MICROSOMAL MEMBRANES FROM ANTIGEN PRESENTING CELLS PRESENT ANTIGENIC PEPTIDES TO T CELLS

A NOVEL APPROACH IN VACCINE DEVELOPMENT

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To my Mum and Dad.

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

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. Nonetheless, vaccination fails to protect against a wide range of acquired chronic infections caused by viruses, such as HIV and HCV, other intracellular pathogens, and cancer. Attempts to combat these diseases are thought to require the induction of the cellular arm of the immune response, in which dendritic cells (DCs) play a key role. Thus, DCs are now considered a promising target/tool when designing new-generation vaccines.

Although mature DCs have the capacity to induce effective primary and secondary immune responses *in vivo*, their use in vaccination strategies is associated with several difficulties; for example, there are limitations involved in the loading of antigen, and in the appropriate maturation of DC *in vitro*.

In this study, we have explored the hypothesis that the use of ER-enriched microsomes isolated from professional antigen presenting cells, such as DCs, can represent an alternative vaccination strategy to those using live DCs.

Endoplasmic reticulum-enriched microsomal membranes (microsomes) isolated from DCs contained high levels of peptide-receptive major histocompatibility complex (MHC) and co-stimulatory molecules. After loading with defined antigenic peptides, injected microsomes mediated MHC class I- and MHC class II-restricted T cell responses.

The microsomal vaccine described and discussed in this thesis protects from a viral infection and was shown to regress an established murine tumor. Therefore, it could represent an exciting new alternative to currently available vaccine strategies.

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ABBREVIATIONS

Ab	Antibody
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
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
MDSC	myeloid derived suppressor cells
MHC	Major Histocompatibility Complex
min	minutes
MP-VV	Vaccinia Virus expressing Influenza A matrix protein
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	tumour growth factor
T_H	Helping effector T cell
TipDCs	tumour-necrosis factor (TNF)/ inducible nitric oxide synthase producing DCs
TLR	Toll-like receptor
Treg	Regulatory T cell
TTAs	tumor-associated antigens
β2m	β-2microglobulin

Acknowledgments

Contributions

In the following result sections the named individuals contributed to the indicated experiments.

Section 2.2: Electron micrograph of microsome preparation (Figure 26) provided by Ping Wang.

Section 2.4: Western blot analysis of co-stimulatory molecules (Figure 30C) was done by Su-ling Li.

Section 2.5: The biotinylated $OVA_{324-340}$ used for these experiments (Figure 32 and 32) was provided by Ping Wang.

Section 2.5: The experiment for the visualization of cross-linked H2-K^b molecules with a radio-labeled modified peptide (Figure 33) was done with the help of Ping Wang.

Section 3.4: The RMA-S/GFP/E19K transfected cell line that was used in these experiments (Figure 39B and 40B) was created in the lab by Salah Mansour.

Section 4.2 and 4.3: The *in vivo* assays of viral infection and tumour were done in collaboration with Su-Ling Li. All the vaccines for these assays were created by me, except where an autologous DC-derived vaccine was used. The *ex vivo* analysis of T cell responses for the viral and tumor assays was done by Su-Ling Li.

Section 5: The HCMV peptide and the human IFNγ ELISpot plates used in this assay were provided by Suzanna McDonald. The HLA-A2 staining of human PBMCs was done with the help of Salah Mansour. The staining of 721.221- and 721.221/A2- derived microsomes was done by Ping Wang (Figure 54).

INTRODUCTION

In 430 BC, a disease struck the city of Athens. Thucydides, who himself had suffered and survived the infection, recorded his observation that people who had recovered from a previous bout of disease could then go on to nurse the sick without contracting the illness for a second time themselves (Figure 1).

Figure 1   **Plague of Athens (430BC).**

The Greek historian left all speculation as to its origin and its causes to other writers and he concentrated on the description of the symptoms. Based on those descriptions, he recognised the difference between resistance and acquired immunity. His open-minded approach of gathering evidence and analysing it in terms of cause and effect without reference to intervention by the gods was the foundation for the development of Medicine.

Figure 2   **Dr Edward Jenner.**

Hundreds of years later, in 1796, Dr Edward Jenner (Figure 2) discovered that inoculating healthy individuals with vaccinia (Figure 3) induced protection against human smallpox, until then an often deadly disease. He didn't have any knowledge of the existence of pathogens, yet his experiments were the introduction to our Vaccines and the basis of Immunology.

Figure 3   **The discovery of vaccination.**

Dr Jenner discovered that milkmaids who came into contact with cowpox seemed to be immune from contracting smallpox. He inoculated patients with discharge of the virus (*vaccinia*) from cows (in Latin cow = vacca, hence the term vaccination) to generate an immune response which cross-reacted and offered protection against smallpox.

Numerous scientists followed up Jenner's work, but it wasn't until the late 19th century that Robert Koch showed that specific diseases were caused by specific pathogens. Taken together, Jenner's experiments and the knowledge generated by Koch and others provided a foundation for Luis Pasteur, who, in the 1880s prepared a vaccine against rabies to treat a boy bitten by a rabid dog. Luis Pasteur knew that what Thucydides and Jenner had previously observed was a mechanism of protection, which he successfully exploited against rabies; the search to understand this mechanism gave birth to Immunology.

From Jenner to this work.

From the time when Jenner introduced the first vaccine, the study of the immune system has provided insight into the mechanisms of protection induced by vaccination. Vaccines aim to provide life-long protection by inducing a prolonged state of immunological readiness, which, normally, can only be reached by engaging pathogen in a full-blown infectious setting (Pennington 2009- *in print*).

The miracle of the immune system is that it consists by cells that are made to recognise every possible antigen before they encounter it. Since there are millions of antigens, we have cells with millions different receptors, which means that for every given antigen only a few numbers of cells have receptors that will recognise it. Upon pathogen recognition, the immune system instucts those cells to multiply and respond so as to provide protection against this pathogen. Following an infectious episode, the immune system has the ability to preserve an increased frequency of protective pathogen-specific cells that upon re-infection are induced to respond rapidly and efficiently (Kaech, Wherry et al. 2002). However, a major weakness of the immune system is the time required between pathogen recognition and the acquirement of protective immune responses. Vaccination capitalises on the specificity and inducibility of immunity and attempts to mimic the interactions between the infectious agent and the immune system. The goal of vaccination is to prepare the immune system in advance to respond faster and better to infectious agents, but in the absence of the very great dangers of disease.

Successful vaccines are now used to control the spread of naturally-occurring diseases such as smallpox, polio, measles, mumps, rubella, influenza, chickenpox, diphtheria, tetanus, pertussis and rabies. Clinically protective vaccines are potent in the induction of neutralizing antibody responses and the importance of protective antibodies is reflected in successful vaccination against certain viruses in childhood. Nonetheless, despite concerted efforts, vaccines remain unavailable for tuberculosis, leprosy, malaria, hepatitis C, leishmania, dengue fever, HIV/AIDS and cancer.

Compelling evidence suggests that attempts to combat these diseases also require the induction of the cellular arm of the immune response. Indeed, T-cell mediated immunity is thought to be essential for eradicating virally infected or malignantly transformed cells. However, all current vaccines that are clinically protective are dependent on neutralizing antibody responses (Letvin 2007), but not exclusively on T cell-mediated immunity (Zinkernagel and Hengartner 2006). Such responses are not sufficient to induce protection against pathogens that cause chronic infections.

Individuals infected with such pathogens not only act as a reservoir for spread of the infectious agent, but chronic infections and cancer in general also increase the risk of subsequent diseases and secondary infections with other pathogens. The inadequacy of our existing vaccines to protect against these diseases underscores the necessity for a new generation of vaccines that aid cell-mediated acquired immunity in addition to humoral immunity. The rationale behind developing those vaccines is based on the concept that during chronic infections or cancer, the critical antigens that induce protection are not appropriately presented to the immune system (Ha, West et al. 2008); a vaccine that provides the right antigen in an immunogenic form to suitably activate the immune system may overcome this deficiency.

The understanding of the function of cell-mediated immune responses has led to exploration of novel vaccines with the use of antigen-armed antigen presenting cells (APCs) against infectious diseases and cancer. Dendritic cells (DCs), the most potent APCs, have been used to elicit protective T cell immune responses to viral infections and cancer in mice and humans with defined antigens (Banchereau and Steinman 1998). Although some initial promising results were reported, clinical applications have been limited. This is largely because of difficulties in the quality control of DC differentiation that led to immunogenic heterogeneity of matured DCs in induction versus suppression of T cell responses, compromising the desired immunological outcome.

This study attempts to investigate the optimal reconstitution of the immune properties of the antigen presenting cell on endoplasmic reticulum derived membranes, namely microsomes. The aim is to overcome the deficiencies of current vaccination approaches and create a vaccine that will promote appropriate and specific combinations of immune responses targeted to particular pathogens, especially those against which the more basic approaches of vaccination have so far failed.

This introduction to this research begins by outlining the structure, the components and the function of the immune system. Next, it concentrates on the characteristics of T cells and their functional specialization during the immune response. Subsequently, it identifies the immune effector mechanisms responsible for protection against acute resolving infections and compares them with the immuno-compromised environment generated during chronic viral infections and cancers. From this, it is possible to infer the major cause of failure of current vaccination to protect from persistent diseases, and propose the required elements of a successful vaccine. The development of new generation vaccines, with particular focus on dendritic cell-based vaccination, is reviewed and the described systems are evaluated. Based on the observations a novel vaccination strategy is proposed.

This thesis describes an alternative approach to current vaccination strategies for both protective and therapeutic applications.

1. The immune system.

The immune system consists of tissues, organs, cells and molecules that work together to facilitate host defence. It detects, distinguishes and eliminates harmful pathogens by orchestrated mechanisms that are collectively called the Immune Response.

1.1 The cellular mediators of the immune system.

All the cells of the immune system originate from pluripotent hematopoietic stem cells in the bone marrow. These highly undifferentiated progenitor cells divide to produce two different types of stem cells with less differentiating plasticity, the common myeloid progenitor and the common lymphoid progenitor, from which all the functional cells of the immune system will develop (Figure 4). 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 NK cells.

Granulocytes, which owe their name to their dense granular cytoplasm, are characterised by a short life span and are detected in increased numbers during immune responses. Mainly involved in the initial stage of the immune response, their role is principally secretory, although neutrophils are also very potent phagocytic cells, together with macrophages and dendritic cells. The latter two, in addition to their phagocytic function, have the capacity to present antigens to T cells and together with B cells they represent the professional antigen presenting cells (APC) of the immune system.

