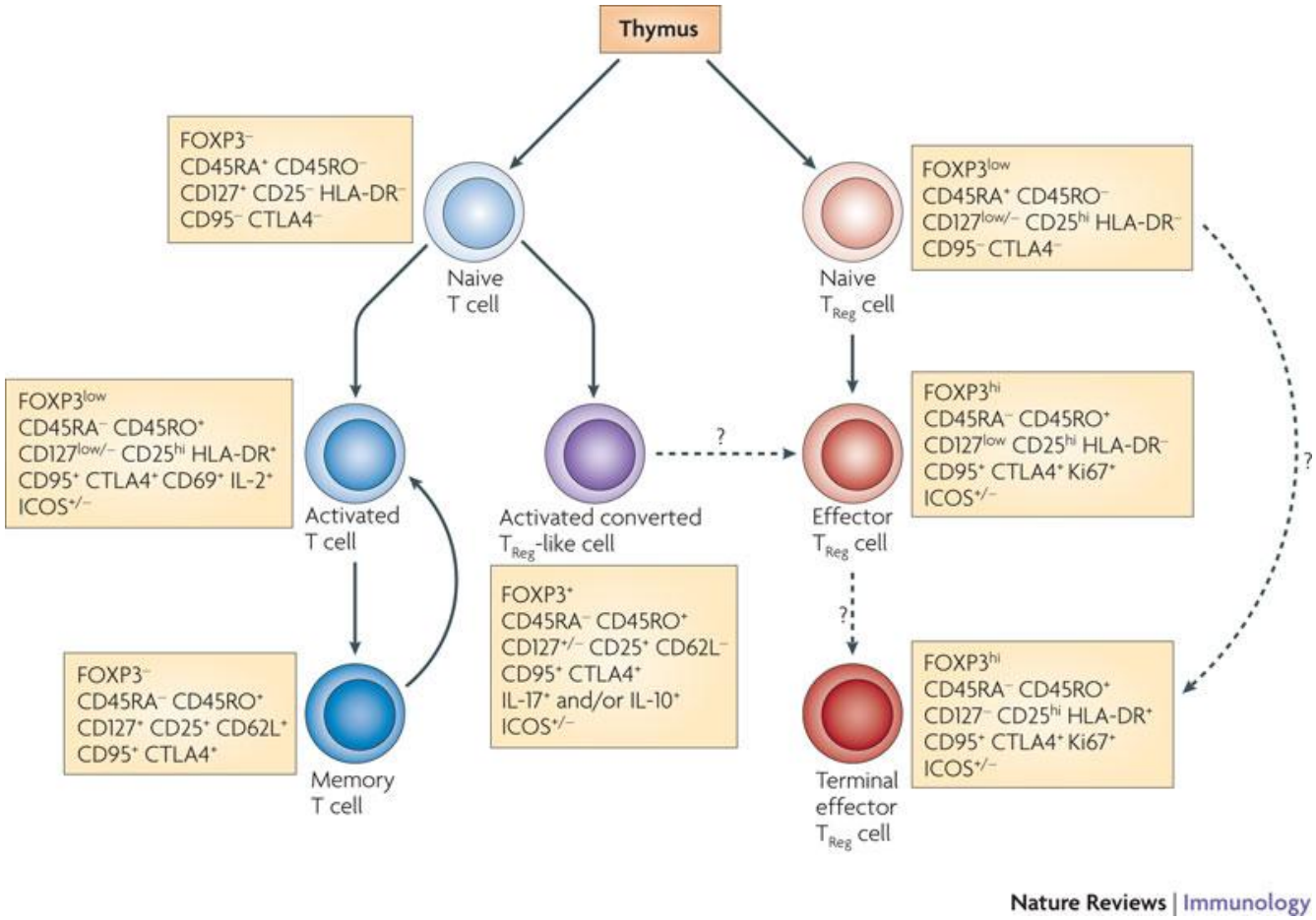

Figure 3.8. FOXP3$^+$ regulatory T cells in the human immune system (Sakaguchi et al, 2010)

One subset of these adaptive regulatory T cells, called T_H3, is found in the mucosal immune system. T_H3 cells produce IL-4, IL-10, and TGFβ which is the main reason to distinguish from T_H2 cells. T_H3 cells may by predominantly of mucosal organ and be activated by the mucosal presentation of antigen. Lack of these cells is linked to autoimmune disease in the gut and to inflammatory bowel disease (Sakaguchi et al. 2006).

Other CD4$^+$ T cells that may regulate the development and differentiation of the helper subtypes are the NKT cells, known as innate-like lymphocytes (ILLs).

They require the recombinases RAG-1 and RAG-2 to produce antigen receptors, and undergo the process of antigen receptor gene rearrangement; therefore these cells belong to adaptive immune system. They arise from the same lymphoid progenitor but their development is distinct from that of the other $CD4^+$ T cells, since it does not depend on the expression of MHC class II. Instead their activation depends on relatively invariant CD1 molecules (Silk, Salio et al. 2008) that are induced in response to infection. Together with other innate-like lymphocytes, such as the γδ T cells, NKT cells are thought to act as intermediates between innate and adaptive immunity.

After antigen/pathogen is cleared most effector cells die, but a few antigen-experienced cells remain for long-term protection. These are known as memory T and B cells, which guard lymphoid organs and patrol peripheral tissues to mount rapid responses on re-exposure to antigen (Sallusto, Lenig et al. 1999). Thus, a successful T cell-mediated immune response has the capacity to clear infection and establish a state of long-term protective immunity to that particular pathogen.

3.5 Immunological memory.

Immunological memory is the ability to provide a rapid reinduction of antigen-specific antibody and effector T cells on subsequent encounters with the same pathogen, thus providing long-lasting and often lifelong protection against it. Although the mechanisms underlying protective immunological memory are still not well understood, the phenomenon has long been recognised and applied to vaccination (Figrue 3.9).

Long-term protective immunity either naturally derived after an infection or after vaccination, involves three key main factors (Rafi Ahmed 13th Congress of Immunology): First, it provides the host with pre-existing neutralizing antibodies which upon re-infection mediate the initial response. Indeed, it is clear from the early days of immunology that antibodies generated by provocation with pathogen can protect from reiterated challenge with pathogen as elegantly demonstrated by

Kitasato and Behring more than 100 years ago (Behring, 1900), (Dorner and Radbruch 2007). Second, it involves long-lived antibody-secreting plasma cells (humoral memory) and long-lived memory B cells capable of reacting quickly to a recurrent antigenic challenge (reactive memory) (Ahmed and Gray 1996). The third key factor of immunological memory is the increased number and longevity of memory T cells, which have the capacity to respond quicker and better than T cells in a primary immune response; as a result they aid a more rapid and efficient elimination of the infectious agent (Sofra, 2009).

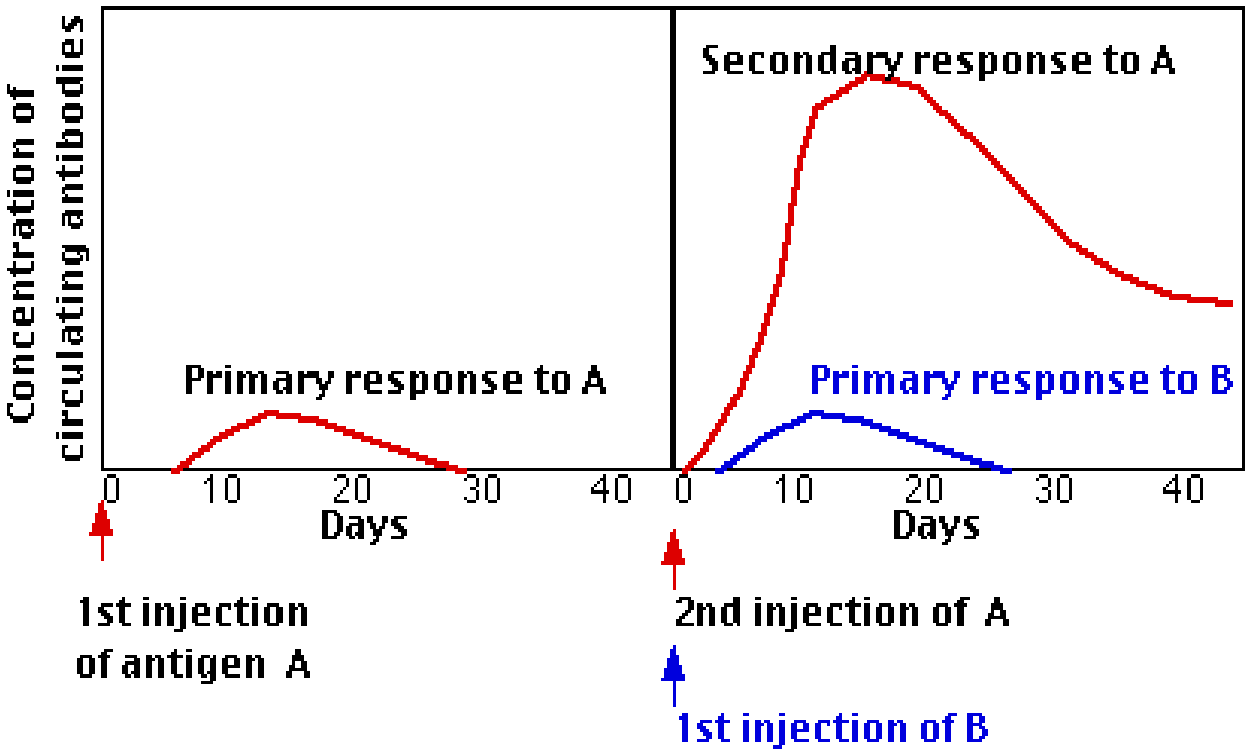

Figure 3.9. Immunological memory

The First encounter with an antigen produces a primary response. Antigen A introduced at day 0 encounters little specific antibody in the serum (Red line). After a lag phase antibody against antigen A appears; its concentration rises to a plateau and then gradually declines which is a typical primary response. After recovering from an infection, a second exposure of antigen A, over the ensuing weeks, months, or even years, a very rapid and intense secondary respond to A occurs. But this response is specific, because there is only a primary response to the new antigen B (Blue line).

3.5.1 Memory T lymphocytes

There is a small group of antigen specific T cells differentiated into long lived memory cells which can survive even after the infection is eradicated and antigens as well as the innate immune reaction to the infectious pathogen are no longer present. These memory T cells can be found in lymphoid organs in mucosal tissues, and in the circulation. There are two subsets of memory T cells. One is central memory cells populate in lymphoid organs and responsible for rapid clonal expansion after re-exposure to antigen. The other called effector memory cells stored in mucosal tissue and mediate rapid effector functions on reintroduction of antigen to these sites. Memory T cells require signals delivered by certain cytokines like IL-7 to keep them stay alive. Although memory T cells cannot continue to produce cytokines to kill infected cells, they still have the ability to recognize antigen and then recover rapidly on encountering it.

To date, it remains to be defined how protective memory is maintained. Generally, there are two fundamentally differing views: One that describes memory maintenance as an inherent special quality of the immune system independent of sustained antigen dependence, and another that illustrates immunological memory as a low-level antigen-driven protective immune response.

3.6 Factors that influence the T cell response.

During the T cell response, the survival and proliferation of T cells are maintained by antigen, co-stimulatory signals from CD28, and cytokines such as IL-2. Numerous mechanisms ensure the generation of a useful T cell response, despite several obstacles. However, initiation of T cell response is mediated by APC that present antigenic peptide-MHC complexes and costimulatory molecules to T cells. And then, the correct type of T cell must respond to antigens form the extracellular and intracellular compartments which worked out perfectly with the help from the specificity of the CD4 and CD8 co-receptors for MHC class II and class I molecules, and by the segregation of extracellular and intracellular protein antigens for display

by MHC class II and class I molecules, respectively. Third, T cells must interact with antigen-bearing APCs long enough to be activated which is accomplished by adhesion molecules to stabilize T cell binding to APCs. Fourth, T cells should respond to microbial antigens but not to harmless proteins. Microbes take the responsibility, because T cell activation requires co-stimulators induced on APCs by microbes. Finally, antigen recognition by a small number of T cells must lead to a large enough response to be effective, which is calculated by several amplification mechanisms that are induced by microbes and activated T cells themselves and lead to enhanced T cell activation.

4 Professional antigen presenting cells

4.1 Function of professional APC

Cells can present antigen peptide associated with MHC molecule to inactive T lymphocytes and activate them for the very first time know as antigen presenting cells (APCs). A variety of cells can function as antigen presenting cells to express MHC class I, but there is a distinguishing feature group which has unique ability to express MHC class II classified as professional antigen presenting cells: dendritic cells, macrophages and B lymphocytes.

Macrophages must be activated by phagocytoses of particulate antigen before they express class II MHC molecules or co-stimulatory membrane molecules such as B7. B cells constitutively express class II MHC molecules but must be activated before they express co-stimulatory molecules. Dendritic cells (DC) are the most effective of the antigen presenting cells, because these cells constitutively express a high level of class II MHC molecules and have co-stimulatory activity, they can activate naïve T_H cells as discussed later in this chapter (Figure 4.1).

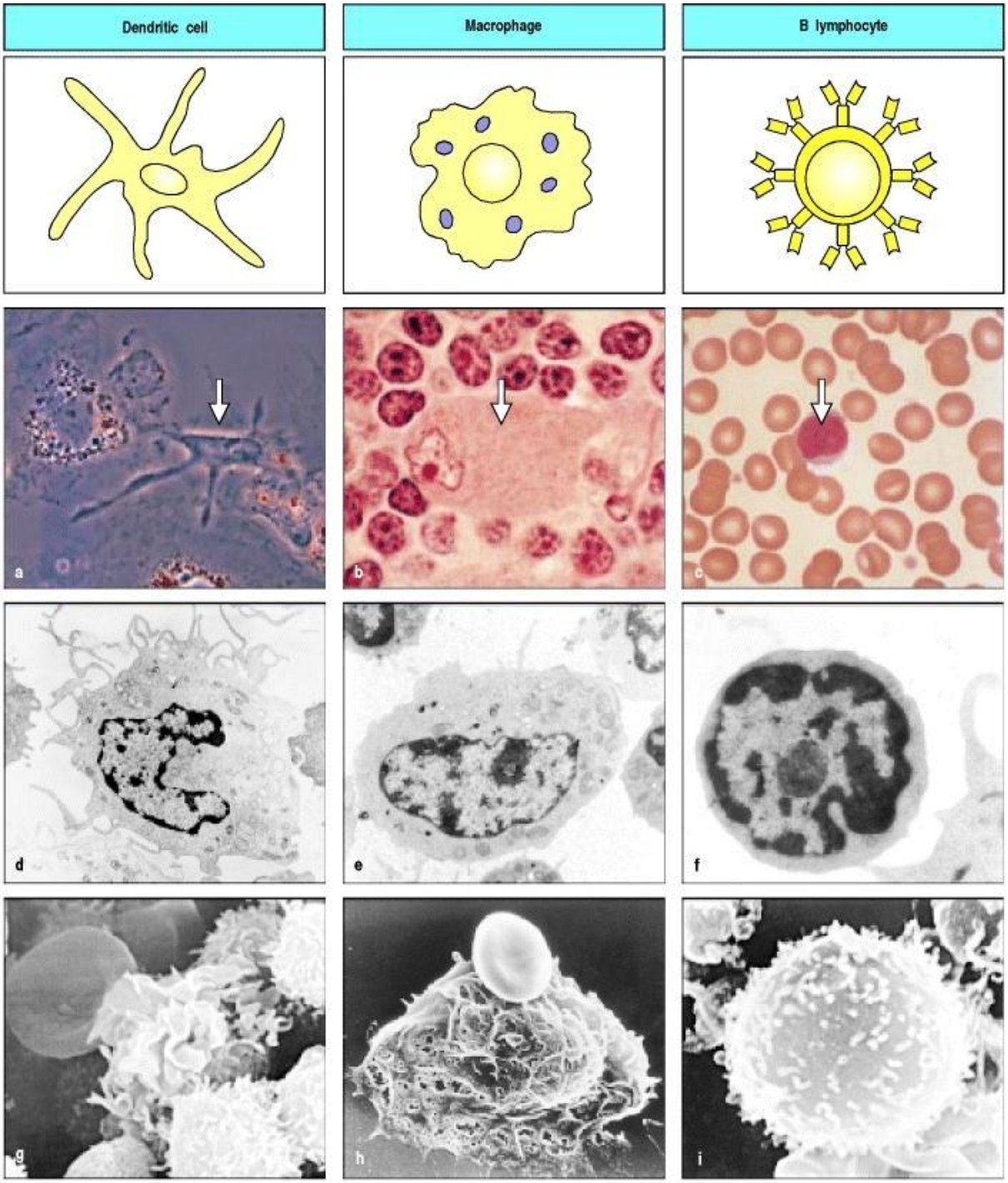

Figure 4.1. Professional antigen presenting cells

There are three different types of professional antigen presenting cells are shown in different models. (From top to bottom).

4.2 Dendritic cell biogenesis, function, *in vitro* generation, antigen loading and subtypes.

DCs represent a unique system of cells that induce, sustain and regulate immune responses (Banchereau and Steinman, 1998). Figure 4.2 shows an electron microscopic image of a DC. DCs originate in the bone marrow from pluripotent $CD34^+$ stem cells and migrate to peripheral tissues through the blood. DC progenitors give rise to myeloid and lymphoid precursors. DCs are distributed in most tissues and, in particular, in tissues that interface with the external environment. There they perform sentinel function for incoming pathogens. Langerhans cells, the first DCs described, are widely distributed to the skin, esophagus, cervix, and buccal epithelia. Interstitial DCs are present in the dermis as well as the interstitium of virtually all tissues except for the brain. Furthermore veiled DCs may be found in the afferent lymph and interdigitating DCs reside in the cortical zone of the lymph nodes and in the spleen (Dannull et al, 2000). The DCs in peripheral tissues are immature but capable of actively taking up antigens by three major pathways, micropinocytosis, phagocytosis and receptor-mediated endocytosis (Paczesny et al, 2003). Macropinocytosis allows uptake of soluble extracellular antigens. In addition, phagocytosis or receptor mediated endocytosis may be initiated by direct, nonopsonic interaction between pathogen, apoptotic cells, or effete body cells and DCs. Receptor mediated uptake may occur via the multilection receptor, the mannose receptors, collectins, toll-like receptors, and scavenger receptors (Colino and Snapper, 2003) (Dannull et al, 2000). Alternatively, antibodies or complement can act as bridging molecules between pathogen and Fc-type or complement- type receptor, thus leading to opsonic uptake (Dannull et al, 2000).

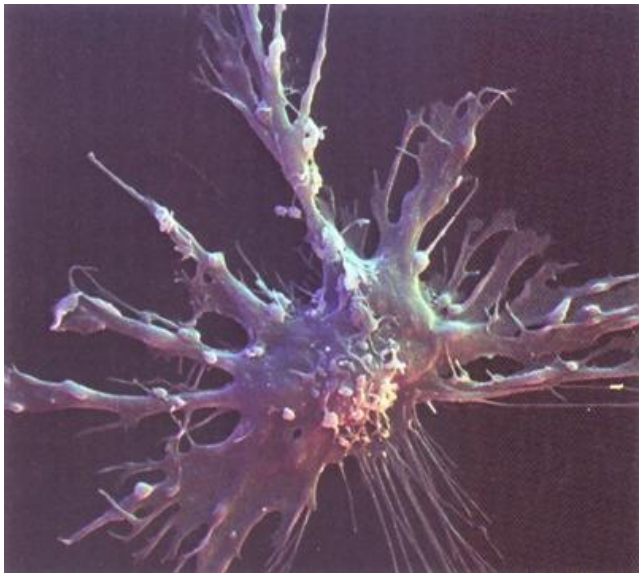

Figure 4.2 Image of a DC

Following uptake of antigens, DCs induce leukocyte recruitment to the site of inflammation through production of chemokines and inflammatory cytokines. Antigen uptake induces maturation, which is complex process with the dual role of transforming immature DCs in the peripheral tissue into cells that will migrate to the secondary lymphoid organs and then act as professional APCs for the priming of naïve T cells (Colino and Snapper, 2003). Toward accomplishing migration, activated DCs lose their phagocytic capacity and tissue adhesive structures; increase their expression of receptors for lymphoid chemokines (i.e. CCR7), and reorganise their cytoskeleton for the acquisition of high cellular mobility (Winzler et al, 1997). Towards the second goal, DCs strongly upregulate their expression of co-stimulatory molecules (i.e. CD40, B7.1, B7.2) and upregulate synthesis and translocation to the surface of MHC molecules complexed with processed antigens (Guermonprez et al, 2002). The ability of DCs to induce a primary immune response is unique among APCs. The mature state of DCs ends by apoptotic cell death in the lymph modes, which is enhanced by immunoinhibitory cytokines such as IL-10.

DCs are also important in immune tolerance (Steinnman et al, 2000) (Steinman et al, 2002) (Steinman et al, 2003). Two mechanisms were created to avoid the immune system attack on the components of self, central and peripheral

tolerance, both of which are maintained by DCs. Central tolerance that occurs in the thymus is dependent on mature thymic DCs which are essential for deletion of newly generated T cells with a receptor that recognises self components (Steinman et al, 2003). However many self antigens may not access the thymus while others are expressed later in life. Hence the need for peripheral tolerance which occurs in lymphoid organs and is mediated by immature DCs. Peripheral tolerance involves induction of T cell anergy or under certain circumstances deletion. Immature DCs within tissues capture the remains of cells that die in the process of physiological tissue turnover. As there is no inflammation accompanying this process the DCs remain immature. There immature DCs, which lack co-stimulatory molecules, migrate to draining lymph nodes where they present the tissue antigens to T cells. T cells presented with antigen in the absence of comstimulation either enter into a state of anergy or get deleted (Steinman et al, 2002) (Steinman et al, 2000). Immature DCs may be critical in development of tolerance towards tumours (Wakkach et al, 2003).

4.3 Cytokine mediated DC differentiation

Dendritic cells are bone marrow derived cells found in most tissues, including lymphoid tissues. Two main functional subsets are distinguished. Conventional dendritic cells take up antigen in peripheral tissue, are activated by contact with pathogens, and travel to the peripheral lymphoid organs, where they are the most potent stimulator of T cell responses. Plasmacytoid dendritic cells also take up and present antigen, but their main function in an infection is to produce large amounts of the antiviral interferons. Both these types of dendritic cells are distinct from the follicular dendritic cell that presents antigen to B cells in lymphoid follicles.

A key characteristic of dendritic cell biology is that the cells differentiate or mature in distinct ways in response to a spectrum of environmental and endogenous stimuli. There is evidence shown either polarized DCs or distinct DC subsets might provide T cells with different signals to determent class of immune response. For example, DCs respond to microbial ligands for pattern recognition receptors (e.g. pathogen-associated molecular patterns recognised by Toll-like receptors), T cell

ligands (e.g. CD40 ligand), innate lymphocytes (e.g. NK cells), and inflammatory cytokines (e.g. TNFα) and upon cell contacts with other DCs.

In the absence of specific maturation stimuli dendritic cells may differentiate by 'default' and can induce tolerance when they capture self or harmless environmental antigens (Hawiger, Masilamani et al. 2004). Generally, induction of tolerance is a consequence of antigen presentation to T cells by phenotypically immature dendritic cells that lack co-stimulatory signals and it can be described as T cell deletion, T cell anergy or the induction of Tregs (Hawiger et al. 2003).

By contrast, in response to infection and inflammation dendritic cells differentiate rapidly to a mature state. During maturation antigen uptake is reduced (Chen et al. 2000), while antigen processing is upregulated by lowering of the pH in endocytic vacuoles, activated lysosomal proteolysis and increased transport of peptide/MHC class II complexes to the cell surface (Trombetta and Mellman 2005). Importantly, the maturing dendritic cell membrane undergoes remodelling, dendrites are formed and membrane associated co-stimulatory molecules are expressed.

The nature of DC maturation, which is regulated by the nature of the stimuli received through pattern recognition receptors, and is also dependant on the type of DC that is responding to these signals, has a major influence on both DC function and subsequent activation of naïve T cells. For example, depending on the type of infection maturing dendritic cells selectively polarise $CD4^+$ T cell differentiation towards T_H1 cells to help resist viruses and tumours, or towards T_H2 cells in response to extracellular bacteria and fungi.

5 Infectious immunity

5.1 Immune responses to viral infection

The viral infection is any type of infection that caused by a virus, which is pathogens composed of a nucleic acid genome enclosed in a protein coat, so it is more difficult to kill than bacteria. Viruses can replicate only in a living cell, as they do not possess the metabolic machinery for independent life. Viruses can be transmitted in numerous ways such as direct contact with infected person, swallowing or inhalation. Effective immune response to viruses requires the coordinated activation of both innate and adaptive immune modules.

Innate immune defense against viruses comprises IFN, NK cells and macrophages (Male et al, 2006). Interferons are pro-inflammatory cytokines that are particularly important in limiting viral infection. Type I interferons, IFNs (α and β), can be produced by many different cell as a response to viral infection, when cell surface or endocytic PRR recognize viral nucleic acids. Pro-inflammatory cytokines such as IL-1 and TNF-α are also potent inducers of IFNα and β production. Most nucleated cells have receptors for type I IFNs. IFN binding to virus-infected or healthy cell leads to expression of more than 300 genes. However, the key mechanisms to prevent viral replication in the cell is activation of enzymes that degrade ssRNA, inhibit mRNA translation, increase production of MHC class I and, in the last instance, trigger apoptosis via Bcl-2. IFNs also activate NK and macrophages (Male et al, 2006) (Takeuchi and Akira 2009). Cells infected with a virus tend to have less MHC class I on their surface, often due to the viral inhibition of antigen presenting machinery. NK cells recognize cells that display less MHC class I, induce them to undergo apoptosis or kill them by perforin and proteases. NK cell also secrete IFNγ (Andoniou et al, 2006).

Macrophages are ubiquitously present in body tissues and are the first line of defense against many pathogens, including viruses. They phagocytize virus-infected cells and produce TNF-α and IFNα (Male et al, 2006).

Adaptive immune response is always required for clearing of viruses. Normally it starts when DC present processed viral antigen on MHC class I to CD8 cells in

peripheral lymphoid organs, however CD4 cell are also of a great importance in viral infection. MHC class I viral antigen presentation can also be carried out by a wide variety of cells, not just DC. CD8 cells differentiate into T_c cells, migrate to the site of infection, recognize virus-infected cells and kill them in perforin- or granzyme-dependent manner or by Fas-FasL interactions (Male et al, 2006). CD4 cells differentiate into T_H1 cells and produce INF-γ that activates macrophages, and TNF-α. TNF-α mediates INF-dependent mechanism of apoptosis in infected cells (Alberts et al, 2008).

Antibodies are also employed during viral infection. They may act alone or with the complement system. They can bind viruses directly and prevent them from entering cells or damage the virus itself. In addition, antibodies bind virus-infected cell thus marking it for recognition by phagocytes (Male et al, 2006). There are two types of viral infection acute infection and chronic infection (Figure 5.1).

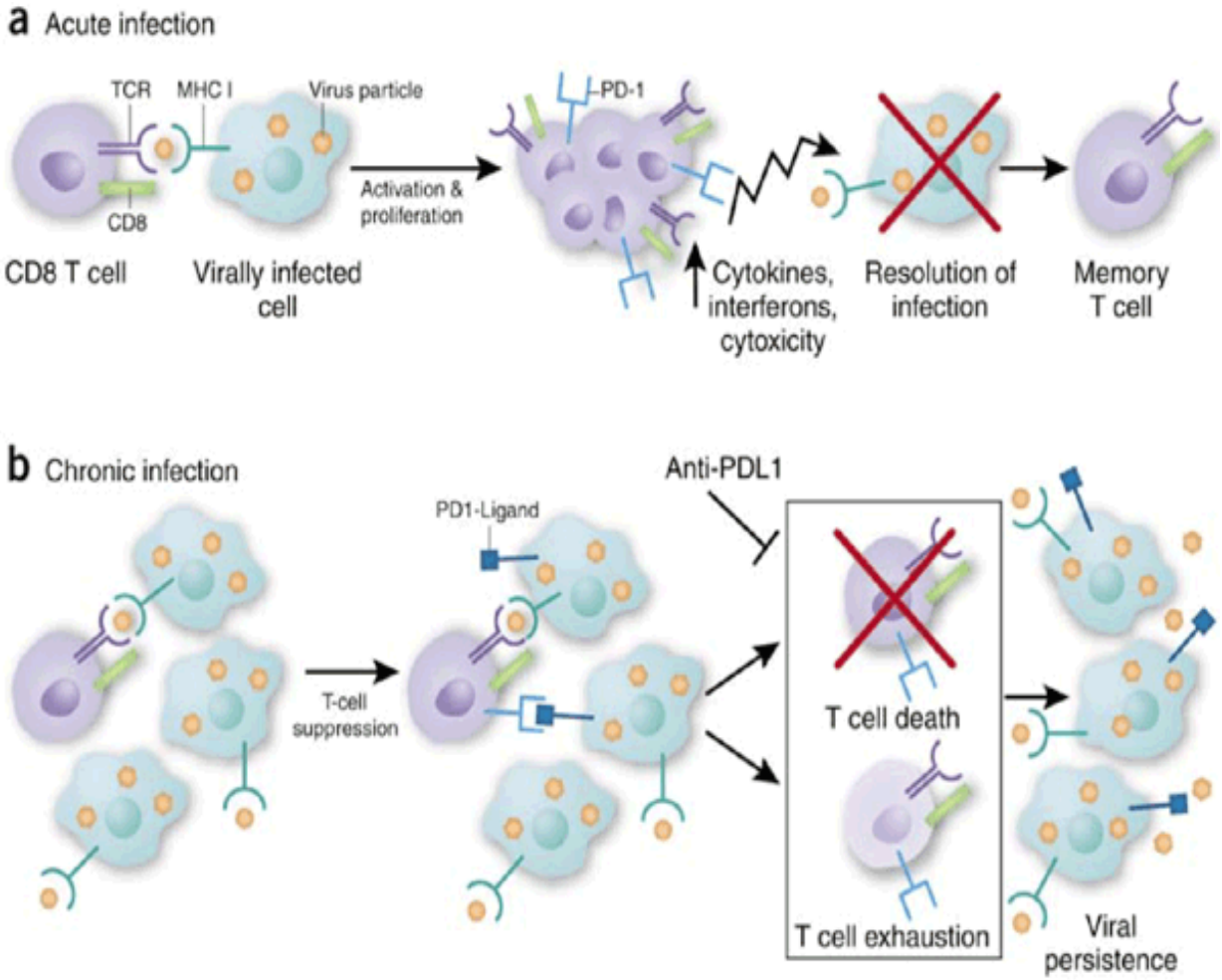

Figure 5.1 Infectious immunity

(**a**) In an acute viral infection, $CD8^+$ T cells become activated in response to viral antigen, proliferate, acquire the ability to produce cytokines and interferons, and kill virally infected cells.
(**b**) During a chronic infection, $CD8^+$ T cells become exhausted, produce fewer cytokines and are less able to kill virally infected cells and control virus levels. (Farrell, 2006)

5.2 Acute viral infection and immune responses

In acute viral infections, the number of infectious agents rapidly increases initiating an innate anti-viral immune response. This takes numerous forms but mainly includes interferon production and natural killer (NK) cell activity, which occurs early after infection and constitutes the first line of defence (Gregoire, Chasson et al. 2007) (Ha, West et al. 2008). As the pathogen is retarded by the components of the innate immune system a concurrent adaptive immune response is triggered; professional antigen presenting cells, such as DCs, process virally-derived antigens, loaded onto MHC, that with the help of co-stimulation result in the activation and expansion of specific effector T cells. Four to seven days after infection, large numbers of activated cytotoxic T cells appear within the secondary lymphoid tissues. At the same time, activated $CD4^+$ T helper cells help B cells to produce specific antibody as both the cellular and humoral adaptive response peak. Systemic viral load is reduced during this stage. Clearance of the infection coincides with elevated cytotoxic T cell activity followed by T cell death as the antigen levels fall. The conclusion of the adaptive response is characterised by a dramatic fall in the numbers of antigen-specific effector T cells, but, also by elevated serum antibody titres that can persist for months and the appearance of antigen-specific memory cells. Typical examples of viruses that result in acute infections are smallpox and yellow fever virus (Miller, van der Most et al. 2008) (Sofra, 2009).

Acute resolving infections leave no residual pathology following an effective adaptive immune response, which, ideally, clears the infection and precludes potential disease by establishing in the host a state of protective immunity against reinfection with the same pathogen.

5.3 Chronic viral infection and immune responses

Consistent to chronic viral infection, there are many virus infection, however often fail to resolve and become chronic which can persist for many years, such as cytomegalovirus, herpes viruses and mycobacterium tuberculosis, have evolved to

coexist with their human hosts, thereby avoiding the generation of efficient protective immunity (Thomas Dörner and Andreas Radbruch, 2007). These infectious agents may exist within the host in a latent state (contained but not eliminated), but when the adaptive immune response is weakened, the pathogen can opportunistically reappear as a virulent systemic infection.

Despite the protection that T cell effector mechanisms provide during the period of acute infection, T cell responses can also be detrimental by destroying inoffensive host cells. As a result, the balance between immunoprotection and immunopathology becomes a very fragile one, as is well-illustrated in the phenotypes of various persistent infections. For example, symptoms in HBV infected patients can range from in-apparent infection to very aggressive hepatitis (Zinkernagel 2003).

5.3.1 Immune defects in chronic infectious diseases

There are two issues for immune defection in chronic infection disease interacted either the virus or it interaction with immune system. For the virus, they can escape from immune surveillance. Some of them focus to destroy the antigen present pathway via MHC class I and II dysfunctional which is appeared in the viral glycoproteins US2 and US3 from human cytomegalovirus (CMV). US2 can bind to MHC class I heavy chain result it dislocation from ER membrane to cytosol, finally degraded by proteasome (Wiertz et al, 1996), which is also demonstrated in MHC class II molecule (Tomazin et al, 1999). US3 also play function to escape, which can associates transiently with MHC class I molecules caused it retention in the ER (Gruhler and Fruh 2000). In addition there are also some kinds of virus can be latency and reactivation in host which will describe latter.

Surprisingly, some of the viruses even contain a special gene which plays a niche specific role during chronic infection to evade immunity. These virus encode genes contribute to evade immunity during acute or chronic infection (Virgin, 2007b). For example which has been demonstrated by Rickinson and Kieff (2007), they found that the latency gene in EBV can specialize to prolong the infection of memory

B cells and to respond to and regulate B cell activation and differentiation. And also they can maintain the viral episome, regulate of B cell antigen receptor signalling and regulate of cell cycle and apoptosis (Herbert et al, 2009). The other speciality of some persist viruses is that they can select a specific cell type to reside. Like genitourinary tract is a particularly place chose by several virus like human T cell leukaemia virus (HTKV), human cytomegalovirus (CMV), Kaposi's sarcoma herpesviurs (KSHV) EBV, HIV, LCMV, HSV, and polyomaviruses (Virgin, 2007b), which is a difficult place for the immune system to surveillance.

In the other hand the immune system also affected by this immune suppressed environment which caused by long time inflammation and suppressed cytokine or receptor which will lead the T cell and B cell responds deficient which will introduce latter. Over all the location, timing, and magnitude of the immune response relative to the speed of virus replication and spread are also the major determinants of the eventual outcome of viral infection (Herbert et al, 2009). Due to the factor that the virus must evade sterilizing immunity, while the immune system must adjust to the continuous presence of viral antigen-driven inflammatory responses in order to limit viral replication to an acceptable level without untoward damage to permanently infected tissues under chronic viral infectious condition, there is a dynamic but metastable equilibrium between the virome and the host immune defence in chronic viral infection.

Major viral mechanisms for chronic infection (Figure 5.2);

1) **Continuous replication**: Viruses in this category include HIV, HBV, and HCV in humans Continuous replication can generate up to 10^{12} particles per day for HBV and HCV (Rehermann and Nascimbeni, 2005). Viruses that persist via continuous replication express potentially antigenic viral proteins that are required for viral assembly and release, resulting in continuous antigenic stimulation of lymphocytes. Continuous replication has other effects on the immune system—low levels of tissue damage and stimulation of inflammatory cytokines and co-stimulatory molecules may alter the normal immune system. Some viruses, however, are very efficient at

avoiding the generation of such inflammatory signals. For example, HBV infection can proceed for weeks without any significant induction of either innate or adaptive immunity in a "stealth" approach to establishing chronic infection (Wieland and Chisari, 2005) (Herbert et al, 2009).

2) **Latency and reactivation**: cell latency is neither slow viral replication nor the presence of viral nucleic acid without the capacity to reactivate. Latent virusesretreat from adaptive immunity into a transcriptionally and antigenically quiescent state. Perhaps the most frightening example of this is HIV, which can survive in memory CD4 T cells in the proviral state without expressing any proteins that can be recognized by the immune system. Similarly, EBV can establish latency in memory B cells with undetectable expression of protein- coding RNAs, at least until the latently infected cell divides (Hochberg et al., 2004) (Herbert et al, 2009).
3) **Invasion of the genome:** the best example is endogenous retroviral elements (ERVs) chronically infect in Mammalian genomes which spread vertically from one host generation to the next as integrated viral genomes or partial genomes in host chromosomes. Some ERVs are replication competent, but many are replication defective. Yet even such defective ERVs can express proteins. Human ERVs from over 30 lineages constitute perhaps 8%–9% of the human genome (Virgin, 2007b). There are two effects on the immune system. First, they can encode B or T cell antigens (Miyazawa et al., 1987) (Wang- Johanning et al., 2008) (Levisetti et al., 2003). Second, ERV-encoded superantigens can shape the T cell repertoire (Meylan et al., 2005) (Sutkowski et al., 2001) (Stauffer et al., 2001) (Herbert et al, 2009).

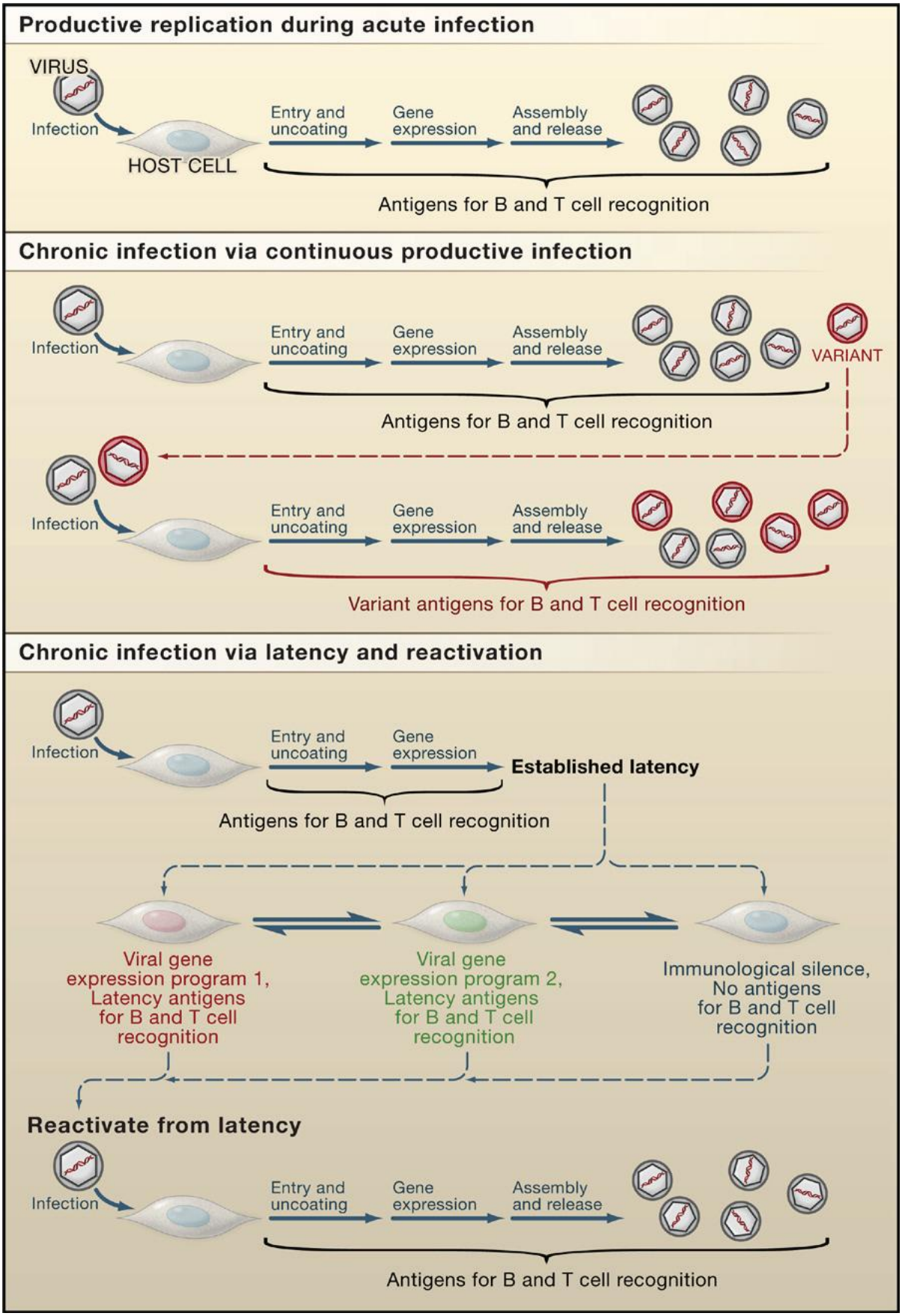

Figure 5.2. Processes of Acute and Chronic Viral Infection

Viruses use a range of strategies for acute and chronic infection. (Top) The strategy used during acute infection results in the expression of antigens associated with the production of new viruses. (Middle) When a virus persists via continuous productive replication, the same antigens expressed during acute infection are produced, but the virus has the opportunity to evolve under immune selection to produce viruses that express neo-antigens and have altered pathogenetic capacities. (Bottom) In contrast to the previous two processes, viruses that persist via latency and reactivation can generate antigens associated with productive replication but also can enter into transcriptional states in which antigens associated with latent infection are expressed. In the most extreme case, no antigens are expressed, resulting in immunological silence. There may be more than one latency-associated gene program, and it is likely that latency gene programs are cell type specific. When these viruses become reactivated, they reinitiate a productive replication program and once again express viral antigens associated with the production of new viruses (Herbert et al, 2009).

Major mechanisms for the unresponsiveness of immune system;

Immune defects in chronic infectious diseases also affect T or B cells response. T cell exhaustion (antigen specific T cells fail to respond to antigens) which is the major type of T cell dysfunction appeared in chronic infectious diseases. These T cells initially develop effector functions, but prolonged or excessive stimulation leads to progressive loss of function over time.

There are at least four mechanisms to limit T cell responses to persisting viruses; first, T cell intrinsic mechanisms including induction of PD1 and CTLA-4 and altered expression of cytokine receptors such as IL-7R and IL-15R. The suppressive receptors, PD1 and CTLA-4 have been found to be induced in antigen specific $CD8^+$ by chronic LCMV infection (Barber et al, 2006) and coexpress in HIV specific $CD4^+$ cells (Kaufmann et al., 2007), while LCMV suppress the expression of IL-7R or IL-15R lead to alter TCR and cytokine receptor signal transduction (Lang et al, 2005). Second, the expression of immunoregulatory cytokines such as IL-10, TGF-β, and possibly additional factors produced by other cells in the environment can modulate and suppress vigorous antiviral T cell responses. It has been found that LCMV infection induced expression of IL-10 which

can induce naïve T cells become iTreg cells, while HCV induces expression of TGF-β that can impact immune responses during persisting infections (Alatrakchi et al., 2007). Third, regulatory cells can modulate antiviral effector T cells. Recent research shows that Tregs are more often associated with ineffective immune responses during chronic infections, including those by Friend leukemia virus (Zelinskyy et al., 2006), HIV (Kinter et al., 2007) (Nilsson et al., 2006), HCV (Boettler et al, 2005) (Ebinuma et al, 2008) (MacDonald et al, 2002), and HBV (Franzese et al, 2005) (Xu et al, 2006). The Treg cells could act through cell-to-cell contact, inhibition of APC maturation, production of immunoregulatory cytokines, or direct inhibition of CD8 T cell effector function. The function of Treg cells functions are enhanced by immunosuppressive chemokine and cytokines such as IL-10 and TGFb (Fantini et al, 2004) (Antonella et al, 2008), thereby creating synergic effect between mechanisms that inhibit T cell effectiveness during chronic viral infection (Herbert et al, 2009).

Finally, defects of APC function have been found in chronic infectious conditions. The quality of T cell stimulation can also be impacted by changes in dendritic cell function or number, as well as differences in antigen presentation by professional versus nonprofessional APC during persisting viral infection. DC as the delegate of APC has been found that monocyte-derived DCs in chronic HCV infection do not respond to maturation stimulation, which will maintain their immature DCs caused impaired antigen presenting function (Susanne et al, 2001).

In addition, defects in memory T cell differentiation during chronic viral infections are not just confined to effector functions, but also include a failure to develop into self-renewing antigen-independent memory T cells which due to reduce expression of CD127 and CD122, the receptor of IL-7 and IL-15 respectively. These factors are essential for the maintenance of memory T cells (Shin and Wherry, 2007).

Similar to viral mediated T cell defects, the effect function of B cells is also reduced in chronic infectious diseases such as deficient responds of B cells during HIV infection. There is a study shows that HIV-specific B cells, but not others, have a low proliferative capacity and express Fc-receptor-like-4 (FCRL4) that can

generate signals that inhibit B cell function (Herbert et al, 2009). There are evidences that alterations in B cell responses, B cell tolerance, and the accumulation of antibody-antigen immune complexes are associated with chronic viral infections and can contribute to disease (Casali and Oldstone, 1983)

5.3.1.1 Immune responses in acute and chronic HBV infection

There are one third of people (about 2 billion) have been infected with hepatitis B virus (HBV) among them about 400million people have become chronically infected. This virus markedly differs in their virological properties and in their immune escape and survival strategies from the other virus. In the acute infection, a rapid viral replication associates with a rapid and strong anti-viral immune response. HBV DNA is detectable in the circulation under one month after infection and keeps control low level of genome equivalents up to 6 weeks until HBV DNA and HBV envelope and surface antigen reach to peak. With the injury of T cell mediated liver, the serum alanine aminotransferase (ALT) level start to rise within 10-15 weeks before most of HBV DNA has been cleared (Rehermann and Nasximbeni, 2005). After acute infection, immune memory is established for protection of further infection.

In contrast to acute infection, the chronic infection has persistent viral load with chronic liver inflammation (Chen et al, 2004). Similar like the other chronic infection disease, chronic hepatitis B infection associates with immune unresponsiveness or low responsiveness (Reignat et al, 2002). The mechanisms for the immune unresponsiveness in chronic HBV infection are largely unknown. It has been found that HBeAg can induce T cell tolerance in transgenic mice (Chenet et al, 2004). The development of viral escape mutations in HBeAg was found which affect humoral immune responses. However, in chronic hepatitis B, T-cell escape mutants are not common (Rehermann et al, 1995) (Rehermann and Nasximbeni, 2005). The unresponsiveness of T cells to chronic HBV infection is largely due to the common mechanisms discovered in most chronic infectious diseases with high viral load such

as over expression of PD1 and CTLA-4 and high levels of inhibitory cytokines (Blackburn et al, 2008).

Recently, it has been demonstrate that Bim express the highest intracellular levels in CTLA-4 high expressed HBV specific $CD8^+$ T cell in chronic HBV infection (Schurich et al, 2011), which suggests that CTLA-4 may provide a pathway for T cell encountering the antigen in liver and then drive it to Bim dependent apoptosis. And also they suggested that only treat the patients with antiviral therapy is not enough, which will not show any affection after 7-12 months later, caused by high level of CTL-4 and Bim (Schurich et al, 2011). So the CLTA-4 blockade could be one of the therapeutic approaches for chronicle HBV therapy in the future.

5.3.2 T cell tolerance

In the T cell compartment, tolerance, or unresponsiveness, to self-antigens is maintained by the deletion of immature T cells that recognize these antigens in the thymus, and by several mechanisms that are operative in the periphery. Peripheral tolerance is attributable to the induction of functional anergy, deletion by apoptosis, and the suppressive actions of regulatory T lymphocytes (Treg) (Abbas et al, 2004). Self-tolerance of T cells is induced and maintained in different compartments of the immune system (Mondino et al, 1996). As mentioned developing T cells can be clonally deleted in the thymus as a result of negative selection, which requires the presence of relevant antoantigens. Peripheral tolerance is therefore a mechanism supplementary to central tolerance. The mechanisms responsible for peripheral $CD8^+$ T cell tolerance can be divided into those acting directly on the responding T cells, such as inactivation or deletion of specific T cell (Walker and Abbas2002) (Steinman et al 2003) and those that act through additional cells or factors, such as regulatory T cells or suppressive cytokines (Sakaguchi et al, 2004) (Hans et al, 2005). In most cases in which natural Treg cells participate in responses to infection, these are chronic infections. The influence of natural Treg cells may favourably affect the outcome or can be harmful to the host, and it most can be infect by the factors which

include the stage of infection, dose of the pathogen and genotype and immunological status of the host as well as the presence of concomitant disease or other infections. In chronic infections, it continually secreted low dose of viral which will finally confuse the body to protect it as a part via Treg. As a result there are only non-responsed antigen specific T cells left in patient body (Figure 5.3).

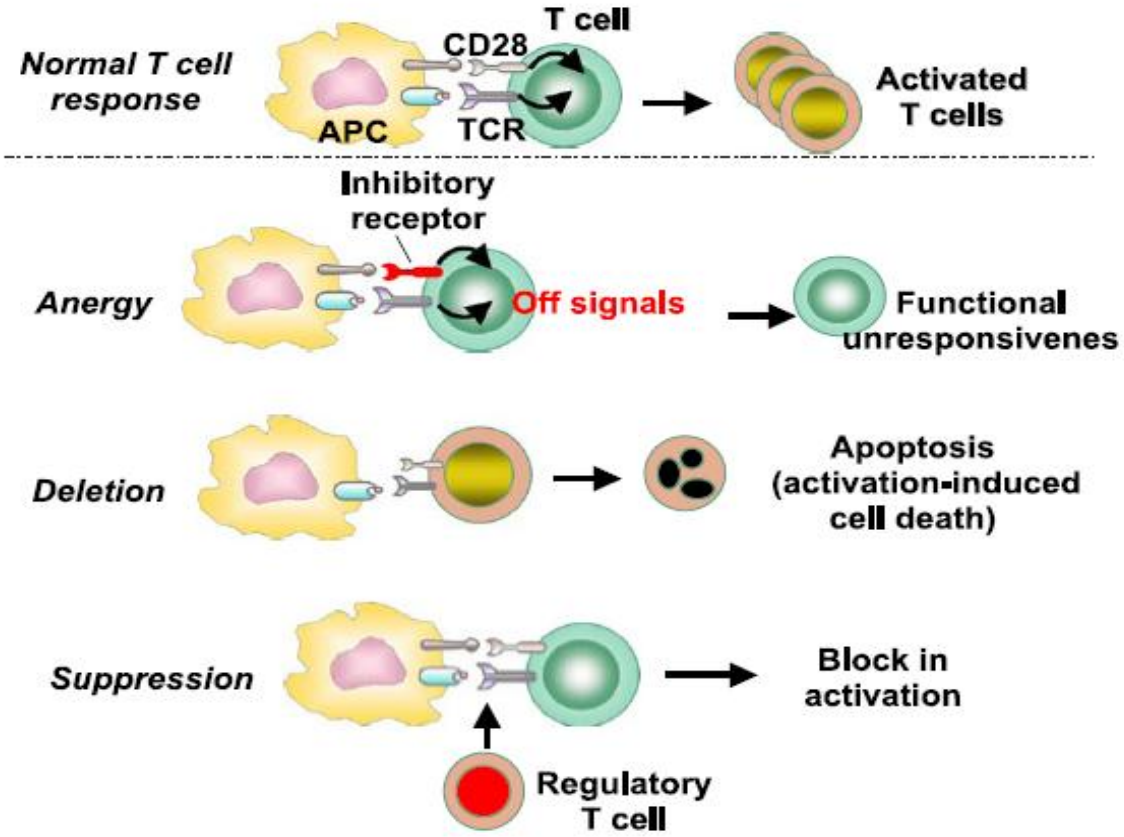

Figure 5.3 Mechanisms of peripheral T cell tolerance.
The mechanisms that maintain tolerance in CD4 T cells are illustrated, and compared with a normal immune response (Abbas et al, 2004).

6. Vaccines

6.1 Immunity is the ability of an organism to resist infection.

Vaccination is the best known and the most successful application of immunological principles to human health. The first vaccine was named after vaccine, the cowpox virus. Jenner pioneered its use 200 years ago. It was the first deliberate scientific attempt to prevent an infectious disease (smallpox), but it was done in complete ignorance of viruses (or indeed any kind of microbe) and immunology. Jenner's achievement was the realization that infection with a bovine analogue of smallpox (vaccinia), which caused cowpox, would provide protective immunity against small pox in humans without the risk of significant disease.

It was not until the work of Pasteur 100 years later that the general principle governing vaccination emerged: altered preparations of microbes could be used to generate enhanced immunity against the fully virulent organism. During his work on chicken cholera he observed that a culture if the responsible bacteria had spoiled and failed to induce the disease in some chickens he was infection with the disease. Upon reusing these healthy chickens he discovered that he could not infect them, even with fresh bacteria; the weakened bacteria had caused the chickens to become immune to the disease, even though they had only caused mild symptoms. After inoculating the infection being fatal, as usual, the chickens recovered completely. Pasteur guessed the recovered animals now might be immune to the disease. He applied this immunization method to anthrax, which affected cattle, and aroused interest in combating other method to anthrax, which affected cattle, and aroused interest in combating other diseases. He publicly claimed he had made the anthrax vaccine by exposing the bacillus to oxygen. Pasteur produced the first vaccine for rabies by growing the virus in rabbits, and then weakening it by drying the affected nerve tissue.

The notion of a weak form of a disease causing immunity to the virulent version was not new; this had been known for a long time for smallpox. Inoculation with smallpox was known to result in far less scarring, and greatly reduces mortality, in comparison to the naturally acquired disease. As mentioned earlier Edward Jenner had also discovered vaccination, using cowpox to give cross-immunity to smallpox (in 1796), and by Pasteur's time this had generally replaced the use of actual smallpox material in inoculation. The difference between smallpox vaccination and cholera and anthrax vaccination was that the weakened form of the latter two disease organisms had been generated artificially, and so a naturally weak form of the disease organism did not need to be found (Figure 6.1).

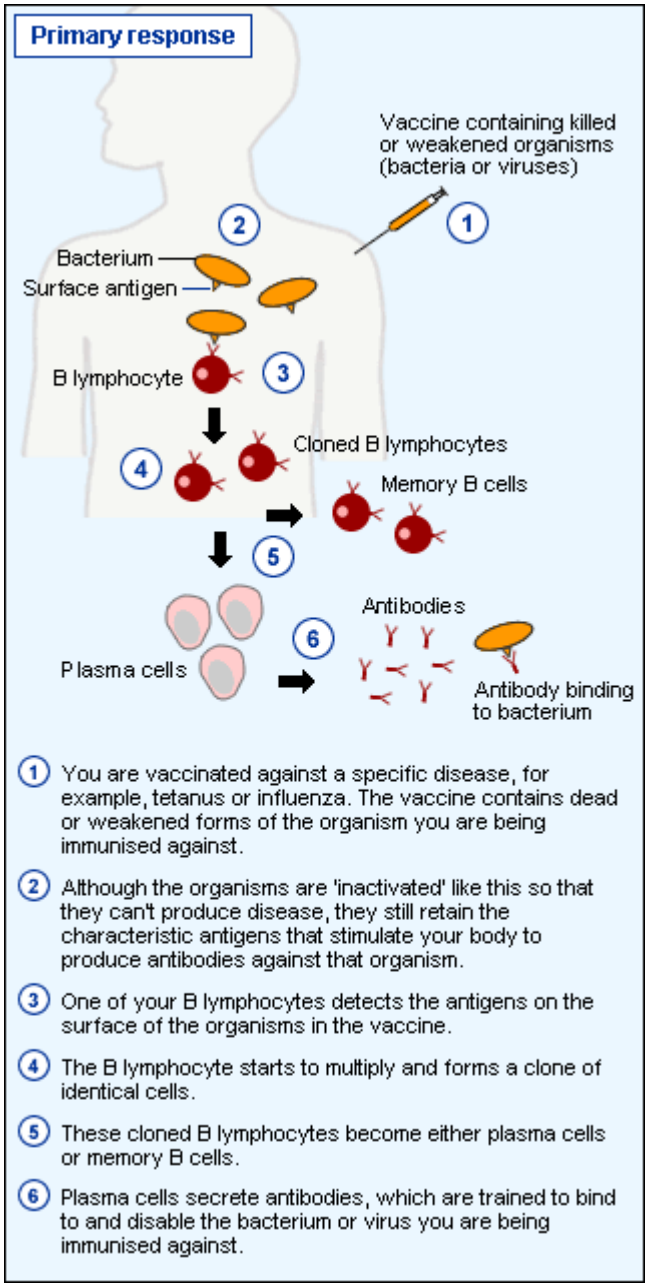

Figure 6.1 Vaccination

The term was originally used by Edward Jenner to describe the process of inoculating patients with discharge of cowpox to protect them from smallpox. Nowadays vaccination applies to the administration of any antigenic material (the vaccine) for the purpose of stimulating active immunity to a disease.

6.2 Antigens used as vaccines

Different vaccination strategies aim to deliver pathogenic protein to antigen presenting cells, that will be processed *in vivo* deliver pathogenic proteins to antigen processing pathways. A major drawback of is that proteins are broken down into many peptides, very few of which are immunogenic. Peptide vaccination offers a solution to this problem, and involves vaccination with characterised T cell specific peptide epitopes that stimulate protective immunity. However, it is not possible to generate MHC class I specific T cell responses by *in vivo* immunisation with free peptides, as free peptides are diluted and degraded too fast once administered. On the other hand, efficient peptide presentation by APCs occurs with endogenously processed antigens (Cresswell et al, 2005) (Zhang and Williams, 2006).

1. **Attenuated pathogen.** Attenuated live vaccines have been highly successful against viruses (e.g. polio, measles, mumps etc) and bacteria (e.g. tuberculosis), generally being easier to attenuate the former than the latter. The aim is to diminish the pathogen's virulence while retaining the desired antigens. Attenuated pathogens are 'changed' pathogens in the sense that are less able to grow and cause disease in their natural host. 'Changed' means that mutations are induced to them. The results of attenuation are widely divergent. An example is the divergence between the three types of live polio vaccine; Type 1 polio has 57 mutations and has almost never reverted to wild type while Type2 and 3 vaccines depend for wild type has occurred, in some cases leading to outbreaks of paralytic poliomyelitis.

2. **Killed/inactivated pathogen.** Killed vaccines are intact but non-living organisms. Some are very effective (rabies and the Salk polio vaccine), some moderately so (typhoid, cholera and influenza), some are of debatable value (Plague and typhus) and some are controversial on the grounds of toxicity. Some of these will undoubtedly be replaced by attenuated or subunit vaccines.

3. **Inactivated toxins and toxoids.** They are the most successful bacterial vaccines. Vaccines against tetanus and diphtheria are based on inactivated exotoxins and constitute the most successful of all bacterial vaccines.

4. **Subunit vaccines.** Aside from toxin-based vaccines which are subunits of their respective microorganisms, a number of other vaccines are employed, which make use of antigens whether purified from microorganisms or produced by recombinant DNA technology. While hepatitis B surface antigen is immunogenic when given with alum adjuvant, the bacterial capsular polysaccharides of Neisseria meningitis, Streptococcus pneumonia and Haemophilus influenza B give lasting protection because helper T cells are not activated by polysaccharide alone. A significant improvement in the efficacy of these vaccines has been obtained by chemically conjugating the purified polysaccharides to protein carriers (e.g. tetanus or diphtheria toxoid), which provide peptide that can be recognised by antigen-specific T cells, thus converting a T cell independent response to a T cell dependent anti-polysaccharide antibody response.

5. **Recombinant vector vaccines.** This class of vaccines utilises attenuated versions of certain microbes as recombinant vectors to express target antigens from other pathogens. Thus the desired gene is incorporated into a vector which can then be injected into the patient, allowed to replicate, express the gene and produce large amounts of antigen in situ. Vaccinia (Kass et al, 1999) is a convenient vector that is large enough to carry several antigens. A number of experimental vaccines using recombinant vaccine have been tested (expressing surface protein from HIV, influenza, etc), though none are yet in routine use. Many other viruses have also been proposed and tested experimentally as vaccine vectors. Viral vector vaccines elicit strong humeral and cell mediated immune response, resulting in immunological memory. They can be targeted by viral tropisms inducing desired immunity and can encode several antigens at the same time. Also they do not interfere with the protection produced by other kinds of vaccines and are relatively inexpensive. On the

other hand, there is always the risk of reversion to virulence and of pathology caused due to immune responses to virus-infected cells.

Attenuated bacteria have the advantage that they have genomes large enough to incorporate many genes from other organisms. They are generally safe and reversions can be controlled. Depending on the bacteria used MHC class I and/or II antigen processing pathway can be targeted. Some bacteria induce both cell mediated and humeral immune responses, including mucosal immunity since some bacteria can survive in the gastrointestinal tract, making this technology attractive for oral immunisations.

6. **DNA vaccine.** A new approach to protect susceptible individuals against a lethal disease is the administration of plasmid DNA by direct inoculations with the intent of inducing an immune response to the protein encoded therein. The beginning of the development of such a vaccine started with the fundamental experiment by Wolf et al (1990). They demonstrated that a single intramuscular (i.m.) inoculation of plasmid DNA encoding the reporter protein β-galactosidase induces the expression of this protein without the use of a delivery vehicle under *in vivo* conditions. Using histochemical staining procedures forβ-galactosidase activity, blue-coloured muscle cells were easily detectable at the site of inoculation 7 days after injection. Moreover, transfected muscle cells were surrounded by infiltrating leukocytes, indicating the activation of a specific immune response against the foreign antigen. Western Blot also showed that there wereβ-galactosidase-specific antibodies.

Other types of injections tried in DNA vaccination include intradermal (i.d), mucosal, and biojector injections as well as direct skin delivery has been reported. The majority of DNA vaccination though involves i.m. or i.d. administration. After i.m. injection DNA is taken up by myocytes and/or professional APCs with subsequent expression of the foreign antigen via the MHC class I pathway. Secreted antigens may be ingested by phagocytes and then presented via the MHC class II pathway. These exogenous antigens may prime both the induction of antibody responses as well as $CD4^+$ T cell activation. However activation of naïve $CD8^+$ T

cells to armed effecter T cells can only occur through MHC class I presentation by bone marrow-derived APCs (and not myocytes).

With the gene gun delivery system, which shoots plasmid DNA on golf particles onto skin via helium gas acceleration, it was demonstrated that DNA is delivered directly into Langerhans cells. Thereafter, these transfected cells migrate to draining lymph nodes presenting the foreign antigen to the immune system (Condon et al, 1996).

In general, DNA vaccines depend upon the delivery route and method of delivery. i.m. inoculation drives immune responses mainly towards the Th1 response (Raz et al, 1996). However the gene gun method requires much less DNA compared to i.m. administration and gives immune responses both of the Th1 and Th2 types (But the mechanism is not clear yet) (Feltquate et al, 1997) (Prayaga et al, 1997). Initial clinical trials indicate that DNA vaccines are currently tested.

The two major advantage of DNA vaccine include that they encode multiple cell-mediated immune responses. In animal studies the vaccination induced a long term protection. Additionally, large-scale manufacturing procedures are available and such vaccines are thermostable and nonvirulent. Finally a more simplified and effective quality control process is allowed.

7. **Peptide vaccines.** Peptide vaccination involves immunisation against usually tumours with identified and characterised immunogenic peptides derived from antigens associated with tumours. Peptide vaccines are either in the form of free peptide administered with adjuvant, peptide-pulsed dendrite cells, or peptides pulsed or incorporated in membrane carriers. Detailed discussion of peptide vaccines will follow in this chapter.

6.3 Adjuvants

Some vaccines contain components that enhance their immunogenicity. These components, called adjuvants, boost the immune response by providing inflammation signals and activate APCs. For example, tetanus toxoid is not immunogenic in the absence of adjuvants, and tetanus toxoid vaccines often contain aluminium salts, which bind polyvalently to the toxoid by ionic interactions and selectively stimulate antibody responses. Pertussis toxin, produced by B. pertussis, has adjuvant properties in its own right and, when given mixed as a toxoid with tetanus and diphtheria toxoids, not only vaccinates against whooping cough but also acts as an adjuvant for the other two toxoids. This mixture makes up the DPT triple vaccine given to infants in the first year of life.

Many important adjuvants are sterile constituents of bacteria, particularly of their cell walls. For example Freund's complete adjuvant, widely used in experimental animals to augment antibody response, is an oil and water emulsion containing killed mycobacteria. A complex glycolipid, muramyl dipeptide, which can be extracted from mycobacterial cell walls or synthesized, contains much of the adjuvant activity of whole killed myxobacteria. Other bacterial adjuvants include killed B. pertussis, bacterial polusaccharides, bacterial heat shock proteins and bacterial DNA. Many of these adjuvants cause quite marked inflammation and are not suitable for use in vaccines for humans.

It is thought that most, if not all, adjuvants act on APCs (especially DCs) and this reflects the importance of these cells in initiating an immune response. Tissue DCs take up antigens from their environment, then migrate into the lymphoid tissue and present these antigens to T cells. They appear to detect the presence of a pathogen in two main ways. A) Ligation and activation of receptors for invading microorganisms. These include receptors of the complement system, Toll-like receptors, and other pattern recognition receptors of the innate immune system. There is much that we don't know about the direct mechanisms of detection of infectious agents. For example APCs get powerfully activated by bacterial DNA

containing unmethylated CpG dinucleotide motifs, bacterial heat shock proteins and muramyl dipeptide and it is not know how they detect them (even though there is indirect evidence that many adjuvants use various Toll-like receptors). APCs upon direct activation, respond by secreting cytokines and expressing co-stimulatory molecules, which in turn stimulate the activation and differentiation of antigen specific T cells. B) The second mechanism of stimulation of DCs by invading organisms is indirect and involves their activation by cytokine signals derived from the inflammatory response triggered by infection. Cytokines such as GM-CSF are particularly effective in activation DCs to express co-stimulatory signals and, in the context of viral infection; DCs also express IFNα and IL-2.

Different types of adjuvants promote different kinds of immune response. For example, pertussis toxin stimulates mucosal immune responses, which are particularly important in defence against organisms entiering through the digestive or respiratory tract. Another vaccine approach is coadministering cytokines. For example IL-12 has been used as an adjuvant to promote protective immunity against the protozoan parasite Leishmania major.

Apart from boosting the immune response adjuvants have a very important role in converting soluble antigens into particulate material, which is more readily ingested by APCs such as macrophages. For instance, the antigen can by absorbed on particles of the adjuvant (such as alum), made particular by emulsification in mineral oils, or incorporated into the colloidal particles of Immune Stimulatory Complexes (ISCOMS). This enhances immunogenicity somewhat but such adjuvants are relatively weak unless they also contain bacteria or bacterial produces.

6.4 IL-2

IL-2 is a 15,000 kDa α-helical cytokine produced predominately by active $CD4^+$ and $CD8^+$ T cells which can active dendritic cells (DC), natural killer (NK) cells and NKT cells. Originally designated T cell growth factor (Morgan et al, 1976), IL-2 supports the growth and proliferation of antigen activated T lymphocytes and

plays a central role in the cascade of cellular events involved in the immune response (Fauci, 1987). IL-2 is rapidly produced by naïve T cell when stimulated by TCR and co-stimulatory molecules via APC. As described, upon activation, resting T cells are induced to express IL-2 receptors and to secrete IL-2. This autocrine stimulation drives the proliferation of activated, receptor-bearing T cells and the generation of antigen specific effector T cell types, including cytotoxic, helper and suppressor T cells (Figure 6.2).

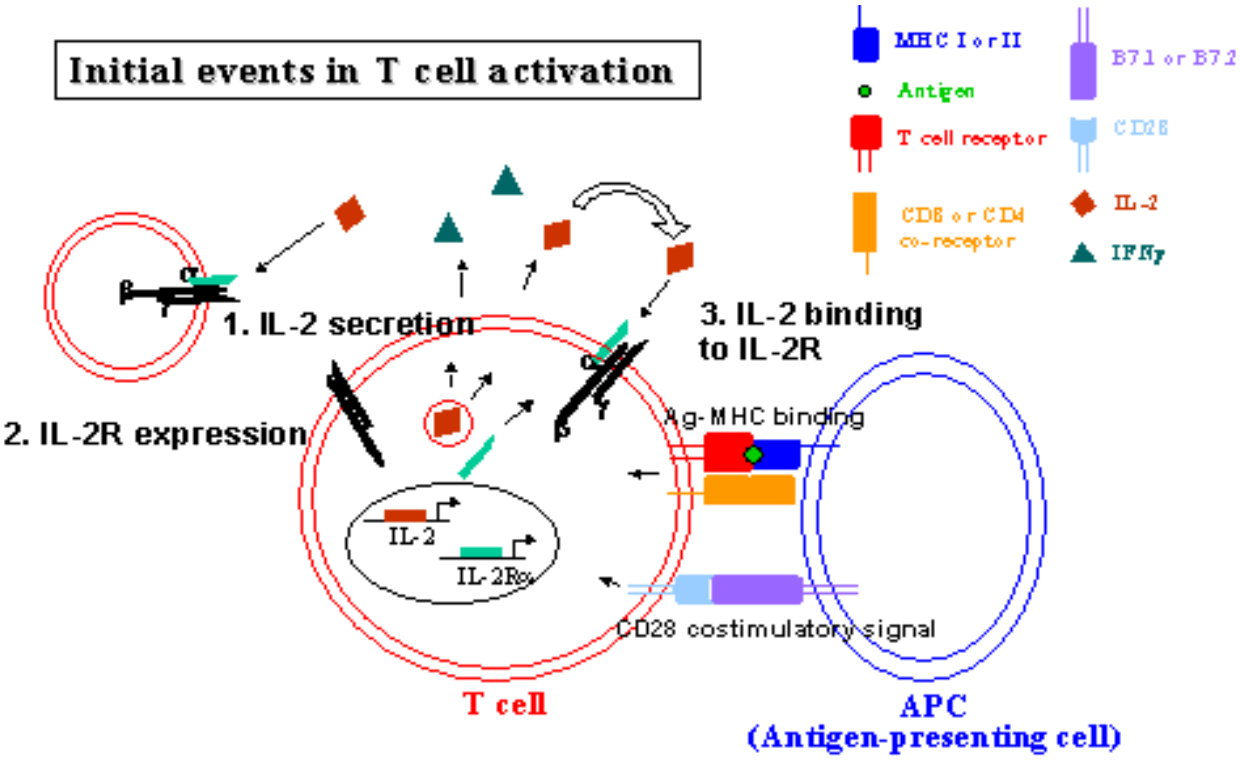

Figure 6.2 CD28 dependent co-stimulation of activated T cells induces expression of the T cell growth factor interleukin-2 and the high affinity IL-2 receptor

6.4.1 IL-2 receptor

The IL-2 receptor is a heterotrimeric protein expressed on the surface of certain immune cells which was the first interleukin receptor described by Kendall Smith. There are three subunits, IL-2Rα (CD25), IL-2Rβ (CD122), and the common gamma chain or γc (CD132). CD122 and CD132 are primarily folded into β-sheet structures as low affinity IL-2R (Nelson and Willerford 1998) (Malek, 2008). Once

all three subunits form together, they will become high affinity IL-2R. Functional structures of IL-2 and IL-2R interaction reveals that each receptor subunit will contact with IL-2, most contact will be at the IL-2/CD25 interface. A strong interaction between CD25, CD122 and CD132 leads to a stable quaternary complex of IL-2, CD25, CD122 and CD132 (Stauber et al, 2006) (Rickert et al, 2005) (Thomas R. Malek, 2008). The IL-2 and IL-2R complex on cell surface is transient (10 to 20 min) and they are rapidly internalized. However, it requires a few hours for IL-2 to drive T cells into cell cycle. It is therefore a continuous expression of IL-2 and its receptor is essential for IL-2 induced T cell proliferation. (Cantrell and Smith. 1984) (Malek, 2008).

CD25 does not express on naïve T cell. Following TCR and co-stimulatory signals, initially a moderate level of CD25 are rapidly induced by after activation of transcription factors of NF-κB, NFAT, AP-1, and CREB/AFT, while after interaction with IL-2, IL-2R mediates activation of Stat-5 pathway leading to a high level expression of CD25. This mechanism is to increase IL-2 binding and hence signalling by activate T cells though enhanced capture of IL-2 by CD25 (Kim et al, 2006) (Malek, 2008).

The initial encounter with specific antigen in the presence of a co-stimulatory signal triggers entry of the T cell into the G1 phase of the cell cycle; at the same time, it also induces the synthesis of IL-2 along with the α chain of the IL-2 receptor, also known as CD25. Resting T cell express a form of this receptor composed of β and γ chains that binds IL-2 with moderate affinity, allowing resting T cells to respond to very high concentrations of IL-2. Association of the α chain with the β and γ heterodimer creates a receptor with a much higher affinity for IL-2, allowing the cell to respond to very low concentrations of IL-2. Binging of IL-2 to the high affinity receptor then triggers progression through the rest of the cell cycle (Figure 6.2).

Activation of $CD8^+$ T cells leads to expression of high affinity IL-2 receptor in lieu of the moderate affinity receptor in resting T cells. It is the binding of IL-2 to its high affinity receptor that initiates cell cycle progression and proliferation. IL-2

may act in an autocrine manner and thus stimulate proliferation into thousands of antigen specific clones. With continued maturation, the T cell develops into an armed effector T cell not requiring co-stimulation to mount an immune attack.

6.4.2 IL-2 signalling pathway

Human IL-2 is a 133 amino acid polypeptide. IL-2 signalling is mediated by IL-2R. The IL-2R α chain primarily increases the affinity of ligand binding, whereas the β and γ chain participate in both ligand binding and signal transduction. There are at least three different pathways in IL-2R signalling system proceeds, which mediate the flow of mitogenic and survival promoting signals (Figure 6.3). First pathways proceeds through protein tyrosine kinase activity, Ras and the MARK (Mitogen-Activated Protein Kinase) cascade, leading to expression of the protooncogenes c-Fos, c-Jun, and Eik1. The Syk, which for the second pathway is responsible for c-Myc gene induction. And the final pathway results in BCL2 (B Cell Leukemia-2) expression, and progression through a Rho, Pl3k (Phosphoinositide-3 Kinase) and Akt/ PKB (Protein Kinase-B) mediated signalling pathway. As the last pathway is also involved in IL-2 promoted regulation of actin cytoskeleton organization (Gomez et al, 1997)

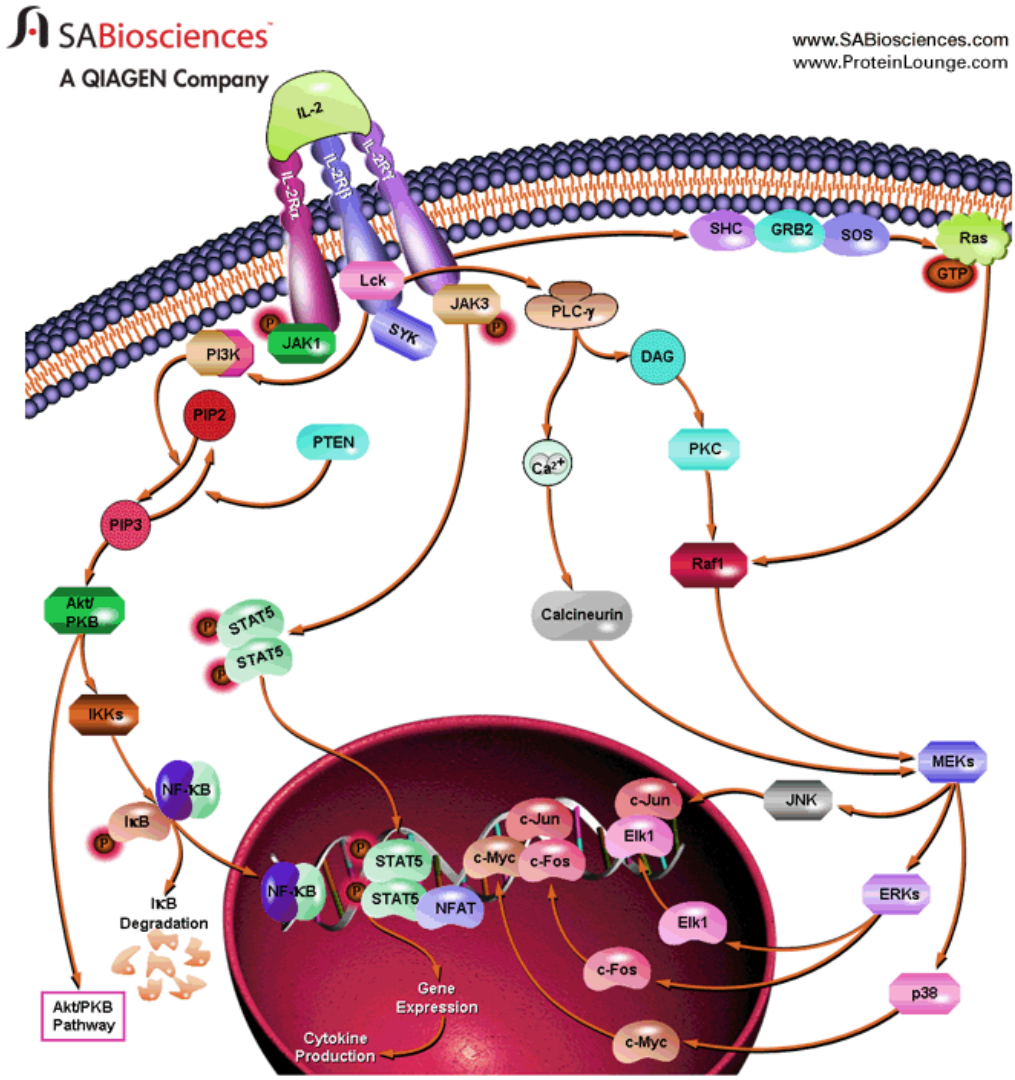

Figure 6.3 IL-2 Signalling pathways

6.4.3 IL-2 function

IL-2 is crucial for *in vitro* proliferation of T cells and is assumed to be the central growth hormone of the immune system. And as most of the data support that IL-2 play essential role in immune tolerance, but this idea is overthrow by IL-2 or IL-2R deficient mice (Sadlack et al, 1995) (Suzuki et al, 1995). This change is occurred when T regulatory cell defect was shown to be responsible for the lethal autoimmunity associated with IL-2/ IL-2R deficient. Most T cells have the potential to secrete IL-2. Treg cells, however, do not produce IL-2, and this represents one of their defining features. In contrast to naïve T cells, the IL-2 proximal promoter in

Treg cells does not undergo chromatin remodeling upon TCR activation (Su et al 2004). Moreover, Foxp3 directly binds to the minimal IL-2 promoter in association with NFAT and further downstream in association with AML1/Runx1, contributing to active transcriptional repression of IL-2. Recent studies indicate that Treg lineage commitment ensues without expression of Foxp3 (Gavin et al 2007) (Lin et al 2007). An important function of Foxp3 is to amplify and fix these pre-existing features required for the suppressive program. Moreover, paracrine IL-2 was suggested as essential for establishing this program (Gavin et al 2007). As the Treg cell suppressive program depends on consistent high levels of Foxp3 (Wan et al 2007) (Gavin et al 2007), a fundamental role of IL-2 likely lies in its ability to increase Foxp3. Another link between IL-2R signaling and expression of important molecules for Treg function is the fact that enforced expression of Foxp3, TGF-β, or CTLA-4 into IL-2- or CD122- deficient T cells controls many aspects of autoimmunity when such cells are present in IL-2- or CD122-deficient mice (Hwang et al, 2004) (Carrier et al, 2007) (Malek, 2008).

6.4.2 IL-2 as an adjuvant in different types of vaccination

As mentioned adjuvant play a key role in vaccination strategies. In many vaccinations delivery system the immune response is significantly boosted by co-administration of IL-2. IL-2 is responsible for promoting cell division in a resting T cell, and it also treat as a key to reverse the tolerance (Essery et al, 1988) (Figure 6.4). They were of interest to determine whether IL-2 could reverse tolerance. They culture T cells with IL-2 after induction of unresponsiveness. Once the T cells are cultured with IL-2 tolerance T cell restore ability to response.

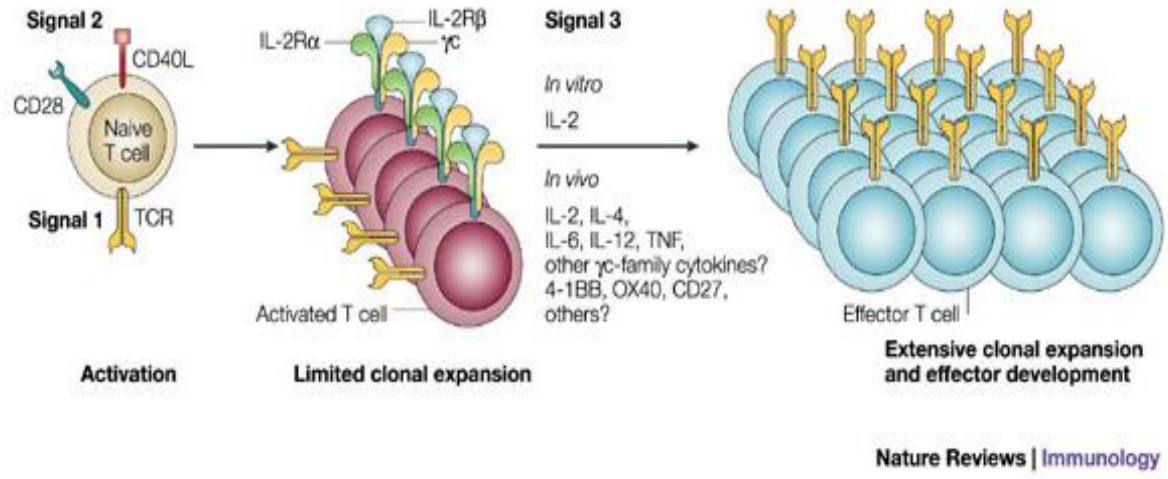

Figure 6.4 IL-2 can reverse tolerance of T cells

Proliferating T cells also produce a variety of other lymphokines that affect other arms of the immune system. B cell specific lymphokines produced by activated T cells (interleukins 4, 5, and 6) act in conjunction with T cell help to activate B cells and to generate mature antibody producing plasma cells and memory B cells (Coffman et al 1988) (Seghal et al. 1987). Another T cell produced lymphokine, interferon γ, induces expression of class II histocompatibility antigens on cells of the monocyte/macrophage lineages and activates these phagocytic and antigen presenting cells (Basham et al, 1984).

In view of these direct and indirect actions of IL-2 in the immune response, it has been postulated that IL-2 may function as a potent adjuvant to vaccination, to increase the specific and durable response to vaccine immunogens.

Recombinant human IL-2 (Rosenberg et al, 1984) (Wang et al, 1984) has been shown to be biologically active in cells of many mammalian species (Fong and Doyle, 1986). Previous work showed that IL-2 can enhance protection against Haemophilus pleuropneumonia in seine when IL-2 is administered systemically in conjunction with intramuscular H. pleuropneumonia vaccination (Anderson et al, 1987). Daily treatment with IL-2, at the time of vaccination and for 4 days thereafter,

was substantially more efficacious than a single IL-2 treatment at the time of vaccination. It was concluded that the continued presence of IL-2, throughout the period of the immune response, is important in the adjuvant effect. This interpretation is consistent with the *in vivo* action of IL-2 during the immune response (Fauci, 1987).

IL-2 was also used as an adjuvant to inactivated rabbits virus vaccine. The National Institutes of Health (NIH) test for rabies vaccine potency (Seligman, 1973) was used to study the effect of daily, systemic IL-2 administration on the potency of inactivated rabies virus vaccine (Nunberg et al, 1989). Nunberg et al (1989) found that daily systemic administration of IL-2 in conjunction with inactivated rabies vaccine can increase the potency of vaccination in out bread mice at least 25-fold, as measured by survival following challenge with virulent rabies virus. The same study revealed that enhanced protection is not correlated with an increase in virus-neutralising antibody titers and suggested that IL-2 acts to increase the cellular immune response to vaccination.

In the recent years IL-2 has been used in conjunction with the highly active antiretroviral therapy (HAARA) against HIV. As IL-2 is the major T cell growth factor that its exogenous administration could help restores immune function in HIV positive patients. This assumption led to the development of therapeutic strategies aimed at modulating IL-2 signal strength for clinical benefit. Indeed, numerous controlled clinical studies in HIV-positive patients with widely varying CD4 counts have now demonstrated that when combined with HAART, IL-2 results in a significant rise in CD4 cell count compared with HAART alone, with only transient or even no associated bursts of HIV plasma viraemia (Kovacs et al, 1996) (Hengge et al, 1998) (David et al, 2001) (Katlama et al, 2002) (Levy et al, 2003). Martinez-Marino et al (2004) reports that IL-2 therapy in combination with HAART leads to significant increases in CD4 numbers that are maintained for six months after discontinuation for the IL-2 treatment. This group also report that CD8 cell non-cytotoxic anti-HIV response was restored among subjects receiving HAART and IL-

2 whereas it declined among those receiving HAART alone and in untreated infected subjects. The percentage of HAART subjects with CD8 cells showing at least 50% suppression of HIV replication increased significantly following IL-2 therapy and persistence for 6 months. According to Marchetti et al (2005), IL-2 administration resulted in a significant increase in CD4 cells, sustained up to one year of follow up, whereas HAART patients experienced a much less relevant and slower rise in CD4 cells. Furthermore Machetti and her group report that clinical recods of the patients enrolled suggested that the accelerated IL-2 driven CD4 cell gain might indeed be effective in preserving an adequate cellular immunity, as no HIV-related clinical eevents were observed among IL-2 treated patients, whereas more than 30% of HAART alone patients presented minor opportunistic infections.

In DNA vaccination against cancer coexpression of cytokine genes together with antigen-encoding genes in DNA vaccination vectors can increase humoral and cellular immune responses and may steer them in a T_H1 or T_H2 direction. In a study by He et al (2005), a plasmid expressing the OVA tumour antigen incorporated into the signal peptide of human IL-2 was tested as a DNA vaccine in a murine model system. Results showed that antigen specific CTL responses were elicited by intramuscular injection of these plasmids. Importantly, compared with the minigene vector expressing the same OVA epitope, IL-2 expression plasmid vaccination was more effective in protection mice from OVA expressing tumour challenge. The improved efficacy appears to result from enhanced antigen presentation as well as the immunostimulatory activity of IL-2.

But IL-2 does get limitation which has been documented. It shows that higher doses of IL-2 appear better clinical responses but cause appreciable toxicity named capillary leak (Waldmann, 2006)

6.5 Other cytokines used as adjuvant in vaccine development.

IL-2 is the first cytokine administered in a vaccine trial. But there are many other cytokine also contributed as the adjuvant to enhance the vaccine response, like

IL-7 and IL-21 for the clinic offered the promise of enhancing anti-tumour responses but with far less systemic toxicity and no expansion of regulatory T cell. Some preclinical studies are also demonstrated that IL-15 could improve T cell and particular NK cell responses too (Christian et al, 2009).

Il-7 can be produces by a variety cells and tissues but not by lymphocytes themselves. It can involved in maintenance and survival of αβ T cells as well as development of B cell and γδ T cells, and also may play a role in DC and monocytes' biology except to support NK cells. IL-7 does not appear to support NK cells. Thus, IL-7 plays a critical role in lymphocyte homeostasis as indicated by markedly diminished lymphocyte counts in IL-7 and IL-7 receptor gene deleted mice and the severe combined immunodeficiency associated with IL-7 receptor mutations in humans. An extensive review of IL-7 biology and signalling can be found elsewhere, but here is focus to point out it as an agent for adjuvant therapy for vaccines (Fry et al, 2002) (Capitini et al, 2009) (Christian et al, 2009). Recently, adjuvant IL-7 was shown to improve vaccine mediated survival in a spontaneously occurring murine tumour model via enhanced Th17 differentiation and reduced T cell-intrinsic inhibitory networks. IL-7 is a very promising agent to enhance overall immune competence and, potentially, tumour specific immune responses. The absence of Treg expansion and the lack of toxicity observed in this clinic would suggest that IL-7 offers definite advantages over IL-2 as an adjuvant. But IL-7 does play a role in either the initiation or maintenance of some leukaemias and lymphomas which described as the potential limitations for IL-7 therapy. Therefore it will need to be used with extreme caution in immunotherapy regimens involving lymphoid malignancies (Korte et al, 1999) (Vudattu et al, 2008) (Christian et al, 2009).

IL-15 is constitutively expressed by a variety of cell types and tissues, but in contrast to IL-2, is mainly membrane bound. IL-2 and IL-15 have pivotal roles in the control of the life and death of lymphocytes. IL-15 and IL-2 exhibit similar immune effects and share the IL-2 receptor subunits IL-2 β and γ except α chain (Waldmann 2006) (Budagian et al, 2006) (Fehniger et al, 2002) (Christian et al, 2009). IL-15 is

required for the differentiation of NK cells and plays a role in maintaining and expanding $CD8^+$ T cells particularly memory subsets, NK cells, NKT cells interferon-killer DCs and γδ T cells. And IL-15 may also have function related to B cells and APCs. So these features of IL-15 biology will have important implication to utilize this cytokine as a therapeutic agent. There have been numerous preclinical studies exploring IL-15 as a vaccine adjuvant. Although the majority has been in infection models, a number of reports of the adjuvant effect of IL-15 have been published. One important observation is that IL-15 can revert tolerant T cells to become effectors (Teague et al, 2006). Adjuvant use of IL-15 can enhance vaccine responses to both dominant and subdominant, tumour antigens (Melchionda et al, 2005). Recently, IL-15 administered after a gene-modified vaccine resulted in enhanced anti-tumour activity in a murine melanoma mode (Basak et al, 2008). Based on the available pre-clinical data, IL-15 would appear to be well suited as an adjuvant to cancer vaccines (Christian et al, 2009).

IL-21 is homologous to IL-15, but the receptor for IL-21 is comprised of a unique subunit designated IL-21Rα and the IL-2Rγ(c). IL-21Rα is expressed on most mature lymphocyte populations (Figure 6.5). Production of IL-21is restricted to activate $CD4^+$ T help cells (Wurster et al, 2002). IL-2 appears to play important roles in modulating responses of lymphocytes to other cytokines. IL-21 has also been shown to induce IL-10 production in models of lupus, suggesting that like IL-2, it can also contribute to immunosuppressive activity (Spolski et al, 2009) (Christian et al, 2009). Besides enhancing IL-2 therapy, IL-21 may also improve the effectiveness of other cytokines and immunotherapies. Combining IFNα and IL-21 increases NK cell and $CD8^+$ T cell mediated cytotoxicity in an experimental model of RCC, leading to inhibition of tumour growth and an increased survival (Eriksen et al, 2009). IL-21 can also significantly augment IL-7 induced expansion of cytotoxic T cells, possibly by preventing the cytokine-induced down-regulation of CD127 on antigen-stimulated T cells, results which suggest that IL-21 may also play a cooperative role with IL-7 in modulating primary $CD8^+$ T cell responses (Liu et al, 2007). Lastly, in regards to a pediatric tumour, vaccinating with IL-21-gene-modified cells in a

syngeneic metastatic neuroblastoma model demonstrated a reduction of microvessels in late metastases from therapeutically vaccinated mice. A role of survivin as a tumour antigen was suggested since a specific T cell response against this antigen was induced (Søndergaard et al, 2007) (Christian et al, 2009).

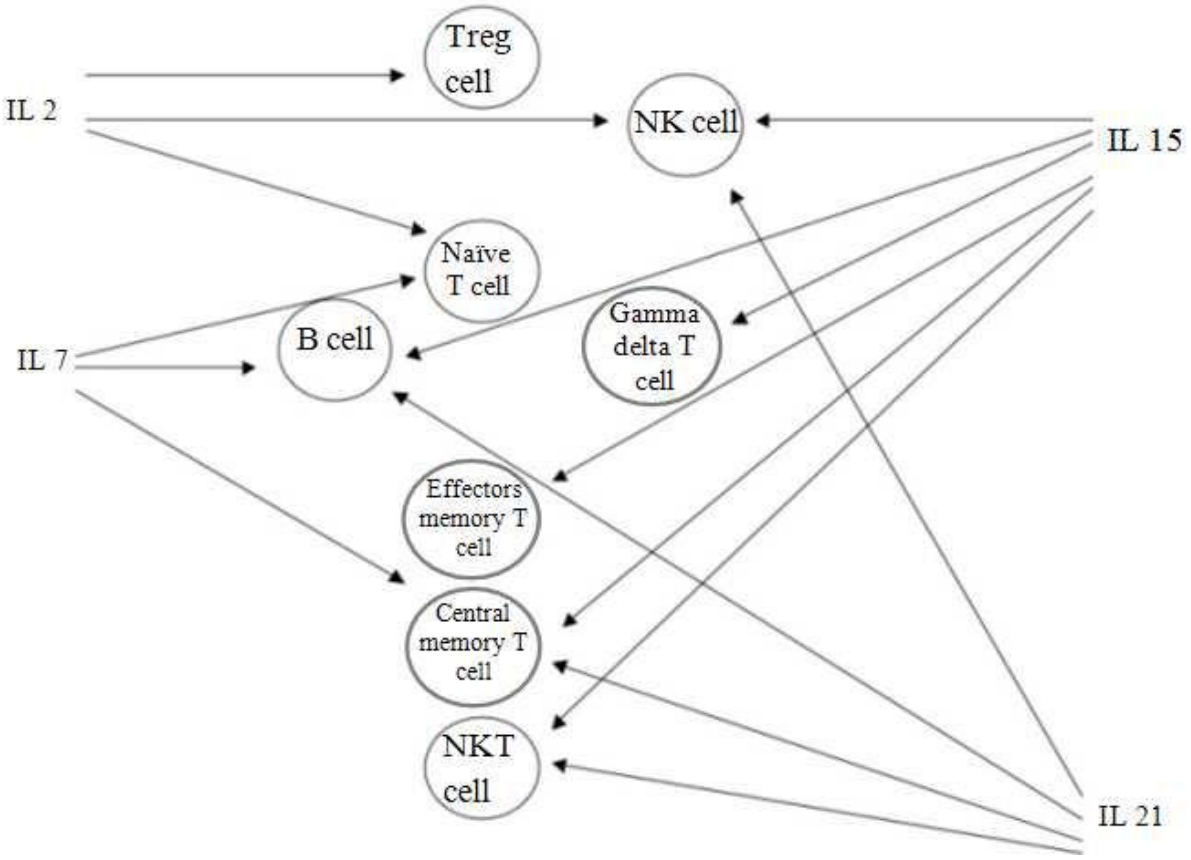

Figure 6.5 Target lymphocyte population for common cytokine receptor γ-chain family cytokines .
There is considered that the effects of stimulatory or inhibitory on lymphocytes depend on the cytokine. IL-21 only increases proliferation of T cells stimulated with anti-CD3 or antigen, but can augment responses to other gamma(c) cytokines. IL-7 acts on developing B cells but not mature cells of this lineage. IL: Interleukin, NK: Natural Killer, NKT: Natural Killer-T cell, Treg: Regulatory T cell (Christian et al, 2009).

6.6 Dendritic cell-based vaccination approaches

There is large literature involving vaccination strategies using DCs loaded ex vivo with antigens. Different sources of DCs for ex vivo loading have been used. 1) Peripheral blood DCs. There are obtained by leukaheresis from blood, followed by

positive or negative selection (Hsu et al, 1996) (Small et al, 2000) (Reichardt et al, 1999). 2) Monocyte-derived DCs. Culture of $CD14^+$ monocytes and plastic adherent mononuclear cells for 7 days in media containing GM-CSF and IL-4, TNF-a, or IL-13 is the most popular culture method (Sallusto and Lanzavecchia, 1994) (Cao et al, 2000) (Morse et al, 1993). 3) $CD34^+$ precursor-derived DCs. The generation of these DCs requires a more substantial cocktail of cytokines, including stem cell factor Flt3L, IL-3, and IL-6, followed by differentiation with IL-4 and GM-CSF (Chen et al, 2001) (Curti et al, 2001). The second and third methods are the most popular methods for *in vitro* generation of DCs for vaccination.

There are six type of ex vivo antigen loading to DCs:

1. **Protein-loaded DCs**. DCs, being professional antigen presenting cells are extremely efficient in taking up proteins. Proteins have the benefit of potentially containing multiple antigenic epitopes, including class II epitopes, and thus avoid MHC restriction. On the other hand proteins are more difficult to synthesis and are less readily available for clinical use. Additionally, proteins are broken down into many epitopes, only few of which are immunogenic. Despite these concerns studies in multiple myeloma inhibitor of DNA binding (Id) proteins loaded DCs have demonstrated that Id- specific T cell responses can be stimulated (Timmerman et al, 2002) (Titzer et al, 2000) (Li et al, 2000). Another concern has been that even though DCs are naturally efficient in taking up proteins, the processing of epitopes derived thereof into MHC class I molecules requires high amounts of exogenous antigen and therefore appears to be rather inefficient (Lanzavecchia, 1996) (Brossart and Bevan, 1997) (Norbury et al, 1997) (Raychaudhuri and Rock, 1998). Therefore many laboratories are involved with the development of methodologies for effective delivery of proteins into the class I processing pathway of DCs. In a report by Laus et al (2000) two proteins (ovalbumin and a fragment of the HER-2/neu protein) were modified through coupling polylysine stretch is proposed to facilitate cellular uptake by neutralising the negative charge of the protein. The fusogenic sequence improves access of the antigen form the endosomal route into the cytosol, thereby boosting

processing of the antigen into MHC class I. addition of the peptide-conjugated antigen to muring APCs resulted in a 10 to 100-fold increase in the efficiency of class I restricted antigen presentation (Laus et al, 2000).

2. DCs loaded with peptides. DCs are known to be able to efficiently take up and process protein antigens and present them in the form of peptide: MHC complexes. However, it was found that peptides can also be loaded to DCs and such peptide-pulsed DCs are potent inducers of immune responses. In fact, direct comparison of peptide- and protein- loaded DCs showed that immunisation with SIINFEKL- loaded DCs resulted in a much stronger H-2Kb-restricted response compare with OVA protein-loading DCs (Met et al, 2003). This could be due to the notion that protein processing leads to the production of numerous peptides, not all of which are immunogenic, whereas the SIINFEKL peptide is reported to be the most immunogenic peptide of the OVA antigen. Advances in peptide- pulsed DC vaccination are discussed below. Walden and his group carried out a research on the efficiency of peptide presentation by peptide-pulsed DCs compared with other peptide-pulsed cell types (Zhen et al, 2006). They found that JY EBV-immortalised B cell line for a comparable number of peptide: MHC complexes on their surfaces. The higher stability of per-existing peptide: HLA molecules on DCs compared with other cell types (Zhen et al, 2004) is the most probable explanation for the less efficient peptide loading and the need for higher concentrations of peptide compared with JY cells. With the use of sodium azide to inhibit all energy- dependent membrane turnover or brefelin A to block export of newly synthesised HLA molecules and thereby decouple peptide loading from conventional MHC class I-specific antigen processing, the efficiency of peptide-loading was reduced. The loading of peptides in the presence of those reagents suggests that a fraction of the peptide: MHC complexes are formed on the cell surface by exchange against peptides already bound to MHC molecules. However the reduces efficiency of peptide loading in the presence of the inhibitor reagents suggests that a fraction of complexes is formed intracellularly (Zhen et al, 2006). Several laboratories have been studying ways it improve peptide loading to DCs. Buschle et al (1997) were

able to show that polyarginine treatment enhanced peptide delivery by more than 2-fold as compared with cells treated with peptide alone; they also showed that polylysine treatment resulted in an approximately 10- fold increase in peptide uptake. In a more recent study it was reported that peptide linking with a C- terminal Lys-Asp-Glu-Leu ER retrieval signal leads in a more efficient and prolonged intracellular MHC class I presentation, in a TAP- and proteasome- independent, but brefelin A-sensitice manner (Wang et al, 2004). The rapid turnover of the peptide: MHC complex also contributes to inefficient peptide presentation. Prolongation of synthetic of synthetic peptide presentation has been reported by Waeckerle- Men et al (2004) employing the peptides encapsulated in biodegradable microspheres.