Mature T and B cells circulate between the blood and the peripheral lymphoid tissues and they are very specific with respect to the antigen they can recognise; this feature makes them the principal mediators of an organised adaptive immune response launched against a specific pathogen. In contrast, NK cells lack this specificity and are thought to recognise a 'missing self' state through sets of activating and inhibitory receptors.

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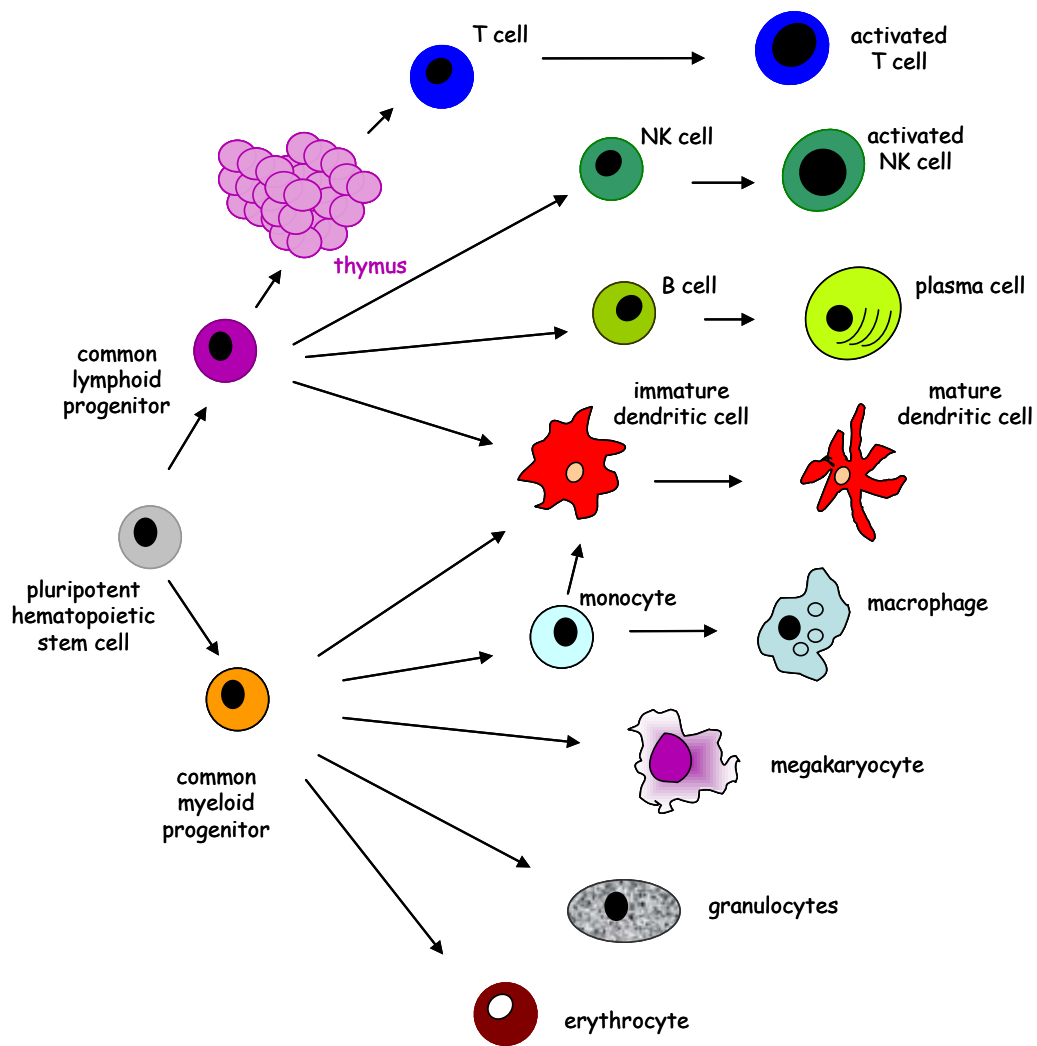
Figure 4   **Origin of cells of the immune system.**

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 (polymorphonuclear leukocytes), megakaryotes (cells that produce platelets) and macropages. Dendrtic cells derive from a common myeloid progenitor, although some dendritic cell subsets derive from a monocyte intermediate or from a lymphoid common progenitor.

1.2 Other mediators of the immune system.

Antibodies or immunoglobulins are proteins that can recognise and bind to whole antigens. They are produced by naïve B cells and activated plasma cells, and they are of generally similar structure, but have unique specificity for antigen. They have the capacity to induce pathogen neutralization, which is the process of inhibiting the infectivity or toxicity of the pathogenic agent, while they also provide specific signals and precise targets to appropriately direct other components of the immune response.

Cytokines are secreted proteins that influence the behaviour of cells via binding to specific receptors on the plasma membrane. Chemokines are proteins with chemoattractant properties that stimulate the directed migration of cells. Cytokines and chemokines create a certain environment at the site of infection that informs the immune response; this environment helps target antigens to the appropriate antigen presenting cells, it initiates APC maturation, it triggers cell migration to the peripheral lymphoid organs and it regulates T cell effector differentiation and antibody mediated immunity.

1.3 The structure of the immune system.

The lymphoid organs are structured tissues containing large numbers of immune cells in a framework of non-lymphoid cells. They can be generally divided into the central (or primary) lymphoid organs, where lymphocytes are generated, and the peripheral (or secondary) lymphoid organs, where adaptive immune responses are initiated and lymphocytes are maintained. Central lymphoid organs are the bone marrow and the thymus in which B cells and T cells develop, respectively. The secondary lymphoid organs are the spleen, the lymph nodes and the 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 mammalian immune response comprises of two phases known as the innate and adaptive immunity.

1.4.1 Innate immune response.

Innate immunity is the first line of host defence against pathogens. It is based on mechanisms that are not antigen-specific (interferons, complement, natural killer cells, activated phagocytes), which compose an important part of host defence (Zinkernagel 2003). The innate immune system has the ability to functionally distinguish harmless self from infectious non-self agents through pattern recognition mechanisms. Innate immunity relies on receptors such as Toll-like receptors (TLR) that evolved to recognize conserved patterns on different classes of pathogens to trigger an inflammatory response that limits pathogen invasion (Janeway and Medzhitov 2002; Akira, Uematsu et al. 2006; Cooper and Alder 2006). Recognition of a conserved pattern, whether in soluble form or as cell surface ligands, provokes activation of these receptors, signalling the presence of 'danger' in the body. In response, macrophages and dendritic cells are triggered to take up, process and present antigens locally or after migrating to nearby lymph nodes where they activate naïve and memory cells. Thus, antigen presentation bridges innate and adaptive immune responses (Figure 5).

Innate immunity employs mechanical, chemical, microbial and cellular means to guard against pathogens. It is an inherent immune strategy that aims to prevent the establishment of infection; even when failing to clear an infection the innate immune response provides information that regulates the ensuing adaptive immune response.
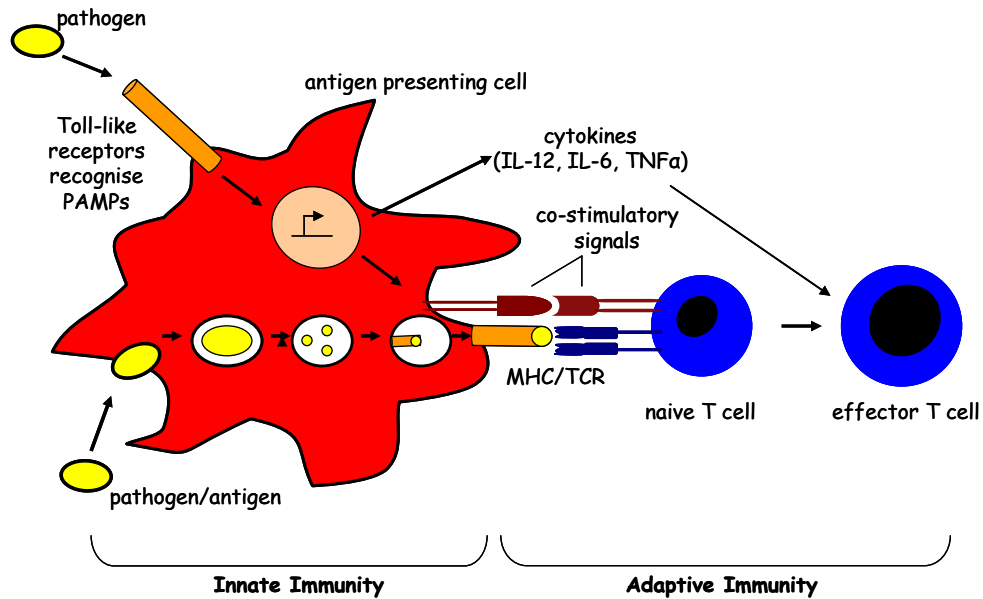
Figure 5   **Innate immunity is critical to adaptive immune response.**

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 deliver antigenic peptides and co-stimulatory signals to naïve T cells. In an inflammatory environment, a naïve T cell that has recognised an antigen differentiates into effector cell that can eliminate the pathogen.

1.4.2 Adaptive immune response.

Adaptive immunity is involved in the elimination of pathogens based on recognition of antigens, which are specific molecules that elicit an immune response, and includes the generation of immunological memory (Akira, Uematsu et al. 2006). Initiated in the peripheral lymphoid organs, the adaptive immune response involves two 'arms' of protection, the cell-mediated response and the humoral response. The cell-mediated response involves T cells instructing largely cellular components of the immune system, such as cytotoxic T cells (CTLs), while the humoral response involves T cell help to B cells that boosts Ab-mediated immune responses (Figure 6).

T cells are divided into two major subtypes; they are cytotoxic T cells and helper T cells, which are recognised by the expression of CD8 and CD4 cell-surface proteins, respectively. CD8$^{(+)}$ T cells recognize antigen presented by MHC class I and respond by elucidating cytolytic function against the antigen-bearing target cells. CD4$^{(+)}$ T cells recognize antigen presented by MHC class II; upon activation they differentiate into various subgroups including those essential for B cell function and expansion of CD8$^{(+)}$ T cells. Their differentiation is determined by the presence of certain cytokines, when activated in the secondary lymphoid organs by DCs.