3. DCs transfected with DNA and mRNA. These methods provide epitopes from endogenously synthesised antigen. DNA and mRNA have the advantage of being easier to manufacture than full-length proteins. Although DCs may be loaded with naked DNA, this approach has low efficiency of transfection, and thus viral vectors are generally employed. However, there have been studies to improve transfection; in a study by Irvine et al, (2000) a cationic peptide (CL22) was used to condense plasmid DNA encoding the antigens of choice to improve transfection efficiency of human and murine DCs. An alternative is to load DCs with mRNA encoding tumour antigens (Boczkwski et al, 1996) (Mitchell and Nair, 2000). mRNA is transfected in its naked form with liposomes or via electroporation. VanTendeloo et al (2001) reported that mRNA-electroporated DCs are more potent in activation antigen specific CTLs than mRNA-liposome transfected DCs. It has been reported that even low levels of transfection are adequate for mRNA-loaded DCs to strongly stimulate antigen specific CTLs (Nair et al, 1998). Importantly, mRNA can be amplified from small amount of tumour, and thus this method may increase applicability of DC based vaccines to patients with minimally available tumours (Heiser et al, 2001). A general advantage of nucleotide transfection of DCs is that endogenously sythesised antigens have better access to the MHC class I pathway.

4. DCs transuded with viral vectors. Several classes of viral vectors have been developed for the delivery of genetic material to DCs. Retroviral vectors can successfully transducer proliferating $CD34^+$ progenitors prior to their differentiation into DCs. These viral vectors carry the risk of oncogenic transformation and have relatively low transduction efficiency (Osada et al, 2006). Lentiviral vectors do not encode viral proteins, thereby minimising their potential to interfere with the function of transduced DCs via the induction of vector –specific immunity (Osada et al, 2006). Lentiviral vectors have been developed with increased safety profiles that prevent the generation of replication- competent recombinants (Firat et al, 2002) (Lizee et al, 2004) (Dull et al, 1998). He et al (2005) directly compared lentiviral vector-transducer DCs with peptide/protein-pulsed DCs by analysing the kinetics and strength of the resultant *in vitro* and *in vivo* antigen-specific immune responses, and demonstrated that the lentivirus stimulated more potent and persistent *in vivo* CTL activity, resulting in superior therapeutic efficacy. Adenoviruses have also been used to transduce DCs and different modifications have been explored to avoid vector immunogenicity and increase safety (Amalfitano et al, 1998) (Rea et al, 2001) (Worgall et al, 2004). Fowlpox vectors have been studied as well, being interesting due to their ability to infect but not replicated within human cells (Brown et al, 2000). Kim et al (1998) reported that fowlpox transduced DCs were able to stimulate $CD8^+$ T lymphocytes *in vitro* from 10 out of 11 patients. Fowlpox vectors with co-stimulatory molecules and antigen have been used to transducer DCs and led in superior antigen presentation capacity (Zhu et al, 2001) (Tasang et al, 2005).

5. DCs loaded with tumour cells or tumour cell lysates. Tumour cells have been explored as a strategy to load DCs, as they contain all the necessary material against which the desired immune response should be directed. In murine models immunisation with DCs that had phagocytosed apoptotic/ necrotic tumour cells resulted in a greater tumour-specific CTL response and successful eradication of lung metastases compared with immunisation with tumour peptide pulsed in many clinical trials, as the processing of the lysate by DCs allows for the targeting of broad variety of unknown proteins (Yu et al, 2004) (Lee et al, 2005) (Chang et al, 2002).

6. **DC- tumour cell fusions**. Another method for delivering the contents of tumour to DCs is the construction of DC-tumour cell fusions. These fused cells express a large repertoire of TAAs, high levels of MHC class I and II molecules, and adhesion/ co-stimulatory molecules, making them strong antigen-presenting platforms (Osada et al, 2006). Preclinical data has demonstrated that DCs fused with tumour cells are potent inducers of tumour-specific immune responses (Gong et al, 2000) (Koido et al, 2004). Polyethylene glycol has long been utilised for fusing cell, and electrofusion has also emerged as an alternative technique (Parkhurst et al, 2003) (Scott-Taylor et al, 2000). Many clinical trials employing DCs fused with autologous tumour or allogeneic tumour cell lines have been initiated, with the results suggesting that hybrid cell vaccination is a safe and well tolerated procedure capable of inducing T cell responses (Martein et al, 2003) (Kikuchi et al, 2001).

6.6.1 Peptide-pulsed DCs in the clinical setting

New methods and technologies allow easier generation and collection of large number of either monocyte- or $CD34^-$ derived autologous DCs from cancer patients (Sallusto and Lanzavecchia, 1994) (Siena et al, 1995). *In vitro* experiments and animal models demonstrated that autologous DCs can effectively present human TAA as loaded proteins or peptide to naïve T cells (Fong and Engleman, 2000). Several clinical trials, mainly on melanoma patients, have been carried out using peptide-pulsed DCs (Figure 6.6).

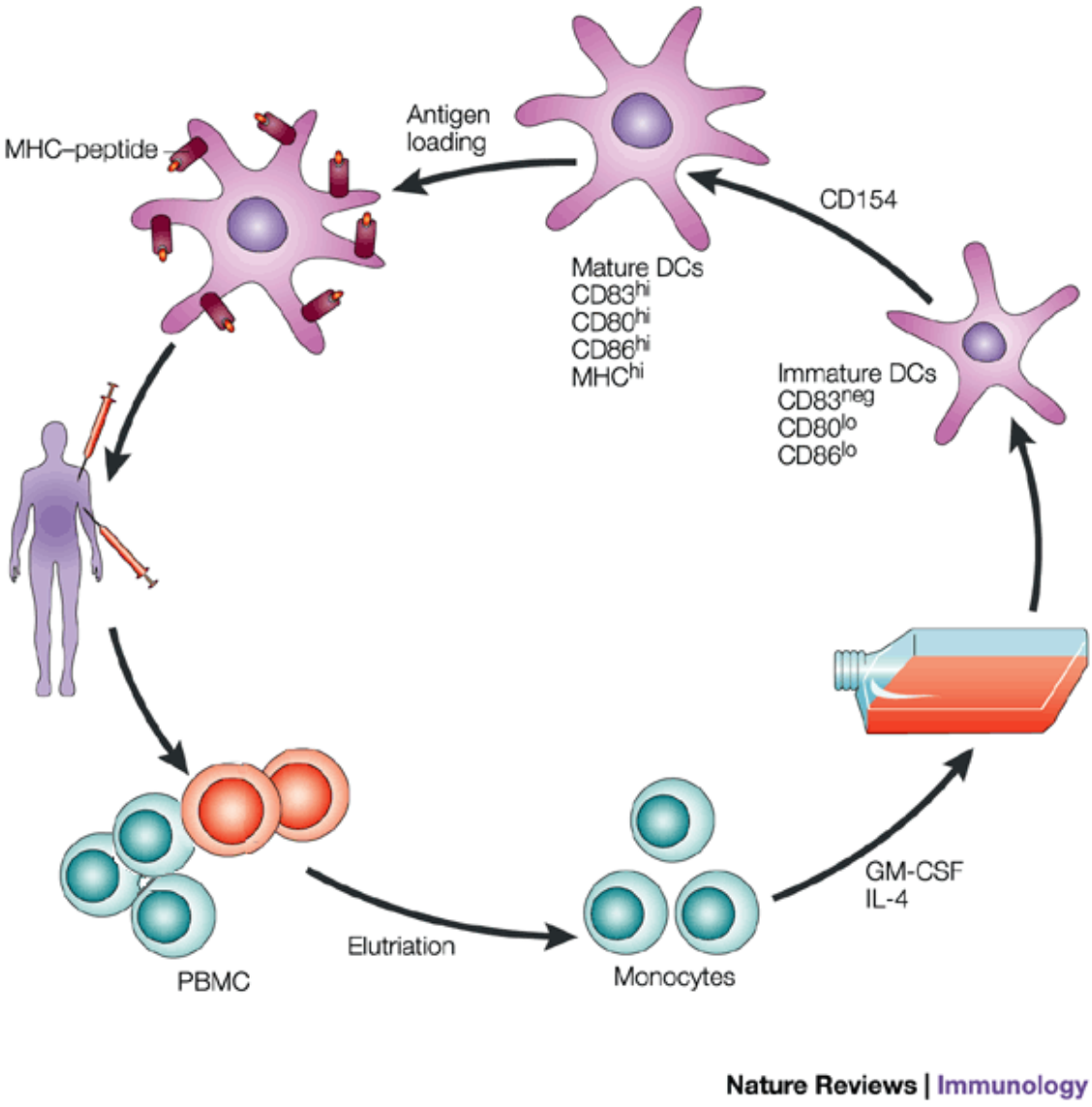

Figure 6.6 Strategy for immunization with autologous peptide-pulsed DCs (Jay et al, 2001)

Monocytes or $CD34^+$ precursors are isolated from patient blood by cytopheresis. Cells are cultured in the presence of various cytokines to differentiate them into immature dendritic cells, which are then loaded with the antigen of interest before or following dendritic cell maturation. Mature antigen-loaded autologous DCs are then administered to patients.

In an early study by Mukherji et al (1995) melanoma patients whose tumour cells express the MAGE-1 gene and who are HLA-A1 + were immunized with a vaccine made of culture autologous APCs (peripheral blood-derived plastic-adherent mononuclear cells culture in GM-CSF) pulsed with the synthetic MAGE-1

nonapeptide EADPTGHSY. Analyses of the nature of the *in vivo* host immune response to the vaccine revealed that the peptide-pulsed APCs are capable of inducing autologous melanoma reactive and peptide-specific CTLs in situ at the immunization site and at distant metastatic disease sites (Mukherji et al, 1995).

In another study (Banchereau et al, 2001) eighteen patients with metastatic melanoma received s.c injections with CD34 progenitor-derived autologous DCs pulsed with peptides derived from four melanoma antigens. Immune responses (expansion of melanoma-specific IFNγ-producing $CD8^+$ T cells in the blood) to one or more peptides were observed in sixteen out of eighteen patients (Banchereau et al, 2001). The overall immunity to melanoma antigens after DC vaccination was associated with a clinical response. In a later follow up study the same group further analysed twelve of the patients to determine whether vaccination with peptide-pulsed CD23-DCs permits expansion of melanoma-specific $CD8^+$ T cells that can yield functional CTLs able to kill melanoma antigen-expressing cells (Paczesny et al, 2004). They showed in nine out of twelve analysed patients the expansion of cytolytic $CD8^+$ T cell precursors specific for melanoma differentiation antigens. Larger follow up studies to assess the immunological and clinical response to peptide-pulsed CD34-DC vaccines are underway.

Peptide-pulsed SC vaccination has also been employed for numerous other clinical trials involving several types of malignancies like multiple myeloma (Titzer et al, 2000), malignant glioma (Yu et al, 2001), nasopharyngeal carcinoma (Lin et al, 2002), chronic myelogenous leukaemia (Takahashi et al 2003), gastrointestinal malignancies (Matsuda et al, 2004), colorectal cancer (Liu et al, 2004), hepatocellular carcinoma (Butterfiled et al, 2006), renal cancer (Wierecky et al, 2006), and breast cancer (Svane et al, 2007).

6.7 Problems of DC based vaccines

DC as the most potent APC has been used to elicit protective T cell immune response to viral infection and cancer. However, so far, there are very limited clinical success (Melief, 2008) (Steinman and Banchereau, 2007) largely due to a lack of knowledge of the ideal antigen-loaded DC for optimal therapy and standardization (Figdor et al, 2004) (Steinman and Banchereau, 2007). One of the major problems is the creation of HLA and peptide complexes on therapeutic DC cells. Although a number of reports demonstrated an induction of CD8 T cell responses by peptide loaded DC cells *in vitro* (Smith et al, 2007) (Melief, 2008), it is impossible to control the quantity and quality of peptide – HLA class I assembly on therapeutic DCs making the therapeutic DCs inconsistent. It is even more difficult to characterise peptide – HLA class II complexes on DCs which is important to induce CD4 helper function required for long lasting CTL responses (Smith et al, 2007) (Melief, 2008).

The second major problem is the immunogenic heterogeneity of the matured of DC matured after derived from monocytes isolated from patient blood *in vitro*. The differentiated DCs have heterogenic population with immunologically active or suppressive function for induction of T cell responses. It has discovered that mature DCs differentiated *in vitro* can produce soluble suppressive factors like IL-10, Transforming growth factor beta (TGF-β) and indoleamine 2,3-dioxigenase (IDO) (Munn et al, 2002) (Lan et al, 2006) (Kobie et al, 2003) (Vassiliki et al, 2009).

The other major problems for live DC therapies are low stability after transfused back into patients, could not be stored for repeated use, therapeutic DC reagents have to be individually prepared from autologous blood monocytes (Fernandez et al, 1998).

In addition to the difficulties to prepare good DC therapeutic reagents, an immune suppressive microenvironment in tumour is also one of the major barriers for a successful anti-cancer immune therapy (Melief, 2008). Numbers of immunosuppressive cytokines, including IL-10 and TGF-b are produced by tumour

or tumour stimulated stromal environment (Kortylewski et al, 2005) (Kortylewski and Yu, 2008). immunoediting (Dunn et al., 2006) (Koebel et al, 2007), in contrast to the activation and maturation factors for DC under infectious condition, the immune suppressive environment induced by tumour negatively affect the function of therapeutic DC cells and effector T cells (Hamzah et al, 2008a) (Hamzah et al, 2008b). In conclusion, the therapeutic DCs manipulated *in vitro* differ functionally from DCs induced by pathogens *in vivo* and the uncontrolled biological functions in therapeutic DCs can severely reduce the efficacy.

To overcome the low stability of therapeutic DCs, cell-free antigen-presenting systems have been reported, including membrane vesicles derived from APC such as exosomes, which are secreted from endosomal compartments of APC and microvesicles derived from plasma membranes of APC after sonication (Kim et al, 2004) (Kovar et al, 2006), However, these vesicles have problems such as difficulty to control the quality and the quantity of pMHCs in the preparation, low yield and have to be prepared from patient cells. Our group has generated a novel vesicle based vaccine by using microsomal vesicles from the ER membranes of APCs (Vassiliki et al, 2009). The data demonstrated that these microsomal membranes not only can be monitored and controlled quantitatively and qualitatively of the level of pMHC with reporter peptides, but also possess a high level of co-stimulatory molecules (Vassiliki et al, 2009).

6.8 Liposomes

Liposomes are colloidal, vesicular structures based on lipid bilayers. Their characteristics depend on the manufacturing protocol and choice of bilayer components. They range from 20nm to 10μm in diameter. The liposomes can be unilamellar (with only one bilayer surrounding an aqueous core) or multilamellar (several bilayers concentrically oriented around an aqueous core). Moreover the choice of bilayer components determines the ‘rigdity’ (or ‘fluidity’) and the charge of the bilayer. For example saturated phospholipids with long acyl chains, such as dipalmitoylphosphatidylcholine, for a rigid, rather impermeable bilayer structure,

while unsatureated phosphatidycholine species from natural sources (egg or soybean phoshatidylcholine) give much more permeable and less stable bilayers. The introduction of positively or negatively charged lipids gives the liposomes a surface charge. Liposome surface can be readily modified. The circulation time of liposomes in the blood stream is dramatically increased by attaching poluethylene glycol (PEG)-units to the bilayer. Alternatively homing molecules can be attached to liposome bilayers to make these structures target site specific. Size, lamellarity, bilayer rigidity, charge and bilayer surface modifications are all parameters that determine the fate of liposomes on the shelf and *in vivo*. Over the years the behaviour of liposomes has been investigated in great detail (Gregoriadis, 1993) (Storm and crommelin, 1997). Algorithms can be used to help the pharmaceutical formulation scientist to select the proper liposome type (Figure 6.7).

Figure 6.7 A drawing of a small spherical liposome seen in cross section

Liposomes are used as carriers for drugs and antigen because they can serve several different purposes. Liposomes can direct a drug to a certain target. Secondly, liposomes can prolong the duration of drug exposure, acting as a slow release reservoir (Oussoren et al, 1999). This has been demonstrated in a number of studies, for example with the antimalarial drug chloroquine or the radical scavenger

superoxide dismutase. Liposomes can protect a drug against degradation (e.g. metabolic degradation). Conversely, liposomes can protect the patient against side effects of the encapsulated drug. For example liposome encapsulation greatly reduces exposure to the heart to doxorubicin and thereby its cardiotoxicity. As liposomes can solubilise lipophilic compounds, this solubilising potential can be used to inject poorly water soluble compounds intravenously. If a fast pharmacological response is desired, then 'fragile' liposomes with 'fluid' bilayers should be selected.

Liposomes can be made tissue or cell specific. Most of the work on liposome targeting has been done with antibodies or antibody fragments attached to the liposome surface (Mastrobattista et al, 1999). However other homing devices have also been considered, e.g. plasminogen-coated liposomes were designed to endothelial cells to block angiogenesis (Koning et al, 2006). Saccharide directed targeting has also been described to direct liposomes to hepatocytes. Targeted liposomes should readily have access to the target site and should not be taken up by macrophages before encountering their target tissue or cells. Therefore nowdays stealth technology is often combined with the attachment of a homing device to the terminal end of the PEG chain that is exposed to the aqueous medium.

In cancer immunotherapy, liposomes have been used to deliver antigen and immunomodulating agents to DCs. Different startgies are being explored to target liposomeal antigens to DCs *in vivo*. In a study by Kawamura and colleaques (2006) IgG conjugated liposomes without attaching PEG were most efficiently endicytosed by DCs. Immunisation of mice with DCs that endocytosed ovalbumin containing IgG liposomes completely prevented the growth of OVA-expressing lymphoma cells. Importantly administration of DCs that endocytosed OVA containing IgG liposomes to the mice with established OVA expressing tumours strongly suppressed tumour growth (Kawamura et al, 2006).

The potential for using cell-deriver liposomal membranes to deliver tumour antigens to DCs recently has been explored using tumour cell derived plasma membrane vesicles (PMVs). Tumour cell derived PMVs are known to retain tumours

antigens from established tumour cell lines. They can be prepared by brief sonication of the cells, followed by separation of the membrane fraction by high speed centrifugation on sucrose (van Broekhoven et al, 2002) (van Broekhoven et al, 2004). The membrane fraction PMVs is then collected and used for vaccine production.

Various tumour vaccine combinations have been prepared recently from tumour cell derived PMVs that have been modified to contain cytokines and engrafted with either T cell co-stimulatory molecules or with ScFv that target the surface markers CD11c and DEC-205 on murine DCs. Importantly, immunological studies showed that PMV preparations containing the co-stimulatory molecules B7.1 and CD40 and IL-2 cytokines can exert potent antitumour responses, including the inhibition of tumour growth and regression of established tumours (van Broekhoven et al, 2002). Later, the same group used an approach where single chain antibody fragments to the DC surface molecules CD11c and DEC-205 were attached to vesicles containing IFNγ or lipopolysaccharide, to target them to DCs (Altin et al, 2004) (van Brokhoven et al, 2004). Such membranes induce dramatic antitumour responses and immunotherapeutic effects when used as a vaccine in the murine tumour model B16-OVA melanoma. Therefore, PMV targeting of antigen and maturation signals directly to DCs *in vivo* represents a much simpler strategy for cancer immunotherapy than antigen loading DCs ex vivo (Altin et al, 2004) (van Broekhoven et al, 2004).

Clinical trials of liposomal vaccines on cancer patients have revealed that liposomes are safe and well tolerated and can induce tumour specific T cell responses (Neidhart et al, 2004) (Neelapu et al, 2004). In a study by Neelapu et al (2004), ten patients with advanced stage follicular lymphoma were vaccinated with liposomal idiotype/IL-2 vaccine. 6 out of 10 patients showed complete remission. However, only a few clinical trials have been carried out, generally with no significant clinical results.

6.9 Dendritic cell-based cell-free vaccination approaches

6.9.1Exosomes

Exosomes are saucer shaped vesicles of 30-100nm in diameter, which are delimited by a lipid bilayer and which float at a density of 1.13-1.19g/ml in sucrose gradients. These vesicles are secreted by various cells in culture (Figure 6.8). Analysis of the protein composition of exosomes that are secreted by various cells reveals the presence of some common proteins, which define exosomes as a bona fide secreted subcellular compartment, as well as the presence of some cell type specific proteins, which could mediate the different functions of exosomes that are produced by different cell types. All of the proteins that have been identified in exosomes are localised in the cell cytosol or endosomal compartments, never in the endoplasmic reticulum, Golgi apparatus, mitochondria or nucleus. Exosomes also contain some plasma membrane proteins, which have been described also in endosomal compartments. These observations are consistent with the proposed origin of exosomes as internal vesicles of late multivesicular compartments (Tery et al, 2002)

The biological functions of exosomes remain unclear. The original role of exosomes was most probably to eliminate undergraded endosomal or lysosomal proteins and mmembranes. Recent results indicate, however, that in different cell types, exosomes might have other functions, such as the stimulation or inactivation of T cells, or the transfer of antigens to DCs. Scientists at the institute Curie proposed a novel mode of functioning of exosomes (Thery et al, 2002); After the uptakge of incoming pathogens in the periphery, immature or maturing DCs generate peptide MHC complexes. Some of these complexes could be secreted on exosomes, and locally sensitize other DCs that have not encountered the pathogen directly, as a result of the effects of inflammation, all of these DCs migtate out of the tissue towards the draning lymph nodes. Although maturing DC seem to secrete fewer exosomes that immature cells, an exchange of exosomes inside the lymph nodes between newly arrived and resident DCs could take place too. Therefore, exosomes

production would increase the number of DCs that bear the revelant peptide MHC complexes, and thereby amplify the magnityde of immune responses. In the absence of inflammation, spontaneous migration of exosomes-bearing DCs could contribute to tolerance induction. Figure 6.8

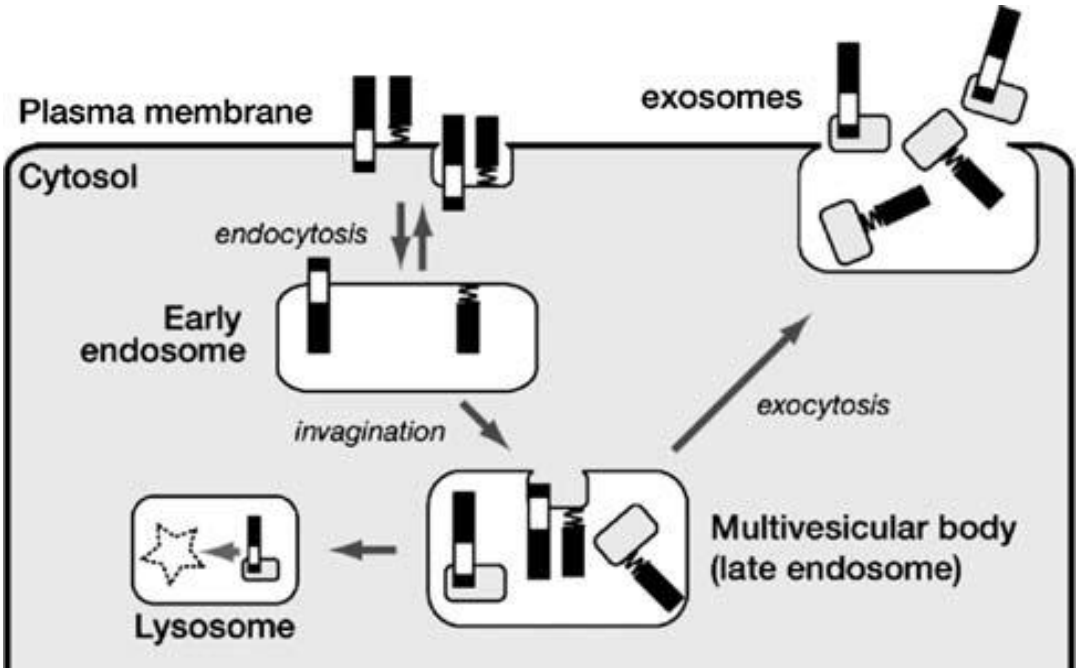

Figure 6.8 The exosome pathway (Laura et al, 2008)

By now results have shown that DC derived exosomes stimulate T cell proliferatio in vitro and have a potent capacity to generate antitumour immune resposes in vivo. All these reported studies have involved in vitro grown mature DCs expanded from precursors with cytokines. However, immature DCs produce higher numbers of exosomes than mature DCs and this is thought to be due to a reduction in endocytosis as DCs mature, associated with reduced formation of multivesiculatr bodies and reduced exosomes formation. In Segura et al (2005) group they found that exosomes secreted by mature DCs are enrighed in MHC class II, B7.2 and ICAM-1, and up to 100fold more potent than exosomes form immature DCs both in vitro and in vivo. Functional analysis using DCderived exosomes from knock out mice showed

that MHC class II and ICAM-1 are required for mature exosomes to prime naïve T cells.

The feasibility of using DC derived exosomes as a cancer therapeutic vaccines has been tested in two Phase I clinical studies in melanoma and lung cancer patients (Escudier et al, 2005) (Morse et al, 2005). Both of these studies highlighted the feasibility of large scale exosomes production and safety of exosomes admistration. However, T cell immunomonitoring did not reveal any significantly strong peptide specific CTL expansion nor clinically significant DTH responses.

6.9.2 Membrane vesicles form APCs

A newly described method involving membrane vesicles for vaccination was published by Kovar et al (2006). They made the hypothesis that since the immunogenicity of DCs presumably reflects their dense expression of MHC/peptide plus high levels of costimulatory molecules, one might expect that the direct immunogenicity of mature DCs could be mimicked by plasma membrane fragments from these cells. In line with this prediction, using a DC line, they shoed that ultracentrifuged vesicles derived from sonicatied of mature DCs contain MHC class I as well as costimulatroy and adhesion molecules such as ICAM-1, B7.1 and B7.2. These vesicels have a round shape and cle=osedly resemble classic exosomes, naïve CD8 T cells were able to bind membrane vesicles from DCs in vitro but only in the presence of peptide. A key finding was that in the presence of specific peptide, sonicated from DCs were strongly stimulatory for CD8 T cells in vitro in the absence of APC. Vesicles from immature DCs were poorly immunogenic. Peptide loaded vesicles led to efficient cells capable of tumour cell elimination. The vesicels, given as a single injection, were immunogenic after either i.v. or s.c. injection. (Figure 6.9).

As discussed earlier, DC derived exosomes are proving a very useful tool for tumour immunotherapy, but their use is limited by low yields, especially from mature DCs. They show that this problem can be overcome simply by degrading DCs into small fragments by sonication. Discarding nuclei and larger debris, the yielded

materal has the form of small membrane vesicels which have the size and morphology of exosomes, and are strongly immunogenic for CD8 T cells when pulsed with peptide.

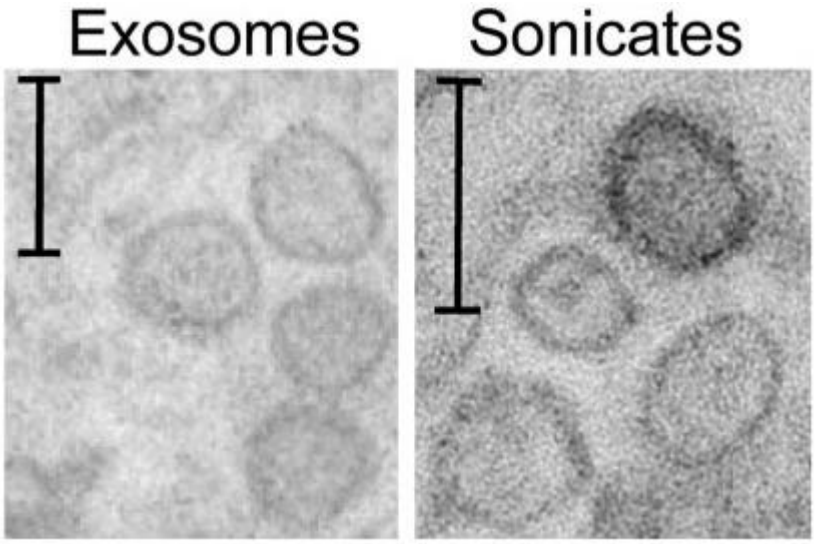

Figure 6.9 EM images of exosomes and sonicates prepared form IFNγ stimulated DCs (Boyman et al. 2006)

How the vesicles are presented to T cells in vivo is unclear. Also unclear is the potential of these vesicles in tumour vaccination in humans; further studies need to be carried out. However it is reported by th eauthors that at least in vitro, vesicles were more immunogenic than exosomes and obtained in much higher yields (Kovar et al, 2006)

7 Proposed system and thesis aims.

From the studies of therapeutic vaccines during last 20 years, it has now been clear that;

1. Manipulation of antigens in vaccine preparation is not enough, therapeutic vaccines have to be able to reverse immune unresponsiveness or enhance antigen specific T cells.

2. Although DCs are most effective antigen presenting cells under infectious conditions *in vivo*, DC vaccines developed from monocytes cannot perform same function as naturally differentiated DCs under infection condition *in vivo.*

3. The best therapeutic vaccines will be ones that can bypass antigen presentation pathways *in vivo* and directly activate antigen specific T cells.

In previous study, we found that the endoplasmic reticular (ER) enriched microsomal membranes (microsomes) isolated from APC can directly activate T cells and induce immune responds for acute virus infection and cancer *in vivo.* In order to apply this strategy for the development of therapeutic vaccine for chronic infectious diseases, we create a nanoAPC able to deliver not only antigen-MHC, but also IL-2 to antigen specific T cells to enhance T cell activation leading to overcome the immune exhaustion.

To develop IL-2-nanoAPC, we constitute a human B cell line 721.221 cells with engineered HLA-A2 and IL-2 specifically inserted into the ER membrane. 721.221 cells express high levels of co-stimulatory molecules, but defect in MHC class I (Shimizu et al, 1988). In order to express IL-2 and HLA-A2 into the ER in 221cells, we fused IL-2 with Tapsin an ER retention protein and HLA-A2 with ER retention signal (Turnquist et al, 2002). With an improved procedure, we produced homogenous vesicles at a nano-size of ~ 500 nm termed as nanoAPC. Assembled with specific antigenic peptides, the IL-2-nanoAPC directly interacted with antigen

specific T cells and induced immune synaptic formation of antigen specific T cells. The IL-2 on nanoAPC did not affect bystander T cells, but potently enhanced activation and effector function of antigen specific T cells. We examined the ability of IL-2-nanoAPC in the induction of viral specific T cell responses in peripheral blood lymphocytes from chronic HBV patients. Assembled with a pool of five HLA-A2 peptides, and four DR and DP associated peptides, IL-2-nanoAPC strongly induced responses of CD4 and CD8 T cells in patient PBMC, while responses were not detected in PBMC following stimulation with peptides or peptide-assembled nanoAPC in the absence of IL-2. Our results demonstrated that IL-2-nanoAPC can be developed as effective therapeutics for the treatment of chronic infectious diseases.

MATERIALS AND METHODS

1. Experimental tools and conditions.

1.1 Animals.

OTI transgenic mice on the C57BL/6 background were kindly provided by Dr. Kioussis D. MRC National Institute for Medical Research, London. C57BL/6 mice were purchased from Harlan UK, (Oxon, England). All animals were maintained in pathogen-free facilities at the Brunel University.

1.2 Cell lines.

The human B-LCL cell line721.221/HLA-A2 cell lines were kindly provided by T. Elliott. The murine DC2.4 line was kindly provided by Dr. Mann D. Southampton, UK. The murine CTL cell line CTLL4 was kindly provided by Dr Gitta Stockinger, NIMR, London. All cell lines were cultured in RPMI 1640 or DMEM supplemented with 10% FBS 1% P/S and 1% L-glutamine if not supplied in medium. Adherent and cells in suspension were pooled and centrifuged at 1200rpm for 5min; the pellet was then resuspended in fresh medium and dispensed into new flasks. The growth medium was changed once per week. Briefly, the old medium was removed, and then the cells were dislodged and dispensed into new flasks. Cells were sub-cultured in a ratio of 1:4, twice a week or as appropriate. All running cultures were maintained in a humidified incubator at 37℃ in 5% CO_2. All cell stocks were stored in freezing medium at $>10^6$cells/ml in liquid nitrogen vapour phase.

1.3 Reagents and antibodies.

All cell culture reagents were from Invitrogen Ltd. (Gibco-BRL, Rockville, MD). Ficoll Pacj was from Amersham (Amersham Biosciences UK Limited, Little Chalfont). FITC-conjugated mouse anti human antibodies to CD54 (ICAM-1), CD80

(EuroBioSciences), and FITC-conjugated Streptavidin; PE-conjugated mouse anti human antibodies to pErk, PD1, HLA A2, , Foxp3, W6/32, FITC-conjugated mouse anti human CD4, APC-conjugated mouse anti human CD8 and PEcy5-conjugated mouse anti human IFNγ were from BD Biosciences. CD8 microbeads (Miltenyi Biotec) were used for isolation of CD8 T cells from spleens of OTI mice, according to the manufacturer's protocol. Goat anti-human IL-2 and anti-human CD3, and W6/32, specific to human MHC class I and MA2.1 specific to HLA A2 were used for immune staining of cells and nanoAPC. Texas Red labelled rabbit anti-goat Ig and FITC labelled goat anti-mouse Ig were used for secondary antibodies.

1.4 Peptides.

Peptides were synthesized by Invitrogen and purified to more than 95% purity. Peptides were reconstituted following the manufacturer's instructions by calculating the hydrophobic and hydrophilic amino acids and accordingly dilute in recommended medium. Specifically, OVA257–264 SIINFEKL CMV pp65 peptide NLVPMVATV HBV HLA-A2 peptide C18-27 FLPSDFFPSV; HBV envelope 183-191 FLLTRILTI; 335-343 WLSLLVPFV; 338-347 LLVPFVQWFV; 348-357 GLSPTVWLSV (Das A et al, 2008) and DR/DP binding peptides HBV Env 180-195 AGFFLLTRILTIPQS; Env 339-354 LVPFVQWFVGLSPTV; and Pol 767-782 AANWILRGTSFVYVP) (Mizukoshi et al, 2004) were reconstituted in DMSO under sterile conditions. Working concentrations of all peptides were prepared in the appropriate cell culture medium.

2. IL-2-tapasin construct

2.1 Construct creation

To establish IL-2-A2-721.221 seed cells, IL-2-tapasin and HLA-A2-E3-19K fusion constructs were constructed by PCR cloning approaches. For IL-2-tapasin, an expression construct of tapasin was modified by substitution of the signal sequence (Li et al 1997) with a 2 x GS linker (GGSG) (Casto and Feng 2000) by PCR cloning. Then, a PCR product of human IL-2 coding region was cloned to upstream of GS linker to create IL-2-GS-tapasin. The sequence of insert contains tapasin and IL-2 shows in figure 1.9. The HLA-A2-E3-19K was constructed by insertion of a 16-aa (LKYKSRRSFIDEKKMP) from E3-19K proteins (Gabathuler and Kvist 1990) to C-terminus of HLA-A2 in an HLA-A2 expression construct (Li et al 2000).

2.1.1 DNA extraction from agarose gel & Restriction enzyme digestion and ligation

After the PCR sample is run on a 7% agarose gel the gel slice is excised, put in an eppendorf tube and its weight is determined. Extraction is carried out using the QIAGEN gel extraction kit and protocol which is carried out in the following way: three volumes of buffer QG to 1 volume of gel. Incubate at 50^{o}C for 10 min while to help dissolve the gel vortex the tube every 2-3 min. The sample is then centrifuged at maximum speed at room temperature for 1 min in the QIAquick spin column on top of a collection tube. After 0.5 ml of buffer QG to column and spin for 1min, the sample will be washed under 0.75 ml PE buffer once. Finally the sample has been dissolved into 30-50ul of EB buffer and then collected into another clean eppendorf tube. 1-2ul of the supernatant is then used test concentration. After same Restriction Enzyme Digest both vector and insert, the vector will be processed under dephosphorylation stage which is the essential process before ligation maximum to avoid vector self ligation. And finally both linearizing vector and insert ligate with T4 ligase and the appropriate buffer at 16^{o}C overnight.

2.1.2 Plasmid extraction

The constructions transfamation into E.coil under heating shock flowing by clone select on LB argos plat and small cultures. The Plasmid extractions from small cultures which are about 1ml-3ml were carried out as follows. After the bacteria cultured overnight, and then are centrifuged for 1min at 13000rpm and the supernatant is removed. The pellet is resuspended in 100ul P1 buffer (Tris 2.5M, EDTA 0.5M, 500ul RNase) and incubated for 5min at room temperature. 200ul of P2 buffer (NaOH 10M, SDS 2.5%) is then added to the mixture, and a further incubation follows for 5min on ice. 15ul P3 (KOAc 3M pH 4.8) are added to the mixture which is then mixed gently and incubated for a further 5min on ice. The mixture is centrifuged at 4℃ at 13000rpm for 5min and the supernatant is transferred to another tube. Add 70% of this volume of isopropanol to the supernatant and after mixing and for centrifuging 5min at 13000rpm the supernatant is discarded and the pellet is washed with 500ul 70% ethanol. The pellet is finally resuspended in water and frozen down at -20℃.

The positive clone will be confirmed by restricted enzyme test and sequence. Chose one positive construct plasmid extraction from big cultures was carried out the QIAGEN plasmid purification kit and protocol.

2.2 Construct plasmid transfection

Construct plasmid were transfected into 721.221 cells using T cell nucleofector solution (Lonza). First Spin 5-10 million cells under 1200rpm for 5 min the supernatant is removed. The pellet is resuspended in 110ul T cell nucleofector solution plus 8ug of DNA and incubated for 2min at room temperature. And then the 60ul of this mixture is transferred to a cuvette and electroporation with program S-018. Immediately add 500ul 37℃ prewarmed complete medium. Finally, add another 8ug of DNA to rest of the mixture and repeat same process as before. The transfectants into complete medium and selected with antibiotic or running sorting couple of days later.

3. Protein analysis.

3.1 Western blot analysis of protein.

3.1.1 Cell lysis

Harvest cells from 1x 75 cm^2 flasks and then wash once with PBS. Resuspend cells in 100ul 1% NP40 lysis buffer and add 1ul PI and 1ul 10mM PMSF following shaking for one hour at 4^oC. Spin down at 14000rpm for 30min at 4^oC and then transfer the supermatant to new tube. After measure protein content via BioRad Dye reagents, the protein sample is ready for western blot analysis.

3.1.2 Gel preparation

Gels were cast and run using the Bio-Rad Western apparatus. The plates were cleaned, the spacers were introduced and the plates clamped in place. The resolving gel was poured first. A 10ml preparation of 10% consists of 2.4ml purified water, 3.35ml 30% acrylamide, 3.75ml 1M Tris pH 8.8, 100µl 10% SDS, 100µl 10% APS and 4µl TEMED. After pouring the gel was covered with isopropanol (~0.5ml) to ensure a level surface to the top of the gel and to aid polymerization. Once set isopropanol was removed and the plates were dried. The stacking gel was then poured and the comp was fitted. A 2.5ml preparation of 5% stacking gel consists of 1.7ml of purified water, 415µl 30% acrylamide, 315µl 1M Tris pH 6.8, 25µl 10% SDS, 25µl 10% APS and 5µl TEMED.

3.1.3 Test sample running

Transfer 25ug protein for each well which need to take required volume of smaple and then mix with smaple buffer and 1ul 1M DTT/10ul sample volume fllowing heating at 95^oC for 5min. The gel is run at 30mA for ~1hour at cool room (4^oC). After running the top plate is removed and the stacking gel is cut away.

3.1.4 Transfer-semi-Dry Blot

The transfer apparatus is then assembled. The bottom plat is covered with 4 sheets of Whatman 3MM paper, then the membrane and gel, 4 more sheets of Whatman 3MM paper. Using a graduated pipette carefully roll out any air bubbles. The sandwich is then placed into the transfer tank with top plate in positive. The transfer of protein to the membrane for 1.5 hours at ~175mA and ~10V was then performed at RT with continuous circulation of the buffer.

3.2 Western blot hybridisation.

Membranes were blocked with 50ml of Western block buffer consisting of 5% w/v non-fat dry milk in TBS, for 1-2 hours at room temperature, before been probed with the corresponding antibodies. Primary antibodies were diluted according to manufacturer's instructions in blocking buffer and incubated for overnight at 4^{o}C with agitation. After 3 washes for 15min with 5ml of wash buffer (0.05% Tween-20 in TBS), the secondary antibodies were diluted according to manufacturer's instructions and added in 5ml block solution. After 1 hour at room temperature, 3 washes for 15min in wash buffer were performed.

4. Preparation of nanoAPC from antigen presenting cells.

4.1 Fractionation of cell contents.

The seed cells were expanded to large volume at a viability of > 99% and used for preparation of nanoAPC. The nanoAPC was prepared by two steps; preparation of the ER enriched microsomes and processing of nano-particles. Human nanoAPC from these transfected 221-A2-IL2 and control 221-A2 cell lines were prepared and purified according to previously described protocols. Mouse nanoAPC isolated from bone-marrow derived DCs (Wang et al. 1996). Specifically, cell cultures were grown to a minimal number of 10^9cells. All the steps during the nanoAPC preparation are performed on ice or at 4 ℃.

Cells were collected, centrifuged at 1500rpm for 5min and washed once with cold PBS. Cells were washed and resuspended in homogenization buffer. After homogenization, the nuclear, mitochondria and larger cell debris were removed by centrifugation at 10 000g. The total microsomes were recovered by centrifugation at 100 000g and subfractionated by flotation in sucrose gradients. The microsomes were layered on top of 5mL of 0.33M sucrose, layered in turn on top of a discontinuous sucrose gradient consisting of 2mL of 2M and 1mL of 2.5M sucrose. Centrifugation in a TH-641 rotor for 1h at 110000g at 4 ℃ yielded a microsome band on top of the 2M sucrose cushion, which was collected and resuspended in RM buffer (250mM Sucrose, 50mM triethanolamine-HCl, 50mM KOAc, 2mM MgOAc2, 1mM DTT). The microsomes were further processed to homogenous nano-particles by sequentially homogenized in a fine cell homogenous (isobiotec) with different cut off size between 6 and 4 microne. The homogenized particles were recovered by centrifugation in a TH-641 rotor for 1 hours at 110 000g at 4^{o}C and then resuspended in RM buffer. After protein assess the nanoAPC was stored at -80^{o}C until use.

5. Labelling and detection of antigens in nanoAPC and cells.

5.1 Flow cytometry.

Flow cytometry analysis was used to detect cell surface or markers on cells and nanoAPC. Single cell suspensions were obtained from homogenised spleens and lymph nodes, or from PBMCs, or from cell cultures.

Cells or nanoAPC after washing, cells were first stained with surface markers with antibodies (0.5mg/ml) were 1 into 10 diluted with FACS media and then incubate for 30min at 4℃ in dark, washed in 1ml FACS media and collected by centrifugation. The stained cells were washed with PBS and fixed according to intracellular staining Kit (BD) after washing step for intracellular staining. Antibodies were diluted according to manufacturer's instructions and added to the pellets, mixed gently and incubated at 4℃ for 30min in dark. The stained cells were washed with PBS, then analysed on a FACS (CantoII). The data were analysed using flowjo (Tree Star, Inc). The isotype Ig was used as background controls for all the staining of both cells and nanoAPC.

5.2 Peptide biotinylation.

DR/DP binding peptides HBV Env 180-195 AGFFLLTRILTIPQS; Env 339-354 LVPFVQWFVGLSPTV; and Pol 767-782 AANWILRGTSFVYVP was biotinylated using the NHS-LC-Biotin reagent (Pierce Chemical). Dissolve FITC in conjugation buffer at a final concentration for 1mg/ml, immediately before use. The reaction was initiated by adding 90µl of 1mM peptides diluted in PBS in the biotin solution. The mixture was allowed to react for 2 hours at room temperature by continuous agitation. Remove excess and hydrolyzed FITC by gel filtration. After analysis the fluorescent biotinylated peptides were stored at -80℃ until use.

5. 3 NanoAPC labelling with chemical fluorescence.

For the detection nanoAPC by fluorescence microscopy nanoAPC were labelled with chemical fluorescence using the FITC1 fluoroTagtm FITC conjugation kit from Sigma FITC1-1KT, according to manufacturer's instructions. For each labelling 1mg of nanoAPC were used. NanoAPC were labelled before or after peptide loading.

Briefly, the contents of one sodium carbonatebicarbonate capsule were dissolved in 50ml of de-ionized water. The pH of the resulting buffer (C0688 at 0.1M) was measured and calibrated at pH 9. NanoAPC suspension was centrifuged at 10000rpm for 5min and the resulting pellet was re-suspended in 200µl of the C0688 buffer. At this stage one vial of FITC was re-constituted in 2ml of C0688 buffer. 50µl of the FITC solution were added drop wise to the nanoAPC suspension on a slow shaker. The mixture was covered in foil to reduce exposure to light and the reaction was allowed for 30min at room temperature, on a shaker. To stop the reaction 50µl of 0.2M glycine pH 8 were added and the experimental tube containing the mixture was stored on ice.

The reaction was centrifuged at 10000rpm for 5min at 4℃ and the supernatatnt was removed. Labelled nanoAPC were re-suspended in 50µl of RM buffer and stored in -80 ℃ and protected from dark until further use.

5.4 Distribution of nanoAPC in lymph nodes

For distribution of nanoAPC in lymph nodes, nanoAPC were prepared from CFSE labeled mouse DC2.4 cells. An aliquot of 20µg of peptide-loaded nanoAPC or FITC labeled Dextran was injected into C57BL/6 mice i.v. After 48 hours, lymph nodes were isolated and processed to single cell suspension. The cellular fraction was collected by concentration at 1000g for 5 min, while cell-free particles were recovered after removing supernatants through microfilter nanoAPC at cutoff size of 3000kd (Millipore Amicon Ultra centrifugal filters) which can recover both

nanoAPC and dextran. The cellular and cell-free particles were stained with PE-labeled CD11c as DC marker and analysed by FACS.

6. Isolation of primary cells.

6.1 Isolation of mononuclear cells from mouse secondary lymphoid organs.

Mouse spleens and lymph nodes were harvested and single cell suspensions were prepared. The cells were pelleted by centrifugation at 1200rpm for 5min at room temperature and the supernatant was discarded by aspiration. The pellets were re-suspended in 5ml of RBC lysis buffer per spleen and incubated at 37^{o}C for 4-5min. The cell suspension was centrifuged at 1200rpm for 5min at room temperature, the supernatant was discarded by aspiration and the resulting cell pellet was re-suspended in the complete medium and incubated at 37^{o}C for 30min to exclude macrophage. The cell suspension was centrifuged at 1200rpm for 5min at room temperature. At this stage, the cells were counted.

6.2 Isolation of mononuclear cells from human peripheral blood.

Mononuclear cells were isolated from human peripheral venous blood by density gradient centrifugation over Ficoll-Histopaque. 15ml of Ficoll hypaque were aliquoted per 50ml test tube in sterile conditions and allowed to equilibrate to room temperature. A dilution of 1/1 in serum free medium was slowly layered on top of the Ficoll-hypaque and centrifuged for 30min at 400 x g centrifuge force in room temperature, with the centrifuge breaks off. After centrifugation the interface of PBMCs was collected, further diluted with serum free medium (approximately 1:2) and centrifuged at 250 x g centrifuge force for 10min at room temperature. The supernatants were then discarded and the remaining cell pellet was resuspended in with serum free medium. The cell suspension was washed with serum free medium and centrifuged at 250 x g centrifuge force for 10min at room temperature. The resulting pellet was resuspended in 10ml of complete medium and the cells were counted.

7. Activation assays.

7.1 Peptide loading of nanoAPC.

NanoAPC suspended in RM buffer were first processed by freeze– thaw (30sec in liquid nitrogen and 5min at 37^{o}C) repeated three times, followed by addition of an equal amount of stripping buffer (0.26M citric acid, 132mM Na_2HPO_4, 2% BSA, pH 5.5) and incubation for five minutes on ice.

For MHC class I loading, 20μg/ml human β_2-microglobin and SIINFEKL peptide at indicated doses were pulsed onto 1-4μg of nanoAPC membranes the mixture was incubated for 5 min on ice followed by neutralization with 1M Tris buffer pH11. And then incubate for one hour at 37 ℃. After loading free peptides and reaction buffers were removed by washing through a filter at cut-size of 3000kd (Millinpore Amicon Ultra centrifugal filters). The loaded nanoAPC were resuspended in serum free medium at 2mg/ml concentration.

For MHC class II, after the freeze-thaw process, the nanoAPC in RM buffer were mixed with equal volume of acidic buffer and peptide at 1uM or as indicated for one hour at 37 ℃. And then neutralization with 1M Tris buffer pH11 following loading excess peptides were removed by washing through a microfilter at cut-size of 3000kd (Millipore Amicon Ultra centrifugal filters). The assembled nanoAPC were resuspended in serum free medium at 2mg/ml.

7.2 Interaction of nanoAPC and T cells

Interaction of SIINFEKL-Kb-nanoAPC or Kb-nanoAPC from CFSE labeled DC2.4 cells and OTI cells were measured by incubation of 10ug SIINFEKL-Kb-nanoAPC or Kb-nanoAPC with 10^6 OTI cells in normal medium incubate at 37^{o}C for 30 minutes. Free-nanoAPC was removed by low speed centrifugation at 1000g for 5 min and washing with cold PBS. Cells were then stained with anti-CD3 antibody followed by DIP counter staining. The samples were analysed by confocal microscopy (ZEISS LSM 510).