Humoral immune responses are initiated by the direct interaction of antigens with receptors on B cells, which leads to B cell differentiation and, in the presence of appropriate T cell help leads to the production of high affinity specific antibodies. Antibody-binding to pathogen and pathogen-derived proteins represents a major mechanism of immune protection.

Mechanism of immunological protection by adaptive immunity
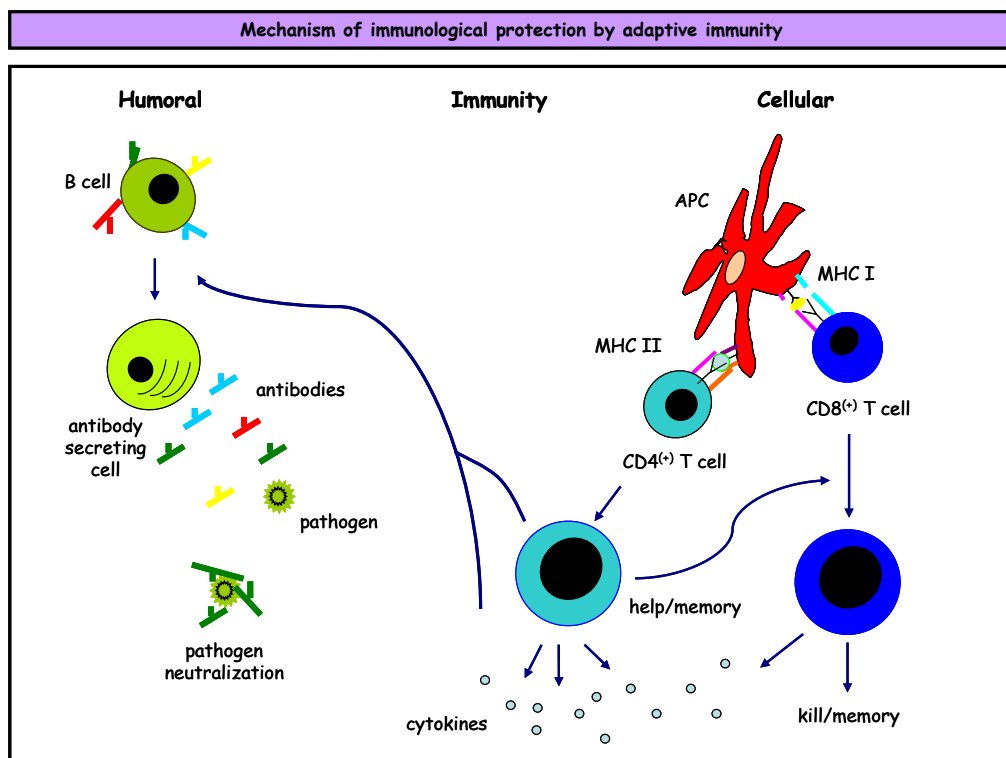
Figure 6   **Adaptive immune response.**

Collectively the immune system provides protection from harmful agents, while permitting tolerance to self-proteins and innocuous antigens, such as food. It is a specialized, continuously evolving mechanism of defence with the capacity to tailor an adaptive immune response appropriate to the pathogen in question, allowing the establishment of life-long protection against that pathogen.

2. Cell-mediated immunity.

Part I- Antigen recognition.

2.1 The major histocompatibility molecules (MHC).

MHC molecules are proteins involved in antigen presentation to T cells by delivering and displaying antigenic peptides on the surface of cell membranes. There are two types of MHC molecules that differ in structure, expression pattern and the source of antigenic peptides which they display. MHC class I molecules are expressed in 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 through x-ray crystallography, from peptide binding studies, and by MHC biosynthesis and antigen processing analysis.

2.2 MHC polymorphism.

Each MHC gene exists as many different alleles that can be co-dominantly expressed in a certain individual. In human they are known as human leukocyte antigens (HLA) and as the H-2 genes in mouse. The organisation of the MHC genes is similar in both species; there are three main class I loci, which are called HLA-A, HLA-B and HLA–C in humans and H2-K, -D and –L in the mouse. There are also three class II loci in humans, which are called HLA-DR, -DP and –DQ, and two class II loci in the mouse, which are called I-A and I–E.

The presence of several different alleles, as well as of various related genes that encode for proteins of similar function, ensures diversity of MHC molecules within an individual and within the population as a whole. In

contrast to MHC-I molecules that are made up by only one heavy chain, the MHC-II consists of two heavy (alpha and ß) chains, increasing its diversity further. The capacity of the MHC to present a wide range of different peptides is a consequence of the high degree of polymorphism.

2.3 The structure of MHC molecules.

Despite substantial sequence diversity, MHC proteins have common structural features. For both MHC-I and MHC-II, the two paired protein domains nearest to the membrane show homology with immunoglobulin domains, whereas the two domains furthest away from the membrane fold together to form a long groove, which is the site where a peptide binds.

2.4 MHC class I molecule.

Mature MHC class I molecules consist of three components: the polymorphic heavy chain (HC), β-2microglobulin (β2m,) and the antigenic peptide, all of which are essential for the formation and stability of a functional MHC class I complex. The heavy chain (43kDa), a transmembrane glycoprotein, is organised into three different structural domains (α-1, α-2, α-3), a transmembrane segment and a cytoplasmic tail (Figure 7). The relatively conserved α-3 domain is proximal to the membrane, and is non-covalently associated with the similarly folded β2m, a soluble, non-polymorphic, non-MHC encoded protein (12kDa). However, the remarkable feature of MHC class I molecules is the distinct structure of the membrane-distal domains, α-1 and α-2, which form the
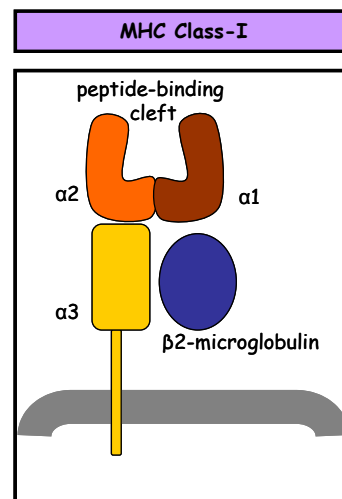
Figure 7 **MHC-I.**

Scematic re-presentation shows the MHC-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. *Image reproduced from Immunobiology 6th edition.*

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peptide-binding site; together with the peptide, this highly polymorphic MHC-I region determines T-cell antigen recognition.

The MHC-I peptide-binding groove can bind a wide variety of peptides (8-10 amino acids long) with high affinity, giving each MHC class I molecule its broad peptide-binding specificity. Differences between allelic MHC variants are reflected by different amino acid sequences in key peptide-interaction sites of the peptide-binding groove. Consequently, different MHC variants preferentially bind different peptides with the same or very similar amino acid residues at two or three particular positions, called anchor residues, along the peptide sequence. The amino acid side chains at these positions insert into pockets of the MHC class I molecule that are lined by polymorphic residues of the heavy chain. Additional amino acid positions, called secondary anchors, can also influence MHC binding.

2.5 MHC class II molecule.

The MHC class II molecule consists of a non-covalently-bound complex of two transmembrane glycoprotein chains, the α-chain (34kDa) and β-chain (29kDa) (Figure 8), which are both encoded within the MHC region of the genome. Each chain has two domains and together they form a four-domain compact structure, similar to that of the MHC class I molecule. The major difference between the two molecules is that for MHC-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
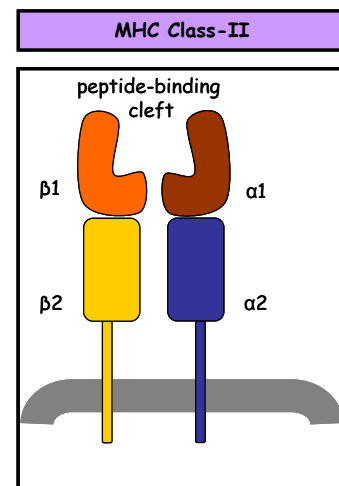
Figure 8   MHC-II.

Schematic re-presentation shows MHC-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. *Image reproduced from Immunobiology 6th edition.*

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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 is longer than of those that bind to MHC class I molecules, in most cases between 13-17 amino acids long. Similarly to MHC class I molecules, the sites of major polymorphism in the MHC class II molecule that determine T-cell antigen recognition 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.

2.6 Peptide/MHC complex.

An important feature of the peptide/MHC complex is that the bound peptide 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.

Newly synthesized MHC class I molecules are held in the ER until they form stable peptide/MHC I complexes. The antigenic fragments derive from proteins found in the cytosol and they are formed by degradation of larger proteins by the proteasome before they enter the ER through the transporters associated with antigen processing (TAP). In the ER, every step of the assembly and forming of the MHC-I molecule undergoes extensive quality control by a wide array of chaperones and specific proteins. Initially, partly folded MHC-I α chains bind to a chaperone protein, calnexin, and form a complex that binds to β2m. The MHC class I α:β2m dimer 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. Tapasin bridges TAP and MHC-I to facilitate the delivery of suitable TAP-associated peptides and their loading on the MHC-I. A stable peptide/MHC class I complex is then allowed to travel to the cell surface.

Conversely, MHC class II molecule is transported from the ER to the lysosome associated with an invariant chain, which protects the peptide binding site and targets delivery of MHC-II molecules to the acidic endosomal compartment. Here, 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 (CLIP) that blocks the MHC-II α:β peptide binding groove. The class II-associated invariant chain peptide (CLIP) remains bound to the MHC-II molecule until it encounters suitable peptides; upon peptide competition it is dissociated or displaced to allow peptide binding to MHC-II. Stable peptide/MHC-II complexes travel to the cell membrane where they are presented to T cells.

Beyond the two well-defined pathways of peptide loading, peptides generated from processing of extracellular proteins can also be presented on MHC class I, by a mechanism known as cross-presentation.

2.7 T cell receptor (TCR).

Antigen recognition is mediated through the T-cell receptor (TCR) on the plasma membrane of T cells that recognise peptide/MHC combinations on antigen presenting cells. T-cell receptors are formed by two transmembrane glycoprotein chains, α and β, which contain highly variable antigen-recognition sites (Figure 9), and they are expressed at the cell surface as a multiprotein complex with the invariant CD3 chains. The rearrangement of antigen-recognition segments in TCR genes results in highly diversified TCR pool sufficient to recognise a plethora of antigens potentially encountered through life time.