7.3 Human study

7.3.1 Experimental subjects

6 CMV serum converted health donors and 57 chronic HBV patients participated in this study. All subjects are HLA A2 positive. Informed consent was obtained, and the study was approved by local ethical committees at the two participating clinics (East London and the City Research Ethics Committee for Barts and The London NHS Trust and Huashan hospital ethics committee, Shanghai China). Out of 26 chronic HBV patients, 47.4% were positive for HBeAg, 18 had raised ALT (>50IU/liter) and high HBV DNA (>10^6 IU/ml) as quantified by real-time PCR assay. 31 patients were HBeAg negative, and positive for serum anti-HBe antibody. All patients were negative for antibodies to hepatitis C virus, HIV-1, and HIV-2.

7.3.2 Isolation of PBMCs and in vitro stimulation

PBMCs were isolated from fresh hepatinized blood by Ficoll-Hypaque density gradient centrifugation and resuspended in RPMI 1640 and 10% heat-inactivated FBS (invitrogen) as mentioned. For some experiments, CD8 positive T cells were isolated with anti-CD8 coated beads (MACS). 5 x 10^5/well PBMC or $CD8^+$ cells in 24-well plates were co-cultured either with antigenic peptide at a concentration of 5uM (the same concentration used for assembling MHC class I in nanoAPC) or 10ug nanoAPC for five days. Cells were cultured with PBS or anti-CD3 coated beads served as negative or positive controls. After five days, 20nM of PMA and Inomycine (sigma) were added together with Golgi stopper (BD) and cultured for 4 hours. Cells are now ready to staining with antibody for FACS analysis.

7.4 Proliferation assays.

To determine CTLLA4 IL-2 dependent cell proliferation 1μCi/ml [3H] Thymidine was added after one days of culture with IL-2-A2-nanoAPC or A2-nanoAPC, recombinant IL-2 severed as positive control. The cells were harvested after 16 hours and tritium incorporation into thymidine was measured.

For [3H] labelling the working surfaces in a radiation controlled area were swept with a tissue and after sweeping 1cm piece of tissue was used to evaluate background radiation. The labelling medium was made at a concentration of 1μCi/ml in RPMR1640. Culture plates were transferred to the radiation culture area after one days of activation assay. Labelling medium was added at 20μl/well; the micro-plate was placed in plastic container and kept in 5% CO_2, at 37℃ for 16 hours. At this stage the working surfaces were tested for radiation levels. Following 16 hours of incubation, the labelled cultures were harvested in a cell harvester or stored at -20℃ until measured.

For harvesting, the orientation of the filter was marked, it was placed with the plate in the harvester and harvesting was initiated. Following harvesting of cultures, the filters were air-dried and melted with scintillation gel. The processed filter was placed in a cassette and the amount of [3H] incorporated by thymidine was measured. Following measurements the filters were disposed as appropriate for disposal of radioactive material.

8. Statistics.

Statistical comparisons were performed using Student's t test; survival was plotted using Kaplan-Meier curves and statistical relevance was determined using log-rank comparison. Unless noted, data were presented as means ± SD of pooled data from four to six independent experiments.

9. List of Buffers.

Complete growth medium: -Culture medium, 10% FBS, 1% Penicillin-Streptomycin–L-glutamine.

Freeze medium: -90% PBS; 10% DMSO.

FACS media: -5% BSA in PBS.

Lysis Buffer: -1% NP40, 5% PMSF, 10ug/ml PI.

PBS: -80.0g NaCl, 11.6g Na_2HPO_4, 2.0g KH_2PO_4, 2.0g KCL, q.s. to 10L; pH to 7.0.

RM buffer: -250mM sucrose, 50mM triethanolamine-HCl, 50mM KOAc, 2mM $MgOAc_2$, 1mM dithiothreitol, pH to 7.2.

Stripping buffer: -0.26M citric acid, 132mM Na_2HPO_4, 2% BSA, pH 5.5.

Neutralization buffer: - 1M Tris buffer pH 11

LB medium: -10g Bactotrypton, 5g Bactoyeast, 7g NaCl q.s. to 1L

LB Argos: -10g Bactotrypton, 5g Bactoyeast, 7g NaCl, 15g Bactoagar q.s. to 1L

P1 buffer: -Tris 2.5M, EDTA 0.5M, 500ul RNase q.s. to 50ml

P2 buffer: -NaOH 10M, SDS 2.5% q.s. to 50ml

P3 buffer: -KOAc 3M pH 4.8

1X TBS: 50 mM Tris.HCl, pH 7.4 and 150 mM NaCl.

Western blot:

Stacking gel: -10ml preparation of 10% consists of 2.4ml purified water, 3.35ml 30% acrylamide, 3.75ml 1M Tris pH 8.8, 100µl 10% SDS, 100µl 10% APS and 4µl TEMED.

Resolving gel: -2.5ml preparation of 5% stacking gel consists of 1.7ml of purified water, 415µl 30% acrylamide, 315µl 1M Tris pH 6.8, 25µl 10% SDS, 25µl 10% APS and 5µl TEMED.

Sample buffer (2x): -125mM Tris pH 6.8, 4% SDS, 20% glycerol, 10% β mercaptoethanol, 4mg BPB/10ml.

Running buffer (10x): -250mM Tris pH 7.5, 192mM glycine, 0.1% SDS.

Transfer buffer: -25mM Tris base pH 8.3, 192mM glycine, 20% methanol.

Blocking buffer: -5% w/v non-fat dry milk powder, 2% w/v BSA, 0.1% v/v Tween-20 in PBS

Wash buffer: -0.1% non-fat dry milk powder, 0.1% Tween-20 in PBS

RESULTS

1 Generation of Seed Cells

1.1 Seed cell selection

In order to apply nanoAPC as a delivery vehicle to develop a therapeutic vaccine for broad patient groups, we selected cell lines that are MHC class I deficient. This allowed me to reconstitute popular MHC class I alleles, such as HLA A2, which are predominantly expressed in a large population (Komlos et al, 2007). The seed cells have to express high levels of co-stimulatory molecules which are essential for optimal T cell activation (Jennifer D Stone et al, 2009). Based on our previous studies, we choose 721.221 cells to develop seed cells (Li et al, 1997). The 721.221 cells are derived from parental 721 cells that are human lymphoblastoid cell lines (LCL) 721. 721 cells are established from peripheral blood lymphocytes (PBLs) which were isolated from heparinised blood of the 721 donor (female age 21-25). To investigate the mechanisms of antigen presentation, Dr Robert DeMars's group developed 721 MHC deficient variants selected after mutagenesis treatment with gamma-irradiation (Shimizu et al, 1988). Table 1.1 describes MHC phenotypes of donor 721 and her parents (Reitnauer et al, 1985). To avoid HLA-C expression in 721 mutated cells, 721.221 have been developed via the following sequence: LCL 721-.45-.144-.184-.184TG3-.221.mutant (Table 1.2). The 721.221 cells were developed from 721.184 TG3, one of the 721 variants, by immunoselection following γ ray (300 rad) (Shimizu et al, 1987). Thus, the 721.221 cells are absence of endogenous HLA-A,-B,-C Ag. The 721.221 cells transfected with MHC class I alleles can express MHC class I Ag at normal levels (Shimizu and DeMars 1989), which demonstrates that the antigen presenting pathways in 721.221 cells are intact. 721.221 cells still retain phenotype of HLA DR, DQ and DP. Since most of the MHC class II associated antigenic peptides can be cross presented by different MHC class II alleles with different affinity, the remaining HLA DR and DP can be used to

produce antigen-MHC class II complexes to induce CD4 helper responses (Haren et al, 2011).

Individuals tested	HLA locus			
	DP	DR	B	A
A[a] (donor of LCL-721)	(p) 4	3	8	1
	(m) 2	1	5	2
M (mother of A)	2	3	8	1
	3	1	5	2
P (father of A)	4	3	8	1
	2	3	13	30

Table 1.1 the HLA types of donor 721 and her parents (Reitnauer et al, 1985)

Cell Line	HLA Ag Phenotype[a]					
LCL 721	A1	Cp[5]	B8	DR3	DQ2	DP4
	A2	Cm	B5	DR1	DQ1	DP2
.114	A1	Cp	B8	DR3	DQ2	DP4
.127	A1	Cp	B8	DR3	DQ2	DP4
	A1	Cp	B8	DR3	DQ2	DP4
.45	A2	Cm	B5	DR1	DQ1	DP2
.144	—	Cm	B5	DR1	DQ1	DP2
.184	—	Cm	—	DR1	DQ1	DP2
.221	—	—	—	DR1	DQ1	DP2
.220	—	(Cm)	—	DR1	DQ1	DP2

Table 1.2 Expression of HLA Ag in HLA Ag loss mutants derived from LCL 721 (Shimizu and DeMars 1989).

In addition to antigen recognition activation of T cells, depends on co-stimulation for initiating adaptive immune responses. Therefore, the expression of co-stimulatory molecules were analysed on 721.221 cells. Both co-stimulatory

molecule B7.1 (CD80) and the adhesion molecule ICAM-1(CD54) were highly expressed on 721.221 cells (Figure. 1.1).

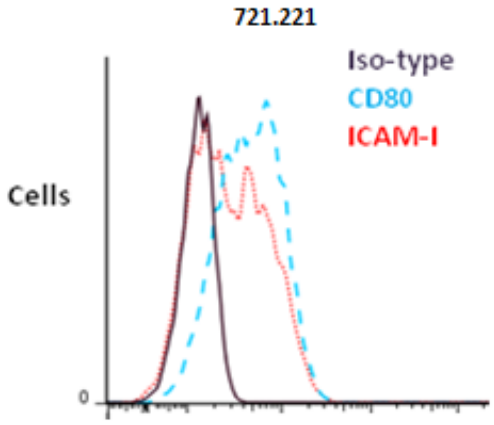

Figure 1.1 expression of co-stimulation molecule in 721.221 seed cells.

721.221 cells were analysed for the co-stimulatory molecules CD80 and adhesion molecule ICAM-1 by staining with corresponding antibodies and analysed on flow cytometry. Isotype Ig was used as background control.

The nanoAPC is prepared from the ER membranes of APC seed cells. Previously, we have demonstrated that ER enriched microsomal membranes from APC have abundant peptide-receptive MHC class I molecules (Sofra et al, 2009). In order to maximize ER-expressing MHC class I molecules, I have used a HLA-A2 transfected 721.221 cell line in which the HLA-A2 is fused with an ER retention signal E3-19K (Figure 1.2). Thus, the transfected HLA-A2 can be retained in the ER. Due to a high level of expression, part of the expressed HLA-A2 is transported to the cell surface which has been detected by HLA-A2 antibody on 721.221-A2 (short for 221-A2) cells surface (Figure. 1.3). This cell line has been used throughout this study.

Figure 1.2 Schematic representation of the carboxy-terminal portion of the E3/19K protein which has KKXX motif at C-terminus.

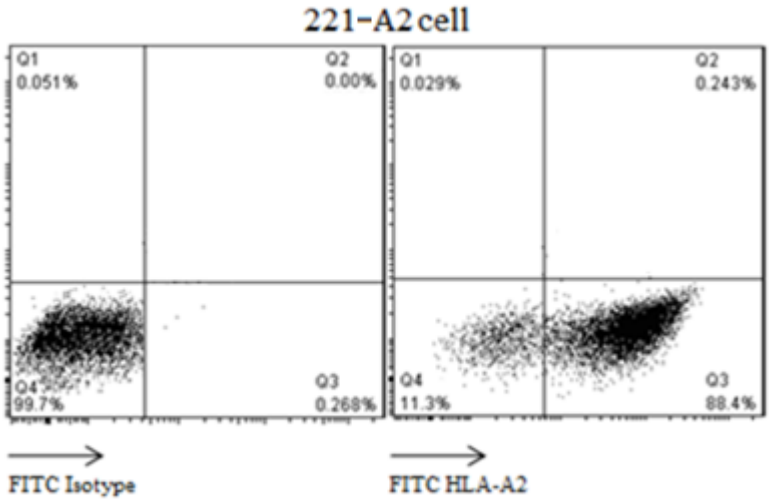

Figure 1.3 Expression of HLA-A2 in 221-A2 cells

221-A2 cells were analysed for HLA-A2 expression by flow cytometry. The FITC isotype Ig was used as background control for the staining.

1.2 Generation of IL-2-tapasin expression construct

1.2.1 IL-2-Tapasin fusion constructs

The major challenge for effective therapeutic vaccines against chronic infectious diseases is the high activation threshold of antigen specific T cells induced by persistent viral antigens resulting in T cell exhaustion during chronic viral infection (Lopes et al, 2011). In order to overcome the inactivation of antigen specific T cells, we designed nanoAPC carrying IL-2 as a bioadjuvant. Previously, in an antigen-induced tolerant model, our group demonstrated that IL-2 can effectively reverse the tolerance (Anderson et al, 2005). In order to achieve this goal, we attempted to create an IL-2 (Figure 1.4) expression construct that can express IL-2 as a fusion protein with the ER chaperon tapasin (Li et al 1997), which can keep IL-2 as an ER-retention molecule in the 221-A2 cells. The ER retention of tapasin (Figure 1.4) is mediated by its C terminal double-lysine motif (KKAE) (Momburg and Tan 2002). Tapasin functions as an ER chaperon specifically for peptide-receptive MHC class I in the ER (Li et al, 1997). Based on the published structure of tapasin, the N-terminus of tapasin is open (Roder et al, 2009). Therefore, we fused C-terminus of IL-2 to the N-terminus of tapasin after deletion of a stop code in IL-2 and signal

sequence of tapasin (Figure 1.5). To create free space for fused IL-2, trip repeated glycine and serine linker (GGSGGS) was introduced between IL-2 and tapasin (Figure. 1.5). The designed fusion molecule has IL-2 with C-termini fused with N-termini of tapasin (the signal sequence of tapasin is deleted) gapped with 2x GS linkers. Thus, the expressed IL-2 is retained in the ER and exposes its functional structure for receptor interaction (Figure. 1.5).

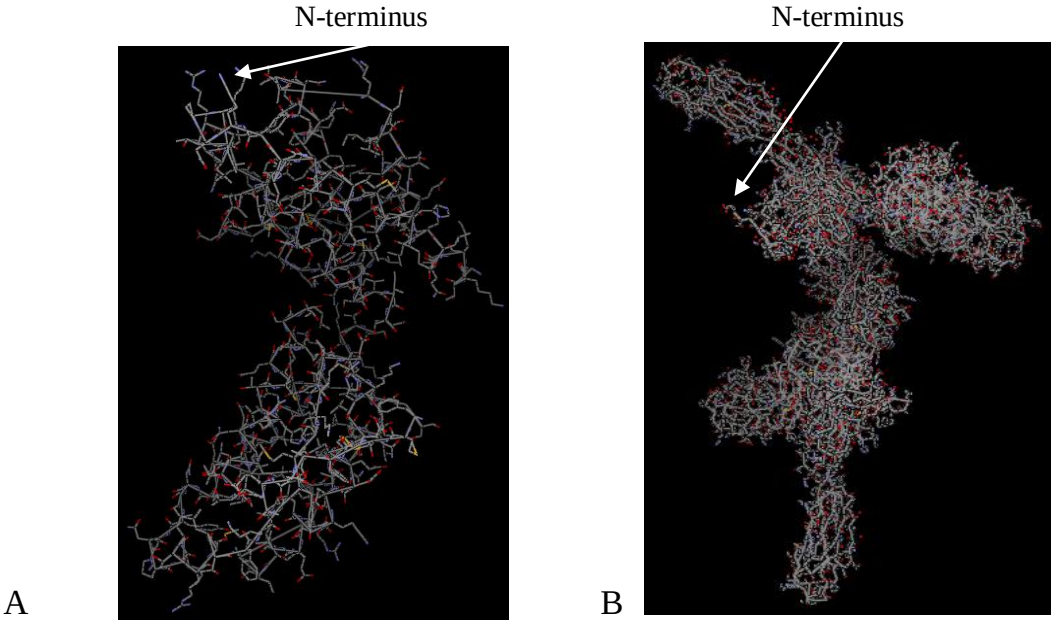

Figure 1.4 the crystal structure of;

A. human interleukin-2 (Arkin et al, 2003)

B. Tapasin ERP57 Heterodimer (Dong et al, 2009)

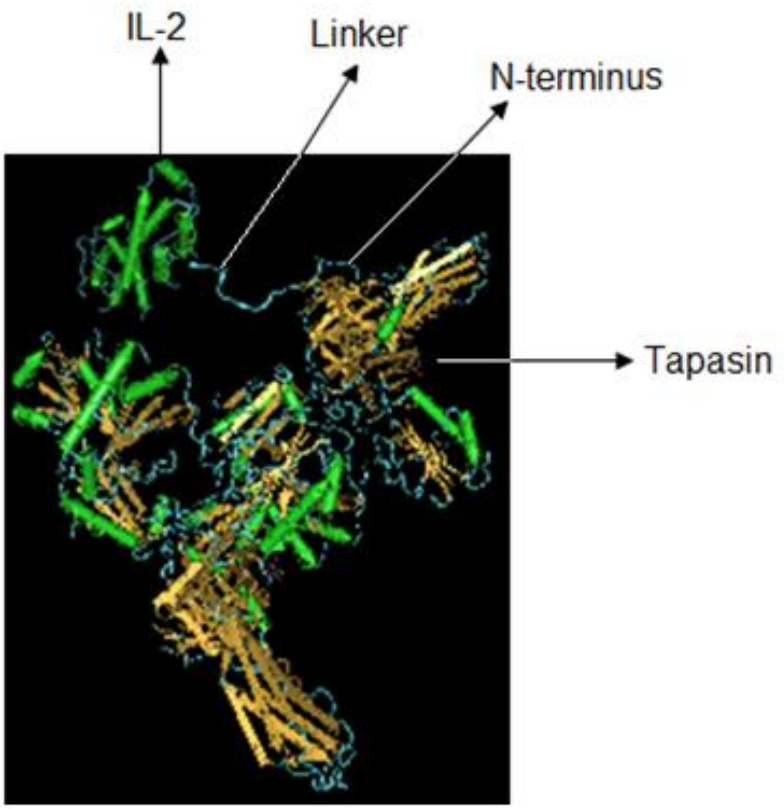

Figure 1.5 Schematic drawing of the backbone of IL-2 fused with Tapasin

It has been demonstrated that tapasin is a MHC class I antigen processing molecule present in the lumen of the ER, which plays an important role in the maturation of MHC class I molecules in the ER lumen (Li et al, 1997). And also it has been introduced as one component of the peptide loading complex; therefore IL-2 fused tapasin may also increase HLA-A2 retention in the ER as peptide receptive molecules (Li et al, 1997).

1.2.2 GFP as a selection mark for transfected seed cells

In order to select IL-2-tapasin transfected seed cells, we used expression constructs with CMV promoter to drive expression of IL-2-tapasin and green fluorescent protein (GFP) as report molecule. GFP has been widely used as selection mark in cell biology and other biological disciplines because it is less harmful when illuminated in living cells (Yuste 2005) (Figure. 1.6). pEGFP-N3 encodes GFP which has been optimised for brighter fluorescence and higher expression in mammalian

cells. The insert of IL-2-tapasin was created by sequential PCR-cloning strategies (Figure 1.7) with proper restriction enzyme sites corresponding to the sites in the vector (Figure 1.8). The final sequence of insert is shown in figure 1.9.

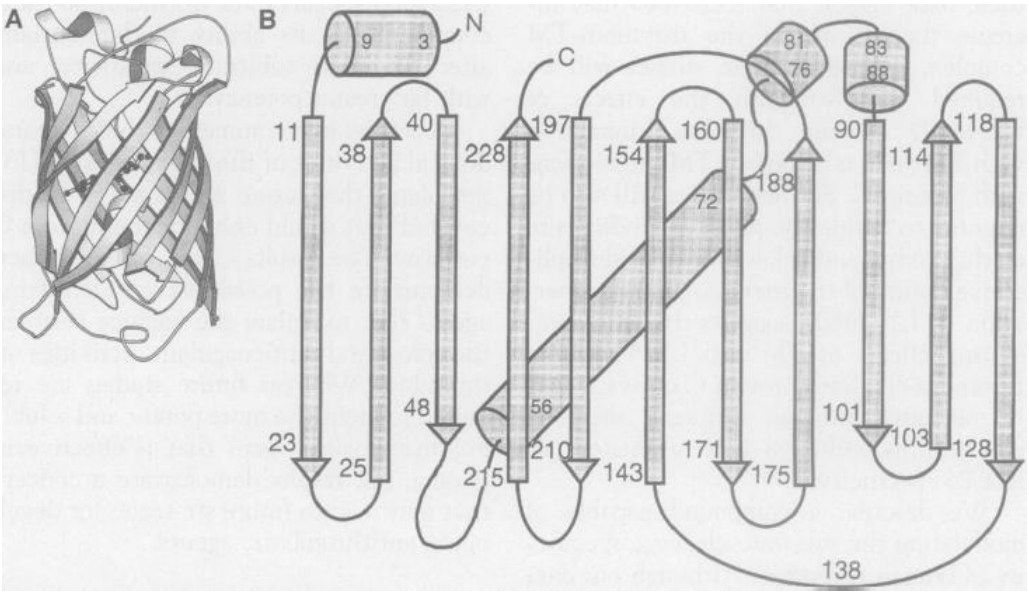

Figure 1.6 (A) schematic drawing of the backbone of GFP produced by the program MOLSCRIPT. The chromophore is shown as a ball and stick model. (B) Schematic drawing of the overall fold of GFP. Approximate residue numbers mark the beginning and ending of the secondary structure elements N, NH_2- terminus; C, COOH-terminus (Ormö et al, 1996).

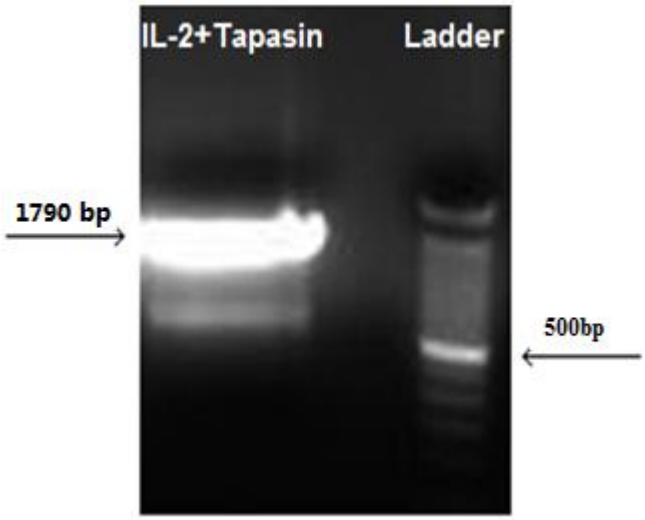

Figure 1.7 Electrophoresis represents the PCR amplified IL-2-Tapasin inserts with BamHI site at two ends.

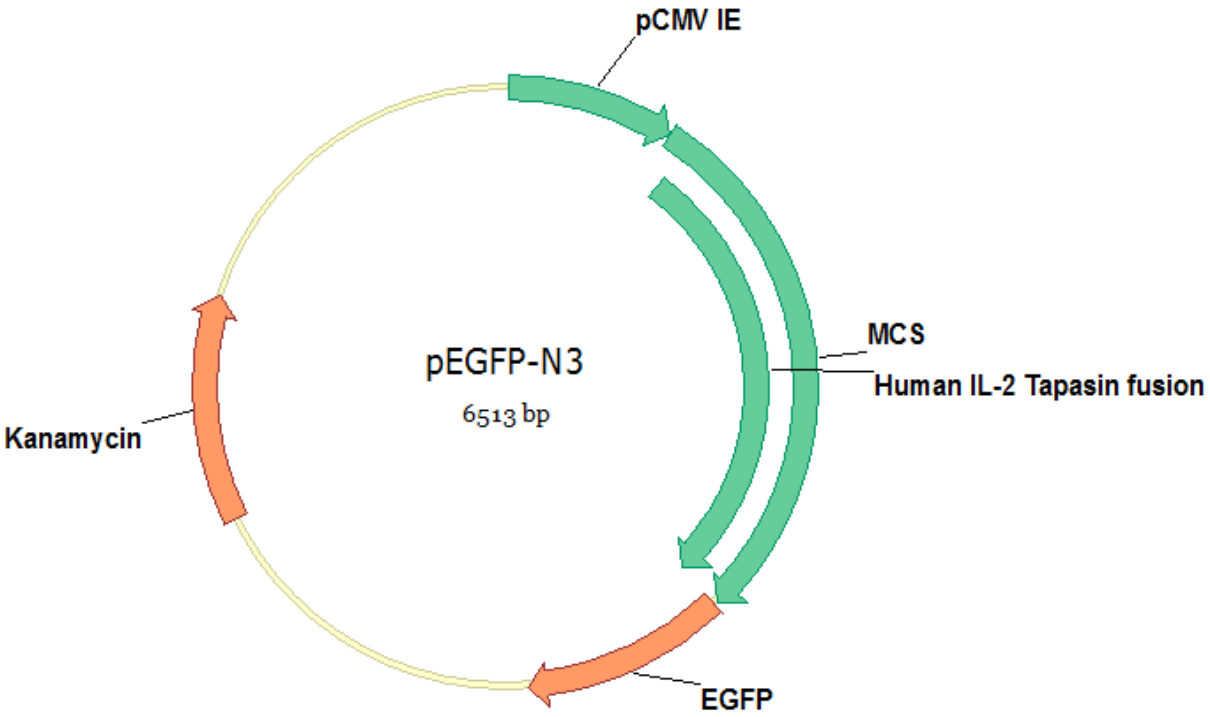

Figure 1.8 The construct map of pEGFP-N3 vector ligate with human IL-2 tapasin

APTSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRNLTFKFYMPKKATEL
KHLOCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCE
YADETATIVESLNRWTTFCQSIIISTLTGGSGGSMKSLSLLLAVALGLATAVS
AGPAVIECWFVEDASGKGLAKRPGALLLRQGPGEPPPRPDLDPELYLSVHDP
AGALQAAFRRYPRGAPAPHCEMSRFVPLPASAKWASGLTPAQNCPRALDGA
WLMVSISSPVLSLSSLLRPQPEPQQEPVLITMATVVLTVLTHTPAPRVRLGQD
ALLDLSFAYMPPTSEAASSLAPGPPPFGLEWRRQHLGKGHLLLAATPGLNGQ
MPAAQEGAVAFAAWDDDEPWGPWTGNGTFWLPRVQPFQEGTYLATIHLPY
LQGQVTLELAVYKPPKVSLMPATLARAAPGEAPPELLCLVSHFYPSGGLEVE
WELRGGPGGRSQKAEGQRWLSALRHHSDGSVSLSGHLQPPPVTTEQHGARY
ACRIHHPSLPASGRSAEVTLEVAGLSGPSLEDSVGLFLSAFLLLGLFKALGWA
AVYLSTCKDSKKKAE

Figure 1.9 Sequence of insert IL-2 (red) and Tapasin (purple) linked with glycine and serine linker (blue)

1.3 Expression of IL-2 in 221-A2

After I transfected IL-2-tapasin construct into 221-A2 seed cells via electroporation, 60% of cells expressed green fluorescent protein (Figure. 1.11). The transfected cells were isolated by cell sorter (Yuste, 2005) (Figure. 1.10). A high level of expression of HLA-A2 and IL-2 in 221-A2-IL-2 cells were revealed by FACS, immunoblotting and fluorescent microscopy with antibodies specific to HLA-A2 and IL-2 (Figure. 1.10, 1.11, 1.12). From the result (Figure. 1.13) also confirm the high expression of co-stimulatory molecule could not be altered by the engineered HLA-A2 and IL-2.

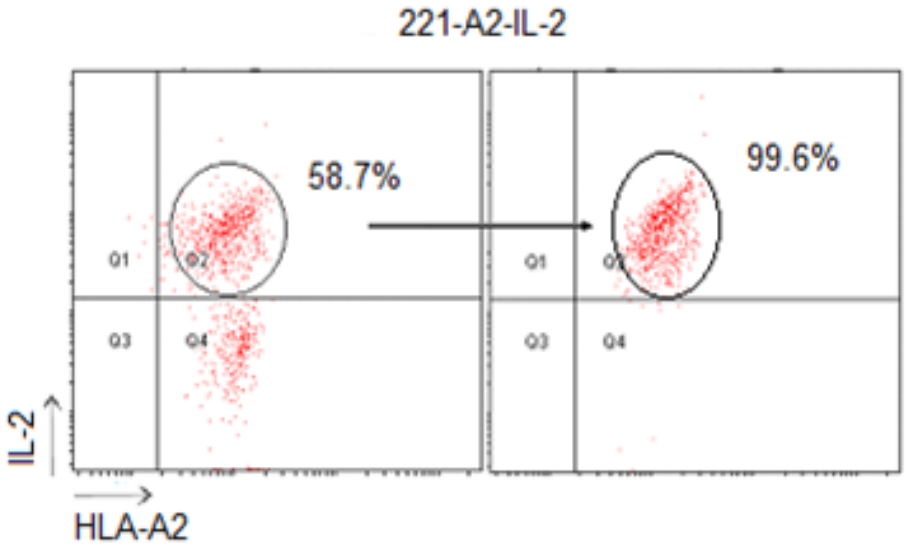

Figure 1.10 IL-2 GFP positive cells were purified by flow cytometric sorting of an IL-2 tapasin construct transfected 221-A2 cells.

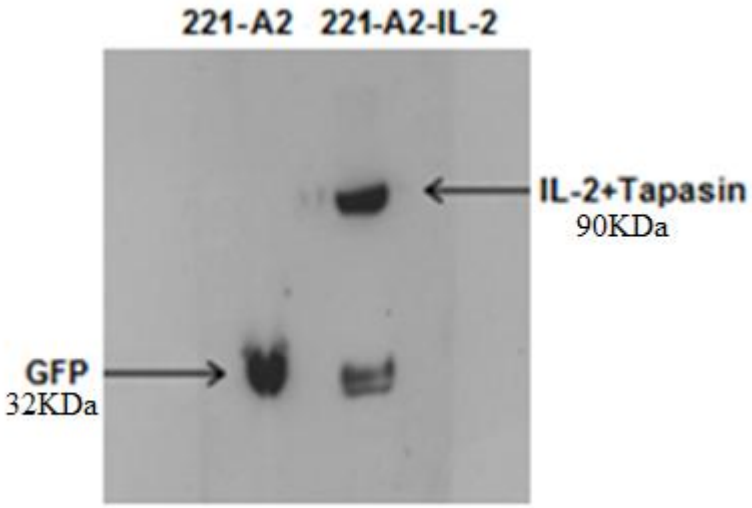

Figure 1.11 Expression of IL-2 in seed cells

Immunoblotting analyses of 20ug lysates from 221-A2 and 221-A2-IL-2 cells respectively with antibody against GFP.

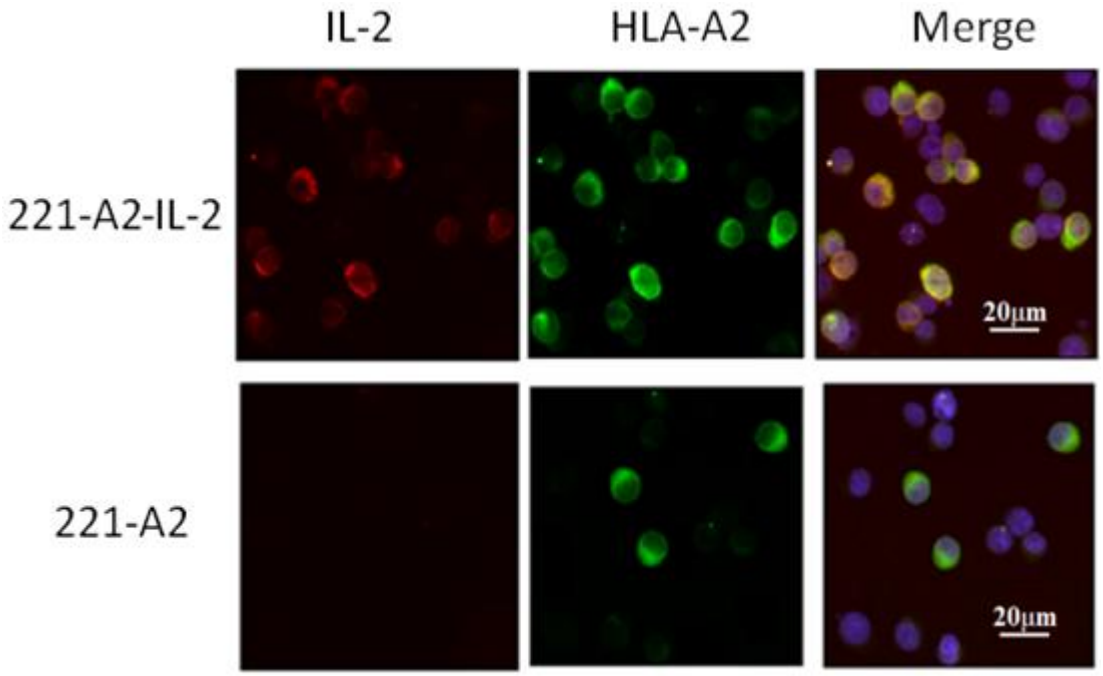

Figure 1.12 Fluorescence microscopy distributes the IL-2 location in IL-2 transfected cell.

Cell nuclei were visualized by DAPI (blue) counter-staining, and Texas Red labelled anti-human IL-2 (red) stained both 221-A2-IL-2 (upper panel) and 221-A2 cells (lower panel).

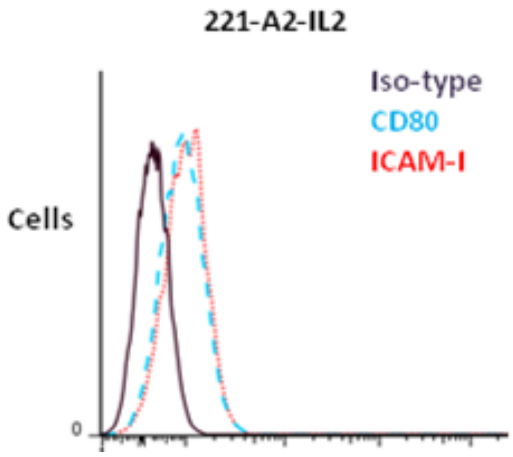

Figure 1.13 expression of co-stimulate molecule in tranfected cells

221-A2-IL-2 cells were analysed for the co-stimulatory molecules CD80 and adhesion molecule ICAM-1 by staining with corresponding antibodies and analysed on flow cytometry. Isotype Ig was used as background control.

2. Preparation of IL-2-A2-nanoAPC

2.1 Growth of large quantity of seed cells

2.1.1 Culture condition and cell growth rate control

In order to obtain high quality and large quantity of seed cells, we started with a small scale, to work out the cell density and survival rates. Under conventional culture conditions with 10% FBS and RPMI 1640 medium, we found that 10^5/ml seeding density is optimal to meet rapid expansion at large volumes and the survival rates will drop when over the density of 1×10^6 cells/ml (Figure. 2.1). Therefore, the cells were expanded by seeding 10^5cells/ml and reseeding after reaching 1×10^6 cells/ml. Thus, the survival rates maintained at > 98%.

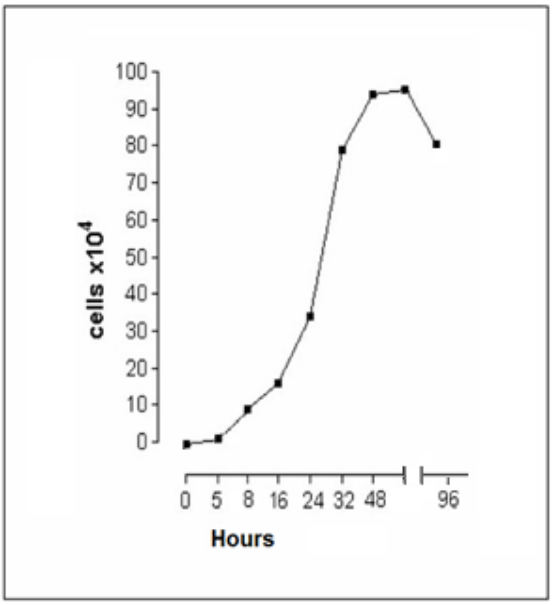

Figure 2.1 S-shaped growth curves of 221-A2-IL-2 cells

In the beginning the cells stay at an initial LAG phase which is the rate of growth or when the cell division is very slow, and then the growth or cell division starts to accelerate into the exponential phase which is when the cells are growing or multiphying rapidly.

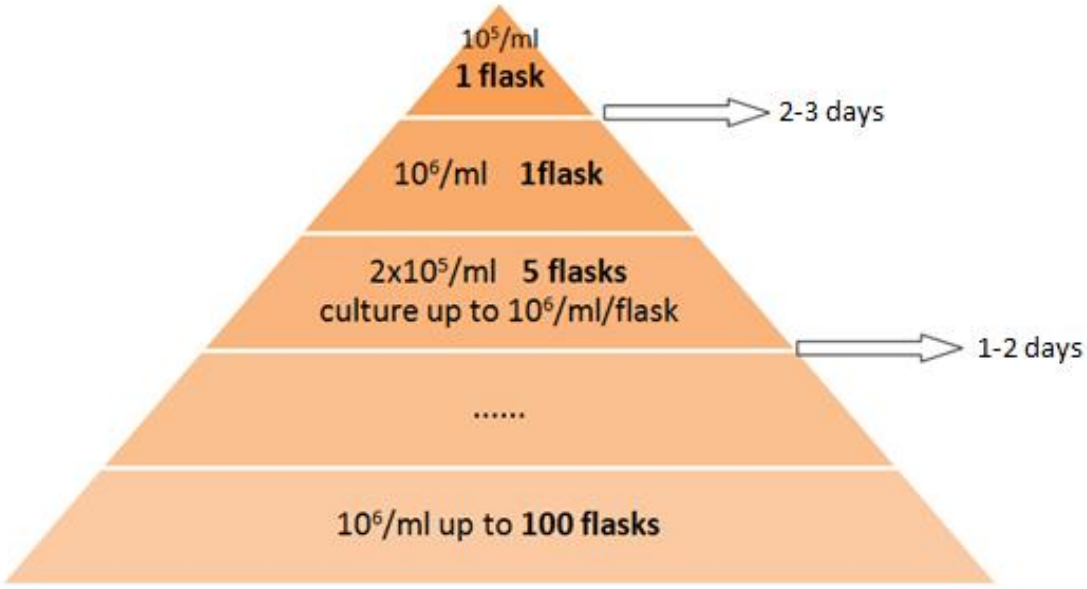

Figure 2.2 Flowchart of cell expansion

At 10^6/ml density, one gram of cells could be harvested from 1000ml culture. After washing, the cells were snap-frozen in the liquid nitrogen and stored at -80℃ until further use. The cell enriched large scale culture did not alter the expression of transfected HLA-A2 and IL-2 molecules (Figure. 2.3).

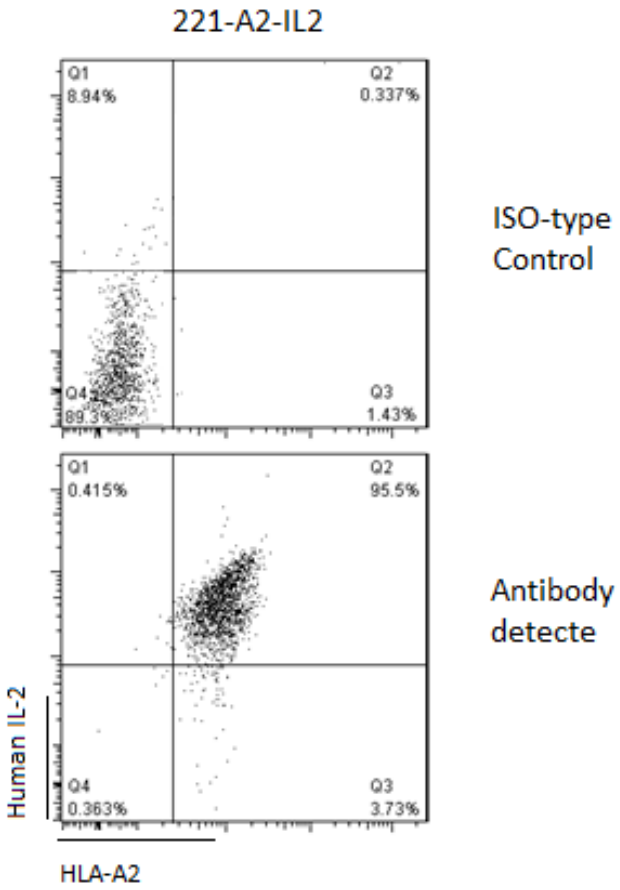

Figure 2.3 IL-2 and HLA-A2 expression on 221-A2-IL-2 cell

Cells were stained with antibodies against IL-2 and HLA-A2 molecule, respectively and then analysed on flow cytometry. The isotype Ig was used as background control.

2.2 IL-2-A2-nanoAPC preparation

To ensure the nanoAPC's quality and function, the temperature was maintained at 4℃ throughout the isolation procedure. We developed protocols that were used to prepare IL-2-A2-nanoAPC at homogenous size of ~500nm and enriched with HLA-A2 and IL-2. Importantly, the prepared nanoAPC have to maintain peptide receptive HLA-A2 molecules and bioactivation of IL-2. Based on previously developed methods for microsome preparation, we used fine homogenizer, 221-A2-IL-2 cells to prepare microsomes and final nanoAPC. To homogenize seed cells, a cut off size of 12 microtron was used to break cells to a mixture of nuclear, mitochondrial and membrane fractions. I used a gradient of sucrose to separate particles based on their individual densities. The generated IL-2-A2-nanoAPC layer

on top of the 2M sucrose cushion was collected (Figure. 2.4). The collected ER and Golgi fractions were finally homogenized at cut off size of 4 microtron to make a homogenous membrane fragments at size of ~ 500nm (Figure. 2.5) (Figure. 2.7). This size is referred as nano-size and can pass micro-vessels and freely distribute to target organs such as peripheral lymph nodes. Our result also confirmed that nanoAPC still maintained high levels of co-stimulatory molecules; CD80 and ICMA-1 (Figure 2.6). The yield of IL-2-A2-nanoAPC is usually about 3-5% of the total APC cell weight.

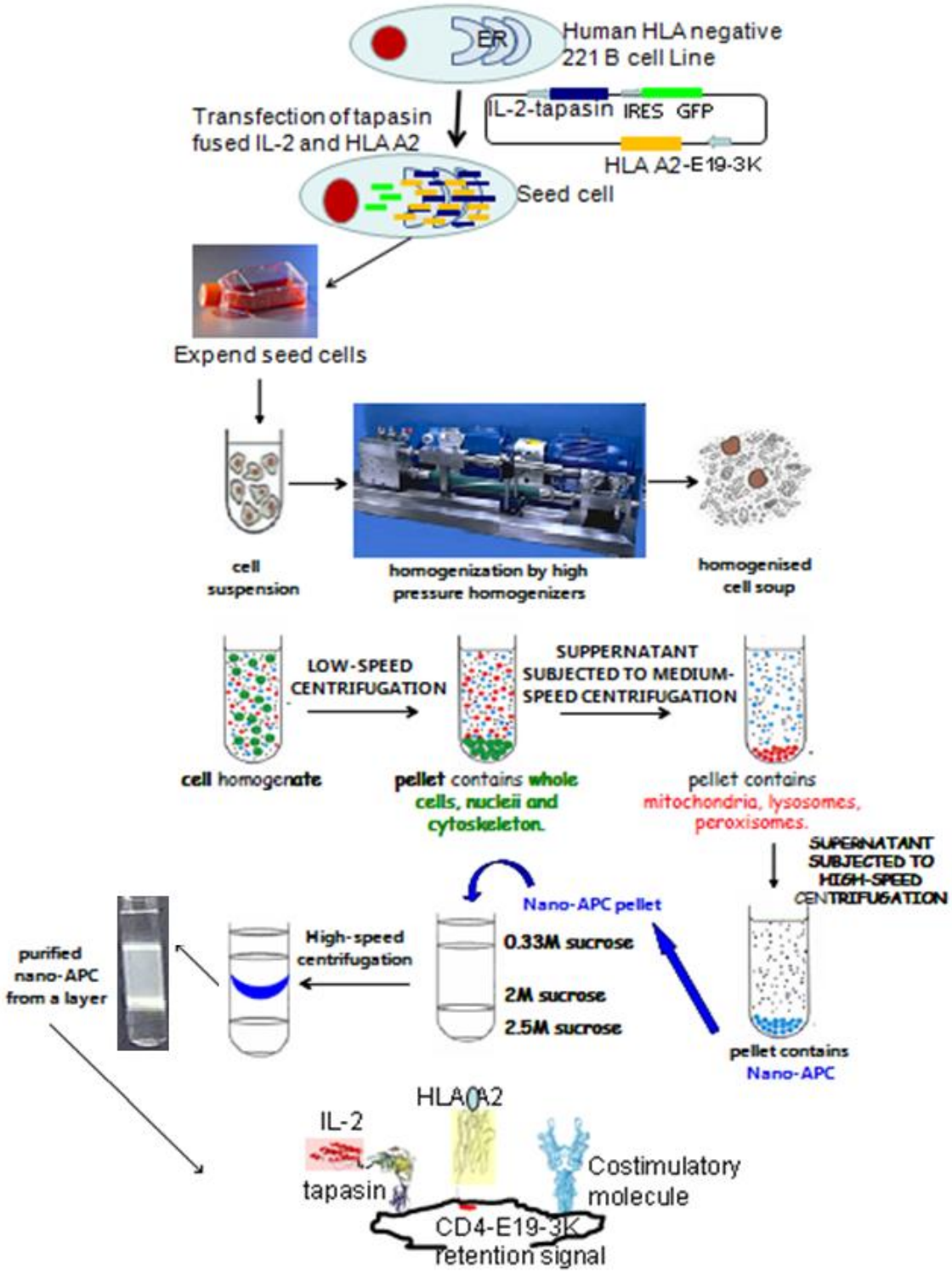

Figure 2.4 IL-2-A2-nanoAPC preparations.

To prepare IL-2-A2-nanoAPC from IL-2 transfected APCs, a cell suspension in a hypertonic solution was placed in a 5ml syringe. Cells were passed though the homogenizer on ice. The nuclear, mitochondrial and larger cell debris was removed from the cell homogenate by centrifugation at 10,000 x g. The total IL-2 nanoAPC was recovered by centrifugation at 100,000 x g and further subfractioned by flotation in discontinuous sucrose gradients.

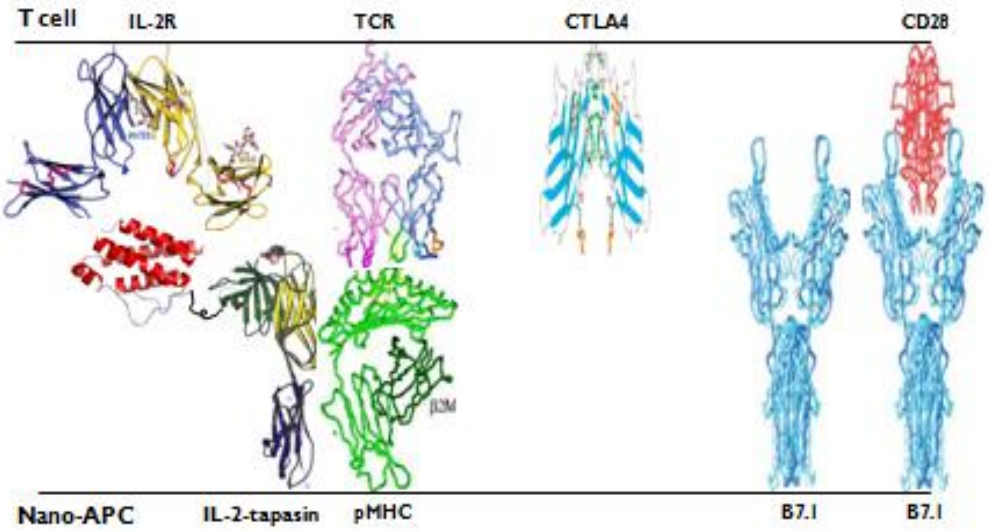

Figure 2.5 IL-2-A2-nanoAPC contained IL-2, HLA-A2 and co-stimulatory molecules
NanoAPC with reconstituted MHC, IL-2-Tapasin and co-stimulatory molecule, which will reactive with T cells working as professional antigen presenting cells.

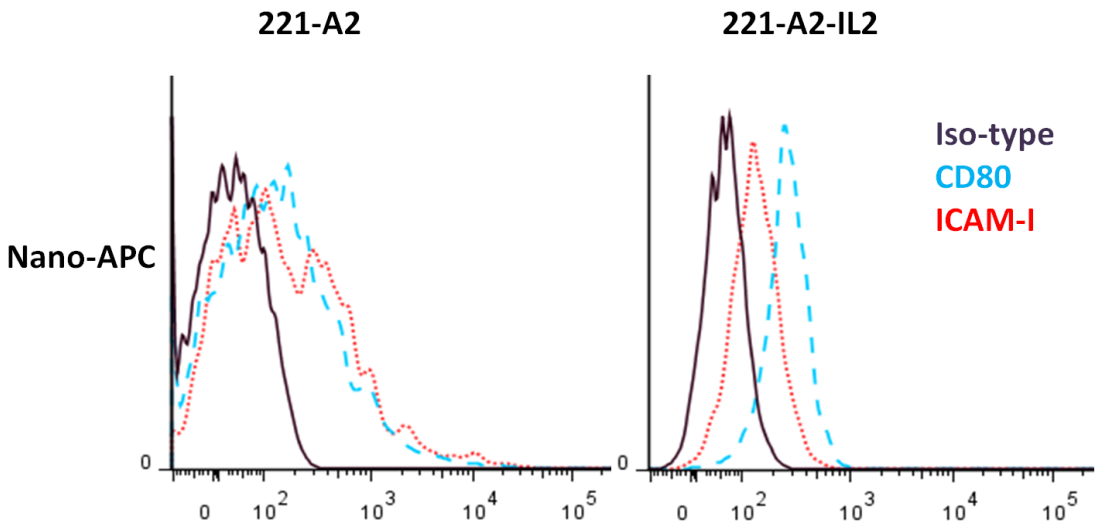

Figure 2.6 expression of co-stimulate molecule in NanoAPC

NanoAPC isolated from either 221-A2 or 221-A2-IL-2 cells were analysed for the co-stimulatory molecules CD80 and adhesion molecule ICAM-1 by staining with corresponding antibodies and analysed on flow cytometry. Isotype Ig was used as background control.

To confirm co-expression of IL-2 and HLA-A2 in nanoAPC, nanoAPC were stained with anti-IL-2 and HLA-A2 antibodies, respectively and analysed by confocal microscopy. Confocal fluorescent micrographs revealed that IL-2 molecule (Red) and HLA-A2 molecule (Green) on nanoAPC prepared from 221-A2-IL-2 seed cells while nanoAPC from 221-A2 seed cells only showed HLA-A2 (Figure 2.7 down panel). This result demonstrated that the IL-2-A2-nanoAPC was of a largely homogenous size, ~500nm, and contained high levels of HLA-A2 and IL-2.

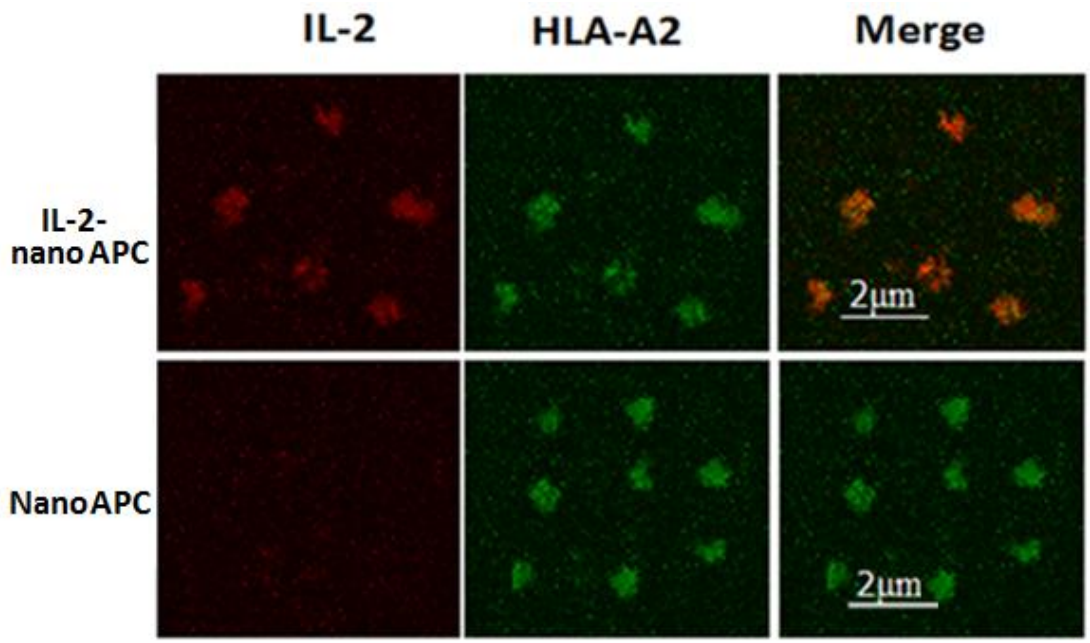

Figure 2.7. Confocal images of GFP expressing nanoAPC

NanoAPC has enriched HLA-A2 and IL-2. NanoAPC was prepared from 221-A2-IL-2 or 221-A2 cells and then stained with antibodies to IL-2 (Red) or HLA-A2 (Green) indicated.

2.3 Analysis of IL-2 bioactivity

Although the IL-2-A2-nanoAPC expressed fused IL-2, it was unclear whether this IL-2 molecule still had their initial biological function. The IL-2-tapasin fusion protein retained IL-2 bioactivity as demonstrated by the proliferation of the IL-2-dependent CTLL4 cell line in response to IL-2-A2-nanoAPC (Figure 2.8A). The pharmacological activity of IL-2 on nanoAPC was ~2 International Unit (IU)/ug nanoAPC (Figure 2.8A). The activity of IL-2 on IL-2-A2-nanoAPC was effectively neutralized by IL-2 neutralizing antibody (Figure 2.8B). Although the activity is about fifty times lower than the ~100 IU per 1ug of recombinant IL-2, the IL-2 on nanoAPC is immobilized which may create an enriched microenvironment for T cells that are specifically interacting with the nanoAPC. This strategy can be applied to other cytokines or bio-active proteins depending on the therapeutic aim.

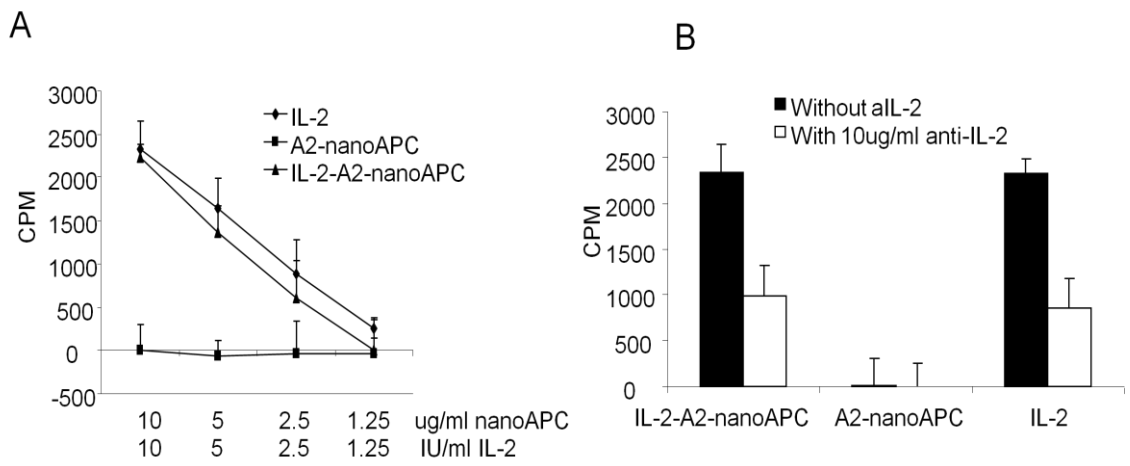

Figure 2.8 IL-2 activity of IL-2-A2-nanoAPC.

Proliferation of CTLL4 was measured and converted to international units after comparing with recombinant IL-2.

(A) A2-nanoAPC engineered with or without IL-2 at different doses was incubated with CTLL4 cells for 24 hours. Proliferation of CTLL4 was measured and converted to international units after comparing with recombinant IL-2.

(B) IL-2 activity was neutralized by anti-IL-2 antibodies. The data presented as mean of triplicate cultures ±SD and are representative of four experiments

3 NanoAPC-associated MHC can bind to antigenic peptides.

3.1 conditions for peptide loading

To explore whether the MHC molecules in ER-enriched nanoAPC from APC can present antigens to T cells, we first examined the ability of MHC molecules in isolated nanoAPC membranes to assemble with peptides.

3.1.1 Inverted nanoAPC's membrane via freeze-thaw to expose the luminal side of nanoAPC

Since assembly of MHC molecules occurs on the luminal side of the ER membrane, the assembly of the MHC molecules present in nanoAPC would occur on the interior surface of the nanoAPC vesicle, which represents the ER lumen (Kvist and Hamann 1990). Thus, in order to achieve maximum access to MHC molecules for peptide-loading we attempted to 'break-open' the nanoAPC membrane, so as to expose the luminal side of nanoAPC, by a repeated freeze-thawing procedure. Based on our findings and previous studies, the repeated freeze-thaw process is applied in order to disrupt the nanoAPC, such that the luminal side of the membrane is exposed. MHC and co-stimulatory molecules are glycoprotein transmembrane proteins naturally exposed to the luminal side of the ER (Kreibich et al, 1978) (Sabatini et al, 1978), which is equivalent to the lumen of the nanoAPC (Kvist and Hamann 1990) (Vassiliki et al, 2009). Therefore, after 'inversion' of nanoAPC by repeated freeze thawing to expose the luminal nanoAPC surface, the MHC glycoproteins should also be exposed, allowing better access of soluble peptides to the peptide-binding site of the MHC molecule (Figure 3.1).

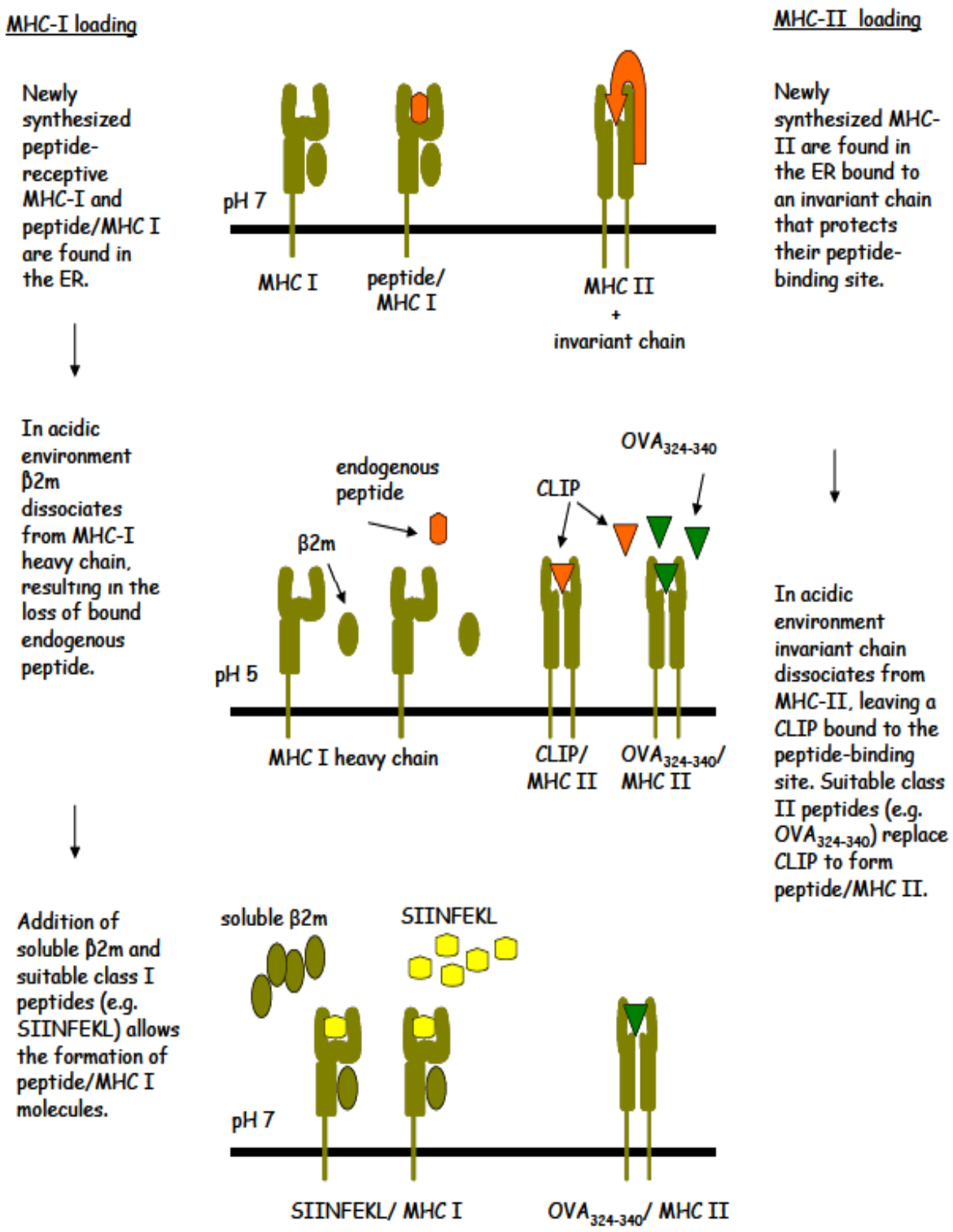

Figure 3.1 Experimental designs for loading of antigenic peptides on MHC class I and MHC class II.

Both MHC molecules are synthesised and matured in the lumen of the ER. Newly synthesized and properly folded MHC class I molecules are retained in the ER until they form stable peptide-MHC class I complexes, while MHC class II molecules associate with an invariant chain and are transported from the ER to the endosome. The invariant chain protects the peptide binding site until it encounters peptide competition (mediated by HLA-DM in human) in the endosomal acidic environment. Peptide loading in an acidic buffer of pH 5 dramatically increased the peptide receptiveness of MHC class II molecules. In addition to generating peptide receptive MHC class II molecules, the acid stripping process also led to a significant increase in peptide-receptive MHC class I molecules, which may be due to dissociation of pre-processed peptides on MHC class I molecules.

3.1.2 Acidic treatment for class I and II peptide loading

Detection of peptide/MHC class I complexes were achieved with the use of specific antibodies. In this experiment we used the H2-K^b molecules in the processed nanoAPC could be loaded with the K^b-specific peptide SIINFEKL, as shown by staining with the SIINFEKL-K^b specific antibody 25-D1.16 (Figure 3.2). It has been shown by our group that the acid-stripping process also led to a significant increase in peptide-receptive MHC class I molecules, which may be due to the dissociation of pre-processed peptides on MHC class I molecules (Vassiliki et al, 2009). The whole process has been clearly presented by the flowchart above (Figure 3.1). As we see that the MHC class I assembly with antigenic peptides takes places in the ER, and from the result it shows that the nanoAPC load with SIINFEKL-K^b specific peptide can be detected and monitored by 25-D1.16 antibody with double mount of the fluorescene means.

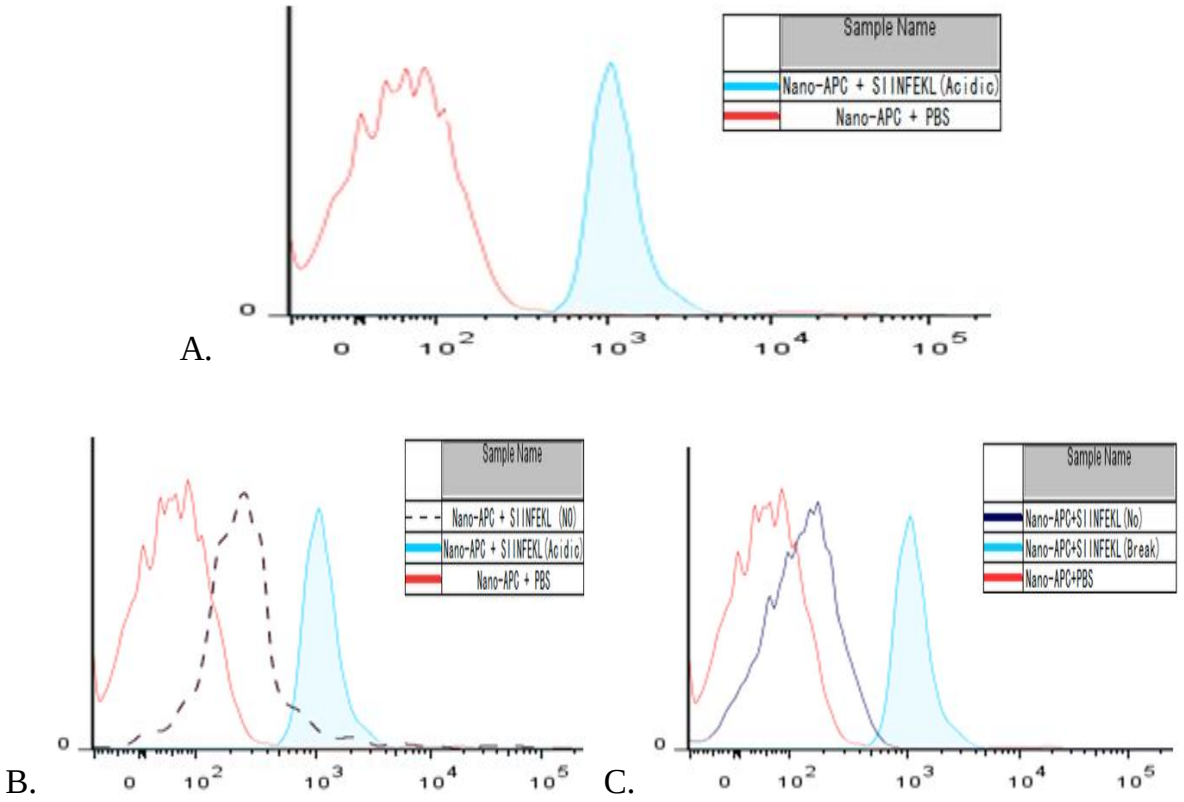

Figure 3.2 Flow cytometric analysis of peptide loading onto MHC molecules in nanoAPC membranes.

NanoAPC from Jaws-II cells were pulsed with either SIINFEKL peptide or PBS. Excess peptides were washed and peptide-loaded nanoAPC were analyzed in comparison to nanoAPC that had been loaded without peptides. :

A. Panel displays the mean fluorescence intensity of PE-labelled 25-D1.16 antibody specific to SIINFEKL-K^b complexes.

B. NanoAPC with or without pretreatment with acid-stripping and

C. NanoAPC with or without freeze-thaw process treatment before peptide loading. NanoAPC without peptide serves as a background control.

We have also developed a method for loading MHC class II antigens onto purified ER vesicles to assemble pMHC class II (Sofra et al, 2009). In this experiment we used three HBV peptides reported to bind to DR and/or DP, and then use FITC biotin to label them by flow cytometry. Analysis of the assembly of HLA DR molecules with peptide showed that HLA DR binding peptides could effectively assemble with DR under acidic conditions (Figure 3.3). 221 cells are deficient in MHC class I, but still express HLA DR and DP molecules. Since MHC class II

molecules are much more promiscuous than MHC class I molecules in terms of selection of their antigenic peptides, as most of the DR associated peptides can also bind to DP to certain degrees (Sadegh-Nasseri et al, 2010), we used the endogenous HLA DR and DP molecules from seed cells to assemble antigenic peptide-MHC class II complexes for activation of effector CD4 helper T cells. Although it shows that the peptide can be loaded on the nanoAPC, however if comparing to naturally processed pMHC in live DCs, the assembled pMHC is still limited. The optimal approach to generate pMHC in nanoAPC is to introduce native antigen protein into seed cells to pMHC processing naturally.

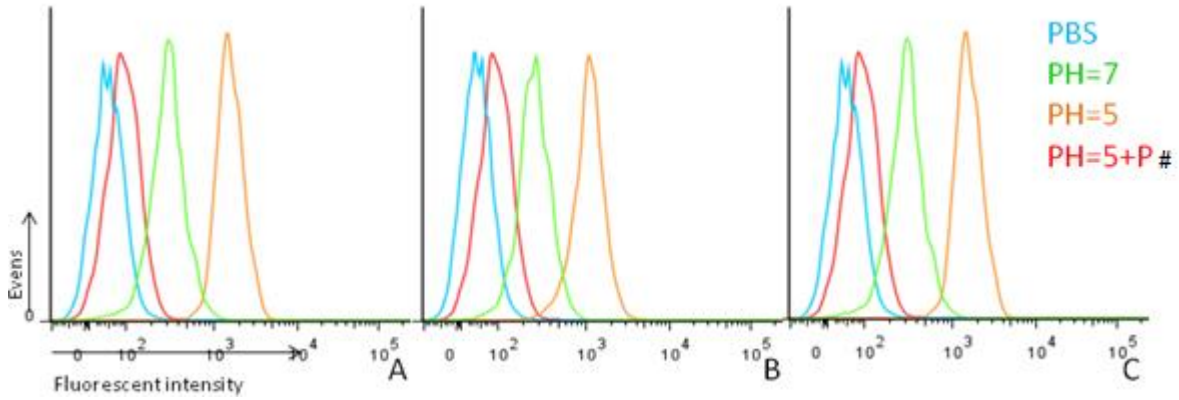

Figure 3.3. Acidic treatment induces peptide-receptive MHC class II

Peptide loaded A2-nanoAPC from 221-A2 cells under pH 7.0 or pH 5.0 conditions. The same nanoAPC with PBS or with 5 times unlabeled peptide serve as a background control.

Fluorescence peptide plus five times unlabelled peptide with A2-nanoAPC under acide condition.

4 Activation of T cells

NanoAPC are designed to directly deliver antigen, co-stimulatory and cytokine signals to antigen specific T cells. Therefore, a sustained interaction between nanoAPC and antigen specific T cells is essential for the T cells to engage IL-2, or other engineered cytokines, effectively.

4.1 Interaction of nanoAPC to OT1 cell

Previously, we have shown that ER vesicles containing pMHC molecules, generated by assembly of the MHC on the vesicles with peptide, can directly activate antigen specific T cells *in vitro* (Sofra et al, 2009). To further investigate whether the interaction of nanoAPC and antigen specific T cells can induce membrane clustering to form stable immune synapses (Fooksman et al, 2010), we examined the interaction of nanoAPC, prepared from the murine DC cell line DC2.4 were processed by repeated freeze-thaw to facilitate inversion of the luminal side, before loading either with SIINFEKL peptides or without. Excess peptides were washed and peptide-loaded nanoAPC were incubated with splenic T cells isolated from OT-I TCR transgenic mice (Jameson et al. 1994), which express TCR specific to SIINFEKL-K^b. T cells without peptide-loaded nanoAPC, were used as a negative control (Figure 4.1).

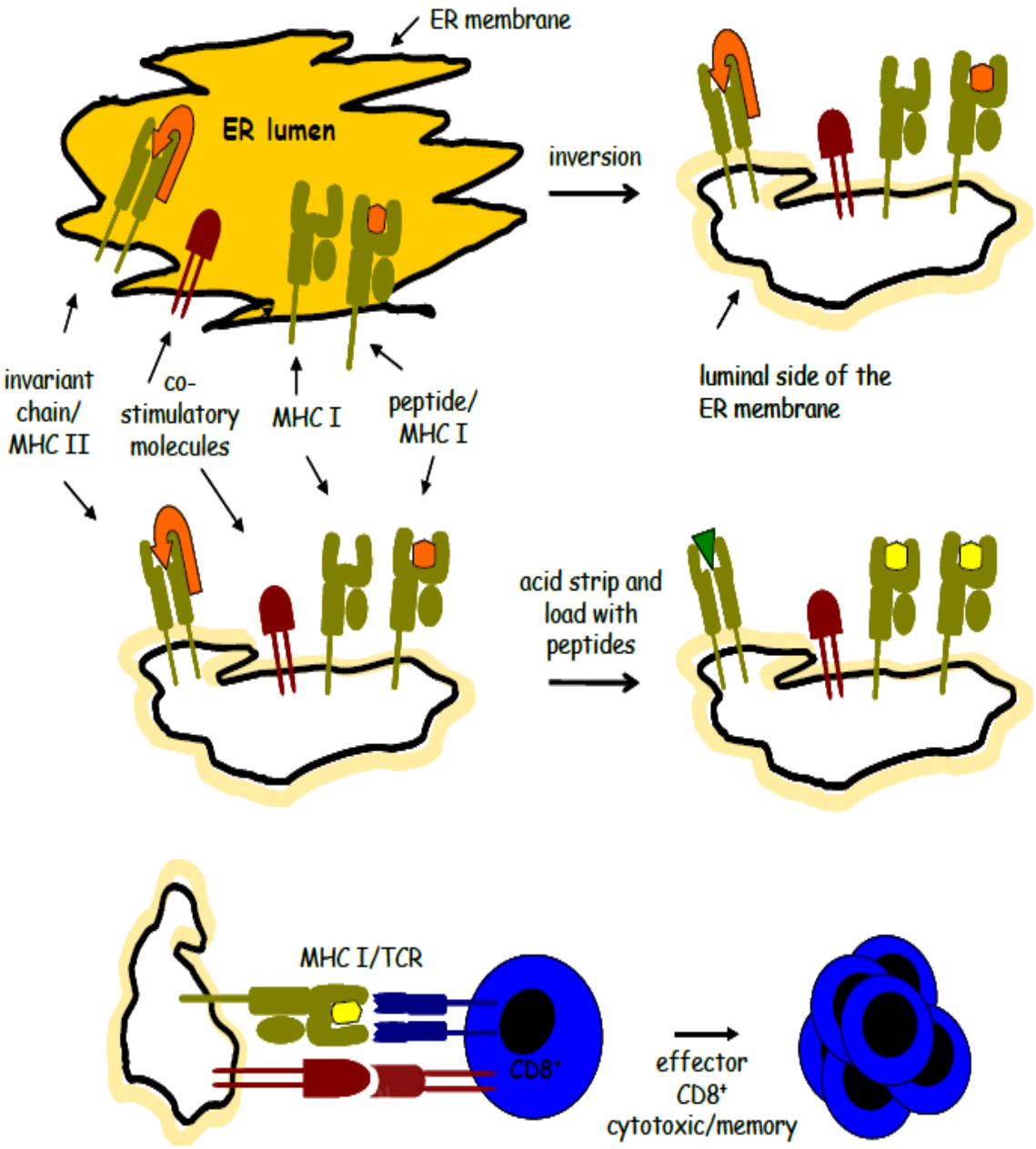

Figure 4.1 Experimental designs for the analysis of T cell responses induced by inverted nanoAPC.

NanoAPC is processed by repeated freeze-thaw so as the luminal side of the membrane is exposed. Endogenous peptides are dissociated from MHC class I, by acid stripping, and peptides of interest are loaded on the nanoAPC.

4.2 Synapse formation

The interaction of nanoAPC and T cells was carried out on an experimental system with nanoAPC derived from mouse DC cell line H-2 Kb positive DC2.4 and Kb binding peptide SIINFEKL. The SIINFEKL loaded nanoAPC were incubated with OTI T cells carrying TCR specific to SIINFEKL-Kb for different periods of time at 30℃. We found that a stable interaction of nanoAPC and T cells formed rapidly after just 30 min incubation, while after less than an hour, nanoAPC induced synapse formation on T cells (Figure. 4.2). The nanoAPC were completely emerged into synapse. The process resembles the membrane processing observed at the contactpoint between live DC and T cells (Fooksman et al, 2010). The nanoAPC associated synapses can facilitate the recruitment of IL-2R into complexes with the T cellbound IL-2-nanoAPC, thus bringing the IL-2R into lipid rafts which can sustain IL-2 activation in effector T cells (Cho et al, 2010). Thus, nanoAPC can fully substitute for live DC, inducing T cell activation and the formation of immune synapses. This creates a stimulatory microenvironment allowing engagement of pMHC, co-stimulatory molecules and engineered bio-adjuvants with their respective receptors on antigen specific T cells. After 6-hours incubation, nanoAPC were infused into T cells a phenomenon observed after interaction of APC and T cells previously. Such interactive process will lead to sustained antigen presentation and bioactivation of engineered bioadjuvants such as IL-2 or IL-7 (Marc et al, 2011).

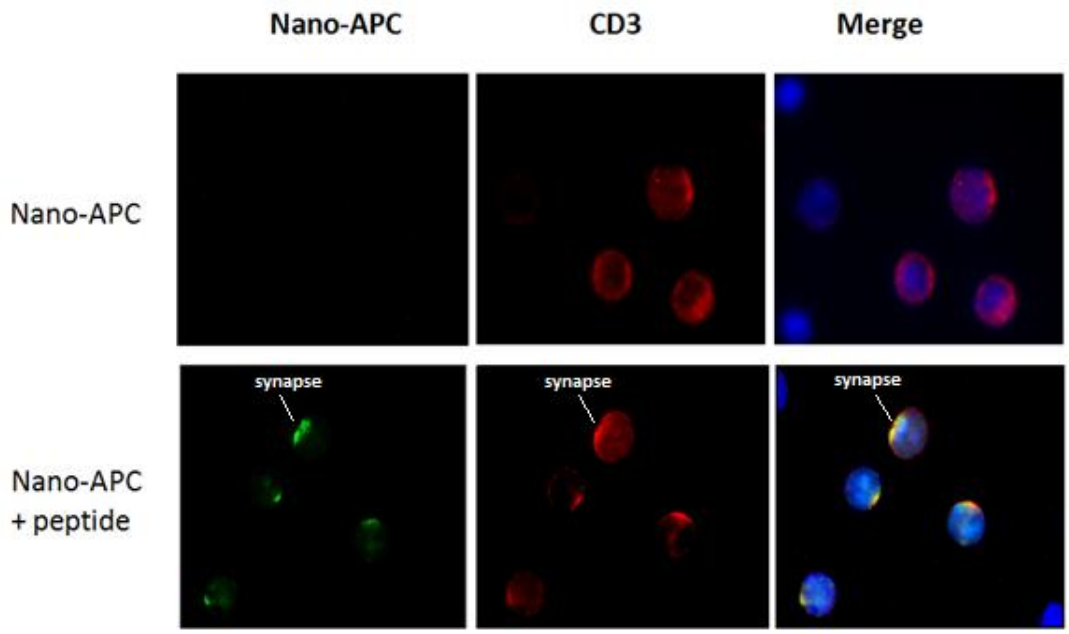

Figure 4.2 NanoAPC interact and activate antigen specific T cells.

NanoAPC labelled with FITC at 10ug/ml was loaded without (upper panel) or with antigenic peptides (low panel) and incubated with antigen specific T cells for 60 min. Free nanoAPC were washed off. The cells were stained with anti-CD3. The nanoAPC interacted with T cells and formed synapse with CD3.

5 Endocytosis of nanoAPC *in vivo*

5.1 NanoAPC do not be endocytozed by DCs in peripheral lymphoid organs

Most reported nano-delivery systems for therapeutic vaccines rely on endogenous DC in patients to process and present antigens to T cells (Nandedkar et al, 2009). However, the nanoAPC, functionally equivalent to live DC, act directly on antigen specific T cells *in vivo* (Sofra et al, 2009). Accumulation of a pharmacokinetic dose of free-nanoAPC in DC rich organs such as lymph nodes and liver will be important to effectively induce activation of antigen specific T cells. Previously, we found that ER membrane vesicles prepared from DC accumulated in the peripheral lymphoid organs and were not endocytosed by DC *in vitro* (Sofra et al, 2009). However, whether the nanoAPC are endocytosed by DC in lymphoid organs is not known.

5.2 Test phagocytic function of DCs for the ER membrance vesicles

To investigate whether nanoAPC remain in lymphoid organs as free nano-particles, nanoAPC prepared from murine DC2.4 cells were labelled with fluorescence and injected i.v. into B6 mice. After 48 hours, the lymph nodes were isolated and separated into cellular and cell-free fractions. The cell-free fragments and DC from the lymph nodes were examined for the presence of free and endocytosed nanoAPC. In contrast to the efficient endocytosis of dextran by DC in lymph nodes, the nanoAPC that accumulated in the lymph nodes remained as free particles (Figure 5.1A). To exclude the possibility that DC activation is required for phagocytosis of the ER membrane vesicles, we infected mice with a vaccinia virus to induce DC activation five days before injection of nanoAPC. Similar levels of free-nanoAPC were detected in lymph node samples from mice pretreated with vaccinia and mice that had not received pretreatment (Figure 5.1B), indicating that the intracellular membrane vesicles from APC were not effectively endocytosed by DC even with a concomitant viral infection. The lack of endocytosis of nanoAPC may be

due to failure of pattern recognition receptors, which enable the DC to discriminate between normal physiological components and pathological particles *in vivo* (Geijtenbeek et al, 2004), to recognise the nanoAPC.

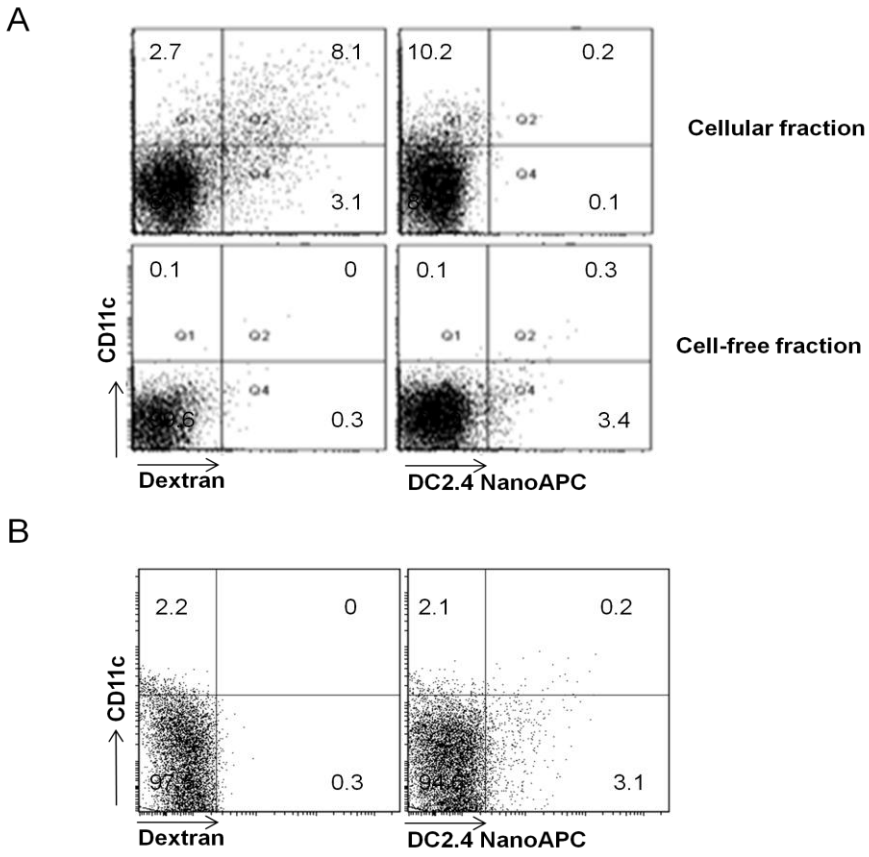

Figure 5.1 NanoAPC are accumulated in lymph nodes as free nano-particles.

A. FTIC labelled Dextran or DC2.4 nanoAPC were injected i.v. at 1mg/kg to C57BL mice. 48-hour post-injection, lymph nodes were suspended as cellular fraction and cell-free fraction. Both were stained with anti-CD11c and analysed by FACS. B. C57BL mice were infected with vaccinia virus at dose of 2 x 10^5 PFU. Five days after infection, FTIC labelled Dextran or DC2.4 nanoAPC were injected i.v. at 1mg/kg. 48-hour post-injection, lymph nodes were suspended and cell-free fraction was stained with anti-CD11c as described in materials and methods.

6. IL-2-A2-nanoAPC present peptides to human T cells *in vitro*.

6.1 Induction of antigen specific $CD8^+$ T cell responses by IL-2-A2-nanoAPC

The design of IL-2-nanoAPC aims to target IL-2 to antigen specific T cells leading to increased T cell activation. To investigate this, IL-2-A2-nanoAPC was assembled with human CMV_{nlv} peptides and used to examine the efficacy for stimulating CMV specific CD8 T cell responses, in peripheral mononuclear cells (PBMC). PBMC isolated from CMV sero-positive and HLA-A2 positive health donors and activation of T cells in the absence of assembled CMV_{nlv} peptides. T cell responses were evaluated by IFNγ production via FACS analysis (Figure 6.1).

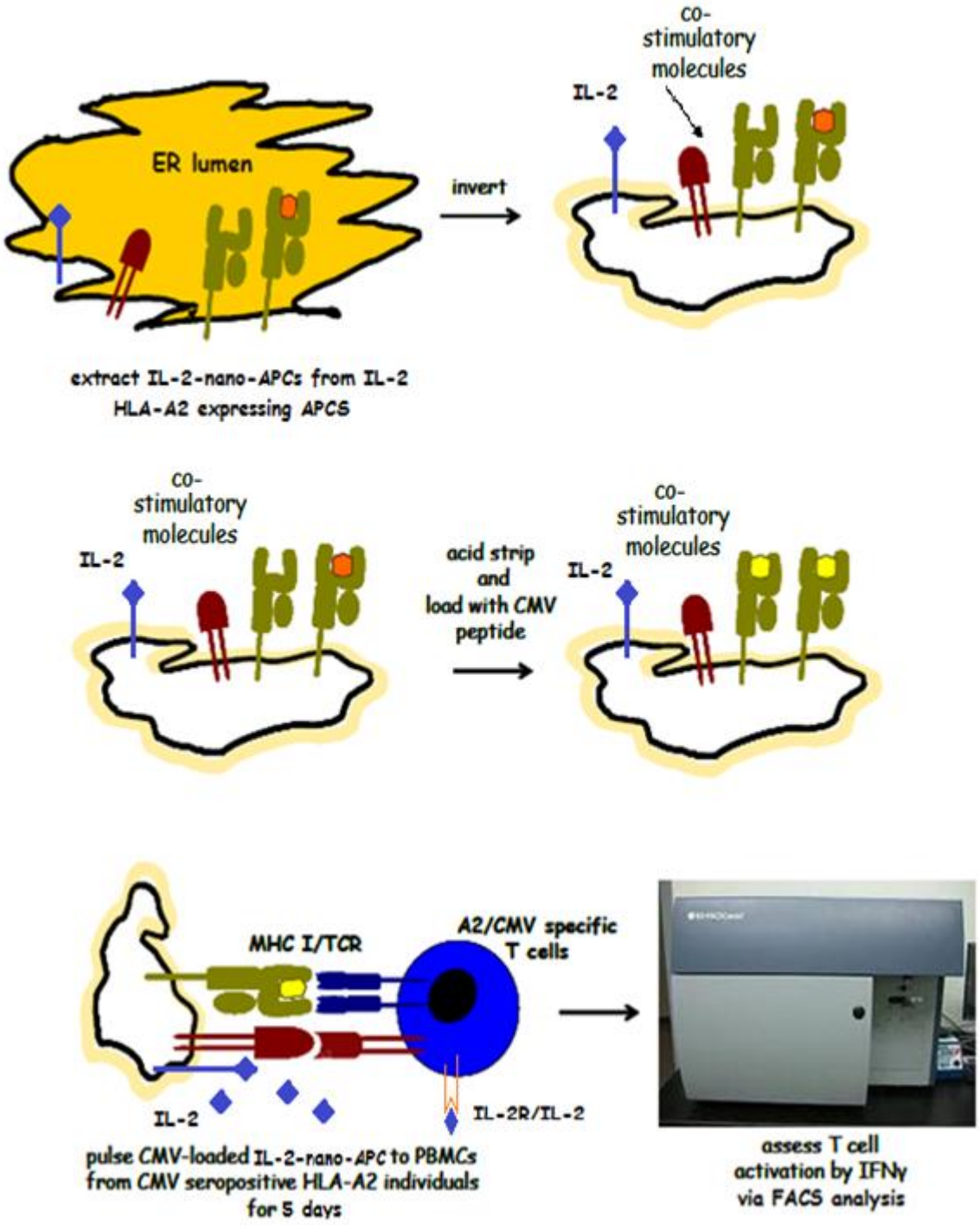

Figure 6.1 Experimental designs for detection of induced memory T cell responses *in vitro* in PBMCs from latently infected individuals by a nanoAPC based vaccine.

IL-2-A2-nanoAPC prepared from IL-2 transfected 221-A2 cells were inverted, stripped and loaded with an HLA-A2-specific CMV peptide that has amino acid sequence 'NLVPMVATV'. CMV peptide-loaded IL-2-A2-nanoAPC was cocultured with PBMC of

CMV serum converted and HLA-A2 positive healthy donors. The presence of reactivated CMV-specific $CD8^+$ T cells was analyzed by IFNγ antibody detection via FACS analysis.

Here we investigate whether IL-2-A2-nanoAPC can induce CMV-specific T cell responses *in vitro* (Figure 6.2). IL-2-A2-nanoAPC are isolated from 221-A2-IL-2 cells, while A2-nanoAPC isolated form 221-A2 cells as control for IL-2 effect though the whole experiment. Human PBMC obtained from HLA-A2 healthy individuals, who were serologically positive for CMV, were co-cultured with IL-2-A2-nanoAPC or A2-nanoAPC loaded with a CMV peptide (amino acid sequence "NLVPMVATV"). Co-cultures with IL-2-A2-nanoAPC without peptide or with 1uM soluble CMV peptide, served as controls. The presence of reactivated CMV-specific $CD8^+$ T cells was indirectly detected by CD8 and IFNγ antibody following analysed analysis by flow cytometry. There is a weak amount of IFNγ in stimulation of PBMC's with soluble CMV peptides which is possibly due to uptake and presentation of the peptide by APC's naturally present in the PBMC's. This result shows (Figure 6.3) that CMV-specific IFNγ-secreting cells can be detected in the samples after stimulation with CMV peptide loaded either IL-2-A2-nanoAPC or A2-nanoAPC. However IL-2-CMV_{nlv}A2-nanoAPC induces significant more strong CD8 T cell responses which can effectively neutralized by human IL-2 antibody (Figure 6.4). To further confirm the antigen-dependent effect of IL-2, the IFNγ producing cells were quantified in CMV_{nlv}pentamer$^+CD8^+$ cells in PBMC after stimulation (Figure 6.5). The antigenic peptide dependent T cell response between IL-2-A2-nanoAPC and T cells demonstrating that this IL-2 in IL-2-A2-nanoAPC only enhances the response of antigen specific T cells but not bystander T cells.

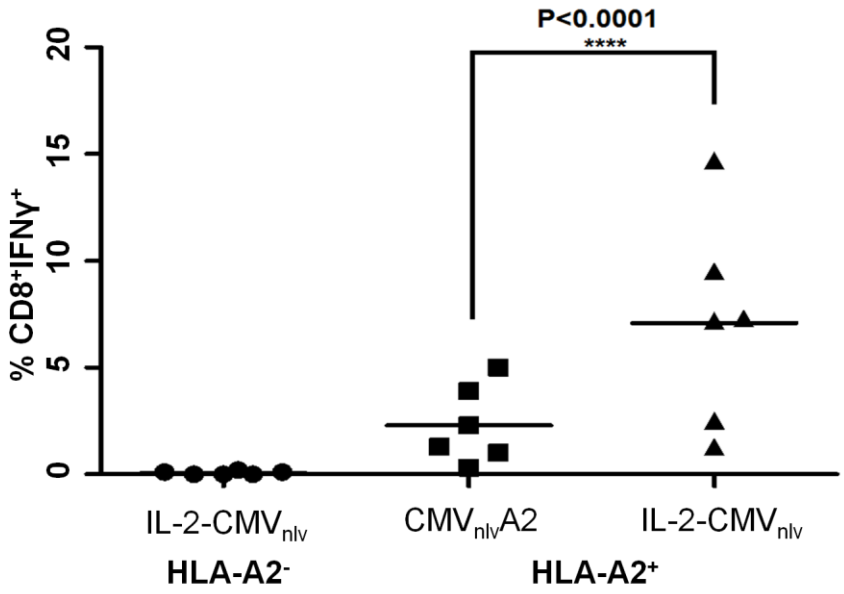

Figure 6.2 Induction of CMV_{nlv} specific CD8 T cell responses.

Six CMV seropositive PBMC's were stimulated with CMV_{nlv}A2-nanoAPC or IL-2-CMV_{nlv}A2-nanoAPC for five days. After re-stimulation with PMA and Inomycine, IFNγ producing CD8 T cells were quantified. Six HLA-A2 negative PBMC's served as controls. Statistical comparisons were performed using Student's t test.

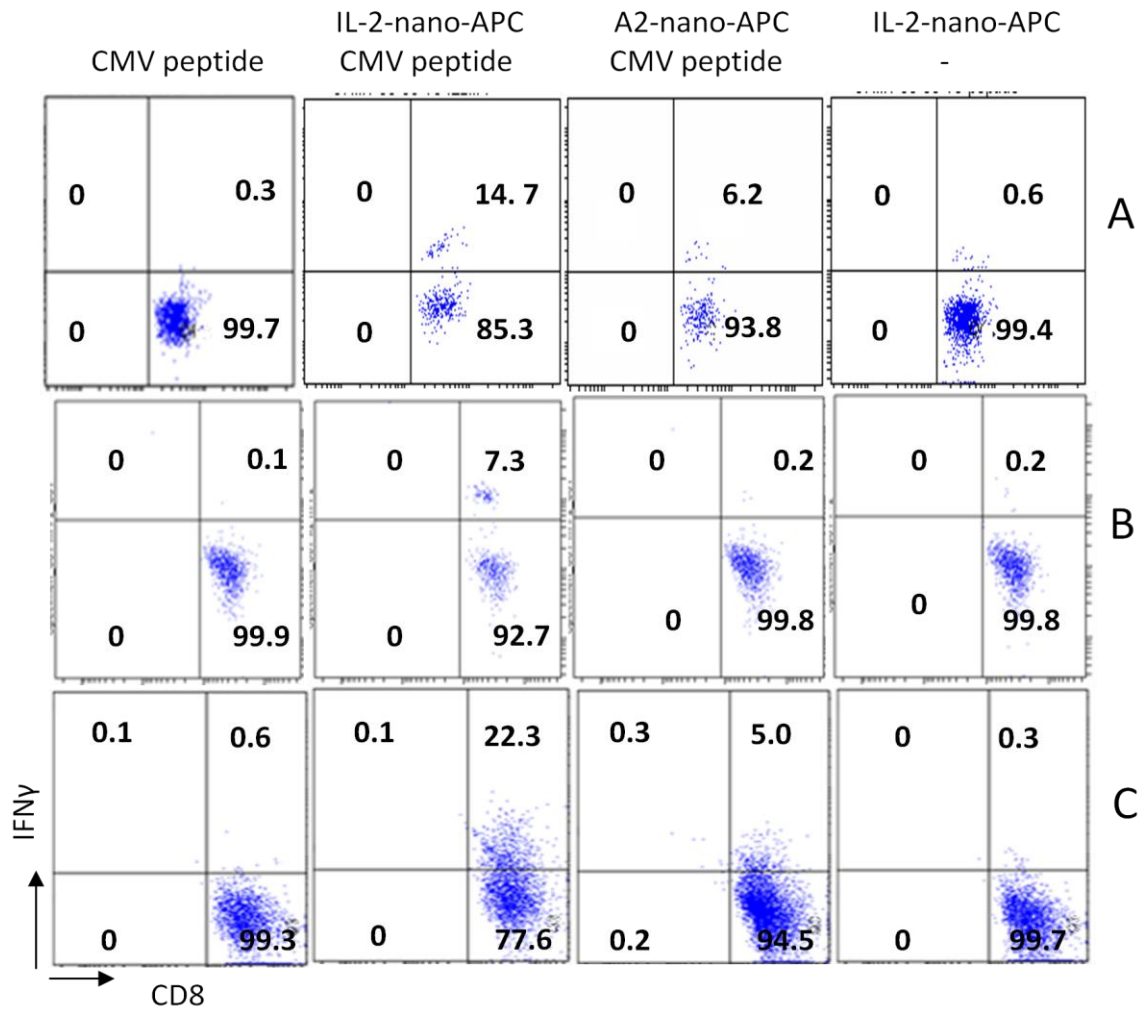

Figure 6.3. IL-2 on nanoAPC enhances responses of antigen specific CD8 T cells.

PBMC from three HLA-A2 positive and CMV serum converted donors (A, B C) were incubated with 1uM CMV_{nlv} peptide or 10ug/ml of IL-2-CMV_{nlv}A2-nanoAPC or 10ug/ml CMV_{nlv}A2-nanoAPC or 10ug/ml IL-2-A2-nanoAPC for five days. After restimulation with PMA and Inomycin for three hours, cells were stained with anti-CD8 and anti-IFNγ. The IFNγ producing cells were measured on CD8 gated cells.

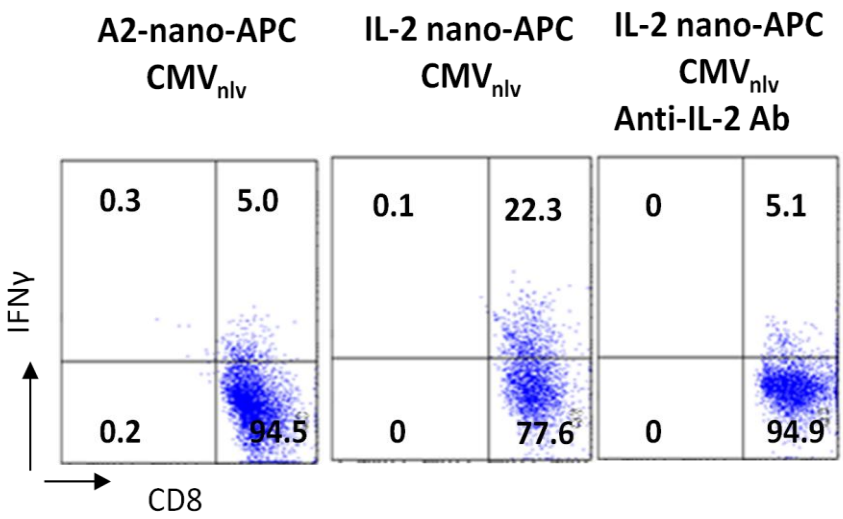

Figure 6.4. IL-2 increases efficacy of nanoAPC on induction of IFNγ producing T cells can be neutralized by IL-2 antibody.

CD8 T cells From CMV serum converted and HLA-A2 positive PBMC were stimulated for 5-days. Cells were stained with anti-IL-2 and IFNγ.

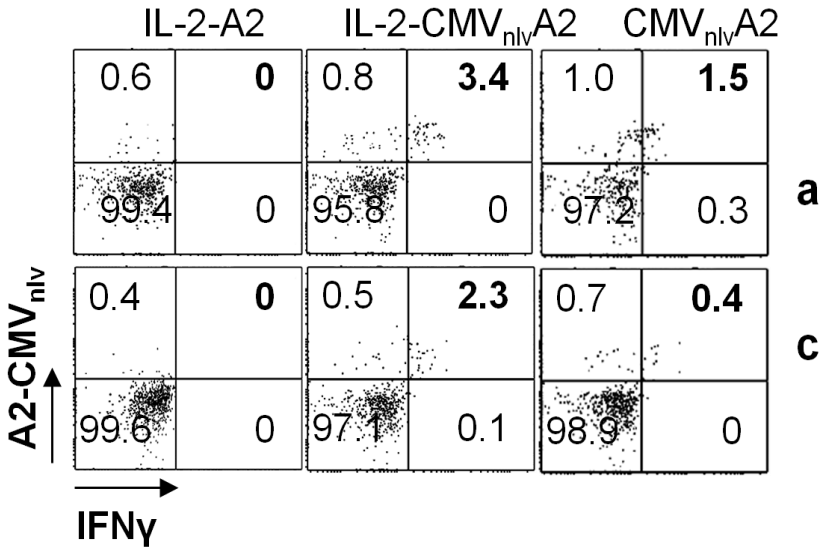

Figure 6.5 IL-2 on nanoAPC enhances the responses of CMV specific CD8 T cells

PBMC's from donor A and C were incubated with 10ug/ml CMV$_{nlv}$A2-nanoAPC or IL-2-CMV$_{nlv}$A2-nanoAPC or IL-2-A2-nanoAPC for five days. After restimulation with PMA and Inomycin for three hours, cells were stained with anti-CD8, anti-IFNγ, CD19 and CMV$_{nlv}$Pentamer. IFNγ producing cells were measured after gating on CMV$_{nlv}$Pentamer^{+}CD8^{+}CD19^{-} cells.