**Figure 9   T cell
          receptor.**

Schematic re-presentation 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. *Image reproduced from Immunobiology 6th edition.*
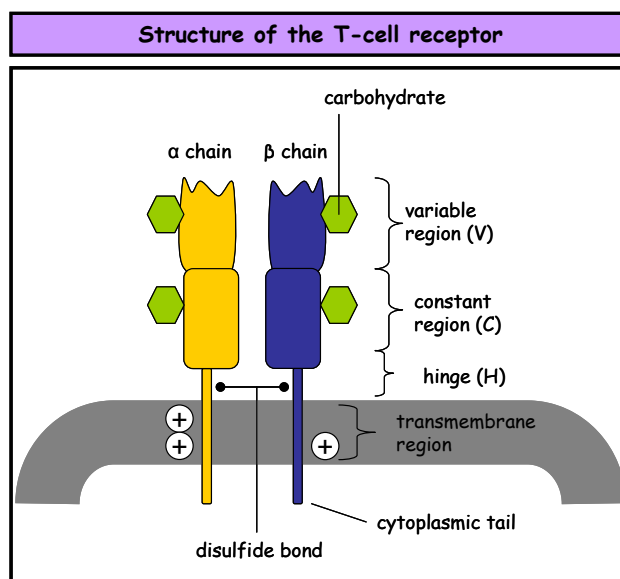
The α:β chains form extracellular disulfide-linked heterodimers responsible for MHC/antigen recognition. The TCR complex is formed with various other components that are required for initiating signalling when the TCR complex binds to peptide/MHC. These components include various CD3 molecules that are; CD3ε, CD3γ and CD3δ. CD3ε can form a dimer with either CD3γ or CD3δ. In addition, the TCR ζ chain also forms a homodimer that is included in the TCR complex. This too is an invariant chain and is critical for TCR signalling.

TCR activation of most TCRαβ$^{(+)}$ T cells is assisted by the CD4 and CD8 co-receptors that bind to MHC class II and MHC class I molecules respectively.

2.8 Co-stimulatory molecules.

Although antigen recognition by the TCR determines the specificity of T cell responses, full activation of T cells requires co-stimulatory signals. Specific signalling through the TCR in the absence of co-stimulatory signals leads to T cell non-responsiveness or 'anergy'. Co-stimulatory signals are delivered by costimulatory molecules on APCs via their cognate receptors on T cells. Different co-stimulatory signalling pathways differentially regulate T

cell activation or suppression; for example B7 on an APC, interacting with CD28 on a T cell provides an activation signal, which is sustained or modified by binding of CD40 ligand on T cell to CD40 on antigen presenting cells, while a CTLA4-B7 interaction inhibits T cell activation. An appropriate combination of co-stimulatory signals is important to induce proper, but not excessive immune responses.

Professional APCs manifest high co-stimulatory capacity during antigen presentation to provide optimal conditions for antigen delivery and for functional activation of naïve T cells. Expression of co-stimulatory molecules on the membrane of APC is a consequence of pattern recognition receptor (PRR) activation in response to pathogen-associated molecular patterns (PAMPs). These act as signals to the immune system that 'danger' is present. These 'danger' signals result in up-regulation of co-stimulatory molecules that leads to activation of naïve T cells.

2.9 The immunological synapse.

The immunological synapse is the name used to describe the specialized communication between an APC and a T cell that consists of the peptide/MHC-TCR interaction as well as other critically important accessory ligands. Advances 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. 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.

The immunological synapse requires hours of stable interaction between a T cell and an APC to result in effective T cell activation (Celli, 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.

Part II- Functional specialization of T cells.

2.10 Naïve T cells recognise specific peptide/MHC combinations on the surface of APCs and become activated to produce armed effector T cells.

Naïve T cells that have never encountered their cognate antigen recognize peptide/MHC complexes on APCs (Figure 10) in secondary lymphoid organs, which triggers T cell clonal expansion and specialised differentiation.

This differentiation of naïve T cells into specialized effector T cells is associated with their enhanced functional potential to orchestrate pathogen clearance, largely under the regulation of cytokines produced by cells of the innate immune system that have been activated in the presence of infection. The activation of naïve T cells in response to antigen and their subsequent proliferation and differentiation constitutes a primary cell-mediated immune response, which not only provides a specialized 'army' of effector T cells, but also generates a state of immunological readiness for protection from subsequent challenge by the same pathogen.
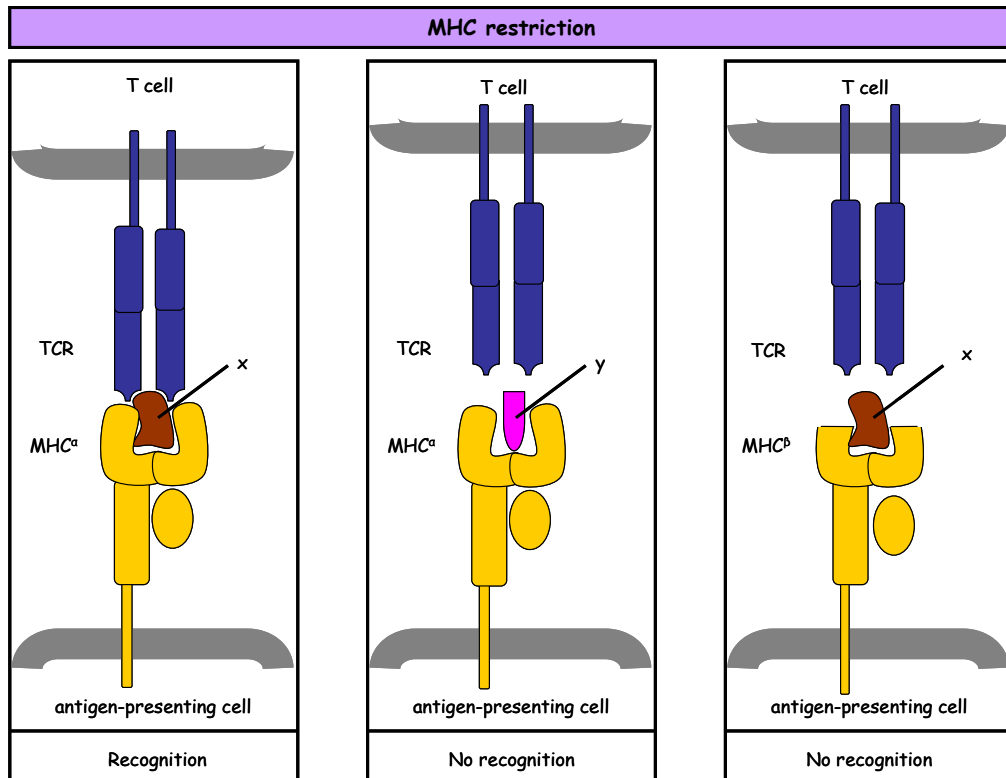
Figure 10   **The recognition of antigens by T cells is MHC restricted.**

Naïve T cells recognise specific complexes of peptide/MHC. For example, a T cell that has a TCR that can recognise antigen x on MHC$^\alpha$ would not recognise the same antigen on a different MHC (e.g. antigen x on MHC$^\beta$), or a different antigen on the same MHC (e.g. antigen y on MHC$^\alpha$). *Image reproduced from Immunobiology 6th edition.*

Once a professional APC has taken up an antigen at the site of infection it migrates to the local lymphoid tissue and undergoes maturation to become a potent activator of naïve T cells. Subsequently, the activated T cells secrete IL-2, which drives them to proliferate and differentiate into armed effector T cells.

Effector T cells fall into 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 are able to view antigens derived from intracellular pathogens in the context of peptide/MHC I complexes on the APC surface and they respond by initiating a cytotoxic response towards the antigen presenting cell that synthesized the antigenic peptides. Effector CD4$^{(+)}$ T cells are able to view

antigens derived from pathogens replicating in intracellular vesicles, as well as extracellular bacteria and toxins, in the context of peptide/MHC II complexes on the APC; in response, they differentiate to produce distinct effector helper or regulatory cell populations.

The naive 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.

In early studies, two classes of CD4$^{(+)}$ T cells were described; T_H1 and T_H2 (Coffman and Carty 1986; Mosmann, Cherwinski et al. 1986). 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 (Hsieh, 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, the generation of cytotoxic T cells and the induction of B cells for the production of opsonizing antibodies.

Conversely, extracellular antigens tend to stimulate the differentiation of T_H2 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 (Shinkai, Mohrs et al. 2002; Min, Prout et al. 2004). Each subset promotes its own development and inhibits the development of the other subset via their secreted cytokines (Fernandez-Botran, 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 T_H1 (e.g. IFNγ) and T_H2 (e.g. IL-4) lineages (McGeachy and Cua 2008). In addition to IL-17, T_H17 cells are characterised by their ability to produce IL-22 and IL-17F (Louten, Boniface et al. 2009). 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 (Infante-Duarte, Horton et al. 2000; Fedele, Stefanelli et al. 2005; Mangan, Harrington et al. 2006) particularly at mucosal surfaces (Monteleone, Pallone et al. 2009). They are thought to contribute to homeostatic maintenance of mucosal tissues such as the gut (Ahern, 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, by suppressing 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_H2 cells.

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

2.11 T cell migration.

The three main effector functions of T cells, namely the capacity to kill, to induce inflammatory responses and to help B cells, depend on the type of cytokines produced, the presence of helper or lytic machinery and their capacity to migrate to appropriate sites of infection. Since T cells respond to pathogens only on direct contact with pathogen-derived antigen, their

migration to the sites where antigen is found is essential and regulated by the expression of adhesion molecules.

2.12 Factors that influence the T cell response.

Commitment of T cells to clonal expansion, differentiation and functional specialization may be regulated by the strength and duration of TCR signalling, which, in turn, is dependent on the context in which the T cell sees antigen; for example, the type of antigen presenting cell or subset of DCs, the density of peptide/MHC complexes on the surface of APCs, the duration of APC-T cell interactions, and the life span of the APC (Lanzavecchia and Sallusto 2001). Other signals, deriving from cytokines and co-stimulatory molecules or from innate immune cells, such as NK or NKT cells, may also influence T cell clonal expansion.