6.2 IL-2-CMV$_{nlv}$A2-nanoAPC can increase CD25 expression on CD8 T cells without influence of Treg cells

It has been mentioned that the IL-2 does have the function to upregulate the expression of CD25 and IL-2Rα chain, which can assemble with IL-2 low affinity receptor, formed by IL-2Rβ and γ chain, switch it to high affinity receptor which is required for IL-2 to induce T cell activation (Malek et al, 2010). The result shows that IL-2 on the IL-2-CMV$_{nlv}$A2-nanoAPC can increase CD25 expression on CMV$_{nlv}$tetramer^{+}CD8^{+} T cells (Figure 6.6). IL-2 is also important for the maintenance and expansion of CD4^{+}CD25^{+}Foxp3^{+} Treg cells, but the result shows that there is no increase of the Treg cells detected in IL-2-CMV$_{nlv}$A2-nanoAPC stimulated PBMC sample (Figure 6.7)

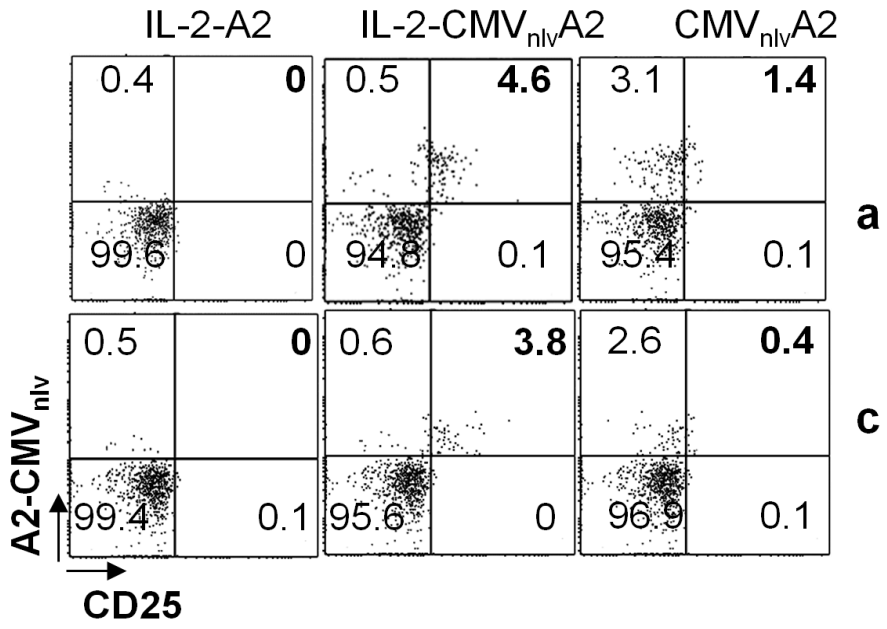

Figure 6.6. IL-2-CMV$_{nlv}$A2-nanoAPC induced CD25 expression on CD8 T cells

PBMC from HLA-A2 positive and CMV serum converted donor were incubated with 10ug/ml CMV$_{nlv}$A2-nanoAPC or IL-2-CMV$_{nlv}$A2-nanoAPC for three days. CD25^{+} cells were quantified after gating on CMV$_{nlv}$Pentamer $^{+}$CD8^{+}CD19^{-} cells via FACS analysis.

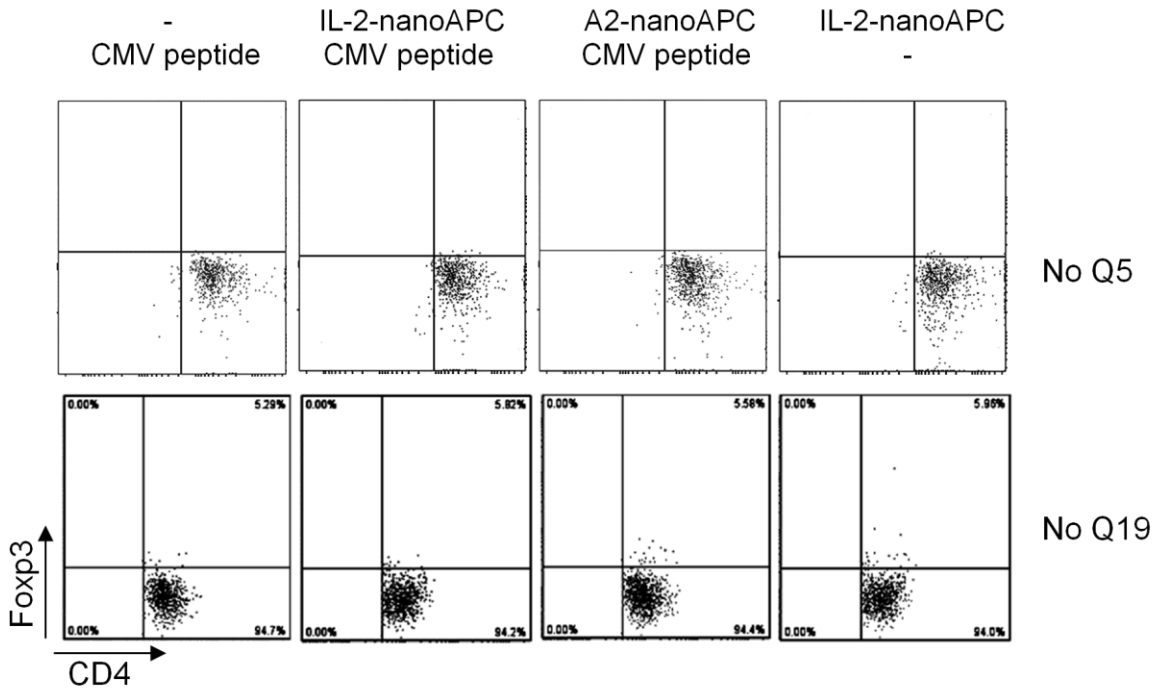

Figure 6.7. Foxp3^{+}CD4^{+} T cells measurement

Foxp3^{+}CD4^{+} T cells were measured in two PBMC's from CMV serum converted donor after stimulation with indicated stimuli for five days.

Overall, IL-2-A2-nanoAPC induced better immune responses in terms of IFNγ secretion compared to the responses induced by the others (Figure 6.3) even better than A2-nanoAPC loaded with same peptide which cannot reach to the similar level. These data indicate that IL-2-A2-nanoAPC can present peptides *in vitro* to induce stronger antigen specific T cell response without affecting any bystander T cells like Treg cells.

7 Examine the efficacy of IL-2-A2-nanoAPC to induce HBV specific responses in PBMC from chronic HBV patients.

The aim is to use IL-2-A2-nanoAPC to reactivate immune responses in chronic infectious patients such as chronic HBV. In chronic HBV infection, the function of HBV specific T cells is impaired and activation threshold is high due to disregulation of TCR signalling (Anderson et al, 2005). Previously studies have shown that the additions of IL-2 can effectively overcomes the tolerance and the activation of T cells towards antigen stimulation.

7.1 Selection of HLA-A2 associated HBV peptides.

We also investigated HBV peptide binding. We chose peptides that have been studied for their ability to induce specific CD8 T cell responses (Das et al, 2008). In order to maximize the response, we selected five HLA-A2 peptides to establish as HLA-A2-HBV peptide pool. The five peptides are HBV core 18-27 FLPSDFFPSV; HBV envelope 183-191 FLLTRILTI; 335-343 WLSLLVPFV; 338-347 LLVPFVQWFV; 348-357 GLSPTVWLSV (Das et al, 2008). To validate whether these five peptides can bind HLA-A2, we did binding assay by using these peptide to compete a known report HLA-A2-binding peptide MP58-66 (YILGKVFTL). The report peptide has been modified by labelling 125I and a crosslinker (Li et al, 2000). According to our previous study, we used 2nm report peptide to bind to HLA-A2 molecules in 10ug nanoAPC while using different concentrations of HBV peptides to complete the binding (Li et al, 2000). Results show that the binding affinity of five peptides was similar (Figure 7.1.). However, none of the five peptides is as good as report peptide (Figure 7.1.). We therefore pooled five peptides to collectively load HLA-A2 molecules in nanoAPC.

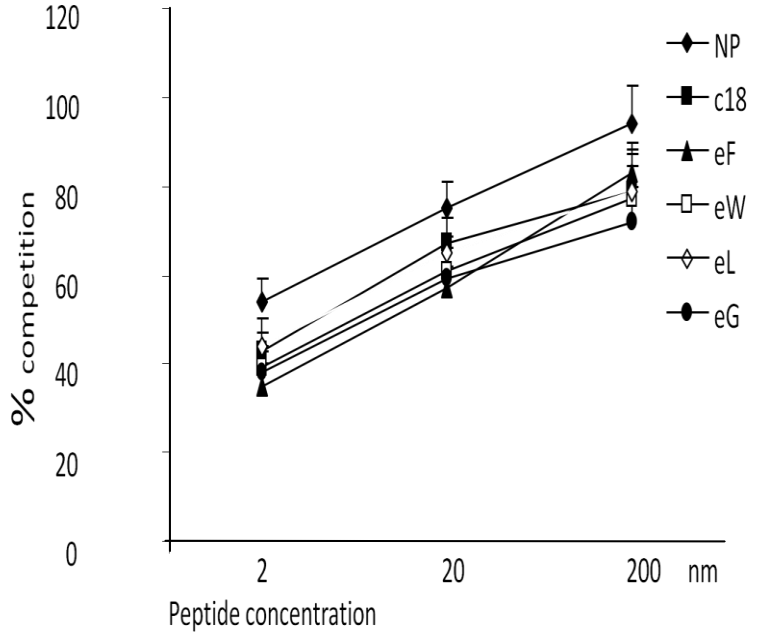

Figure 7.1. Five HBV peptides reported for induction of HLA-A2 restricted CD8 T cell responses in HBV patients were analysed for their binding to HLA-A2 on IL-2-A2-nanoAPC by a competition assay.

Different concentration of peptides were used to compete 125I-labeled HLA-A2-reporter NP peptide and percentage of reporter peptide competed for binding to HLA-A2 were measured. With a similar binding affinity, these five peptides were mixed and pooled at concentration of 1uM each used to assemble HBVA2-nanoAPC or IL-2-HBVA2-nanoAPC.

7.2 Selection of DR/DP binding peptides from HBV.

In order to induce both CD4 and CD8 T cells, we selected peptides (HBV Env 180-195 AGFFLLTRILTIPQS; Env 339-354 LVPFVQWFVGLSPTV; and Pol 767-782 AANWILRGTSFVYVP) (Mizukoshi et al, 2004) that have been tested to interact DR and DP molecules that expressed in IL-2-nanoAPC. To examine the binding affinity, we labelled peptides with fluorescent marker and incubated labelled peptides to nanoAPC. After removal of free peptides, the peptide binding was detected by FACS analysis. The results showed that all these three peptides all can associate with DR and DP molecules under acidic conditions (Figure 7.2). Similar to HLA-A2 peptides, we pooled three peptides and loaded all three onto DR and/or DP molecules in nanoAPC.

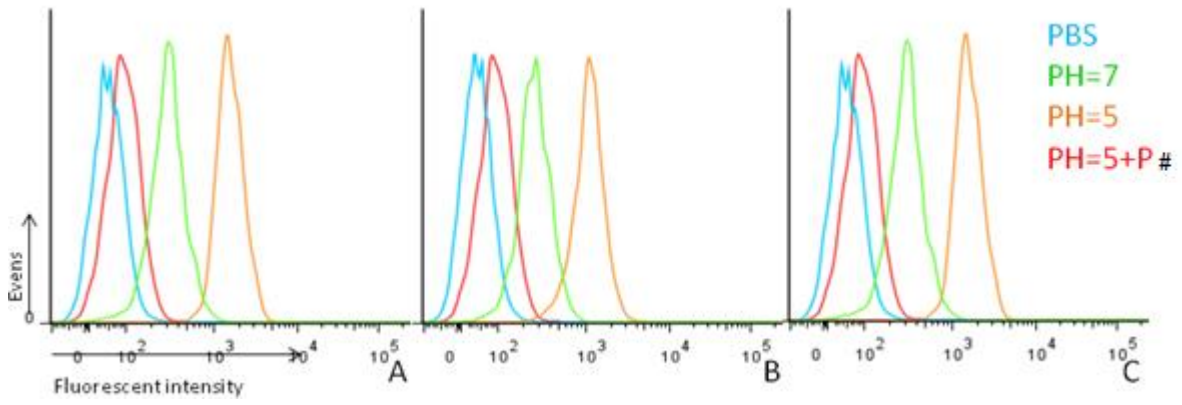

Figure 7.2. Acidic treatment induces peptide-receptive MHC class II

Peptide loaded A2-nanoAPC from 221-A2 cells under pH 7.0 or pH 5.0 conditions. The same nanoAPC with PBS or with 5 times unlabeled peptide serve as a background control.

Fluorescence peptide plus five times unlabelled peptide with A2-nanoAPC under acide condition.

7.3 IL-2-A2-nanoAPCs induce strong CD8 T cell responses in peripheral lymphocytes from chronic HBV patients

We previously found that IL-2 can effectively reverse tolerance induced by antigen persistence (Anderson et al, 2005). T cells from chronic HBV patients show reduced production of IL-2 in response to TCR ligation (Frebel et al, 2010), which is consistent with the defective induction of IL-2 in T cells rendered tolerant by persistent antigen stimulation (Anderson et al, 2005). To investigate whether IL-2-A2-nanoAPC can enhance responses of virus specific CD8 T cells from chronic HBV patients, we developed HBV specific IL-2-HBVA2-nanoAPC by assembling a pool of five HBV peptides, which have been found to induce HBV specific CD8 T cell responses in HLA-A2 positive HBV patients (Das et al, 2008), with the HLA-A2 molecules on the IL-2-A2-nanoAPC. Therefore a pool containing equal amounts (5uM) of each peptide was used for assembly of IL-2-HBVA2-nanoAPC and HBVA2-nanoAPC. Peripheral lymphocytes from 57 HLA-A2 positive chronic HBV patients, with viral loads ranging from 3×10^3 to 10^{13} IU/ml (Table 7.1), were used to investigate CD8 T cell responses. We detected minimal CD8 T cell responses, as measured by the proportion of IFNγ producing CD8 T cells, after incubation with the pool of the five HBV peptides (Figure 7.4.). The HBVA2-nanoAPC induced better responses in about 20% of patients (Figure 7.4. table 7.1 and 2). However, IL-2-HBVA2-nanoAPC induced the strongest responses, with lymphocytes from most patients showing a strong CD8 T cell response (Figure 7.4. table 7.1 and 2). Consistently, IL-2-A2-nanoAPC, in the absence of antigenic peptide, did not induce IFNγ production by CD8 T cells in any of the patient samples (Figure 7.4). Furthermore, we could not detect an increase in the proportion of $Foxp3^+CD4^+$ cells after stimulation with IL-2-HBVA2-nanoAPC or IL-2-A2-nanoAPC (Figure 7.5.).

CD8	Overall HBV	QMUL†	Shanghai
Number	57	20	37
Median age (range)	36 (17-69)	40 (17-69)	33 (18-52)
Sex (M:F ratio) %male	(42:15) 73.7%	(13:7) 65%	(29:8) 78.4%
Viral load (Log; range)	6.35 (3.27-9.96)	5.94 (3.27- 9.96)	6.57 (4.31-9.13)
Median ALT (IU/liter; range)	120.2 (20-430)	81.7 (30-211)	141 (20-430)
HBeAg status (% positive)	47.4%	25%	59.5%

Table 7.1. Clinical characteristics of chronic HBV patients investigated with IL-2-A2-nanoAPC

HBV patients stimulate with HBV class I peptide pool load on IL-2-A2-nanoAPC or A2-nanoAPC

†Queen Mary University of London

CD4	Overall HBV	QMUL†	Shanghai
Number	28	6	22
Median age (range)	35.4 (19-69)	45.8 (28-69)	32.5 (19-52)
Sex (M:F ratio) %male	(18:10) 64.3%	(4:2) 66.7%	(14:8) 63.6%
Viral load (Log; range)	6.57 (3.27-9.96)	5.00 (3.27- 9.96)	7.00 (5.29-9.13)
Median ALT (IU/liter; range)	110.4 (20-430)	42 (31-97)	129 (20-206)
HBeAg status (% positive)	46.4%	33.3%	50.5%

Table 7.2. Clinical characteristics of chronic HBV patients investigated with IL-2-DR/DP-nanoAPC

HBV patients stimulate with HBV class II peptide pool load on IL-2-DR/DP-nanoAPC or DR/DP-nanoAPC

†Queen Mary University of London

	HBVA2-nanoAPC	IL-2-HBVA2-nanoAPC
No respond	77.2%	35%
Respond >0.5% CD8	13/57=22.8% (0.6-6.2)	37/57=65% (0.7-16.4)
Respond >0.5% CD4	9/28=32% (0.6-4)	18/28=64.3% (0.6-5.6)
IL-2/A2>2‡ CD8	-	70.3%
IL-2/A2>2‡ CD4	-	46.4%

Table 7.3. Antigen specific T cell response ratio comparison between HBVA2-nanoAPC and IL-2-HBVA2-nanoAPC

The data in the bracket shows the range

‡ IL-2-HBVA2-nanoAPC induce more than twice response compare to HBVA2-nanoAPC

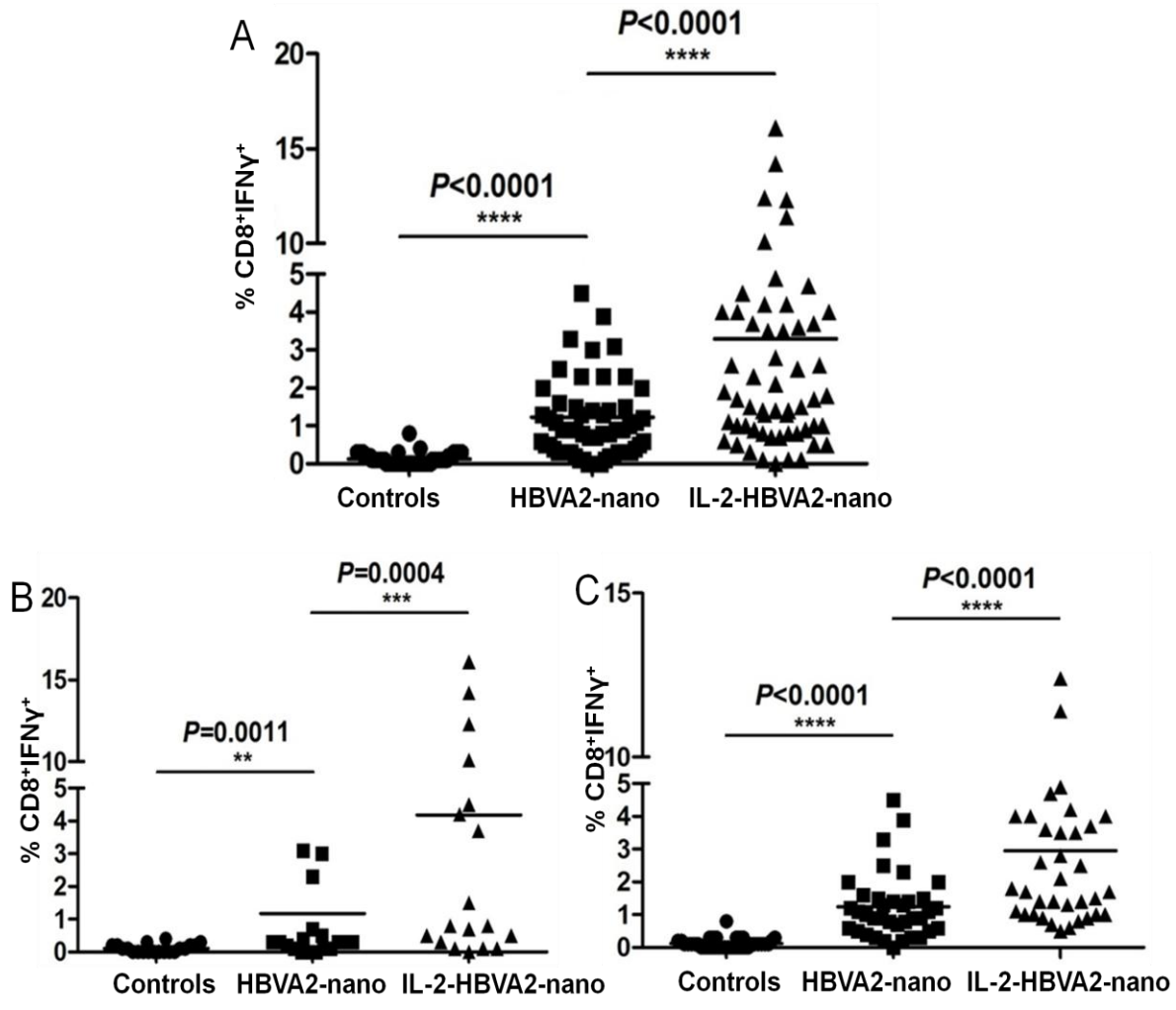

Figure 7.3. Percentages of IFNγ producing CD8 T cells induced in

A. Total 57 HLA-A2 positive chronic HBV patients
B. 20 HLA-A2 positive chronic HBV patients from Barts and the London hospital care ethics review board and
C. 37 HLA-A2 positive chronic HBV patients from Huashan hospital ethics committee, Shanghai China

Statistical comparisons were performed using Student's t test.

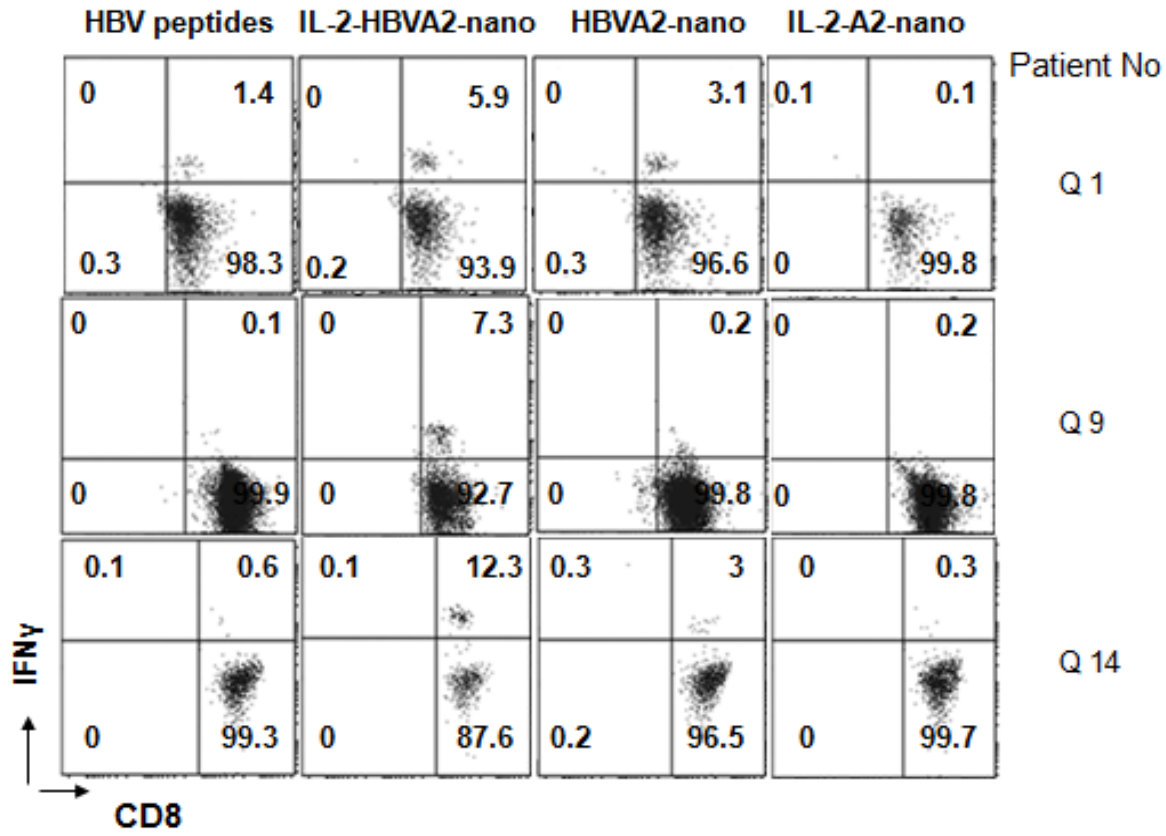

Figure 7.4. IL-2-HBVA2-nanoAPC enhance HBV specific CD8 T cell responses

PBMCs from chronic HBV patients were incubated with the pool of five HBV peptides at concentration of 1uM each or 10ug/ml IL-2-HBVA2-nanoAPC or 10ug/ml HBVA2-nanoAPC or IL-2-A2-nanoAPC for five days. After restimulation with PMA and Inomycin for three hours, IFNγ producing CD8 T cells were quantified after gating on CD8 cells

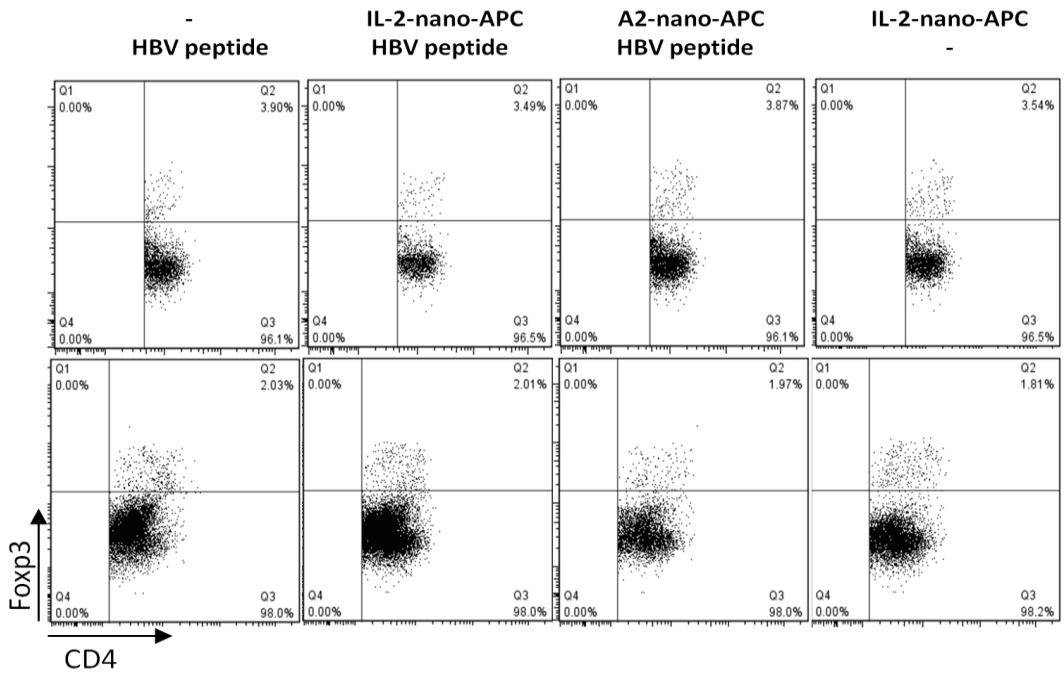

Figure 7.5. Foxp3$^+$CD4$^+$ T cells measurement

Foxp3$^+$CD4$^+$ T cells were measured in two PBMCs from chronic HBV patients after stimulation with indicated stimuli for five days.

7.4 IL-2-HBVA2-nanoAPC improve TCR signalling and suppress expression of inhibit receptor in HBV specific CD8 T cells from chronic HBV patients

In addition, expression of PD1, an inhibitory co-stimulatory molecule, was increased in CD8 T cells from chronic HBV, HCV and HIV patients (Keir et al, 2007). To investigate whether the enhanced CD8 T cell responses induced by IL-2-HBVA2-nanoAPC resulted from improved TCR signalling and/or reduced expression of PD1, we test the expression of PD1 on IFNγ producing CD8 T cells induced by either HBVA2-nanoAPC or IL-2-HBVA2-nanoAPC. To further prove that IL-2 on IL-2-A2-nanoAPC can reverse tolerance which was induced by persistent antigenic stimulation, effectively increases the threshold for T cell receptor activation in response to antigen stimulation by suppressing TCR proximal signalling pathways (O'meill et al, 2004) (Anderson et al, 2005) we examined activation of extracellular signal-regulated protein kinase (Erk), which is repressed in tolerant T cells (Anderson et al, 2005). The results demonstrated that the levels of Erk

activation were enhanced in responding CD8 T cells from chronic HBV patients stimulated with IL-2-HBVA2-nanoAPC compared to those stimulated by HBVA2-nanoAPC (Figure 7.6.), suggesting that IL-2 can antagonize T cell tolerance by enhancing TCR proximal signals. In addition to increased TCR signalling, the expression of PD1 was reduced on IFNγ producing CD8 T cells induced by IL-2-HBVA2-nanoAPC compared to those stimulated by HBVA2-nanoAPC (Figure 7.6.). Thus, we have provided direct evidence to demonstrate that IL-2 on nanoAPC effectively reduces the TCR activation threshold and the expression of negative regulators in virus specific CD8 T cells; the two important factors needed to overcome T cell tolerance.

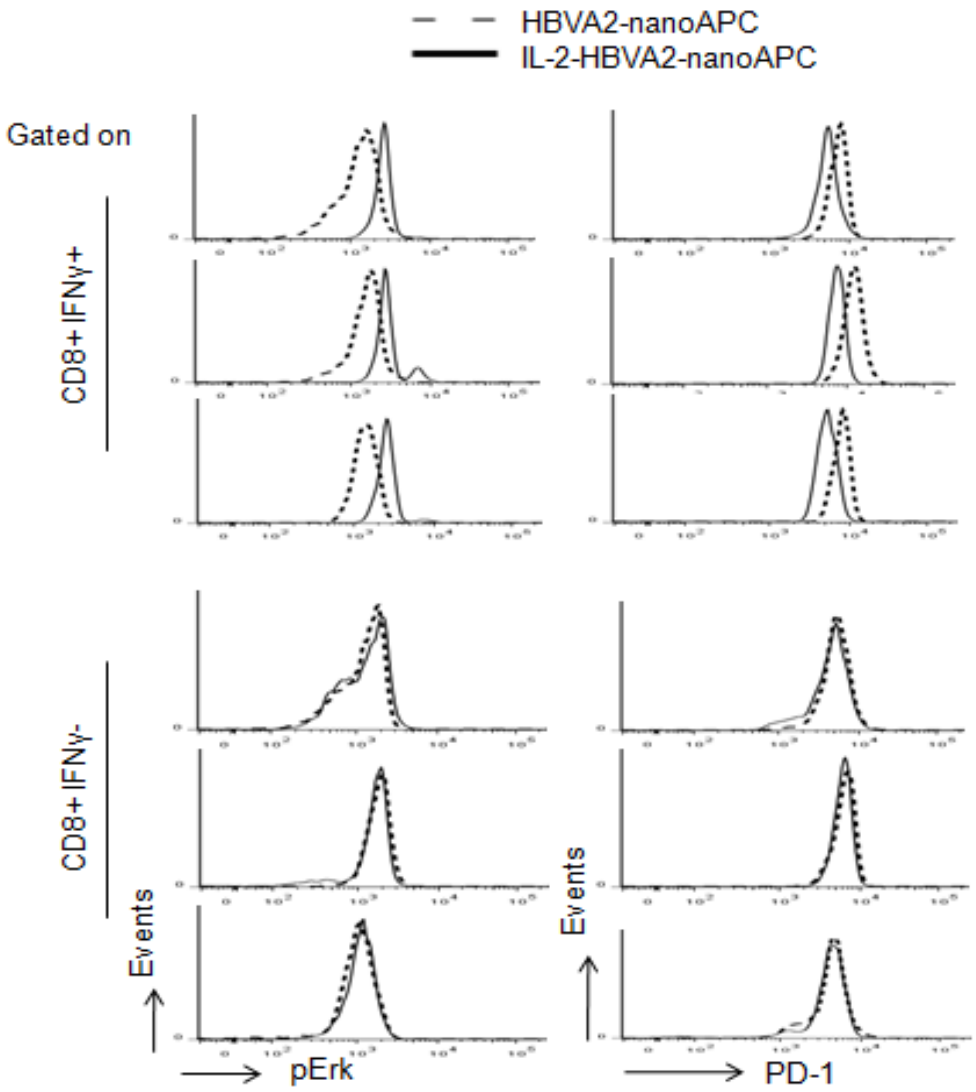

Figure 7.6. IL-2 can enhance T cell activation and suppress expression of PD1 on HBV specific CD8 T cells.

PBMCs from three chronic HBV patients, which showed weak responses to HBVA2-nanoAPC, but strong responses to IL-2-HBVA2-nanoAPC, were incubated with 10ug/ml of IL-2-HBVA2-nanoAPC or HBVA2-nanoAPC for five days. After restimulation with PMA and Inomycine for three hours, cells were stained with anti-CD8, anti-IFNγ, anti-PD1 and anti-phospho-Erk. IFNγ positive or IFNγ negative CD8 T cells were gated for analysis of PD1 expression or Erk phosphorylation.

7.5 IL-2-nanoAPC increase CD4 T cell responses to viral antigens

It has been proved that CD4 T cell tolerance is similar to CD8 cells, and also it has been reported that CD4 helper function is essential for the development and maintenance of CD8 T cell effector function. I have shown that MHC class II molecules in isolated ER vesicles can assemble with antigenic peptides under acidic conditions. 221 cells are deficient in MHC class I, but still express HLA DR and DP molecules (Shimizu et al, 1989). Three HBV peptides reported to bind to DR and/or DP (Mizukoshi et al, 2004) were analysed for their ability to assemble with nanoAPC from 221-A2 and 221-A2-IL-2 cells. Consistent with previous findings (Mizukoshi et al, 2004), specific binding of all three peptides to nanoAPC were detected under acidic conditions (Figure 7.7.). Pools of the three peptides were assembled with DR and/or DP molecules on either nanoAPC or IL-2-nanoAPC to create HBVDR/DP-nanoAPC or IL-2-HBVDR/DP-nanoAPC, respectively. Peptide alone did not induce IFNγ producing CD4 T cells in peripheral lymphocyte populations isolated from chronic HBV patients (Figure 7.7.). HBVDR/DP-nanoAPC induced a weak response in 8% patient samples (Figure 7.7.), while significant CD4 T cell responses were induced in more than 50% of patient samples treated with IL-2-HBVDR/DP-nanoAPC (Table 7.3.) (Figure 7.7.). Again, we did not find an increased proportion of $Foxp3^{+}$CD4 T cells in peripheral lymphocytes from chronic HBV patients after culture with IL-2-HBVDR/DP-nanoAPC (Figure 7.8.).

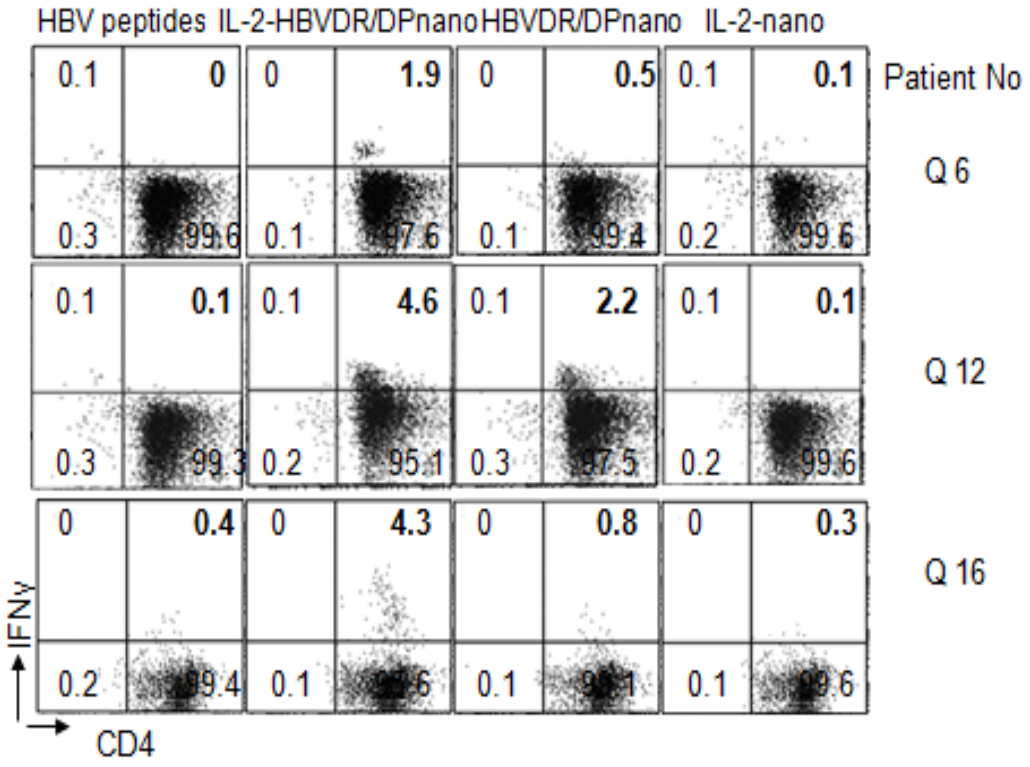

Figure 7.7. IL-2-HBVDR/DP-nanoAPC induce CD4 T cell responses in PBMCs from chronic HBV patients

Induction of IFNγ producing CD4 T cells in PBMCs from chronic HBV patients. PBMCs were incubated with 10ug/ml of IL-2-HBVDR/DP-nanoAPC or HBVDR/DP-nanoAPC or IL-2-A2-nanoAPC for five days. After restimulation with PMA and Inomycin for three hours, IFNγ producing CD4 T cells were quantified after gating CD4 cells.

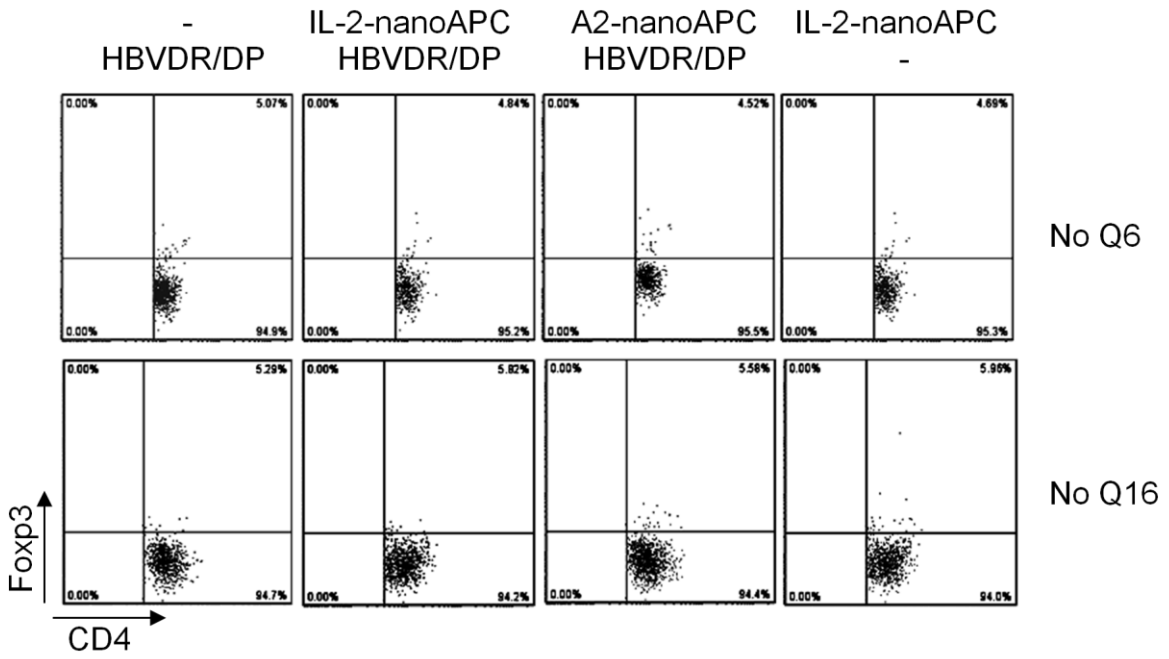

Figure 7.8 Foxp3^{+}CD4^{+} T cells were measured in PBMCs from chronic HBV patients after stimulation with indicated stimuli for five days.

The presented data were from patients' number Q6 and Q16, who were among the patients shown CD4 T cell responses towards IL-2-HBVDR/DP-nanoAPC stimulation

7.6 IL-2-nanoAPC induced CD4 T cell responses leading to stronger viral antigens CD8 T cell responses

To investigate whether the induced Th1 CD4 responses can enhance viral antigen specific CD8 T cell responses, patient lymphocytes were stimulated with both IL-2-HBVDR/DP-nanoAPC and IL-2-HBVA2-nanoAPC. The results showed that the induction of Th1 CD4 T cells increased the proportion of IFNγ producing CD8 T cells in response to IL-2-HBVA2-nanoAPC (Figure 7.9.), suggesting that CD4 responses are indeed important for the expansion of effector CD8 T cells. Therefore, the presence of IL-2 on nanoAPC is important to drive optimal CD4 and CD8 T cell responses against HBV in lymphocytes from chronic HBV patients.

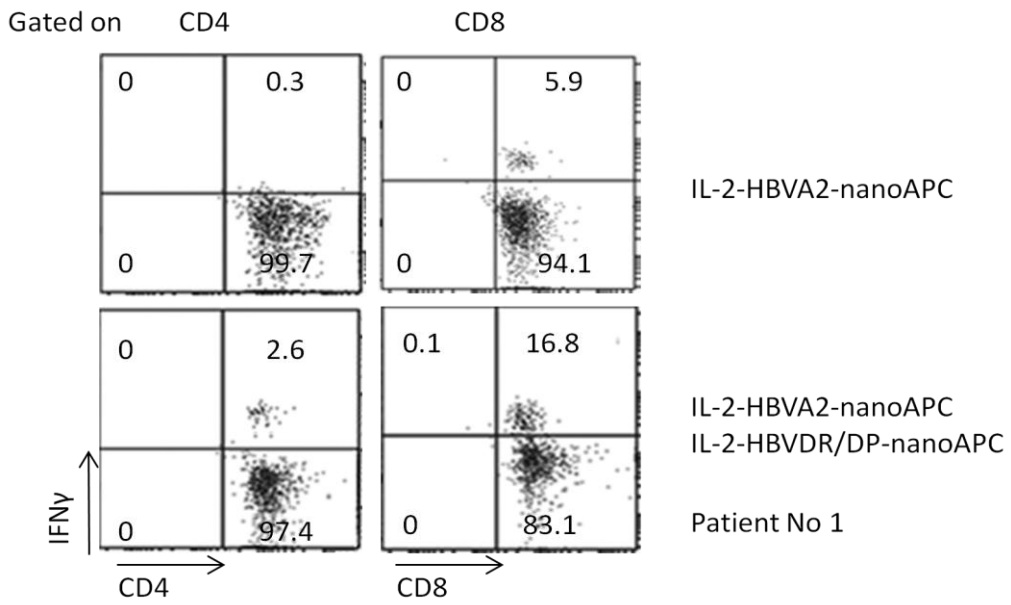

Figure 7.9. IL-2-HBVDR/DP-nanoAPC induces CD4 T cell responses in PBMCs from chronic HBV patients leading to the enhanced responses of HBV-specific CD8 T cells. PBMCs from chronic HBV patients were incubated with 20ug/ml IL-2-HBVA2-nanoAPC or 10ug/ml IL-2-HBVA2-nanoAPC and 10ug/ml IL-2-HBVDR/DP-nanoAPC for five days. After restimulation with PMA and Inomycin, IFNγ producing CD4 or CD8 cells were quantified after gating on CD4 or CD8 cells.

DISCUSSION

The study of the immune system has provided insight in the mechanism of protection induced by vaccination; primarily that most clinically protective vaccines are potent in generating neutralizing antibody responses. Nonetheless, vaccination fails to protect against a wide range of acquired chronic infections caused by viruses, such as HIV, HBV and HCV, intracellular pathogens, and cancer. Attempts to combat these diseases are thought to also require the induction of the cellular arm of the immune response, in which dendritic cells (DCs) play a key role. Thus, DCs are considered a promising target/tool when designing new-generation vaccines (Farkas et al. 2005) (Clay et al. 2006). But these are associated with several difficulties. For example, there are limitations involved in the loading of antigen, and in the appropriate maturation of DC *in vitro.* Furthermore, the complexity of DC subsets in relation to the induction versus suppression of T cell activation *in vivo* severely limits DC- based vaccine applications (Hawiger et al. 2003). To overcome the difficulties of DC therapies, we have developed a microsome based DC vaccine by using ER-enriched microsomes isolated from professional antigen presenting cells, such as DCs (Sofra et al, 2009). This approach not only preserves antigen presenting function of DC, but also avoids unrelated function of DC such as inhibitory function and soluble factors such as IL10, TGF-β and IDO (Sofra et al, 2009). However, it has been clear that poor immune response against viral infection is one of major courses of chronic infectious diseases. It is therefore ineffective if the therapeutic vaccine delivers only conventional antigen and co-stimulatory signal. Based on our microsomal vaccine, here, we have explored the hypothesis by selectively delivering IL-2 with HLA-antigen complexes to antigen specific T cells in patients with chronic infectious diseases.

1. Defective immunoresponses in chronic infectious diseases

Our approach is based on the recent understanding of immunological defects in chronic infectious diseases. The studies of chronic HBV infection demonstrate that the persistent high load of virus can lead to severe immune tolerance (Frebel et al, 2010). The T cells from chronic HBV are tolerant to viral antigens and fail to produce effective cytokines such as IL-2, TNFα and IFNγ in response to viral stimulation. The viral persistence in chronic HBV patients leads to reduction of T cell population and the proliferation potential of effector T cells (Frebel et al 2010). It has been suggested that the deficiency is due to both cellular intrinsic and extrinsic mechanisms. The expression of inhibitory receptors such as CTLA4 and PD1 on antigen specific T cells, and regulatory cytokines such as IL-10, and the possible increases of Treg cells are found in chronic HBV, HCV and HIV patients (Frebel et al 2010) (Keir et al 2007) (Racanelli et al 2007). Recently, the downregulation of TCR proximal signalling pathways has been found in T cells from chronic HBV patients (Maini and Schurich 2010) (Das et al, 2008); compelling support for the notion that persistent antigen engagement is at least one of the mechanisms for the induction of virus specific T cell tolerance. The unresponsiveness of viral specific T cells has been found as a generic mechanism partly responsible for the chronic infections of HIV, HBV and HCV (Keir et al 2007), suggesting that the induction of T cell tolerance is due to persistent engagement of T cells with viral antigens, rather than the function of specific viral molecules.

Together with the findings from chronic infectious diseases and animal models, it indicates that viral induced tolerance is not due to loss of antigen specific T cells, but rather downregulation of proximal TCR signalling pathways following antigen stimulation (Anderson et al, 2005) (Frebel et al, 2010). Such tolerance cannot be reversed by an increase in the amount of antigen presented by activated dendritic cells and this cannot reverse tolerance (Anderson et al, 2005). Thus the priority to develop a therapeutic vaccine for chronic infectious diseases is its capability to reverse the function of antigen specific T cells.

2. Immunotherapeutics for increasing immunogenicity in chronic infectious diseases

To overcome the tolerance of viral specific T cells, approaches to increase the function of APC have been investigated such as increase of co-stimulatory signalling by treating DCs with α-galactosylceramide which binds to CD1, CD40-specific antibody and TLR ligands such TLR9 ligand (Paulsson et al, 2007). Although some of these modifications have increased efficacy in induction of HBV HCV specific T cells in vivo in mouse models, the use of autologous DC and dysfunction of DCs in chronic infectious condition (Encke et al, 2005) (Farag et al, 2010) have limited their clinical applications. The nanoAPC with reconstitute IL-2 and HLA-A2 not only deliver the bio-adjuvant and antigen directly to T cells, but also can be broadly applied to patient groups sharing HLA alleles. Using cytokines as bio-adjvenat to immunetherapy the chronic infections have found that IL-7, IL-15 and IL-2 can effectively improve the anti-viral immune response (Ha et al, 2008). IL-7 and IL-15 are required for survival and homeostatic proliferation of memory T cells (Meldhionda et al, 2005). IL-7 has been found to suppress Socs3 (suppressor of cytokine signalling 3) expressions in effector T cells (Marc et al, 2011). Socs3 is suppressor of cytokine signalling (Yoshimura et al, 2007). Thus, the inhibition of Socs3 results in induction of IL-22 and IL-6 that is required for anti-viral responses and protection of liver pathology (Marc et al, 2011). Although unlike IL-2, IL-7 induces less organ pathology, the function of IL-7 is not limited to viral specific T cells. Therefore, it may be less effective under the chronic infectious condition which the immune system is largely deregulated with high levels of Treg cells and inflammatory cytokine production (Frebel et al 2010).