2.13 The quality of T cell response.

A T cell response is fundamentally characterised by its magnitude, which generally refers to a quantitative measurement of the response and is commonly represented as the number of antigen-specific T cells generated or as the bulk measurement of a specific effector function. However, the lack of any consistent correlations between the number of antigen-specific $CD8^{(+)}$ T cells and control of either viral replication or tumour growth indicates that not all such T cells are equivalent in disease settings and that protective efficacy depends on qualitative rather than quantitative parameters (Appay, Douek et al. 2008). The quality of a T cell response is distinct from the quantity of antigen-specific T cells; it is rather determined by combinations of T cell functions, including their ability to proliferate or induce proliferation of other cells (for example through the secretion of growth factors), to organize immune responses (by secreting chemoattractants), and to carry out effector functions by directly killing infected cells through cytolytic mechanisms or secretion of cytokines. T cell responses with a better quality (that is, a greater degree of multi-functionality) have been shown to correlate with disease non-progression and protection in some models (Seder, Darrah et al. 2008).

Ultimately, the functional specialization of T cells is relevant to immunity only if protection against disease caused by an infection is guaranteed at a particular time by certain immune effector molecules and/or cells (Zinkernagel RM, 1996).

2.14 Immunological memory.

The Greek historian Thucydides notes in his records the practical importance of immunological memory, that is, the protection of the host to re-infection. The observation that individuals infected with a particular virus are subsequently resistant against the same infection has since been confirmed in various natural experiments. Although the mechanisms underlying protective immunological memory are still not well understood, the phenomenon has long been recognised and applied to vaccination.

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.

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. Managing immune responses to fight infections and tumours.

Part I- Immunization strategies for the induction of protective immunity.

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

The human immune system is a complex network of cells and molecules capable of mounting highly specific, potent protective responses against a broad range of invading pathogens (Pennington 2009- *in print*). The state of resistance to a pathogen, which is called immunity, can be innate from inherited qualities or it can be acquired by immunization actively or passively, naturally or artificially.

Passive immunity is induced by the transfer of pre-made elements of the immune system from one organism to another. For example, during natural passive immunisation maternal antibodies are being transferred from mother to child via the placenta or the milk to help protection during early life (Zinkernagel 2001). Artificially, passive immunity is acquired by administering sera containing antibodies from animals or humans. This type of artificial immunization is useful because it induces immunity very quickly, but its duration is short because the transferred antibodies are essentially proteins that are naturally broken down. Therefore, it is applied as an emergency treatment to poisons such as tetanus or snake venom, or during outbreaks of a particular disease.

Active immunity, which consists of cell-mediated T cell-dependent responses and humoral B cell-dependent immunoglobulin production, is acquired naturally during an infection or artificially by vaccination. Although originally the term was 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
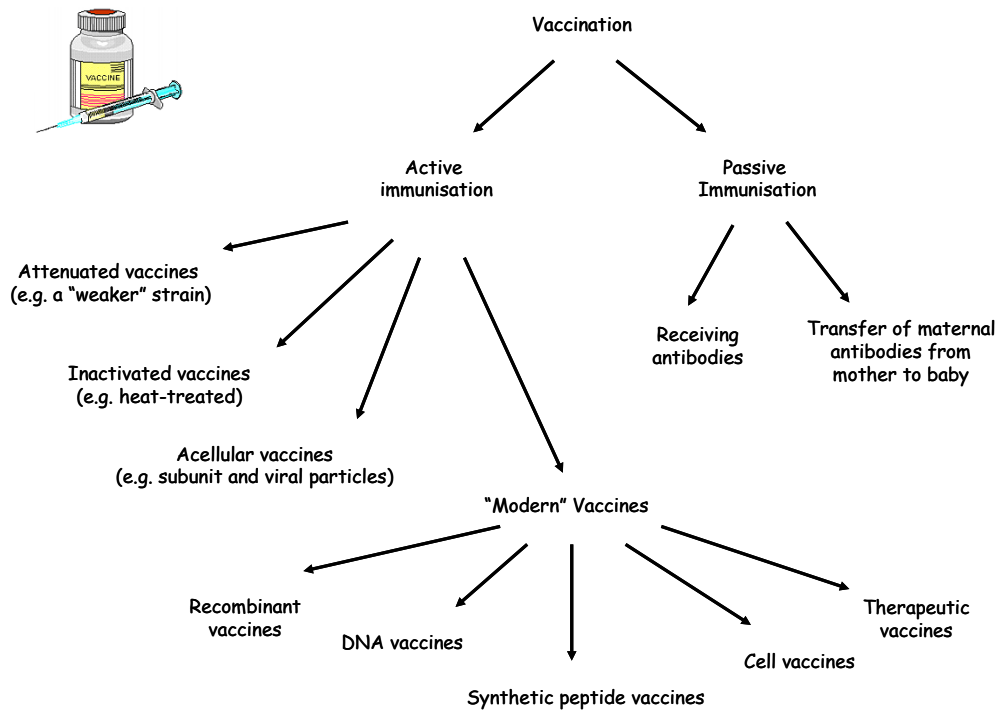
Figure 11   Vaccination.

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.

(the vaccine) for the purpose of stimulating active immunity to a disease (Figure 11).

3.2 Protective immunity can be induced by vaccination.

Vaccines are a major accomplishment of medicine; they have eradicated naturally-occurring diseases such as smallpox, whilst they successfully control the spread of others such as polio, typhus, measles, mumps, rubella, influenza, chickenpox, diphtheria, tetanus, pertussis and rabies. The success of protective vaccines lies in that they tackle a main inherent weakness of the immune system; the extended time required between antigen recognition and generation of protective adaptive immune responses if innate mechanisms fail. Vaccination helps to limit this window of vulnerability in which pathogens could cause death and disease; by using the

immune system's natural specificity and inducibility it prepares it in advance to respond faster and better when infection strikes (Bloom and Ahmed 1998). However, despite concerted efforts, conventional vaccines are not effective for several infectious diseases that kill millions of people every year such as tuberculosis, leprosy, leishmania, dengue fever, malaria, hepatitis C and HIV/AIDS, and for cancer.

3.3 Classic vaccines.

The general principle behind the invention of the safe and effective vaccines is the empirical observation that pathogen-specific antigens from a non-pathogenic form of the infectious agent can stimulate a protective immune response that is cross-reactive to the actual pathogen. For example, vaccinia is a bovine analogue of smallpox that can provide protective immunity against smallpox in humans without causing significant disease. The first vaccines developed were whole pathogen vaccines, either in live-attenuated form or killed.

Live attenuated pathogens such as the vaccines used to immunize children against measles, mumps, and rubella retain their immunogenicity but with greatly decreased virulence. These preparations have the advantage of entering both MHC class I and MHC class II pathways of antigen presentation, inducing potent vaccine protection by both cell-mediated and humoral immunity. The infectious agent has the ability to multiply in the host and provide antigenic stimulation, which means that less antigenic material is needed in the vaccine to induce protection and that one injection is usually sufficient to provide long-term protection. However, attenuated viral vaccines carry the risk of reversion to more virulent forms as well as that of interference by related viruses, and they can cause lethal systemic infections to immunosuppressed individuals.

A safer approach are killed vaccines that consist of heat- or chemically- inactivated pathogens, such the ones used in the Salk polio vaccine as well as for cholera, influenza and plague. They are effective against

some extracellular bacteria that are well controlled by humoral responses, but stimulate poor cell-mediated responses due to limited MHC-I presentation. Since the inactivated pathogen cannot replicate, a large amount of it, administered in periodic boosters, is required for stimulating and maintaining immunity. An additional disadvantage is that killed vaccines for viruses that mutate rapidly, such as influenza, are not protective unless they are manufactured regularly to include the new antigenic specificities of the mutated viral strains.

3.4 'Second generation' vaccines.

In order to overcome these difficulties, 'second generation' vaccines were developed. Advanced technology allowed the identification and purification of components or products of pathogens that would be as effective as the whole organism, leading to the development of acellular vaccines, such as subunit and virus-like particle vaccines. Examples have included vaccines that consist of purified protein antigens such as inactivated bacterial toxins or toxoids (e.g. against tetanus and diphtheria), vaccines derived from viral particles such as capsular polysaccharides, either alone (e.g. against HPV) or conjugated to a protein carrier (e.g. against bacterial meningitis), and vaccines that use recombinant protein components such as the hepatitis B surface antigen (e.g. against HBV).

Viral particles are more immunogenic than subunit vaccines, because they have a more stable multi-covalent structure compared to purified proteins that may not retain their native conformation and, consequently, can result in the production of antibodies that may not recognise the pathogen. Generally, acellular vaccines are safer than those based on whole organisms but often fail to stimulate a full range of immune responses because, like killed vaccines, they have limited access to appropriate pathways of antigen presentation. Thus, they invariably require the correct choice of an immunogenic protein carrier or adjuvant to ensure sufficient immunogenicity.

3.5 Existing vaccines are good for the protection against acute viral infections.

Interestingly, all vaccines that work today and are proven to protect, including the classical childhood vaccines (e.g. measles, mumps, and rubella), are those that induce potent neutralizing antibody responses of apparently long duration against pathogens that cause acute infectious episodes. Acute infections, usually caused by highly cytopathic infectious agents, are generally better controlled by soluble diffusible factors including T cell–dependent cytokines and by specific neutralizing antibodies, while antiviral CD8$^{(+)}$ T cell responses may also play an important role (Miller, van der Most et al. 2008). Highly cytopathic viruses and bacteria usually exhibit various epitopes that induce antibodies of numerous specificities, which include neutralizing antibodies that are protective (Bachmann, Kalinke et al. 1997). Overall, protective vaccination does not necessarily prevent re-infection, but reduces its severity so as to prevent disease (Bloom and Ahmed 1998). This imitates the mechanism of protection by immunity; it doesn't refer to sterile protection, but it represents a balance of optimal immunity against the various cytopathic infections, while avoiding excessive immunologically mediated tissue damage (Zinkernagel 2003).

3.6 Existing vaccines are inefficient for protection against chronic infections.

By contrast to the success of our vaccines against acutely cytopathic agents, chronic infections (e.g. HIV) have been much more difficult to control. Although there is insufficient information on the correlates of protective immunity against pandemics of persistent infections such as HCV and HIV, facultative intracellular bacteria such as mycobacteria, and intracellular parasites, evidence suggests that those pathogens are usually controlled initially by T cells, with antibodies playing a possible but inconclusive role (Ciurea, Klenerman et al. 2000). Indeed, in several persistent infections including tuberculosis, leprosy, and perhaps HIV in some sero-

negative AIDS-resistant patients, it has been empirically noted that T cell memory occasionally provides efficient protection against reinfection.

3.7 'Third generation' vaccines and therapeutic vaccination aim to mobilize the immune system against persistent infections and cancer.

Improved understanding of the mechanisms involved in the immunity against persistent infections and advances in molecular biology have permitted the development of 'third generation' vaccines, which aim to combat 'resistant diseases'. Furthermore, a clearer insight of how the immune system functions and where is weakened during persistent diseases has led to the concept of therapeutic vaccination, which could control or cure chronic infectious diseases and cancer by rectifying and/or generating proper immune responses. Similar to persistent microbial and viral infections, optimizing the cell-mediated immune responses through vaccination is thought to be the major element of successful immunotherapy strategies for cancer.

3.8 Application of recombinant DNA technology for the development of novel vaccines.

Recombinant DNA technology allows the use of bacterial, viral or other vectors for the production of large quantities of the desired antigen, which is then purified and injected as subunit vaccine; the only example of such vaccine licensed for use in humans is the recombinant HBV vaccine. Novel vaccination approaches use recombinant DNA technology for selective attenuation of pathogens; an application that could prove useful for viruses that undergo frequent antigenic shifts such as influenza, or for bacteria that produce pathogenic enzymes such as typhoid and salmonella. An alternative vaccination approach aimed to protect from multiple pathogens uses DNA-recombinant-attenuated micro-organisms as vectors to carry antigenic particles from several diverse organisms. They are known as hybrid vaccines and examples include both bacterial vectors (e.g. attenuated strains of

salmonella carrying antigens from Listeria, Leishmania, Schistosoma) and viral vectors (e.g. vaccinia virus that express genes from HSV).

Overall, recombinant technology allows the selection for safe antigens and vectors, while proteins expressed on a live organism are more likely to preserve their native conformation. Recombinant vaccines induce both cellular and humoral immunity as they are live organisms and they can be engineered so as reversion to a wild type of pathogen is impossible. However, reliable laboratory markers of attenuation and virulence are still lacking. Moreover, vaccination efficiency of these novel applications depends on the analytical power of recombinant DNA methods, since the genes encoding for protective antigens must be located, cloned and successfully expressed in the new vector.

3.9 Protective immunity can be induced by injecting DNA encoding immunogens.

DNA-based vaccines involve immunization with DNA vectors that encode for immunogens, rather than with the immunogen itself. They induce cell-mediated and humoral immune responses by generating antigenic proteins *in situ* by the host, in a manner similar to that seen with live-attenuated vaccines. However, in contrast to these original vaccines, the DNA-based vectors do not have the potential to cause disease and are being designed to deliver only the desired genetic sequences that encode for immunogenic antigens. Due to the immunogenicity of the DNA itself, their inherent stability and their low manufacturing cost, DNA vaccines have become an appealing tool in designing effective vaccination against diseases caused by persistent viruses (e.g. HIV), intracellular bacteria (e.g. tuberculosis), parasites (e.g. malaria) and cancer.

Nonetheless, the mechanisms that underlie DNA-based vaccination are not yet fully understood. Important questions include the method of T cell priming, the pathways of antigen presentation employed by APCs (e.g. direct through plasmid transfection, indirect through cross-presentation, or both) and

the duration of the antigen presentation. For example, extended immuno-stimulation by the foreign antigen could potentially provoke chronic inflammation or autoantibody production, while, as with all gene therapy, the possibility that the vaccine's DNA could integrate into and interfere with host chromosomes is an ongoing concern.

3.10 Synthetic peptides of protective antigens can stimulate protective immune responses.

Elucidation of the crystal structure of the peptide/MHC complex (Stern and Wiley 1994), along with the discovery of anchor-residue sequence motifs that account for the binding specificity of peptides to MHC (Rammensee 1995), has provided the visual and mechanistic tools to help understand how T cells recognize antigens in the form of short peptides. The stability of the peptide/MHC complex allows the purification and sequence analysis of naturally occurring antigenic peptides, expressed by infected or tumour cells, that enables antigen fragment and sequence prediction and, subsequently, synthesis of potential vaccine candidates. Selection of peptides for use in vaccination can be achieved through biochemical identification, by genetic approaches, or with bioinformatics, and it is applied both for MHC class I and MHC class II peptide complexes, although the latter imposes more difficulties in binding prediction due to the open structure of the binding groove.

Peptide-based vaccination allows utilization of one or a cocktail of selected peptide fragments, which can be formulated to become more immunogenic by simple sequence modification. The peptide-induced immune response is generated solely against the selected antigen and, in contrast to recombinant or attenuated viral vaccines it doesn't carry the risk of mutations that revert the vaccine to more virulent forms. It is a method characterized by simple manufacturing technology, while the peptide products are easy to preserve/store and their supply is broadly available.

A major drawback in injecting soluble peptides is lack of immunogenicity, whilst in some studies such vaccination approaches have even been shown to induce tolerance, presumably due to peptide uptake and presentation by immature antigen presenting cells that fail to deliver the co-stimulatory and cytokine signals (e.g. IL-12 and/or IFNα) required for T cell activation (Mescher, Agarwal et al. 2007). To counter this, the use of adjuvants and suitable carrier systems has proved essential in peptide-based vaccination; carriers provide protection for the peptides from protease degradation, while adjuvants support T cell activation by increasing the expression of co-stimulatory ligands and stimulating IL-12 and/or Type I IFN production by DCs through engagement of TLRs. One such system involves the use of immune-stimulatory complexes (ISCOMs), which are lipid carriers that also act as adjuvants but have minimal toxicity. By fusing with the plasma membrane of an antigen presenting cell, ISCOMs deliver peptides and proteins in the cell cytoplasm, allowing them to enter the MHC class I pathway for induction of CD8$^{(+)}$ T cell responses (Sanders, Brown et al. 2005). Such approaches permit the 'indirect' presentation of the selected peptides by APCs, thus overcoming restriction of accessible MHC-I molecules occupied by endogenous antigen and generating cytotoxic T cell (CTL) responses through cross-priming.

Nevertheless, the quality of the induced CTL response to peptides is hard to predict, because synthetic peptides can represent cryptic epitopes that would not be naturally generated. Furthermore, individual peptides can be useful only in patients with appropriate HLA molecules capable of presenting that peptide; it is therefore a necessity in peptide vaccination to manufacture several different epitopes to match various HLA alleles.

To date, several strategies have been implemented in peptide vaccination, both to improve immunogenicity and to steer the immune system toward desired types of responses.

3.11 Antigen presenting cells can be used to modulate the immune response.

Another area of intense research in modern vaccine development is cell-based vaccination. Approaches, which are discussed in detail later, are mainly focused on the use of autologous dendritic cells and their derived particles with aim to maximise the induction of potent T and B cell responses.

Part II- Different infections present different problems to the immune system.

Despite success in the lab, results of the new-generation vaccines from the clinic have been generally disappointing, and much effort is being divested into vaccine research. This thesis describes the development of a new vaccine strategy, using microsomes, that attempts to overcome the problems faced by many of the vaccines described above. These problems are discussed in more detail below.

3.12 Acute resolving infections.

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
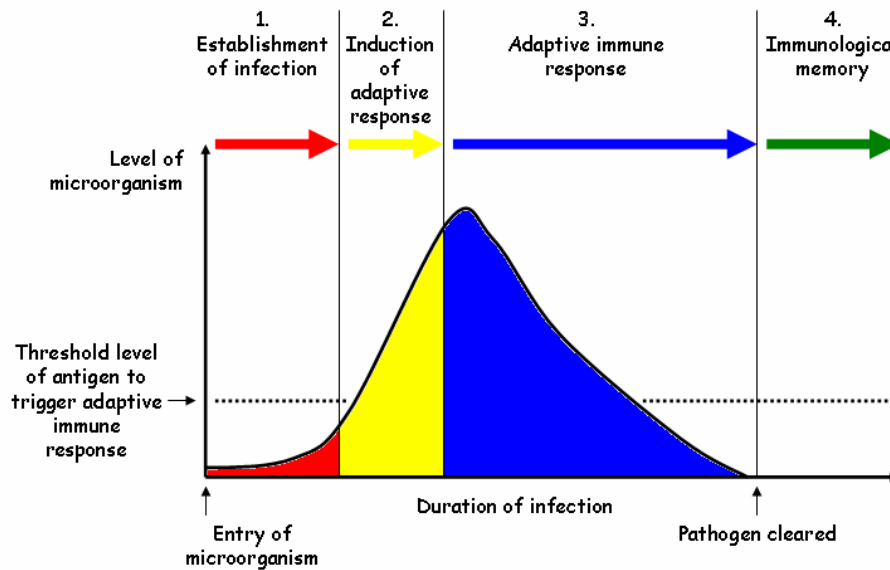
Figure 12 **The course of a typical acute infection that is cleared by an adaptive immune response.**

1. Following entry of the microorganism in the body the level of the infectious agent increases as the pathogen replicates.
2. An adaptive immune response is initiated when the numbers of pathogen exceed the threshold level of antigen required to intiate an adaptive immune response.
3. Effector cells and other components of the adaptive immunity start clearing the pathogen.
4. When the infection is cleared that adaptive immune responses cease establishing the host with immunological memory that ideally will provide lasting protection against re-infection with the same pathogen.
Image reproduced from Immunobiology 6th edition.

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).

Acute resolving infections (Figure 12) 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.

3.13 Latent and chronic persistent infections.

Non-acutely cytopathic pathogens, 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.

When a latent pathogen reappears as an infectious and continuously replicating agent it leads to chronic persistent infection which progresses slowly and lasts a long time. Chronic infections, such as acquired immune deficiency syndrome (AIDS) and hepatitis C, result from pathogens that escape acute immune responses because they don't provide enough antigen or they don't provide antigen at the right place to trigger adequate stimulation of the immune system. Using various strategies to avoid recognition by the immune system as well as mechanisms to suppress immune responses (Johnson and Desrosiers 2002; Hilleman 2004; Gale and Foy 2005) these pathogenic agents have evolved strategies to persist in the infected host. Notably, the immune response during chronic infections is altered or impaired through impaired DC function, induction of regulatory T cells (Tregs) and production of immunosuppressive cytokines (Ha, West et al. 2008).