3. IL-2 as adjuvant for immunotherapy

IL-2 is the most potent stimulatory cytokine for T cells and is required for memory T cell function (Malek 2008). It has been used as a bio-adjuvant to overcome low immunogenicity of therapeutic vaccine in the treatment of chronic infectious diseases (Ha et al 2008) (Fearon 2007). Previously, our group discovered that IL-2 can effectively reverse T cell tolerance induced by antigen persistence (Anderson et al, 2005). Therefore, among cytokines used for immune therapy, IL-2 is the best choice for a promising strategy to augment the efficacy of therapeutic vaccination against chronic viral infections. However, application of IL-2 as a bio-adjuvant for immunotherapy is hampered by organ pathology induced by therapeutic doses and the expansion of Treg cells leading to the suppression of effector T cells (Malek and Castro 2010). IL-2 is required for expansion of Treg cells and IL-2 treatment for HIV induces immune suppressive effect due to the overexpansion of Treg cells (Weiss et al, 2010).

Our approach to use immobilized IL-2 incorporated into the nanoAPC effectively delivers the IL-2 to antigen specific T cells that are engaging pMHC on APC. By this, the therapeutic dose of IL-2 to enhance activation of antigen specific T cells is much less than the therapeutic dose of soluble IL-2 used for systemic administration (Bernsen et al, 1999). IL-2 activation depends on the formation of its receptor. In resting T cells, only IL-2 receptor β and γ chains are expressed while IL-2 receptor α chain is effectively induced by pMHC stimulation (Malek and Castro 2010). We have shown that nanoAPC can induce synapse formation that can induce strong TCR signalling and subsequently the expression of IL-2 receptor α chain (Malek and Castro 2010). Thus, the IL-2 on nanoAPC can effectively interact with high affinity receptor on antigen specific T cells. Moreover, it has been shown that IL-2 can autocrinely induce expression of high affinity receptor on antigen stimulated T cells (Ascherman et al, 1997). Consistently, the stimulation of CMV specific T cells by IL-2-CMV-A2nanoAPC effectively induces CD25 expression on CMV tetramer positive T cells. Thus, the IL-2-nanoAPC creates a microenvironment

for antigen specific T cells to effectively engage high doses of IL-2 locally. IL-2 forms four helical proteins that dock on the trimetric receptor surface. Since the IL-2 on nanoAPC is preferentially targeting antigen specific T cells, therefore, it does not significantly affect bystander T cells and Treg as shown in both systems of CMV and HBV.

In order to maintain the function of structure of IL-2, We optimized the IL-2-tapasin structure to remain IL-2 exposed by addition of due GGS linker sequence. The amount of the IL-2 expressed in nanoAPC expresses a similar activity to that of recombinant IL-2 (~ 100IU/ng) suggesting that the engineered IL-2 maintains its activity on nanoAPC. We found that the IL-2 on nanoAPC is much more stable than soluble recombinant IL-2 (data not shown). It may be due to the stable structure after being immobilized on nanoAPC membranes. IL-2 can sustain its function after internalization by T cells following interaction with its high affinity receptor (Malek and Castro 2010). We found that nanoAPC engraphed into the synapse can be internalized by T cells. Thus, the IL-2 can remain active in complex with its receptor after internalization. This approach can be applied with other cytokines or even combination of different cytokines if they are beneficial for optimizing viral specific T cell activation.

4. Nano-sized antigen presenting cells (nanoAPC)

Nano-particles have been used to develop therapeutic vaccines for chronic infectious diseases (Nandedkar, 2009) (Ansari et al, 2011). Most of the nano-particles are developed by synthesis of chemical reagents or viral particles or microbial after engineered with antigens (Nandedkar, 2009). So far, the reported nano-vaccine aim to introduce antigen to DC in vivo and activate DC to stimulate T cells (Cruz et al, 2011). However, the high level of viral load in chronic infectious diseases indicates that the immune unresponsiveness to virus is not resulted from lack of antigens, but the defects in both innate and adaptive immune systems including function of DC (Frebel et al, 2010). It is therefore the induction of exogenous antigen to DC may not be effective for reactivation of defected immune

system. Some of the nano-vaccine aim to deliver pMHC to T cells by coupling folded recombinant pMHC to surface of particles (Cruz et al, 2011). Due to the nature of MHC-peptide folding process, only limited number of antigen peptides can effectively induce MHC class I folding and the conformation of folded pMHC on particles may differ from physiologically processed pMHC in APC. The nanoAPC are derived from APC with abundant peptide receptive MHC class I molecules that semi-folded under physiological condition. It is therefore effectively assembled with peptides. We have shown that all the HLA-A2 peptides used in this study can effectively bind to HLA-A2 molecules in the nanoAPC. In addition, the engineered IL-2 is also processed naturally in APC seed cells. It is therefore nanoAPC present physiological pMHC and IL-2 to T cells. In addition, nanoAPC have perfect biocompatibility with nature components including co-stimulatory molecules synthesised in APC cells. At a nanosize, we demonstrate that nanoAPC can effectively distribute to peripheral lymphoid organs. Importantly, unlike nano-particles, nanoAPC are not effectively endocytosed by DCs in lymph nodes. This is also largely due to the physiological pattern of nanoAPC that are not recognized by pattern reorganization receptors on DC (Namswskar, 2009).

In previous report, HBV antigenic peptides can induce viral specific T cells in PBMC (Das et al, 2008). However, these reports were using IL-2 in stimulatory cultures (Das et al, 2008). We have discovered that even a minimal dose of IL-2 (20IU) can globally activate T cell activation and proliferation. It is therefore in the absence of IL-2 we could not detect significant T cell responses by antigen peptides alone. The induced responses are only observed in some cases with peptide assembled nanoAPC. The significant responses are only detected in IL-2-nanoAPC which is consistent with our previous observation that IL-2 can effectively reverse responses of tolerant T cells to antigen stimulation (Anderson et al, 2005).

5. T_H1 helper cells are important for the induction of effective responses of viral specific CD8 T cells

We however confirmed that an induced T_H1 response is important for a maximum response of CD8 T cells to viral antigens. We tried two different approaches to induce both CD4 and CD8 responses by either separately assembling MHC class I and MHC class II on nanoAPC then mixed together for stimulation of PBMC and also assembling sequentially the pMHC class I and class II on same nanoAPC. Results showed that the pMHC class II can induce strong CD4 T cell response when stimulated by IL-2-nanoAPC and the induced CD4 T cell response can enhance the response of HBV specific CD8 T cells. This finding indicate; 1. IL-2 is important for the induction of viral specific CD4 T cells and 2. T_H responses are important for the development and maintenance of CD8 T cell function (Yang et al, 2010). However, in all the cases analysed, the proportion of CD4 cells that responded was lower than the proportion of responding CD8 cells. This may be due to the fact that CD8 T cells are more sensitive to IL-2 than CD4 T cells (Li et al, 2000). Another possibility, is that the level of MHC class II molecules expressed on nanoAPC prepared from 721.221 seed cells is lower than the level of HLA-A2 (Gabathuler and Kvist 1990) or that the pMHC class II complexes, generated by assembly with the selected HBV peptides, are not optimal for induction of CD4 T cell responses. It is therefore important for further development to detail screen the MHC class II binding peptides from HBV viral peptide databases. We have tried different parameters to assemble pMHC class I in order to obtain maximum loading. We found a pre-acidic treatment reported for increasing peptide receptive MHC class I by striping bound peptides is not effective. This suggests that the MHC class I in nanoAPC derived from the ER membranes are effectively peptide receptive. However, when we compared SIINFEKL-Kb complexes on nanoAPC prepared from OVA transfected APC and SIINFEKL loaded nanoAPC, the level of pMHC is much higher in nanoAPC if the pMHC is processed naturally. I therefore propose that instead of loading antigenic peptide in vitro onto nanoAPC, the antigen has to be engineered into the seed cells and let the seed cells to process pMHC complexes.

The effect cytokines induced by antigen peptides in CD8 T cells from chronic HBV patients have been investigated and a differential expression of IL-2 and IFNγ was discovered with defective IL-2 expression, but induction of IFNγ (Frebel et al 2010). We however did not detect significant increase of IL-2 producing CD8 T cells after five day stimulation. This may be due to the addition of IL-2 into the culture in reported studies and also to the time for detection of IL-2 as the reported results are from peptide stimulated T cells in short time (less than 48 hours) (Schurich et al, 2011).

6. IL-2 enhances T cell responses by reciprocally regulating expression of PD1 and TCR signalling

One of the major disorders of viral specific immune response in chronic infectious diseases such as HIV, HCV and HBV is the elevated expression of PD-1 on viral specific T cells (Das et al, 2008). Although it remains unknown by which mechanism the PD-1 has reported that the expression of PD-1 can effectively induce Batf expression, an AP1 repressor and the expressed Batf can subsequently inhibit TCR mediated AP1 activation (Quiqley et al, 2010). PD1 expression has been found in T cells from chronic HBV patients (Keir et al, 2008). We have now demonstrated that stimulation of viral specific T cells from chronic HBV patients with IL-2 and pMHC delivered by nano-APC can effectively reduce expression of PD1. Our data not only supports previous findings of induced expression of PD1 on T cells by viral persistence (Keir et al, 2008) and also demonstrates a possible mechanism for IL-2 to repress PD1 expression on effector T cells. In addition, we could also engineer membrane bound single chain antibodies to PD1 and/or CTLA4 to further reduce negative co-stimulatory signals on viral specific T cells.

One of the major mechanisms for viral specific T cell to be tolerant is the impairment of TCR signalling in chronic infectious diseases (Das et al, 2008). We have previously demonstrated that persistent stimulation with high affinity viral antigens can effectively induce T cell tolerance with severe impaired TCR signalling which resemble the findings from chronic infections (Anderson et al, 2005). In

chronic HBV conditions, a defective expression of CD3ζ downregulate was discovered on CD8 T cells (Das et al, 2008). The reduced expression of CD3 ζ associates with defective function of CD8 induced by anti-CD3 (Das et al, 2008). It has been found that the reduced expression of CD3 ζ is due to persistent infections. In another investigation, Bim has been found to be induced in T cells from chronic HBV, but not acute infected patients (Schurich et al, 2011). Bim is a pro-apoptotic protein and expression can repress TCR signalling (Hale et al, 2011). A generous defect in TCR signalling is a common disorder of T cells in chronic infections largely due to the viral persistence (Maini and Schurich 2010) (Das et al, 2008). In a tolerant model induced by persistant antigen stimulation, we have demonstrated that IL-2 can effectively reverse the tolerance and restore full responses to tolerogenic antigens (Anderson et al, 2005). Consistently, we have now demonstrated that IL-2 together with pMHC on IL-2 nanoAPC can enhance TCR signalling, a therapeutic mechanism to reverse T cell tolerance.

7. NanoAPC are biocompatible as live APC and accumulate in peripheral lymphoid organs

Unlike reported nano-vaccine, nanoAPC affects directly on antigen specific T cells in vivo (Nandedkar, 2009). It is therefore important that nanoAPC remain as free particles in peripheral lymphoid organs. Previously, we demonstrated that nanoAPC are not effectively endocytosed by DCs in vitro (Sofra et al, 2009). Here, we further confirmed that the nanoAPC largely remain as free particles in the lymph nodes and accumulated for more than 48 hours. This is important not only for induction of memory T cells in lymphoid organs and also for activation of viral specific T cells in pathological organs such as liver in chronic HBV with a high level of lymphatic infiltration. Although we do not know why the nanoAPC are resistant to endocytosis, the lack of pattern recognition molecules those are recognized by pattern recognition receptors on DC may be the reason (Geijtenbeek et al, 2004). In addition to the tolerance to endocytosis, nanoAPC are also highly accumulated in peripheral lymphoid organs (Sofra et al, 2009). This may result from high levels of

homing receptors inherited from seed APC cells (Sachstein, 2005). Nevertheless, the nanoAPC have great advantages in lymphoid distribution and sustained free particles comparing to synthesised nano-vaccines.

8. NanoAPC induce immune synapse on antigen specific T cells

Synpatic formation on the interactive parts between DC and antigen specific T cells is the functional architectures of effective antigen recognition (Fooksman et al, 2010). The clustering of multi surface molecules including pMHC-TCR, co-stimulatory molecules are required for the initiation of potent TCR signalling. The nanoAPC can effectively induce synapthesis formation on antigen specific T cells. Importantly, the membrane clustering leads to the engrapher of whole nanoAPC into T cells, a phenotype observed in the process of synapthses after interaction between DC and antigen specific T cells (Fooksman et al, 2010). The endocytosis of synapesis is important to sustain the interaction of pMHC-TCR and co-stimulatory molecules in the T cells (Fooksman et al, 2010). It has been reported that an interaction of IL-2 with high affinity IL-2 receptor on T cells can also induce endocytosis of IL-2-IL-2R complex which is also important for a sustained IL-2 stimulation. Thus, the endocytosis of nanoAPC can result in the sustained stimulation of both TCR and IL-2 receptor, a maximum induction of viral specific T cells which is critical for the reversed immune responses in chronic infectious conditions.

9 NanoAPC can be applied as vaccine delivery system.

In addition to the flexible reconstitution of selected bio-active molecules and physiolocially processed therapeutic molecules, the nanoAPC created from MHC class I negative seed APC cells (721.221) gives advantages to freely reconstitute MHC class I alleles. As statistics reviewed, there are a few of most common MHC class I alleles that are distributed in a large population (Janeway et al, 2001). It is therefore, reconstitution of these most common MHC class I alleles that can make nanoAPC broadly used by large population of patients. Another major advantage of

nanoAPC is the sustained activity after storing at -80^{o}C, which is not only possible for pharmaceutics production but also maintains consistent between benches easier.

10 Future considerations/ work

The ultimate aim of nanoAPC is to deliver engineered bio-adjuvant to antigen specific T cells, but not to bystander T cells and Treg cells. It is therefore important that reconstituted pMHC on nanoAPC can effectively engage antigen specific T cells in vivo. To achieve this, high affinity and broad spectrum pMHC on nanoAPC can effectively induce comprehensive responses against viral antigens and subsequently deliver engineered adjuvant to antigen specific T cells. We demonstrated in this study that pMHC assembled by the loading of exogenous peptides to MHC class I in vitro on nanoAPC or live DC is much lower than that processed physiologically in APC after forced expression of antigen into the APC. It is therefore important in future development that viral antigen has to be transfected into seed cells and allow the pMHC to be assembled before isolation of nanoAPC. The efficacy of nanoAPC with engineered bio-adjuvant has to be tested in clinical trials and the induction of viral specific CD4 and CD8 T cells and viral clearance have to be examined in vivo.

11 Conclusions:

To overcome the unresponsiveness of viral specific T cells induced by viral persistence in chronic infectious patients, in this study, we merged advantages from nano-technology, bio-adjuvant, antigen presenting cells and selected viral antigenic peptides in the development of therapeutic vaccines for chronic infectious diseases (Nandedkar, 2009) (Ha et al, 2008) to develop a novel form of nano-vaccine, nanoAPC, to pMHC, co-stimulatory signals and IL-2 or other engineered bio-adjuvant to viral specific T cells, but not bystander T cells and Treg cells. The selection of MHC class I defective human B cell line as seed cells allows us to reconstitute the most popular MHC class I alleles into nanoAPC. Thus, the therapeutic vaccines can apply to large population sharing same MHC class I alleles.

Derived from natural APC, the nanoAPC serve native APC function and effectively induce functional synapses on antigen specific T cells. Therefore, I conclude that nanoAPC serve as a most effective delivery platform for the development of therapeutic vaccines for chronic infectious diseases or cancer in which the T cells are tolerant to antigens.

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APPENDIX

Table 1. Administration of stimulatory cytokines to boost exhausted T cells during chronic infection

(Ha et al, 2008)

Cytokine	General effect on T cells	Treatment	Infection	Effect	References
IL-2	• Expansion of recently activated T cells • Expansion and maintenance of Tregs • Induction of AICD • Upregulation of Bcl-2	IL-2	LCMV	• ↑Virus-specific T cells • ↓Viral load	•Blattman et al, 2003
		IL-2+ Anti-retroviral drugs	HIV*	• ↑CD4 T cells Virus-specific T cells (ND) • Viral load (no effect)	•Aladdin et al, 2001 • Levy et al, 1999 • Kovacs et al, 1996
		IL-2+ Therapeutic vaccine +	HIV*	• ↑Virus-specific T cells • ↓Lower viral load†	•Levy et al, 2005
		Anti-retroviral drugs	HIV‡	• Virus-specific T cells (no effect) • Viral load (no effect)	• Hardy et al, 2007 • Kilby et al, 2006 • Smith et al, 2007

			SIV‡	• ↑Virus-specific T cells • ↓Lower viral load †	•Tryniszewska et al, 2002
IL-7	• Maintenance of memory • T cells Survival of IL-7Rα^{Hi} effector T cells • Upregulation of Bcl-2	IL-7	SIV	• ↑CD4 and CD8 T cells Virus-specific T cells (ND) • Viral load (no effect)	• Beq et al, 2006 •Nugeyte et al, 2003 •Fry et al, 2003
		IL-7 + Therapeutic vaccine + Anti-retroviral drugs	SIV‡	• Virus-specific T cells (no effect) • Viral load (no effect)	•Hryniewicz et al, 2007
IL-15	• Maintenance of memory T cells • Survival of IL-7Rα^{Low} effector T cells • Upregulation of Bcl-2	IL-15+ Anti-retroviral drugs	SIV*	• ↑Virus-specific T cells	•Picker et al, 2006
		IL-15+ Therapeutic vaccine + Anti-retroviral drugs	SIV‡	• Virus-specific T cells (no effect) • ↑Viral load†	•Hryniewicz et al, 2007

***Compared with anti-retroviral therapy alone.**
†After cessation of anti-retroviral therapy.
‡Compared with therapeutic vaccine 1 anti-retroviral therapy.
ND not determined.

Table 2 Different types of immunotherapy

Type of vaccine	Origin/generation	Antigen loading	Characteristics/ advantages	Disadvantages	Reference
Free peptides	• Synthetic immunogenic peptides from TAAs		• Administered with or without adjuvants. • Induction of CTL responses in vivo and in clinical trials. • No induction of immunity with free peptides in the absence of adjuvant in clinical models. • Therapeutic vaccination with IFA is effective in a few animal models. • Some clinical trials showed some clinical responses against melanoma. • Easy and inexpensive production of	• Can induce tolerant. • Inconclusive clinical results, limited clinical responses. • Ineffective prophylactic vaccination against non-virally induced cancers in vivo models. • Some clinical trials showed no clinical response. • Require uptake by patients DCs, which can be compromised.	• Schulz et al, 1991 • Mandelboim et al, 1995 • Wang et al, 1999 • Jager et al, 1996 • Jager et al, 2000 • Gjertsen et al, 1997 • Gjertsen et al, 2001

			peptides. • Simple peptides administration in a clinical setting.		
Dendritic Cells	• Monocyte-derived DCs. • $CD34^+$ precursor-derived DCs. • Peripheral blood DCs. (Maturation is induced by culturing with cytokines.)	• DCs pulsed with peptides. • DCs pulsed with proteins. • DCs loaded with DNA and mRNA • DCs loaded with viral vectors. • DCs loaded with tumour cell lysates. • DC-tumour cell fusions.	• Professional APCs for potent T cell induction. • Superior than other methods in tumour models. • Strong CTL induction in animal models. • Effective prophylactic and therapeutic vaccination in mouse models (eg, melanoma, HPV). • Expansion of tumour specific CTLs and regressions in cancer patients. • Can also induce Th cell responses. • In i.v. administration DCs	• Problematic long-term storage. • Time consuming and labour intensive. • Expensive. • On a large scale this approach will be problematic. • DCs generated ex vivo home poorly in the lymph nodes after s.c. injection (although the few cells that reach the draining lymph nodes generate T cell responses). • Even though they can stimulate T cell responses, these are often not accompanied with significant clinical	• Mayordomo et al, 1995 • Zitvogel et al, 1996 • Bellone et al, 2000 • Soares et al, 2001 • Eggert et al, 1999 • Mullins et al, 2003 • Saha et al, 2007 • Nestle et al, 1998 • Thurner et al, 1999 • Banchereau et al, 2001 • Schuler-Thurner et al, 2002 • Mandic et al, 2005 • Londge et al, 2000 • Thomas-Kaskel et al, 2006

			home to the spleen and do not reach the lymph nodes (effective in lung cancer model but not for subcutaneous tumour). • S.C. injection leads to mixed homing to spleen and lymph nodes. • Safe and well tolerated for clinical use. • Have shown clinical responses to melanoma, prostate cancer, and metastatic renal cell carcinoma...	responses. In general clinical results are unsatisfactory.	
Liposomes	• Manufactured in the lab from phospholipids. • Tumour cell-derived liposomes.	• TAA or TAA-derived peptide incorporation during preparation.	• Can be modified for site-specific delivery, can be targeted to DCs. • Efficiently endocytosed by	• Very few clinical trials with cellular but no significant clinical response. • Require uptake by patients DCs,	• Kawamura et al, 2006 • Van Broekhoven et al, 2004 • Van Broekhoven et al, 2002

		• Tumour cell-derived liposomes retain antigenic properties from original tumour cells.	DCs, • Can be prepared to contain cytokines and DC maturation signals. • Offer antigen protection against degradation. • Cell free vaccine (simpler). • Can be rapidly available and in large quantities. • Induce strong CTL responses in animals. • Provide prophylactic immunity in animal models against tumour challenge. • Therapeutic effects in animal models inducing regression of established tumours. • Better than free peptides. • Clinical response to	which can be compromised.	• Altin et al, 2004 • Neelapu et al, 2004 • Neidhart et al, 2004

			B cell lymphoma.		
ISCOMs	• Manufactured in the lab from cholesterol, saponin and phospholipids.	• Proteins and peptides incorporate into iscomes during preparation. • Cationic antigens naturally associate to ISCOMATRIX when admixed.	• Cell-free. • Fuse with DCs unloading their contents. • Available in large quantities. • Protect antigen from degradation. • Contain saponin which is highly immunogenic. • Promote high antibody levels and strong CTL and Th cell responses in animal models. • Induced antibody and $CD8^+$ T cell response against HPV16 protein in a clinical trial. • Better than free peptides. • Safe for clinical use.	• One clinical trial reported against cancer with no significant clinical response. • Require uptake by patients DCs, which can be compromised.	• Barr and Mitchell, 1996 • Claasen and Osterhaus, 1992 • Maloy et al, 1995 • Lenarczyk et al, 2004 • Stewart et al, 2004 • Frazer et al, 2004
Exosomes	• Secreted from DCs or tumour cells and obtained from culture	• Exosomes pulsed with peptides.	• Naturally secreted and endocytosed by DCs, so efficient	• Low yields from mature DCs, which limits their	• Zitvogel et al, 1998 • Andre et al, 2004

	supernatants.	• Pulsing peptides to DCs before exosome production and purification.	uptake for vaccination purposes. • Contain MHC class I and II. • Contain co-stimulatory/adhesion molecules. • Require mDCs from antigen presentation. • Induce potent CTL responses in animal models. • Only exosomes from mDCs are efficiently immunogenic. • Both prophylactic and therapeutic immunity in animal models. • As efficient as DCs and more efficient than peptides in animal models. • Cell-free. • Can be stored for prolonged periods	clinical use. compromised • Require uptake by patients DCs, which can be compromised. • Only a couple of phase I trials at the moment, with no clinical and limited cellular response.	• Chaput et al, 2004 • Segura et al, 2005 • Escudier et al, 2005 • Morse et al, 2005 • Andre et al, 2002 • Dai et al, 2006 • Chen et al, 2006

			in vitro. • Safe and well tolerated.		
Membrane Vesicles from DCs	• Membrane vesicles obtained by ultracentrifugation of sonication-disrupted DCs.	• Administered together with peptides.	• Cell-free therefore simple. • Can be obtained in high yields. • Contain MHC class I and co-stimulatory/adhesion molecules. • Can stimulate T cells in the absence of APCs in vitro. • Prime $CD8^+$ T cells in vivo. • Induce a prophylactic response against tumour challenge in vivo.	• No clinical results available. • System not well characterized.	• Kovar et al, 2006
Microsomes	• ER membrane obtained from lysed APCs by rounds of ultracentrifugation.	• Pulsed with peptides.	• Cell-free, therefore simpler. • Can be obtained in high yields. • Microsomes efficiently bind peptides. • Directly stimulate	• No clinical results available.	• Sofra et al, 2009

			T cell in vitro, without the presence of APCs. • Contain MHC class I and II. • Contain co-stimulatory/adhesion molecules. • Induce peptide-specific $CD8^+$ responses in vivo.		
IL-2-nanoAPC	• ER membrane obtained from lysed IL-2 and HLA-A2 transfected 721.221 cell line by rounds of ultracentrifugation.	• Pulsed with peptides.	• Continue each advantage from microsome • Delivery both antigen peptide and bio-adjuvant IL-2 to antigen T cell • Broad application to HLA shared patient groups • IL-2 direct active on antigen T cell no effect to the rest T cells or Treg cells • Nano-size free pass and accumulate	• Limitation of high affinity and broad spectrum pMHC	• Manuscript send to Journal of Immunology

			into peripheral lymph nodes • Formed in a part of immune synapse to induce T cell responds • More than 50% patient samples shown positive responds for the IL-2-nanoAPC with peptide in the HBV clinical trail		

Table 3 Strategles Applies to Vaccine Development and Their Featurres

Vaccine Type	Structure	Replication	Mark	Production			Administration	Immunity		Safety		Notes
				Cell Culture	Chemical Synthesis	Recombinant DNA		Humoral	Cell Mediated			
Classic Killed	Particles Microorganisms	N	N	*			**	*	*	+-	Uncomplete inactivation	Yes, if pathogens can be easily cultured
Classic Live attenuated	Microorganisms	Y	N	*			*	*	*	+-	Rescue of Viulence In vivo recombination	Yes, if pathogens can be easily cultured
Subunit	Single proteins	N	Y	*	*	*	***	*	?	+	Yes, if properly purified	New adjuvants and formulations
Peptides	Carrier-conjugated peptide	N	Y		*		***	*	?	+	Yes, highly purified	Long R&D study Also polyvalent
Recombinant antigens	Single proteins Carrier-conjugated	N	Y			*	***	*	?	++	Yes, highly purified	
Micelles Liposomes	Antigens embedded In lipid Bilayer	N	Y	*	*	*	**	*	?	++	Yes, highly purified	Long R&D development Coexpression of antigens and/or cytokines
ISCOMs	Particles	N	Y	*	*	*	**	*	*	++	Yes, highly purified	
VLPs	Particles	N	Y			*	**	*	*	++	Yes, no toxic	

											effects	
Recombinant Live vectors	Particles Microorganisms	Y	Y			*	*	*	*	+-	Rescue of virulence Recombination with WT virus	Long R&D development Coexpression of antigens and/or cytokines
Naked DNA	Plasmid DNA, RNA, Lipsomes Gold Microbullets	N	Y			*	**	*	*	++	Applicable to gene therapy	Easy to prepare Thermostable Avirulent

Table 4 Patient characterization and CD8 T cell response induced by nanoAPC for each patient

Subject	ALT (IU/liter)	HBeAg	Viral load (log)	Sex	Age	IL-2M-I	M-I	Control
						$CD8^+$ $IFN\gamma^+$ (%)		
1	39	N	5.28	F	36	5.9	3.1	0
2	37	N	3.27	M	44	0.8	0.3	0.1
3	24	N	4.58	M	69	0.8	0.1	0
4	53	N	5.98	M	23	10.1	0.7	0.2
5	32	P	7.99	F	35	0.1	0	0
6	24	N	5.84	M	42	0.5	0.4	0
7	36	N	4.67	F	44	3.7	0.3	0.3
8	127	N	4.63	M	26	1.5	0.3	0.4
9	65	N	7.14	F	58	7.3	0.2	0.2
10	30	P	7.45	M	17	0.1	0	0.1
11	211	N	5.22	M	33	4.2	2.3	0.1
12	97	P	4.05	F	56	0.3	0.1	0
13	206	N	5.27	M	51	16.1	5.4	0.2
14	45	P	7.31	M	18	12.3	3	0.3
15	202	N	8.37	F	27	0.1	0.1	0
16	31	P	9.96	M	28	4.5	0.5	0.1
17	79	N	5.58	M	52	0.5	0.3	0
18	44	N	5.11	M	42	0.7	0.3	0
19	169	N	6.65	F	36	14.2	6.2	0.2
20	83	N	4.49	M	57	0	0	0
21	27	P	5.36	M	27	1.4	1.4	0.1
22	135	p	7.23	F	52	0.5	0.3	0
23	430	p	4.68	M	33	11.4	4.5	0.3
24	110	P	7.54	M	22	4	2.5	0.1
25	143	N	7.03	F	32	3.5	2	0.1
26	188	N	6.13	M	19	1.8	1.6	0
27	194	P	7.05	F	41	3.5	1.5	0.1
28	121	N	5.79	M	37	1.7	1.4	0.1
29	267	N	9.13	M	22	1	1	0
30	126	P	6.23	M	19	1	0.3	0
31	20	P	7.04	F	40	1.4	0.5	0
32	313	N	6.32	M	38	1	0.9	0.1
33	212	P	9.12	M	47	1.5	0.2	0
34	122	P	7.15	M	32	1.7	1.2	0.1
35	42	N	5.21	M	44	1.3	1.3	0.3

36	35	N	7.24	M	20	4.2	1.3	0.3
37	23	N	7.24	F	37	1.1	0.8	0.2
38	73	N	5.58	M	23	1.4	1.2	0.3
39	46	N	6.09	F	38	0.8	0.3	0.8
40	310	N	7.61	M	34	4	0.4	0.1
41	76	N	7.14	M	46	0.6	0	0.1
42	362	P	4.31	M	32	2.6	0.7	0
43	211	N	7.54	M	33	1	0.6	0.1
44	79	N	6.23	F	52	3.7	0.2	0
45	140	N	5.38	M	40	12.4	1.1	0
46	170	P	5.42	M	27	7.8	0.5	0
47	35	P	6.34	M	26	4	2	0
48	120	P	5.49	M	29	3.6	3.3	0
49	278	P	5.83	M	40	4.9	2.3	0.3
50	40	P	5.87	M	25	0.9	1.5	0
51	46	P	7.38	M	18	2.1	1.1	0.3
52	80	P	7.24	F	27	5.6	0.9	0
53	423	P	5.63	M	33	2.5	0.8	0.2
54	43	P	7.23	M	18	4.7	3.9	0.2
55	86	P	7.42	M	30	2.8	0.9	0.3
56	47	P	7.86	M	41	0.9	0.9	0.1
57	42	P	6.1	M	28	0.7	0.6	0

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IL-2–Engineered nano-APC Effectively Activates Viral Antigen-Mediated T Cell Responses from Chronic Hepatitis B Virus-Infected Patients

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Impaired function of virus-specific T cells resulting from virus persistence is one of the major mechanisms underlying the development of chronic hepatitis B viral infection. Previously, we found that IL-2 can restore the effector function of T cells rendered tolerant by Ag persistence. However, systemic administration of IL-2 induces organ pathology and expansion of T regulatory cells. In this study, we show that nano-APC with engineered HLA alleles and IL-2 deliver peptide–MHC complexes, costimulatory molecules, and IL-2 to Ag-responding T cells, resulting in enhanced expression of CD25 and activation of TCR signaling pathways, while suppressing PD-1 expression on viral-responding CD8 T cells from chronic hepatitis B virus patients. The enhanced activation of CD4 and CD8 T cells induced by IL-2–nano-APC was Ag dependent and IL-2–nano-APC did not affect T regulatory cells. At a size of 500 nm, the nano-APC effectively induce immune synapse formation on Ag-specific T cells and accumulate as free particles in the lymphoid organs. These attributes of IL-2–nano-APC or other bioadjuvant-engineered nano-APC have profound implications for their use as a therapeutic strategy in the treatment of chronic hepatitis B virus infection or other chronic viral diseases. ***The Journal of Immunology*****, 2012, 188: 1534–1543.**

Hepatitis B virus (HBV) is a noncytopathic hepadnavirus that can cause acute and chronic hepatitis (1). Approximately 350 million people worldwide are chronically infected with HBV, which greatly increases the risk of hepatocellular carcinoma and causes >1 million deaths annually (1). Despite recent advances in the development of immunotherapies for chronic HBV infection, effective induction of virus-specific T cell activation in persistent HBV infections remains a challenge (2). One of the major problems for the development of therapeutic vaccines for chronic HBV infection is the functional defects in T cells resulting from continual stimulation of these T cells by persistent viral Ags (3, 4). The functional properties lost by the antiviral T cells can range from the failure to produce effector cytokines, such as IL-2, TNF-α and IFN-γ, to complete deficiency in cytokine production and proliferation ability, in addition to a reduction in virus-specific T cell populations (4). These deficiencies are due to both cell intrinsic and extrinsic mechanisms, including the expression of inhibitory receptors, such as CTLA4 and PD-1; regulatory cytokines, such as IL-10; and possibly increased numbers of T regulatory (Treg) cells (4–6). The unresponsiveness of virus-specific T cells has been found to be a generic mechanism partly responsible for chronic HIV, HBV, and hepatitis C virus (HCV) infections (5), suggesting that the induction of T cell tolerance is due to persistent engagement of T cells with viral Ags, rather than the function of specific viral molecules. Therapeutic interventions to counter the effects of T cell tolerance, and overcome the immunosuppressive environment resulting from high viral Ag load, have aimed to boost T cell responses via administration of defined Ags in combination with blocking Abs to IL-10 or PD-1 or with cytokines enhancing T cell activation, such as IL-2 and IL-7 (7–9). Recently, downregulation of TCR-proximal signaling pathways was found in T cells from chronic HBV patients (3, 10), compelling support for the notion that persistent Ag engagement is at least one of the mechanisms for the induction of virus-specific T cell tolerance. Ag-induced immune tolerance has been well studied in many animal models (11). After repeated Ag stimulation, effector T cells become tolerant (12). We found that tolerance is not due to loss of Ag-specific T cells, but rather downregulation of proximal TCR signaling pathways following Ag stimulation (13, 14), which resembles the findings from chronic viral infections (4). We found that an increase in the amount of Ag presented by activated dendritic cells (DC) cannot reverse tolerance (13). However, addition of IL-2 can effectively overcome tolerance and restore the full activation of T cells in response to Ag stimulation (13). It has been found that liver sinusoidal endothelial cells can induce tolerance of HBV-specific CD8 T cells through interaction between B7-H1 on liver

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Abbreviations used in this article: DC, dendritic cell; ER, endoplasmic reticulum; HBeAg, hepatitis B e Ag; HBV, hepatitis B virus; HCV, hepatitis C virus; MP, matrix protein; pMHC, peptide MHC; RM, rough microsomes; Treg, T regulatory.

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sinusoidal endothelial cells and programmed cell death-1 on CD8 T cells, and such tolerance can be effectively overcome by IL-2 (15). IL-2 has been used as a bioadjuvant to overcome the low immunogenicity of therapeutic vaccines for the treatment of cancer and chronic infectious diseases (16, 17). Although IL-2 is a potent activator of memory and effector T cells, systemic administration of high doses of IL-2 not only induces severe side effects, but may also promote Treg function, which can further increase the activation threshold of Ag-specific T cells (16).

To use IL-2 to overcome the T cell exhaustion induced in chronic HBV infection while avoiding the side effects of systemic administration, we have now developed a novel therapeutic vaccine (nano-APC) that delivers Ag and IL-2 to Ag-responding T cells, but has little effect on bystander T cells. The nano-APC are prepared from the endoplasmic reticulum (ER) membranes of a MHC-deficient human B cell line, 721.221 (18), which expresses high levels of costimulatory molecules and genetically engineered ER-retained MHC I alleles and IL-2 constructs. After assembly with antigenic peptide in vitro, the nano-APC directly interact with Ag-specific T cells and induce formation of immune synapses and expression of the high-affinity IL-2R on T cells. The IL-2 delivered by nano-APC enhanced T cell responses and effector function, but did not affect bystander T cells or Treg cells. When assembled with a pool of HLA-A2–associated HBV peptides and HBV peptides associated with HLA-DR and DP, IL-2–nano-APC induced strong CD4 and CD8 T cell responses in peripheral lymphocytes from chronic HBV patients. Our results demonstrate that IL-2–nano-APC, which deliver both Ag and IL-2 to Ag-responding T cells, can significantly increase functional antiviral responses, thereby overcoming the immune tolerance induced by persistent viral load.

Materials and Methods

Mice and cell lines

OTI transgenic mice on the C57BL/6 background were provided by D. Kioussis (Medical Research Council, National Institute for Medical Research, London, U.K.). All CD8 T cells in OTI mice carry same TCR recognizing OVA residues 257–264 in the context of $H2K^b$ (19). C57BL/6 mice were purchased from Harlan UK (Oxon, U.K.). All animals were maintained in pathogen-free facilities at the Brunel University. The DC2.4 DC line was provided by D. Mann (Southampton, U.K.). The 721.221 cells have been described in our previous reports (20). CTLL4 was provided by G. Stockinger (National Institute of Medical Research, London, U.K.). All lines were cultured in RPMI 1640 with 10% FBS (Invitrogen).

Abs, flow cytometry, and confocal microscopy

FITC-conjugated Abs to CD54 and CD80 were from EuroBioSciences, and FITC-conjugated streptavidin, and PE-conjugated Abs to pERK, PD-1, HLA A2, W6/32, Foxp3, FITC-conjugated CD4, FITC-conjugated CD19, allophycocyanin-conjugated CD8, and PEcy5-conjugated IFN-γ were from BD Biosciences. PE-conjugated A2-CMV_{nlv}pentamer was from ProImmune (Oxford, U.K.). Abs against tapasin and E3-19K retention signal were described previously (21, 22). For all flow cytometry data, the median fluorescence intensity from three experiments was presented. Isotype Abs were used as background controls for all experiments. The side-scatter and forward-scatter settings were the same for cells and microsomes. CD8 microbeads (Miltenyi Biotec) were used for isolation of CD8 T cells from total splenocytes from OTI TCR transgenic mice, according to the manufacturer's protocol. Goat anti-human IL-2; anti-human CD3; W6/32, specific to human MHC I; and MA2.1, specific to HLA A2, Abs were used for immune staining of cells and nano-APC. Texas Red-labeled rabbit anti-goat Ig and FITC-labeled goat anti-mouse Ig secondary Abs were used, and samples were analyzed by confocal microscopy (ZEISS LSM 510).

Peptides, peptide modification, and binding assay

Peptides were synthesized by Invitrogen and purified to >95% purity. The ε-amino group of the lysine in the influenza-A matrix protein (MP), M58–64G58YF62K (YILGKVFTL) peptide was synthesized by Invitrogen and was covalently modified by a photoreactive cross-linker and labeled with iodination (^{125}I), as described previously (23). The N termini of the HBV peptides, HBV Env 180–195 AGFFLLTRILTIPQS, Env 339–354 LVPFVQWFVGLSPTV, and Pol 767–782 AANWILRGTSFVYVP (24), were synthesized by Invitrogen, and labeled and purified with a FITC-labeling kit (Pierce). For the HLA-A2–binding assay, 2 nM modified MP peptides was incubated with IL-2–A2–nano-APC in the absence or presence of unlabeled competing peptides, HBV C18-27 FLPSDFFPSV, HBV envelope 183–191 FLLTRILTI, 335–343 WLSLLVPFV, 338–347 LLVPFVQWFV, 348–357 GLSPTVWLSV, CMV pp65 matrix protein epitope NLVPMVATV, and MP, at various concentrations, as described previously. HBV peptides were described in previous publication (10). CMV NLVPMVATV and OVA SIINFEKL peptides were synthesized by Invitrogen. After the removal of free peptides, HLA-A2 was precipitated with the W6/32 Ab, and the binding was measured by detection of radioactivity using a gamma counter (Beckman). For the DR/DP-binding peptides (synthesized by Invitrogen), HBV Env 180–195 AGFFLLTRILTIPQS, Env 339–354 LVPFVQWFVGLSPTV, and Pol 767–782 AANWILRGTSFVYVP (24), 20 nM FITC-labeled peptides were incubated with IL-2–A2–nano-APC in acidic condition (23). After removal of free peptides, samples were analyzed by flow cytometry. The binding specificity was confirmed by competition assays using unlabeled peptides.

Preparation of nano-APC

To establish IL-2-A2-721.221 seed cells, IL-2-tapasin and HLA-A2-E3-19K fusion constructs were constructed by PCR-cloning approaches. For IL-2-tapasin, atapasin expression construct was modified by substitution of the signal sequence (22) by a 2× GS linker (GGSG) (25) using PCR cloning. Then, a PCR product containing the human IL-2 coding region was cloned upstream of the GS linker to create IL-2-GS-tapasin. HLA-A2-E3-19K was constructed by insertion of a 16-aa (LKYKSRRSFIDEKKMP) sequence from the E3-19K protein (21) downstream of the C terminus of HLA-A2 in a HLA-A2 expression construct (26). The constructs were transfected into 721.221 cells, as described in our previous publications (26). The seed cells were expanded to yield a large volume of cells with >99% viability and then used for preparation of nano-APC. The preparation of nano-APC was a two-step process, consisting of preparation of ER-enriched microsomes and processing of nano-particles. Microsomes from cells were prepared and purified, as described in our previous publication (23). Briefly, cells were washed and resuspended in homogenization buffer. After homogenization, tonicity was restored to 0.15 M NaCl. The nuclei, mitochondria, and larger cell debris were removed by centrifugation at 10,000 × *g*. Total microsomes were recovered by centrifugation at 100,000 × *g* and subfractionated by flotation in sucrose gradients. The microsomes were layered on top of 5 ml 0.33 M sucrose, which was, in turn, layered on top of a discontinuous sucrose gradient consisting of 2 ml 2 M and 1 ml 2.5 M sucrose. Centrifugation in a TH-641 rotor for 16 h at 110,000 × *g* at 4˚C yielded a microsome band on top of the 2 M sucrose cushion, which was collected and resuspended in rough microsomes (RM) buffer (250 mM sucrose, 50 mM triethanolamine-HCl, 50 mM KOAc, 2 mM $MgOAc_2$, and 1 mM DTT). The microsomes were further processed to homogenous nano-particles by sequential homogenization using an Isobiotec cell homogenizer (Heidelberg) with a cutoff size between 6 and 4 μm. The homogenized particles were recovered by centrifugation in a TH-641 rotor for 24 h at 110,000 × *g* at 4˚C and then resuspended in RM buffer at a concentration of ~4 μg/ul. The nano-APC were stored at −80˚C until use.

Assembly of peptides with MHC molecules on nano-APC

The nano-APC in RM buffer were first processed by a freeze-thaw procedure (three cycles of 30 s in liquid nitrogen, followed by 5 min at 37˚C) three times. For assembly of MHC I, the nano-APC were mixed with peptides at 1 μM concentration and human β_2-microglobulin (5 μg/ml; M4890; Sigma-Aldrich) under acidic conditions (0.26 M citric acid, 132 mM Na_2HPO_4, 2% BSA [pH 3]). The mixture was incubated for 5 min on ice, followed by neutralization with 1 M Tris buffer (pH 7.5). The neutralized mixtures were incubated for 30 min at 37˚C. Free peptides and reaction buffers were then removed by washing through Amicon Ultra centrifugal filters with a cutoff size of 3000 kDa (Millipore Amicon Ultra centrifugal filters). The loaded nano-APC were resuspended in RM buffer at a 2 mg/ml concentration. For MHC II loading, after the freeze-thaw process, the microsomes in RM buffer were mixed with an equal volume of acidic buffer (0.26 M citric acid, 132 mM Na_2HPO_4, 2% BSA [pH 3]) containing peptide at 1 μM concentration or as indicated for 30 min at 37˚C. After loading, free peptides were removed by washing through Amicon Ultra centrifugal filters. The assembled nano-APC were resuspended in RM buffer at 2 mg/ml.

Human study

Experimental subjects. Five CMV serum-converted healthy donors and 57 chronic HBV patients participated in this study. All subjects were HLA-A2

positive. Informed consent was obtained, and the study was approved by the local ethics committees at the two participating clinics (East London and City Research Ethics Committee for Barts and The London National Health Service Trust, and Huashan Hospital Ethics Committee, Shanghai, China). Of 57 chronic HBV patients, 47.4% were positive for hepatitis B e Ag (HBeAg), whereas 18 had raised alanine aminotransferase (>50 IU/l) and high HBV DNA ($>10^6$ IU/ml), as quantified by real-time PCR assay. Thirty-one patients were HBeAg negative and positive for serum anti-hepatitis B e Ab. All patients were negative for Abs to HCV, HIV-1, and HIV-2.

Isolation of PBMCs and in vitro stimulation. PBMCs were isolated from fresh heparinized blood by Ficoll-Hypaque density gradient centrifugation and resuspended in RPMI 1640 containing 10% heat-inactivated FBS (Invitrogen). For some experiments, CD8-positive T cells were isolated with anti-CD8–coated beads (Miltenyi Biotec). A total of 5×10^5/well PBMC or $CD8^+$ cells in 24-well plates was cocultured either with antigenic peptide at a concentration of 5 μM (the same concentration used for assembling MHC I on nano-APC) or 10 μg nano-APC for 5 d. Cells were cultured with PBS, or anti-CD3–coated beads served as negative or positive controls. After 5 d, 20 nM PMA and ionomycin (Sigma-Aldrich) were added together with GolgiStop (BD Biosciences) and cultured for 3 h. Cells were then harvested and washed with cold PBS. After washing, cells were first stained with surface markers, and then washed with PBS and fixed for intracellular staining with selected Abs or isotype controls using an intracellular staining kit (BD Biosciences), according to the manufacturer's instructions. The stained cells were washed with PBS, and then analyzed on a Canto II flow cytometry. The data were analyzed using FlowJo (Tree Star).

Interaction of nano-APC and T cells, and in vivo distribution of nano-APC in lymph nodes

The interaction between nano-APC and OTI cells was visualized by incubation of 10 μg SIINFEKL–Kb–nano-APC or Kb–nano-APC, prepared from CFSE-labeled DC2.4 cells, with 10^6 OTI cells in normal medium for 15 min. Free nano-APC were removed by low-speed centrifugation at 1000 × *g* for 5 min and washing with cold PBC. Cells were then stained with anti-CD3 Ab, followed by DAPI counterstaining. The samples were analyzed by confocal microscopy (ZEISS LSM 510).

For analysis of the distribution of nano-APC in lymph nodes, nano-APC were prepared from CFSE-labeled mouse DC2.4 cells. An aliquot of 20 μg peptide-loaded nano-APC or FITC-labeled dextran was injected into C57BL/6 mice i.v. After 48 h, lymph nodes were isolated and processed to single-cell suspensions. The samples were centrifuged at 1000 × *g* for 5 min, and the pellet containing the cellular fraction was collected. Cell-free particles were recovered from the supernatant by centrifugation through a microfilter with a cutoff size of 3000 kDa (Millipore Amicon Ultra centrifugal filters), which can recover both nano-APC and dextran. The cellular and cell-free particles were stained with PE-labeled CD11c as a DC marker and analyzed by FACS.

Statistics

Statistical comparisons were performed using the Student *t* test; unless otherwise noted, data were presented as the means ± SD of pooled data from four to six independent experiments.

Results

IL-2–nano-APC

To reverse the impairment in viral-specific T cell function induced by virus persistence (4), we developed IL-2–nano-APC with the aim to deliver high doses of peptide MHC (pMHC), IL-2, and costimulatory molecules directly to Ag-specific T cells. We selected the HLA-I–deficient human B cell line 721.221 (18) as seed cells for the nano-APC. The 721.221 cells express high levels of costimulatory molecules (Fig. 1*A*) and HLA-DR and DP molecules (18). We engineered ER-retained HLA-I alleles (in this study, HLA-A2 was used) and IL-2 by transfecting HLA-A2–ER retention signal, a 16-aa–long fragment from the C-terminal end of E19 3K protein (21), and IL-2-tapasin, an ER-retained membrane protein (20, 22), fusion constructs into the 721.221 cells. The 16-aa ER-retention signal from E19 3K and tapasin effectively retained the HLA-A2 and IL-2 fusion proteins in the ER membranes, as shown by the ER-staining pattern with anti-A2 and anti–IL-2 Abs (Fig. 1*B*). The engineered HLA-A2 and IL-2 did not alter the expression of costimulatory molecules (data not shown). The HLA-A2– and IL-2–expressing 721.221 cells were used for preparation of nano-APC (IL-2–A2–nano-APC), as described in *Materials and Methods*. The IL-2–A2–nano-APC were of a largely homogenous size, ~500 nm, and contained high levels of HLA-A2, IL-2, and costimulatory molecules (Fig. 1*B*, 1*C*). The IL-2–tapasin fusion protein retained IL-2 bioactivity, as demonstrated by the proliferation of the IL-2–dependent CTLL4 cell line in response to IL-2–A2–nano-APC (Fig. 2*A*). The pharmacological activity of IL-2 on nano-APC was ~2 IU/μg nano-APC (Fig. 2*A*). The activity of IL-2 on IL-2–A2–nano-APC was effectively neutralized by IL-2–neutralizing Ab (Fig. 2*B*). Although the activity is about 50 times lower than the ~100 IU/1 μg rIL-2, the IL-2 on nano-APC is immobilized, which may create an enriched microenvironment for T cells that are specifically interacting with nano-APC. This strategy can be applied to other cytokines or bioactive proteins depending on the therapeutic aim. Previously, we have demonstrated that ER membranes derived from APC contain peptide-receptive MHC I and abundant costimulatory molecules, which can assemble with antigenic peptides and induce protective immune responses (23). Consistent with this, the A2 molecules on IL-2–A2–nano-APC effectively assembled with A2-binding peptides (Fig. 1*D*), as shown by reduced binding of known A2 peptide MP58–64 to IL-2–A2–nano-APC after incubation together with CMV_{nlv} peptide in a competition assay. We have also developed a method for loading MHC II Ags onto purified ER vesicles to assemble pMHC II (24). Analysis of the assembly of HLA-DR molecules with peptide showed that HLA-DR–binding peptides could effectively assemble with DR under acidic conditions (Fig. 1*E*). Because MHC II molecules are much more promiscuous than MHC I molecules in terms of selection of their antigenic peptides, as most of the DR-associated peptides can also bind to DP to certain degrees (27), we used the endogenous HLA-DR and DP molecules from seed cells to assemble antigenic pMHC class II complexes for activation of effector CD4 Th cells. Thus, at a size of ~500 nm, the IL-2–nano-APC are designed to deliver pMHC, IL-2, and costimulatory molecules to T cells.