Chronic persistent viruses and bacteria can interfere at various stages of the adaptive immune response with evasion mechanisms that either function independently within different immune cells or work cooperatively. Intracellular viral or microbial proteins can directly interfere with antigen presentation by altering the expression of MHC or costimulatory molecules, resulting in inappropriate presentation of critical antigens to the immune system. Furthermore, the persistent presentation of pathogen-derived antigens

on APCs may induce deletion of corresponding T cells or mutation of the antigenic epitope. Alternatively, prolonged exposure to these antigens can lead to a progressive exhaustion of T cells, which is characterised by the loss of protective cytokine production, cytotoxicity and proliferation and the upregulation of inhibitory molecules, such as PD1 and CTLA4 (Ha, West et al. 2008). As a result of their impaired function, T cells fail to further control the infection. Moreover, the establishment of viral persistence is supported by an immunosuppressive and regulatory environment, which is induced in the early stages of chronic infection (Brooks, Teyton et al. 2005).

Chronic infections are largely non- or low-cytopathic, often slow in kinetics, and have a tendency to persist (Zinkernagel 2003). Persistent pathogens do not kill the immunocompetent host rapidly, but rather tend to establish a balanced state of infection-immunity, where they coexist in low numbers together with low grade cell-mediated and humoral immune responses.

Cytotoxic T cells are crucial for the efficient control of non-cytopathic agents that cause extra-lymphatic infections that persist in the periphery, for example in neurons, epithelial cells, or granulomas. A notable exception is HBV, a non-cytopathic but much less variable DNA virus, against which a polyclonal neutralizing antibody response protects very efficiently. However, lessons learned from TB or leprosy suggest that T cell protective responses during these infections usually depend on continually-activated effector T cells to contain re-occurrence, spread, and expansion of infection (Zinkernagel 2003). Non-replicating antigen preserved in the form of immune complexes on antigen presenting cells is perhaps sufficient to maintain CD4$^{(+)}$ T cell memory and, consequently, antibody production. However, protective CD8$^{(+)}$ T cell memory requires persistent infection that feeds T cell activation, since the MHC class I pathway of peptide loading is dependant on constitutive intracellular synthesis and generation of peptides (Yewdell and Haeryfar 2005).

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. To avoid the resulting immunopathology that would otherwise lead to disease or death, the immune system contains such unbalanced cytotoxic T cell responses against too many infected host cells by negatively selecting these repertoires (Chisari and Ferrari 1995; Zinkernagel 1996). 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).

To exacerbate this complexity, persistent pathogens exploit mechanisms that control the balance of infection (immunopathology) versus immunity (immunoprotection). Their immune evasion strategies often preclude infection of macrophages and antigen-presenting cells and therefore antigen presented by MHC-I may not reach draining lymph nodes to induce CD8$^{(+)}$ T cell effector responses. Even if antigen arrives at the draining lymph nodes in phagocytosed form to induce CD4$^{(+)}$ T cell and subsequent humoral immunity, antibodies may not be effective in reaching the certain peripheral organs and often not sufficient to eradicate the infected cells.

The difficulties of the immune system in dealing with such strictly peripheral extra-lymphatic events are reflected in our failure, so far, to produce successful vaccination for many persistent infections (Figure 13).

Immunoprotection vs Immunopathology

pathology

protection

protective vaccines
(acute high-cytopathic infectious agents)

protection   pathology

vaccines not necessary
(non-acutely cytopathic agents)

protection

pathology

vaccines we need
(low- or non- cytopathic agents, usually
persistent extra-lymphatically)
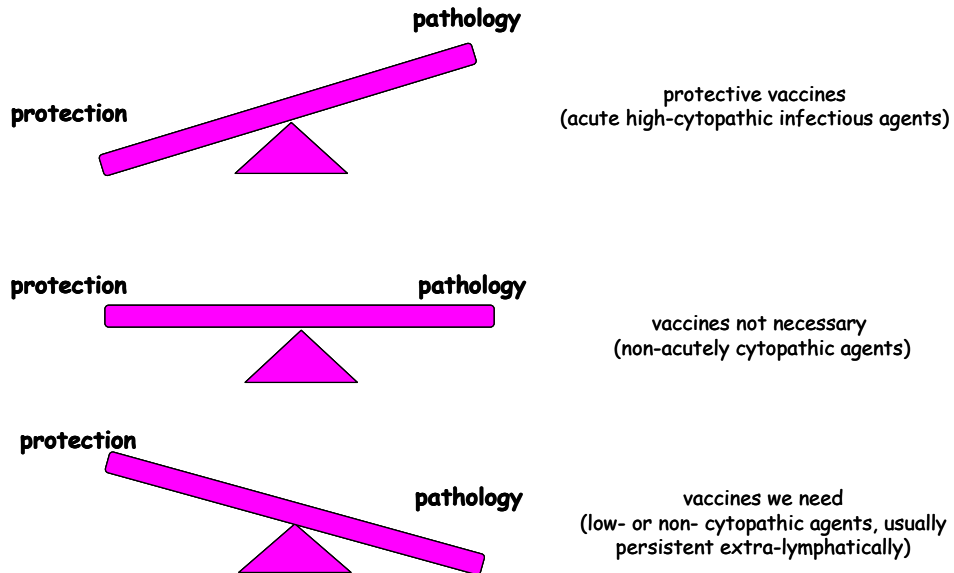
Figure 13   Balance between immunoprotection and immunopathology.

Our protective vaccines imitate the mechanism of protection against acute resolving infections. Acute high-cytopathic infectious agents 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.

Persistent infections are largely non- or low-cytopathic, often slow in kinetics, and have a tendency to persist. Persistent pathogens will not aim to kill the immunocompetent host rapidly, but rather tend to establish a balanced state of infection-immunity, where they coexist in low numbers together with an active immune antibody and T cell response. Vaccines are not necessasary for protection.

When the immune system cannot balance optimal resistance against the infection and excessive immunologically-mediated tissue damage, is the actual immune response that results in immunopathology. These infections result in disease and necessitate vaccines, which are not yet available.

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3.14 Cancer.

Cancers are caused by the progressive growth and cell division of a single transformed cell. The idea of a defence system recognizing cancer cells as foreign was first postulated by Paul Ehrlich at the beginning of the last century and supported by Burnet's and Thomas' hypothesis *of tumour-immune surveillance* (Burnet FM, 1970) (Croci, Zacarias Fluck et al. 2007). This theory proposed that tumours appeared more frequently than are observed, but were eliminated efficiently by the immune system before they were clinically detectable. However, in spite of the presence of an effective immune system, tumours arise and develop from normal tissues and invade surrounding and distant sites. To date, the question of whether immunosurveillance against tumours truly exists is yet to be resolved.

Pathogens consist of a variety of foreign proteins many of which can be recognised by the immune system as 'non-self' and/or dangerous. In contrast, tumour cells are derived from host cells and differ only slightly, limiting the number of antigens available to trigger an immune response. Often, tumour antigens are not expressed on the surface of tumour cells and are therefore inaccessible for recognition by antibodies. Moreover, the tumour environment doesn't induce efficient DC maturation. Consequently, antigen presentation at the tumour site or tumour-associated draining lymph nodes of potential tumour antigens by immature DCs that lack costimulatory molecules (Figure 14) leads to anergy and tolerance of the corresponding tumour-primed T cells, hampering the exquisite specificity of the immune system to attack cancer. Even if effector T cells are efficiently primed, cancerous lesions promote growth and angiogenesis, producing an abnormal tumour vasculature that prevents the smooth entry of tumour-specific effector cells into vascularized cancer nodules (Melief 2008).
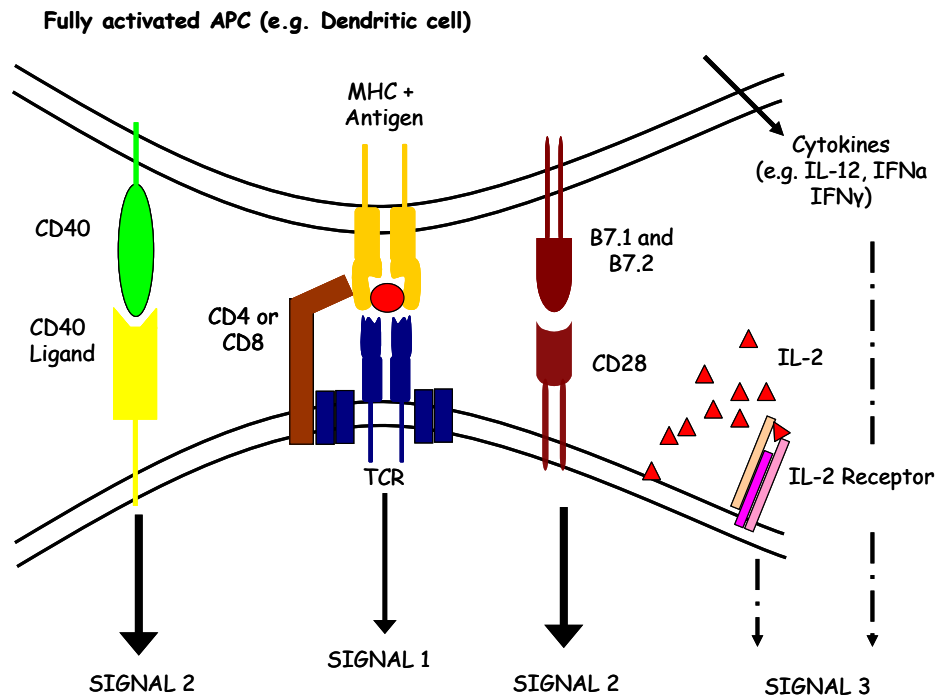
Figure 14   **Priming of effector T cells requires three signals.**

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. By contrast, in the absence of all three signals activation of T cell leads to anergy or tolerance. *Image inspired by Dr. D.Pennington's lectures.*

Malignant cells demonstrate multiple strategies that evade the immune response to promote tumour survival. Oncongenic protein expression in tumour cells leads to over-expression of STAT3, production of inflammatory chemokines (e.g. CCL2, CCL5, CXC) and expression of various immunosuppressive cytokines (e.g. IL-10 or TGFβ) (Melief 2008). Chemokines support tumour growth by promoting the migration and invasion of tumour cells (Soria and Ben-Baruch 2008), while also mediate recruitment of immunosuppressive leukocyte infiltrates such as myeloid derived suppressor sells (MDSCs), tumour-associated macrophages and immature dendritic cells into the tumour stroma. STAT3 upregulation further induces expression of immunosuppressive cytokines, drives suppression of T_H1 cell immune responses and causes impairment of antigen presentation by tumour-associated DCs by reducing their expression of costimulatory and MHC class II molecules (Kortylewski and Yu 2008). Cross-presentation of tumour

antigens by insufficiently activated DCs induces anergic T cells or deletion of tumour-responding effector T cells, and the expansion of regulatory T cells in tumour-draining lymph nodes (Ghiringhelli, Puig et al. 2005) instead of tumour immunity.