The IL-2 on IL-2–nano-APC enhances activation of Ag-responding T cells, but does not affect bystander T cells

The IL-2–nano-APC are designed to deliver IL-2, Ag, and costimulatory molecules to Ag-specific T cells. To investigate whether the IL-2 on nano-APC can affect both Ag-specific and bystander T cells, A2–nano-APC and IL-2–A2–nano-APC were assembled with CMV_{nlv} peptide to generate CMV_{nlv}A2–nano-APC and IL-2–CMV_{nlv}A2–nano-APC, which were used to stimulate CD8 T cells isolated from peripheral lymphocytes of CMV-seropositive and HLA-A2–positive healthy donors. In contrast to treatment with free 1 μM CMV_{nlv} peptide, which induced low CD8 T cell response, CMV_{nlv}A2–nano-APC could induce detectable Ag-specific CD8 responses (Fig. 3*A*). However, IL-2–CMV_{nlv}A2–nano-APC induced far stronger CD8 T cell responses (Fig. 3*A*). These enhanced CD8 responses induced by IL-2–CMV_{nlv}A2–nano-APC could be effectively neutralized by anti–IL-2 Ab (Fig. 3*B*). Although we analyzed IFN-γ–producing T cells after a 3-h restimulation with PMA and ionomycin, in the absence of antigenic peptide, IL-2–A2–nano-APC did not induce a T cell response, suggesting that the IFN-γ–producing cells were Ag-responding T cells (Fig. 3*A*). To further confirm the Ag-dependent effect of IL-2, the IFN-γ–producing cells were quantified in CMV_{nlv}tetramer$^+CD8^+$ cells in PBMC after stimulation. Consistently, an enhanced response of Ag-specific CD8 T cells was detected specifically after stimulation with IL-2–CMV_{nlv}A2–

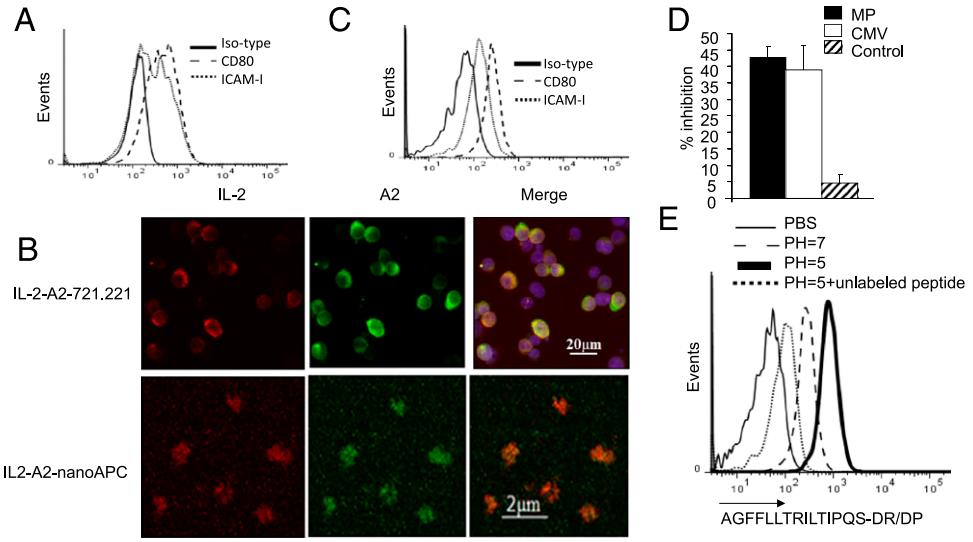

FIGURE 1. Characterization of IL-2–A2–nano-APC assembled with MHC I and II peptides. *A*, 721.221 seed cells express the costimulatory molecules CD80 and ICMA1. *B*, IL-2-A2-721.221 cells and IL-2–A2–nano-APC were stained with anti–IL-2 (red) and A2 (green). Nuclei were visualized by DAPI counterstaining. *C*, Costimulatory molecules CD80 and ICMA-1 on IL-2–A2–nano-APC. *D*, Binding of A2-associated peptides. Binding of MP58–64 and CMV_{nlv} peptides to A2 in IL-2–A2–nano-APC was measured by competing with ^{125}I-labeled MP58–64 peptides at the same 20 nM concentration. HBV Env 180–195 AGFFLLTRILTIPQS peptide served as control. *E*, Assembly of HLA-DR with HBV Env 180–195 AGFFLLTRILTIPQS on IL-2–A2–nano-APC. Binding of HBV Env 180–195 to IL-2–A2–nano-APC was measured under different pH. Binding was measured by detection of FITC-labeled HBV Env 180–195 on IL-2–A2–nano-APC after removal of excess peptides at the end of the binding reaction. PBS and addition of unlabeled peptide as controllers.

nano-APC (Fig. 3*C*). We compared Ag-specific responses of CD8 T cells induced by IL-2–CMV_{nlv}A2–nano-APC and CMV_{nlv}A2–nano-APC plus 20 IU/ml soluble IL-2. Although soluble IL-2 did increase IFN-γ–producing CD8 T cells, most of the IFN-γ–producing CD8 T cells in PBMC stimulated by CMV_{nlv}A2–nano-APC and soluble IL-2 were CMV_{nlv}A2-tetramer negative (Fig. 3*D*). In contrast, IL-2–CMV_{nlv}A2–nano-APC effectively induced responses of CMV_{nlv}-specific CD8 T cells (Fig. 3*D*). These results demonstrate that the overall dose of IL-2 delivered by IL-2–CMV_{nlv}A2–nano-APC is not sufficient for a pharmacological effect on bystander T cells. We found that in the absence of Ag, a minimum of 50 IU/ml IL-2 is required to induce any detectable T cell response (data not shown), whereas the IL-2 dose equivalent of 10 μg/ml IL-2–CMV_{nlv}A2–nano-APC is ~10 IU/ml. It is known that IL-2 upregulates expression of CD25, the IL-2Ra chain that is required for assembly of the high-affinity IL-2R needed for IL-2 to induce T cell activation (28) on preactivated T cells (28). Indeed, in addition to induction of high levels of CD8 T cell responses, IL-2–CMV_{nlv}A2–nano-APC also induced CD25 expression on CMV_{nlv}tetramer$^+$CD8$^+$ T cells (Fig. 3*E*). IL-2 is also important for the maintenance and expansion of CD4$^+$CD25$^+$ Foxp3$^+$Treg cells (28). However, we did not observe an increase in the proportion of Foxp3$^+$CD4$^+$Treg cells in PBMCs after stimulation with IL-2–A2–nano-APC (data not shown). These findings suggest that nano-APC can selectively deliver IL-2, together with pMHC and costimulatory molecules, to Ag-specific T cells.

IL-2–A2–nano-APC induce strong CD8 T cell responses in peripheral lymphocytes from chronic HBV patients

Functional impairment of virus-specific T cells due to persistent viral Ags is one of the important mechanisms leading to, and maintaining, chronic HBV infection (3, 10). We previously found that IL-2 can effectively reverse tolerance induced by Ag persistence (13). T cells from chronic HBV patients show reduced production of IL-2 in response to TCR ligation (4, 10), which is consistent with the defective induction of IL-2 in T cells rendered tolerant by persistent Ag stimulation (13). To investigate whether IL-2–A2–nano-APC can enhance responses of virus-specific CD8 T cells from chronic HBV patients, we developed HBV-specific IL-2–HBVA2–nano-APC by assembling a pool of five HBV peptides, which have been found to induce HBV-specific CD8 T cell responses in A2-positive HBV patients (10), with the A2 molecules on the IL-2–A2–nano-APC. We found that the five HBV peptides had similar binding affinities for A2, as measured by competition assays with a known A2-binding peptide: a modified influenza MP peptide YILGKVFTL (26) (Fig. 4*A*). Therefore, a pool containing equal amounts (5 μM) of each peptide was used for assembly of IL-2–HBVA2–nano-APC and HBVA2–nano-APC. Peripheral lymphocytes from 57 HLA-A2–positive chronic HBV patients, with viral loads ranging from 3×10^3 to 10^{13} IU/ml (Table I), were used to investigate CD8 T cell responses. We detected low CD8 T cell responses,

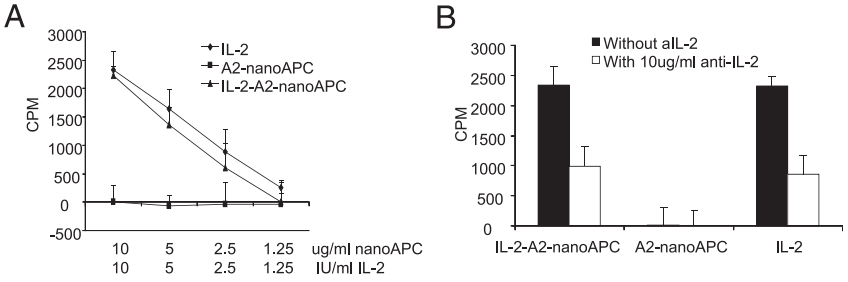

FIGURE 2. Bioactivity of IL-2 on IL-2–A2–nano-APC. The bioactivity of IL-2 was measured by stimulation of the IL-2–dependent CTLL4 cell line. A2–nano-APC and human rIL-2 served as negative and positive control, respectively. *A*, Dose-dependent induction of CTLL4 proliferation by IL-2–A2–nano-APC. *B*, The activity of IL-2 on IL-2–A2–nano-APC can be neutralized by anti–IL-2 Ab.

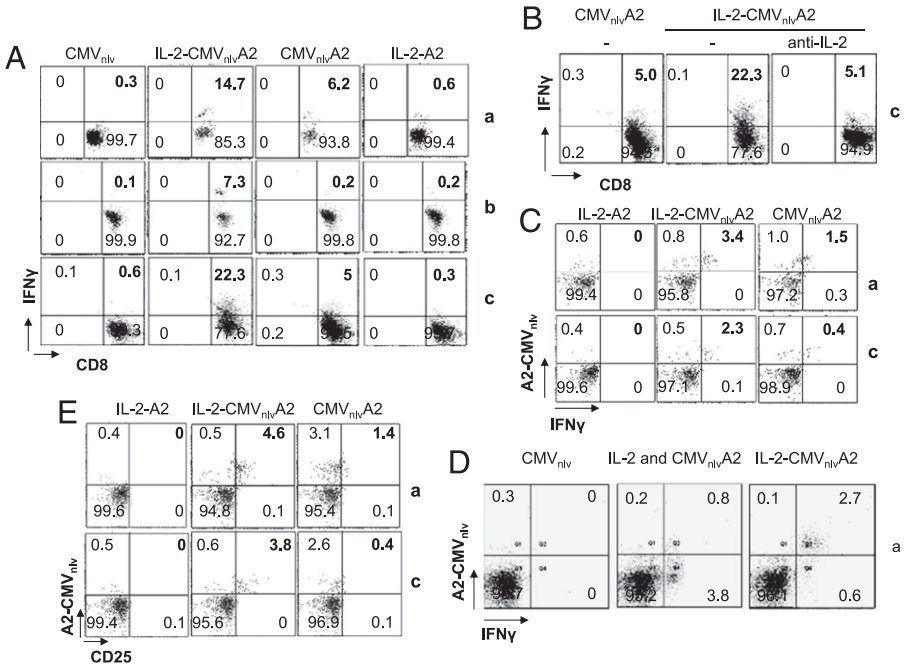

FIGURE 3. IL-2–nano-APC enhances the responses of Ag-responding CD8 T cells. *A*, PBMCs from three A2-positive and CMV-seropositive donors (*A*–*C*) were incubated with 1 μM CMV_{nlv} peptide, 10 μg/ml IL-2–CMV_{nlv}A2–nano-APC, 10 μg/ml CMV_{nlv}A2–nano-APC, or 10 μg/ml IL-2–A2–nano-APC for 5 d. After restimulation with PMA and ionomycin for 3 h, cells were stained with anti-CD8 and anti–IFN-γ. IFN-γ–producing cells were measured after gating on CD8 cells. *B*, Part of PBMC from donor C was incubated with 10 μg/ml IL-2–CMV_{nlv}A2–nano-APC in the presence of IL-2 neutralization Ab. *C*, PBMCs from donors A and C were incubated with 10 μg/ml CMV_{nlv}A2–nano-APC, IL-2–CMV_{nlv}A2–nano-APC, or IL-2–A2–nano-APC for 5 d. After restimulation with PMA and ionomycin for 3 h, cells were stained with anti-CD8, anti–IFN-γ, CD19, and CMV_{nlv}pentamer. IFN-γ–producing cells were measured after gating on CMV_{nlv}pentamer$^+$CD8$^+$CD19$^-$ cells. *D*, PBMCs of donor A were stimulated either with 10 μg/ml IL-2–CMV_{nlv}A2–nano-APC for 5 d or with 20 IU/ml IL-2 and 10 μg/ml CMV_{nlv}A2–nano-APC for 3 d, and then restimulated with same doses of IL-2 for another 2 d. IFN-γ–producing and CMV_{nlv}pentamer-positive cells were quantified on gated CD8 cells. *E*, PBMCs of donors A and C were stimulated with 10 μg/ml CMV_{nlv}A2–nano-APC, IL-2–CMV_{nlv}A2–nano-APC, or IL-2–A2–nano-APC for 3 d. CD25$^+$ cells were quantified after gating on CMV_{nlv}pentamer$^+$CD8$^+$CD19$^-$ cells.

as measured by the proportion of IFN-γ–producing CD8 T cells, after incubation with the pool of the five HBV peptides (Fig. 4*B*). The HBVA2–nano-APC induced better responses in ∼50% of patients (Fig. 4*B*, 4*C*, Supplemental Fig. 1, Supplemental Tables 1, 2). However, IL-2–HBVA2–nano-APC induced the strongest responses, with lymphocytes from most patients showing a strong CD8 T cell response (Fig. 4*B*, 4*C*, Supplemental Tables 1, 2). The increased responses by IL-2–HBVA2–nano-APC were not significantly related to viral load, HBVeAg, age, and sex (Supplemental Tables 1, 2), suggesting that IL-2–HBVA2–nano-APC improved effector function of viral-specific T cells rather than induced immune responses of naive T cells. Consistently, IL-2–A2–nano-APC, in the absence of antigenic peptide, did not induce IFN-γ production by CD8 T cells in any of the patient samples (Fig. 4*B*). Furthermore, we could not detect an increase in the proportion of Foxp3$^+$CD4$^+$ cells after stimulation with IL-2–HBVA2–nano-APC or IL-2–A2–nano-APC (data not shown). Thus, taken together with the induction of CMV_{nlv}-specific CD8 T cell responses, we have demonstrated that the addition of IL-2 into nano-APC effectively enhanced the response of Ag-responding T cells, which may be essential to overcome virus-induced T cell tolerance and the tolerogenic environment in chronic HBV patients.

IL-2–HBVA2–nano-APC improve TCR signaling and suppress the expression of PD-1 on HBV-responding CD8 T cells from chronic HBV patients

Evidence of T cell exhaustion has been found in both virus-specific and nonspecific CD8 T cells in chronic HBV patients (3, 10). Exhaustion induced by persistent antigenic stimulation effectively increases the threshold for TCR activation in response to Ag stimulation by suppressing TCR-proximal signaling pathways (12, 13). In addition, expression of PD-1, an inhibitory costimulatory molecule, was increased in CD8 T cells from chronic HBV, HCV, and HIV patients (5, 29). To investigate whether the enhanced CD8 T cell responses induced by IL-2–HBVA2–nano-APC resulted from improved TCR signaling and/or reduced expression of PD-1, we examined activation of ERK, which is repressed in tolerant T cells (13), and the expression of PD-1 on IFN-γ–producing CD8 T cells induced by either HBVA2–nano-APC or IL-2–HBVA2–nano-APC. The results demonstrated that the levels of ERK activation were enhanced in responding CD8 T cells from chronic HBV patients stimulated with IL-2–HBVA2–nano-APC compared with those stimulated by HBVA2–nano-APC (Fig. 5), suggesting that IL-2 can antagonize T cell tolerance by enhancing TCR-proximal signals. In addition to increased TCR signaling, the expression of PD-1 was reduced on IFN-γ–producing CD8 T cells induced by IL-2–HBVA2–nano-APC compared with those stimulated by HBVA2–nano-APC (Fig. 5). Although the cells were restimulated with PMA and ionomycin for 3 h before analysis, a similar level of pERK and PD-1 in IFN-γ–negative T cells suggests that IL-2–HBVA2–nano-APC improves TCR signaling and represses PD-1 expression on Ag-responding T cells compared with HBVA2–nano-APC. These results suggest that IL-2 on nano-APC reduces the TCR activation threshold and the expression of negative regulators in viral-responding CD8 T cells, two important factors needed to overcome T cell tolerance.

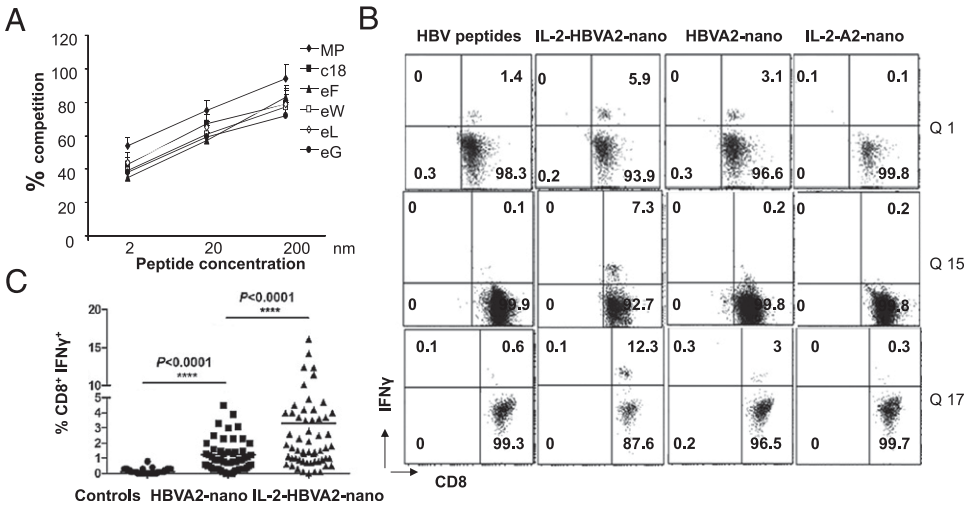

FIGURE 4. HBV-specific CD8 T cell responses were induced by delivering IL-2 to viral-specific T cells. *A*, Five HBV peptides reported to induce HLA-A2–restricted CD8 T cell responses in HBV patients (7) were analyzed for their ability to bind to HLA-A2 on IL-2–A2–nano-APC by a competition assay. Different concentration of these peptides was used in a competition assay with a ^{125}I-labeled A2-reporter MP peptide, and the percentage of reporter peptide that failed to bind to A2 was measured. Due to their similar binding affinities, these five peptides were pooled at a concentration of 1 μM each, and used to assemble HBVA2–nano-APC or IL-2–HBVA2–nano-APC. *B*, PBMCs from chronic HBV patients were incubated with the pool of five HBV peptides at concentration of 1 μM each or 10 μg/ml IL-2–HBVA2–nano-APC, 10 μg/ml HBVA2–nano-APC, or IL-2–A2–nano-APC for 5 d. After restimulation with PMA and ionomycin for 3 h, IFN-γ–producing CD8 T cells were quantified after gating on CD8 cells. *C*, Percentages of IFN-γ–producing CD8 T cells induced in 57 A2-positive chronic HBV patients. The CD8 T cell responses induced by IL-2–HBVA2–nano-APC or HBVA2–nano-APC for each patient were summarized in Supplemental Table 1.

IL-2–nano-APC increase CD4 T cell responses to viral Ags, leading to stronger CD8 T cell responses

Like CD8 T cells, virus-specific CD4 T cells are functionally defective in chronic HBV patients (4), consistent with our finding that persistent antigenic stimulation can effectively induce CD4 T cell tolerance. It has been reported that CD4 helper function is essential for the development and maintenance of CD8 T cell effector function (30). Previously, we have demonstrated that MHC II molecules in isolated ER vesicles can assemble with antigenic peptides under acidic conditions (23). The 721.221 cells are deficient in MHC I, but still express HLA-DR and DP molecules (18). Three HBV peptides reported to bind to DR and/or DP (24) were analyzed for their ability to assemble with nano-APC from A2-721.221 and IL-2-A2-721.221 cells. Consistent with previous findings (24), specific binding of all three peptides to nano-APC was detected under acidic conditions (Fig. 6*A*). A pool of the three peptides was assembled with DR and/or DP molecules on either nano-APC or IL-2–nano-APC to create HBVDR/DP–nano-APC or IL-2–HBVDR/DP–nano-APC, respectively. Peptide alone did not induce IFN-γ–producing CD4 T cells in peripheral lymphocyte populations isolated from chronic HBV patients (Fig. 6*B*, Supplemental Table 3). HBVDR/DP–nano-APC induced a weak response in a few patient samples (Fig. 6*B*), whereas significant CD4 T cell responses were induced in >50% of patient samples treated with IL-2–HBVDR/DP–nano-APC (Fig. 6*B*, Supplemental Table 3). Again, we did not find an increased proportion of Foxp3$^+$CD4$^+$ T cells in peripheral lymphocytes from chronic HBV patients after culture with IL-2–HBVDR/DP–nano-APC (data not shown). To investigate whether the induced Th1 CD4 responses can enhance viral Ag-responding CD8 T cell responses, patient lymphocytes were stimulated with both IL-2–HBVDR/DP–nano-APC and IL-2–HBVA2–nano-APC. The results showed that the induction of Th1 CD4 T cells increased the proportion of IFN-γ–producing CD8 T cells in response to IL-2–HBVA2–nano-APC (Fig. 6*C*), suggesting that CD4 responses are indeed important for the expansion of effector CD8 T cells. Therefore, the presence of IL-2 on nano-APC is important to drive optimal CD4 and CD8 T cell responses against HBV in lymphocytes from chronic HBV patients.

nano-APC form stable interactions with Ag-specific T cells as part of immune synapses

nano-APC are designed to directly deliver Ag, costimulatory, and cytokine signals to Ag-specific T cells. Therefore, a sustained interaction between nano-APC and Ag-specific T cells is essential for the T cells to engage IL-2, or other engineered cytokines, effectively. Previously, we have shown that ER vesicles containing pMHC molecules, generated by assembly of the MHC on the vesicles with

Table I. Clinical characteristics of chronic HBV patients

	Overall HBV	Queen Mary University of London	Shanghai
Number	57	20	37
Median age (range)	36 (17–69)	40 (17–69)	33 (18–52)
Sex (M:F ratio) % male	(42:15) 73.7	(13:7) 65	(29:8) 78.4
Viral load (log; range)	6.35 (3.27–9.96)	5.94 (3.27–9.96)	6.57 (4.31–9.13)
Median alanine aminotransferase (IU/l; range)	120.2 (20–430)	81.7 (30–211)	141 (20–430)
HBeAg status (% positive)	47.4	25	59.5

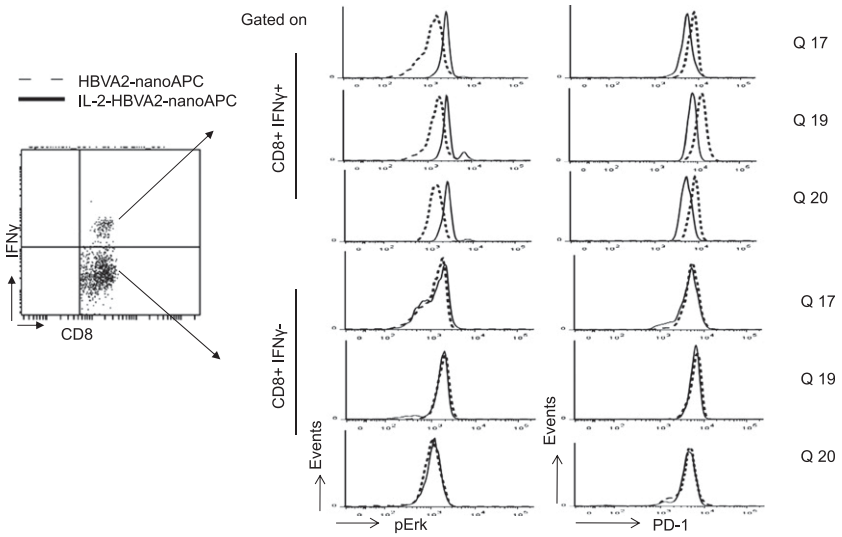

FIGURE 5. Enhanced TCR signaling and reduced expression of PD-1 on HBV-specific CD8 T cells. PBMCs from three chronic HBV patients, which showed weak responses to HBVA2–nano-APC, but strong responses to IL-2–HBVA2–nano-APC, were incubated with 10 μg/ml IL-2–HBVA2–nano-APC or HBVA2–nano-APC for 5 d. After restimulation with PMA and ionomycin for 3 h, cells were stained with anti-CD8, anti–IFN-γ, anti–PD-1, and anti–phospho-ERK. IFN-γ–positive or IFN-γ–negative CD8 T cells were gated for analysis of PD-1 expression or ERK phosphorylation.

peptide, can directly activate Ag-specific T cells in vitro (23). To further investigate whether the interaction of nano-APC and Ag-specific T cells can induce membrane clustering to form stable immune synapses (31), we examined the interaction of nano-APC, prepared from the murine DC cell line DC2.4 and assembled with peptide to form SIINFEKL–Kb complexes, with SIINFEKL-Kb–specific OTI T cells (23). An Ag-dependent interaction between nano-APC and T cells induced changes in the TCR distribution on the T cell membrane, leading to clustering of TCR complexes with nano-APC to form synapses (Fig. 7*A*). The process resembles the membrane processing observed at the contact point between live DC and T cells (31). The nano-APC–associated synapses can facilitate the recruitment of IL-2R into complexes with the T cell-bound IL-2–nano-APC, thus bringing the IL-2R into lipid rafts, which can sustain IL-2 activation in effector T cells (32). Thus, nano-APC can fully substitute for live DC, inducing T cell activation and the formation of immune synapses; this creates a stimulatory microenvironment allowing engagement of pMHC, costimulatory molecules, and engineered bioadjuvants with their respective receptors on Ag-specific T cells.

nano-APC are not endocytosed by DC in peripheral lymphoid organs

Most reported nano-delivery systems for therapeutic vaccines rely on endogenous DC in patients to process and present Ags to T cells (33). However, the nano-APC, functionally equivalent to live DC, act directly on Ag-specific T cells in vivo (23). Accumulation of a pharmacokinetic dose of free nano-APC in DC-rich organs such as lymph nodes and liver will be important to effectively induce activation of Ag-specific T cells. Previously, we found that ER membrane vesicles prepared from DC accumulated in the peripheral lymphoid organs and were not endocytosed by DC in vitro (23). However, whether the nano-APC are endocytosed by DC in lymphoid organs is not known. To investigate whether nano-APC remain in lymphoid organs as free nano-particles, nano-APC prepared from murine DC2.4 cells were labeled with fluorescence

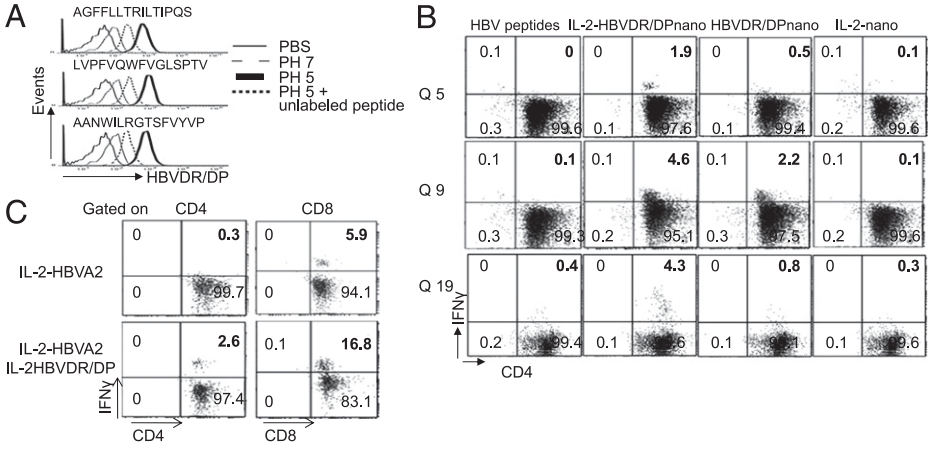

FIGURE 6. CD4 Th function is important for the responses of HBV-specific CD8 T cells. *A*, Three HBV peptides reported to bind to DR and/or DP and induce CD4 T cell responses in HBV patients (25) were examined for their ability to interact with DR and/or DP on nano-APC. A total of 100 nM of these FITC-labeled peptides was incubated with 10 μg IL-2–A2–nano-APC under different pH. After removal of unbound peptides, binding to nano-APC was assessed by flow cytometry. The specificity of binding was measured by addition of unlabeled peptide at a concentration of 500 nm. These three peptides were pooled at 1 μM each used to assemble with DR and/or DP on nano-APC. *B*, Induction of IFN-γ–producing CD4 T cells in PBMCs from chronic HBV patients. PBMCs from indicated patients were incubated with 10 μg/ml IL-2–HBVDR/DP–nano-APC, HBVDR/DP–nano-APC, or IL-2–A2–nano-APC for 5 d. After restimulation with PMA and ionomycin for 3 h, IFN-γ–producing CD4 T cells were quantified after gating CD4 cells. *C*, PBMCs from chronic HBV patient were incubated with 20 μg/ml IL-2–HBVA2–nano-APC or 10 μg/ml IL-2–HBVA2–nano-APC and 10 μg/ml IL-2–HBVDR/DP–nano-APC for 5 d. After restimulation with PMA and ionomycin, IFN-γ–producing CD4 or CD8 cells were quantified after gating on CD4 or CD8 cells. The presented data are from patient Q1 (Supplemental Table 1).

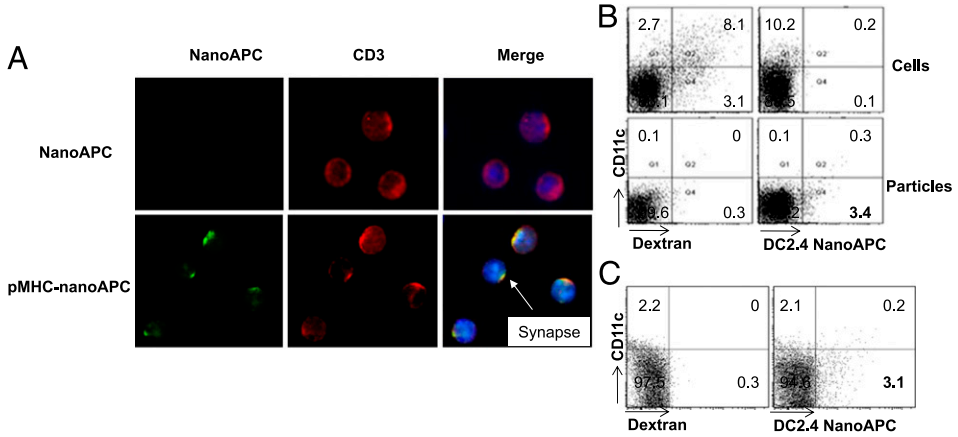

FIGURE 7. Nano-APC induce immune synapse formation on Ag-specific T cells and remain as free particles in lymphoid organs in vivo. *A*, Kb–nano-APC, prepared from murine DC2.4 cells, were labeled with FITC and assembled with or without SIINFEKL peptides. SIINFEKL-Kb–specific OTI CD8 T cells were incubated with SIINFEKL–Kb–nano-APC or Kb–nano-APC for 30 min. After removal of unbound nano-APC, cells were stained with anti-CD3 Ab, counterstained with DAPI, and assessed for immune synapse formation by confocal microscopy. *B*, FITC-labeled dextran or DC2.4-derived nano-APC was injected i.v. at 1 mg/kg to C57BL/6 mice. Forty-eight hours postinjection, lymph nodes were isolated and separated into cellular and cell-free fractions. Both fractions were stained with anti-CD11c and then analyzed by flow cytometry. *C*, C57BL/6 mice were infected with vaccinia virus at dose of 2×10^5 PFU. Five days postinfection, FITC-labeled dextran or DC2.4-derived nano-APC were injected i.v. at 1 mg/kg. Forty-eight hours postinjection, lymph nodes were isolated and the cell-free fraction was stained with anti-CD11c and analyzed by flow cytometry.

and injected i.v. into B6 mice. After 48 h, the lymph nodes were isolated and separated into cellular and cell-free fractions. The cell-free fragments and DC from the lymph nodes were examined for the presence of free and endocytosed nano-APC. In contrast to the efficient endocytosis of dextran by DC in lymph nodes, the nano-APC that accumulated in the lymph nodes remained as free particles (Fig. 7*B*). To exclude the possibility that DC activation is required for phagocytosis of the ER membrane vesicles, we infected mice with a vaccinia virus to induce DC activation 5 d before injection of nano-APC. Similar levels of free nano-APC were detected in lymph node samples from mice pretreated with vaccinia and mice that had not received pretreatment (Fig. 7*C*), indicating that the intracellular membrane vesicles from APC were not effectively endocytosed by DC even with a concomitant viral infection. The lack of endocytosis of nano-APC may be due to failure of pattern recognition receptors, which enable the DC to discriminate between normal physiological components and pathological particles in vivo (34), to recognize the nano-APC.

Discussion

T cells are essential for the clearance of HBV postinfection (30). Like most chronic viral infections, persistent HBV infection results in functional defects in virus-specific T cells largely due to immune tolerance (unresponsiveness) induced by continual stimulation by viral Ags and the tolerogenic environment induced by HBV (4). The unresponsiveness of virus-specific T cells results from downregulation of TCR signaling pathways and upregulation of suppressive molecules such as PD-1 (5), tolerogenic mechanisms common to many tolerance models induced by persistent antigenic stimulation (12, 13). Although IL-2 is an important cytokine in modulation for effective function of T cells and it is required for the expansion of effector T cells and effector function of memory T cells (28), application of IL-2 as a bioadjuvant for immunotherapy is hampered by organ pathology induced by therapeutic doses and the expansion of Treg cells, leading to the suppression of effector T cells (28). By immobilizing IL-2 on membrane nano-particles prepared from bioengineered APC, we have shown that IL-2–nano-APC can potently induce effector functions in virus-responding T cells, by providing an immunostimulatory microenvironment to Ag-specific T cells and delivering IL-2 to Ag-responding T cells, without the activation of bystander T cells or organ pathology. The impaired T cell function in chronic HBV infection resembles, in many respects, T cell tolerance induced by Ag persistence (4, 12, 13). Indeed, studies have shown that the proximal TCR signaling molecule CD3ζ is downregulated, whereas the expression of the inhibitory molecule PD-1 is upregulated in CD8 T cells from chronic HBV patients (10). IL-2 on nano-APC is able to enhance TCR-proximal signaling and downregulates PD-1 expression on virus-responding CD8 T cells from chronic HBV patients, which could effectively reverse tolerance, as demonstrated by induction of IFN-γ–producing CD8 T cells in lymphocytes from chronic HBV patients. In addition to TCR signaling, MAPK activation can directly result from IL-2R signaling (28). It has been found that the activation of MAPK and PI3K through Shc recruited by the IL-2R is independent on STAT5 signaling in effector T cells, which differs from that in Treg cells, and is important for the expansion of activated CD8 T cells (35). We have demonstrated that nano-APC can induce CD25 expression and immune synapse formation, which not only execute the function to induce T cell activation, but also bring engineered bioadjuvant such as IL-2 stably into signalsomes of effector T cells (36). The increased expression of CD25 on CMV Ag-specific CD8 T cells by IL-2–CMV_{nlv}A2–nano-APC is consistent with the well-known observation that IL-2 can induce CD25 expression on preactivated CD8 T cells. Thus, together with pMHC and costimulatory molecules, the selective delivery of IL-2 is important to induce activation of HBV-responding T cells from chronic HBV patients. As the overall pharmacological dose remains low, the IL-2–nano-APC do not activate Treg cells, indicating that this approach can be adapted for use with other bioadjuvants.

Expression of PD-1 is low in memory CD8 T cells, but it is upregulated in chronic viral infections; this leads to defects in proliferation and production of effector cytokines, which facilitate viral persistence (37). The enhancement of HBV-Ag–dependent CD8 T cell effector function by IL-2–HBVA2–nano-APC was associated with downregulation of PD-1, suggesting that the expression of PD-1 on T cells may be associated with impaired function of those T cells. Indeed, PD-1 expression correlates with

viral load, declining CD4 count, and impaired function of CD8 T cells in HIV infection, and with CD8 T cell exhaustion in chronic HCV infection (37).

The function of CD4 T cells is essential for an effective and long lasting antiviral CD8 response and viral clearance (30). Persistent viral infection can also induce CD4 T cell tolerance via mechanisms similar to those that induce CD8 T cell tolerance (13). We found that IL-2–HBVDR/DP–nano-APC induced strong CD4 T cell responses, which further enhanced the response of HBV-responding CD8 T cells to IL-2–HBVA2–nano-APC, in lymphocytes from chronic HBV patients. However, in all the cases analyzed, the proportion of CD4 cells that responded was lower than the proportion of responding CD8 cells. This may be due to the fact that CD8 T cells are more sensitive to IL-2 than CD4 T cells (28). Other possible reasons are that the level of MHC II molecules expressed on nano-APC prepared from 721.221 seed cells is lower than the level of HLA-A2 (18) or that the pMHC II complexes, generated by assembly with the selected HBV peptides, are not optimal for induction of CD4 T cell responses. We have developed effective screening methods to define the binding affinity of peptides to MHC I or II on nano-APC, which can be used to select optimal viral antigenic peptides from viral peptide databases (23).

Nano-particles prepared from synthetic materials or genetically engineered microbes have been used to deliver Ags to DC for induction of antiviral or anticancer immune responses (33). In contrast to these particles, nano-APC are prepared from the ER membranes of bioengineered APC. Therefore, they are not only more biocompatible than synthetic nano-particles or microbars, but also deliver therapeutic molecules that are physiologically synthesized by APC seed cells. Thus, the IL-2 on IL-2–nano-APC is more stable than free IL-2 in vivo, and maintains its physiological conformation, allowing optimal interaction with the IL-2R (data not shown). Unlike other nano-particle–based vaccines, nano-APC directly activate T cells. The nano-APC mimic live DC to induce lipid raft clustering on T cells and formation of an immunological synapse, which is essential for T cell activation. Furthermore, using HLA-I–negative 721.221 cells as seed cells allows us to specifically express selected HLA alleles, allowing construction of HLA allele-matched nano-APC for individual patient populations.

Previously, we observed nano-APC homing to T cell areas of peripheral lymphoid organs, largely due to the expression of homing receptors by the cells from which the nano-APC are derived (23). We have now further demonstrated that nano-APC are not effectively endocytosed by DC in vivo, which is important, as it allows the nano-APC to remain as free particles in peripheral lymphoid organs. The absence of endocytosis may be due to the lack of molecules on nano-APC recognized by DC pattern recognition molecules (34).

In chronic HBV infection, T cells are continuously confronted with moderate to high levels of viral Ags (4), which, in combination with the induced immunosuppressive microenvironment resulting from high viral load and dysregulated immune responses, lead to the downregulation of T cell activation and, subsequently, reduction of effector function (4). Therefore, one of the major challenges for immunotherapy against chronic infectious diseases, such as HBV, is the development of delivery vehicles targeting Ag-specific T cells that provide not only Ag, but also designed bioadjuvant(s) that can restore effector function. Our results demonstrate that the ability to deliver bioadjuvant, as well as pMHC, to virus-specific T cells, to generate specific nano-APC for each individual HLA-shared patient group, and to directly activate Ag-specific T cells are advantages of nano-APC over current nano-particles used for immunotherapy of chronic HBV and other chronic viral infections.

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Disclosures

The authors have no financial conflicts of interest.

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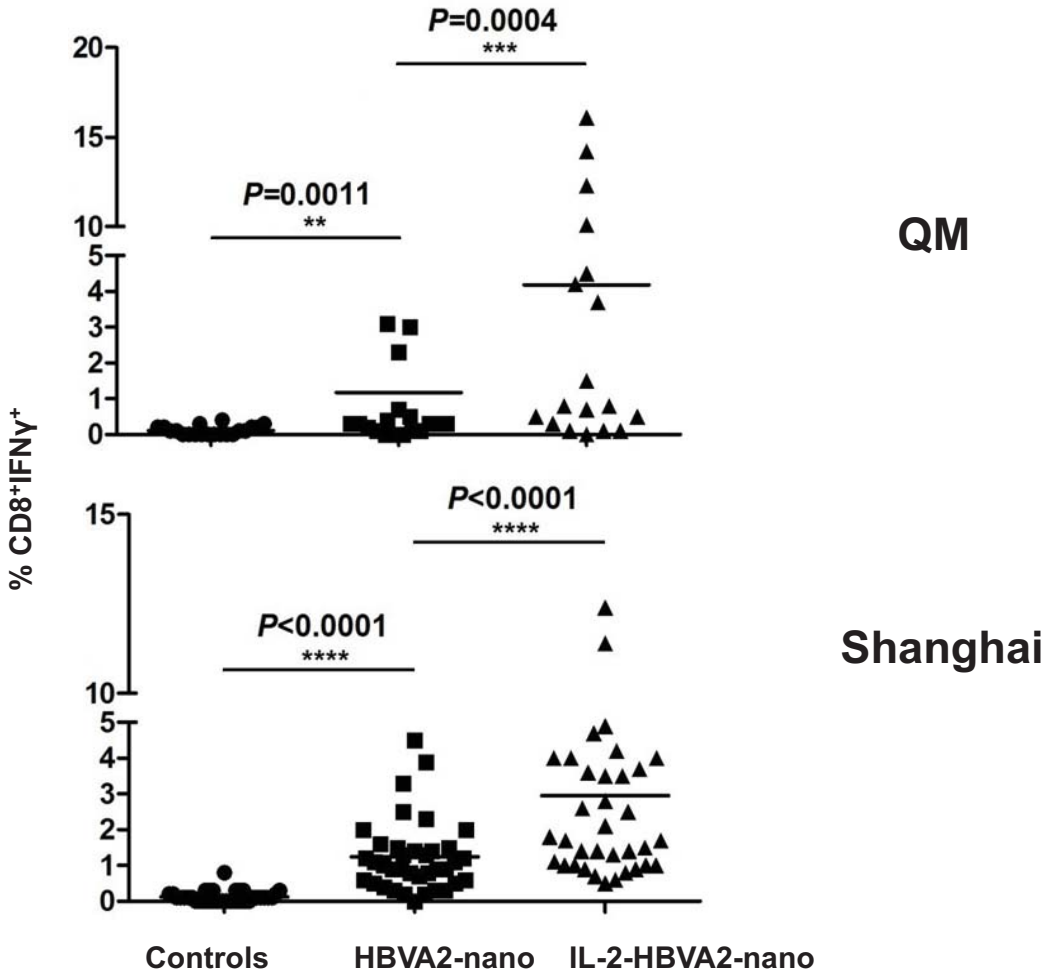

Supplementary data Fig. 1. Summary of CD8 T cell responses from two clinical studies of HLA A2 positive chronic HBV patients induced by HBVA2-nanoAPC or IL-2-HBVA2-nanoAPC. Unstimualted PBMC from same patient served as controls.

Subject	ALT	HBeAg	Viral load	Sex	Age	IL-2M-I	M-I	Control
Low viral load	(IU/liter)		(log)			CD8$^+$IFNγ$^+$(%)		
Q1	39	N	5.28	F	36	5.9	3.1	0.1
Q2	37	N	3.27	M	44	0.8	0.3	0.1
Q3	24	N	4.58	M	69	0.8	0.1	0
Q4	53	N	5.98	M	23	10.1	0.7	0.2
Q5	24	N	5.84	M	42	0.5	0.4	0
Q6	36	N	4.67	F	44	3.7	0.3	0.3
Q7	127	N	4.63	M	26	1.5	0.3	0.4
Q8	211	N	5.22	M	33	4.2	2.3	0.1
Q9	97	P	4.05	F	56	0.3	0.1	0
Q10	206	N	5.27	M	51	16.1	5.4	0.2
Q11	79	N	5.58	M	52	0.5	0.3	0
Q12	44	N	5.11	M	42	0.7	0.3	0
Q13	83	N	4.49	M	57	0	0	0
Average	81.54	P/N=1/12	4.92	F/M=3/10	44.23			
High viral load								
Q14	32	P	7.99	F	35	0.1	0	0
Q15	65	N	7.14	F	58	7.3	0.2	0.2
Q16	30	P	7.45	M	17	0.1	0	0.1
Q17	45	P	7.31	M	18	12.3	3	0.3
Q18	202	N	8.37	F	27	0.1	0.1	0
Q19	31	P	9.96	M	28	4.5	0.5	0.1
Q20	169	N	6.65	F	36	14.2	6.2	0.2
Average	82	P/N=4/3	7.84	F/M=4/3	31.29			
Total Average	81.7	P/N=5/15	5.94	F/M=7/13	39.7			

Supplementary data Table 1. Patient characterization and CD8 T cell response induced by nanoAPC for each patient from QM study.

Subject Low viral load	ALT (IU/liter)	HBeAg	Viral load (log)	Sex	Age	IL-2M-I	M-I	Control
						$CD8^+IFN\gamma^+$ (%)		
F1	430	P	4.68	M	33	11.4	4.5	0.3
F2	121	N	5.79	M	37	1.7	1.4	0.1
F3	42	N	5.21	M	44	1.3	1.3	0.3
F4	73	N	5.58	M	23	1.4	1.2	0.3
F5	362	P	4.31	M	32	2.6	0.7	0
F6	140	N	5.38	M	40	12.4	1.1	0
F7	170	P	5.42	M	27	7.8	0.5	0
F8	120	P	5.49	M	29	3.6	3.3	0
F9	278	P	5.83	M	40	4.9	2.3	0.3
F10	40	P	5.87	M	25	0.9	1.5	0
F11	423	P	5.63	M	33	2.5	0.8	0.2
Average	199.91	P/N=7/4	5.38	F/M=0/11	33			
High viral load								
F1 2	135	P	7.23	F	52	0.5	0.3	0
F13	110	P	7.54	M	22	4	2.5	0.1
F14	143	N	7.03	F	32	3.5	2	0.1
F15	188	N	6.13	M	19	1.8	1.6	0
F16	194	P	7.05	F	41	3.5	1.5	0.1
F17	267	N	9.13	M	22	1	1	0
F18	126	P	6.23	M	19	1	0.3	0
F19	20	P	7.04	F	40	1.4	0.5	0
F20	313	N	6.32	M	38	1	0.9	0.1
F21	212	P	9.12	M	47	1.5	0.2	0
F22	122	P	7.15	M	32	1.7	1.2	0.1
F23	35	N	7.24	M	20	4.2	1.3	0.3
F24	23	N	7.24	F	37	1.1	0.8	0.2
F25	46	N	6.09	F	38	0.8	0.3	0.8
F26	310	N	7.61	M	34	4	0.4	0.1
F27	76	N	7.14	M	46	0.6	0	0.1
F28	211	N	7.54	M	33	1	0.6	0.1
F29	79	N	6.23	F	52	3.7	0.2	0
F30	35	P	6.34	M	26	4	2	0
F31	46	P	7.38	M	18	2.1	1.1	0.3
F32	80	P	7.24	F	27	5.6	0.9	0
F33	43	P	7.23	M	18	4.7	3.9	0.2
F34	86	P	7.42	M	30	2.8	0.9	0.3
F35	47	P	7.86	M	41	0.9	0.9	0.1
F36	42	P	6.1	M	28	0.7	0.6	0
F37	140	P	6.5	M	32	0.3	0.1	0
Average	120.35	P/N=15/11	7.12	F/M=8/18	32.46			
Total Average	141.11	P/N=22/15	6.61	F/M=8/29	32.64			

Supplementary data Table 2. Patient characterization and CD8 T cell response induced by nanoAPC for each patient from Shanghai study.

Subject	ALT	HBeAg	Viral load	Sex	Age	IL-2M-II	M-II	Control
Low viral load	(IU/liter)		(log)			$CD4^+IFN\gamma^+$ (%)		
Q1	39	N	5.28	F	36	2.6	0.5	0
Q3	24	N	4.58	M	69	1.4	0.2	0.1
Q5	24	N	5.84	M	42	1.9	0.5	0
Q9	97	P	4.05	F	56	4.6	2.2	0.1
F4	73	N	5.58	M	23	0.9	0.8	0
F5	362	P	4.31	M	32	0.6	0.2	0
F8	120	P	5.49	M	29	0.9	0.6	0.1
F10	40	P	5.87	M	25	1.7	0.3	0
Average	97.38	P/N=4/4	5.13	F/M=2/6	39			
High viral load								
Q19	31	P	9.96	M	28	4.3	0.8	0.4
F12	135	P	7.23	F	52	0.6	0.2	0.2
F13	110	P	7.54	M	22	3.6	1.5	0
F14	143	N	7.03	F	32	5.6	2.9	0.1
F18	126	P	6.23	M	19	0.9	0.2	0
F19	20	P	7.04	F	40	1	0.3	0
F20	313	N	6.32	M	38	0.7	0.6	0
F21	212	P	9.12	M	47	0.5	0.2	0
F23	35	N	7.24	M	20	1.9	0.5	0.1
F24	23	N	7.24	F	37	0.5	0.3	0
F25	46	N	6.09	F	38	0.7	0.1	0.1
F26	310	N	7.61	M	34	3.9	1.9	0.2
F27	76	N	7.14	M	46	0.3	0.5	0.1
F28	211	N	7.54	M	33	2.7	3	0.2
F29	79	N	6.23	F	52	1.4	1.7	0
F30	35	P	6.34	M	26	0.8	0.1	0.1
F31	46	P	7.38	M	18	3.4	3.2	0
F35	47	P	7.86	M	41	2.9	2.7	0.3
F36	42	P	6.1	M	28	3.5	2.4	0.1
F37	140	P	6.5	M	32	0.1	0.1	0
Average	109	P/N=11/9	7.19	F/M=6/14	34.15			
Total Average	108.15	P/N=15/13	6.65	F/M=8/20	35.52			

Supplementary data Table 3. Patient characterization and CD4 T cell response induced by nanoAPC for each patient included in this study.