Indeed, the requirement and differentiation of sub-optimally functional dendritic cells in the tumour microenvironment are among the main factors that account for tumour escape.

Part III- The elements of a successful vaccine.

3.15 Requirements for a successful vaccine.

Taking these observations into account, a successful vaccine against diseases caused by persistent infections and cancers must aim to provide T cell–mediated memory and protection; maintain long term immunity, without causing disease. A good vaccine should allow periodical or continuous generation of MHC class I–presented peptides in secondary lymphoid tissues to activate $CD8^{(+)}$ T cells against peripherally located intracellular infections (Yewdell and Haeryfar 2005). Moreover, it should provide sufficient antigens and in appropriate form to be taken up by APCs and macrophages in order to activate T cells to produce macrophage activating soluble mediators such as IFN, TNF, and other interleukins such as IL-12 (Zinkernagel 2003; Yewdell and Haeryfar 2005).

The antigen used for vaccination should be carefully selected taking into consideration that persisting viruses are highly variable (Davenport, Price et al. 2007). Moreover, naturally immunodominant antigens may not represent the optimal targets for effective immunity (Friedrich, Valentine et al. 2007). Indeed, reported evidence suggests that vaccine-induced T cells generally recognize native antigen less efficiently (Appay, Speiser et al. 2006) and are therefore less effective in the face of their real targets (Stuge, Holmes et al. 2004). Nonetheless, the robust generation and maintenance of highly specific

CD8$^{(+)}$ T cells is not as straight forward as the simple administration of relevant antigen at the right doses. Other variables, including the timing and intensity of the antigen delivery schedule, the local environment at the site of inoculation and the choice of appropriate vectors are also required to produce an optimised response (Appay, Douek et al. 2008).

Although induction of CD4$^{(+)}$ T cells is important to support initiation of CD8$^{(+)}$ T cell responses, issues related to specific pathological contexts should be carefully evaluated when developing novel vaccine candidates. For example, CD4$^{(+)}$ T cells can serve as preferential targets for some viruses, such as HIV, thus their activation may promote rather than control viral load (Douek, Brenchley et al. 2002), (Pennington 2009- *in print)*. Additionally, CD4$^{(+)}$ T cell can be stimulated to generate regulatory CD4$^{(+)}$ T cell populations, thus compromising a cytotoxic immune response against virally infected or oncogenic cells (Appay, Douek et al. 2008).

Clearly, the concept of therapeutic vaccination implies that the host immune system is still competent for eliciting an immune response after vaccination. Exhausted T cells surrounded by an immunosuppressive environment are likely to be unresponsive to antigen provided by therapeutic vaccine, except if this is combined to alleviate inhibitory and suppressive factors. Therefore, successful immunotherapy should not only have the capacity to produce amplifying immune responses against the corresponding pathogens but, also must involve mechanisms that are able to break antigen-specific tolerance if this has occurred.

Ultimately, an efficient vaccination strategy against the diseases which we don't have vaccines for should establish: long-lived, antigen-specific plasma cells that produce neutralizing antibody, persisting CD4$^{(+)}$ and CD8$^{(+)}$ T cells, and effective migration of pathogen-specific T and B cells to sites of infection, especially mucosal sites (Pulendran and Ahmed 2006). This, in turn, should provide long-lived immunological protection in order to prevent disease, or reduce the severity of symptoms.

3.16 Additional requirements for cancer vaccines.

A successful cancer vaccine should first identify tumour antigen targets that discriminate cancer cells from normal cells. Several tumour-associated antigens (TTAs) that are presented by MHC to T cells and induce anti-tumour immune responses have been identified and are classified in six categories: These are strictly tumour-specific antigens (e.g. CDK-4 gene in melanoma) that result during oncogenesis from mutations in a particular gene that is associated with the tumour, testis-specific antigens (e.g. MEGE-1 gene in melanoma), tissue-specific antigens (e.g. tyrosinase enzyme in melanoma), strongly over-expressed antigens (e.g. HER-2/ neu in breast carcinoma), molecules that display abnormal post-translational modifications (e.g. MUC-1 in breast carcinoma) and viral proteins that cause cancer (E6 & E7 of human papilloma virus in cervical carcinoma). Tumour-associated antigens are able to activate tumour-specific T cells and are therefore promising targets for use in immunotherapy.

Second, the form in which antigen is administered should be considered (i.e. peptide, protein, DNA, or RNA), with the aim of efficiently delivering these tumour antigens to the secondary lymphoid organs in appropriate amounts and within a specific time frame. Third, the selection of appropriate adjuvants that imitate optimal conditions for immune stimulation is vital. These adjuvants condition the microenvironment (of both lymphoid tissue and the tumour) to alleviate DC suppression and licence them to generate effector T cells, while facilitating recruitment and activation of innate immune mediators.

In summary, to achieve eradication of the tumour we need to develop methods sufficient to break self-tolerance to many tumour antigens, while overcoming mechanisms by which tumours evade the host immune response.

3.17 The route of vaccine administration is an important determinant of success.

The route of immunization can influence the compartmentalization and the nature of the induced immune response, due to the presence of distinct antigen-presenting cells at different inoculation sites. Vaccines can be administrated through mucosal, intramuscular or intravenous inoculation. Rationally, mimicking the natural route of entry of the pathogen against which the vaccination is directed is likely to be the most effective way to stimulate an appropriate immune response.

Since many pathogenic agents enter the body and establish infection at mucosal surfaces numerous studies have focused on the mechanisms by which these organisms stimulate mucosal immunity. For example, mucosal-associated lymphoid tissue is the primary site of viral entry and replication for HIV; thus, delivering vaccines to this compartment should target early viral dissemination. An example of a vaccine that is administered via the pathogen's normal route of entry is the oral polio vaccine, which protects at the site of entry by stimulating mucosal immunity and, subsequently, the production of neutralizing antibodies.

A desirable feature of vaccine-induced immunity influenced by the route of administration is the subsequent localization of effector T cells to the site of likely pathogen exposure. Mucosal immunizations appear to lead to the induction of T cells with higher antigen sensitivities compared with subcutaneous vaccination in animal models (Belyakov, Isakov et al. 2007). Indeed, recent research has associated the presence of poly-functional CD8[(+)] T cells in the mucosal-associated lymphoid tissue with slow HIV disease progression (Critchfield, Lemongello et al. 2007). Likewise, to arrest the growth of solid tumours, it is necessary to induce the effective migration of tumour antigen-specific T cells to the tumour site. The induction of highly antigen-sensitive T cells in specific compartments may also compensate for local regulatory mechanisms that compromise the full potential of antigen-specific cellular immunity (Appay, Douek et al. 2008). In addition to the

efficiency of the immune response, the route of delivery has been shown to also affect the nature of the immune response. For example gene-gun intradermal delivery favours T_H2 responses, while intramuscular administration tends to induce more T_H1 differentiation (Torres, Iwasaki et al. 1997).

Finally, a critical consideration when choosing the administration route for a vaccine is the smooth delivery of the antigen to the targeted antigen presenting cells and, subsequently, the efficient uptake of intact vaccine into the antigen processing pathways. The importance of this issue is reflected in that the majority of the current vaccines are administered by injection rather than via the oral route, in order to avoid vaccine digestion in the gut and preserve the structure of the antigen from proteolytic degradation on its way to the antigen presenting cell.

Part IV- Current attempts to make better vaccines.

Generally, new generation vaccines are being designed to generate immunity beyond that induced by pathogens themselves, especially against persistent viral infections. An improved understanding of developing immune responses has led to the identification of key intervention points in the design of novel experimental vaccines. For example many new strategies attempt to induce the cell-mediated arm of the adaptive immune response. Thus, they are targeted to several components of cellular immunity. There are many considerations relevant to the design of these vaccines; these will be discussed here.

3.18 Improvement of immune responses through selection/enhancement of optimal epitopes for T cell activation.

Classical vaccination strategies that use live attenuated viruses are often not protective for viruses that cause persistent chronic infections,

because they have evolved under the selective pressure of the host immune system and, as a result, are unlikely to carry optimal antigenic epitopes. Similarly, tumours thrive once T cells specific for the most immunogenic epitopes of the tumour cells are deleted due to tolerizing mechanisms. Thus, the above observations suggest that epitope enhancement, the process of altering the sequences of certain viral or tumour epitopes to improve their immunogenicity, would be beneficial for vaccine design.

One way of enhancing the immunogenicity of an epitope is by increasing the peptide binding affinity for MHC molecules. This can be achieved by artificially improving the anchor residues of the peptide or by screening peptide libraries for peptide sequences that best bind to MHC. Converting a subdominant peptide into a dominant one makes it more competitive for the available MHC molecules and potentially increases the level of stable peptide/MHC complexes on the surface of an antigen presenting cell, which is crucial in improving the potency of a vaccine. Alternatively, epitope enhancement can involve the modification of amino acid sequences that would improve the affinity of the peptide/MHC complex for the TCR, in order to effectively activate 'low-avidity' CTLs that are less sensitive in antigen recognition. This is beneficial in cases where 'high-avidity' TCRs have been deleted due to self-tolerance mechanisms. Finally, epitope enhancement can be achieved by constructing chimeric peptide sequences to elicit more broadly cross-reactive T cells.

Epitope enhancement is advantageous as it can be used to improve the immunogenicity not only in peptide vaccination, but also in any form of vaccine in which such T cell epitopes occur, including DNA, recombinant protein, viral vector and attenuated viral vaccines.

3.19 Selection of protective CTLs can improve the biological outcome of immune response.

Compelling evidence showing that $CD8^{(+)}$ T cells are critically important in the host response to viral infections and cancers, resulted in them

becoming a significant target for vaccine design. Detailed attempts to identify protective T cells have highlighted 'functional avidity' as a crucial determinant of T cell efficacy. In those studies, 'functional avidity', which is also defined as 'antigen sensitivity', is dependent on the biological functional outcome instead of the antigen binding affinity at the cellular level (Appay, Douek et al. 2008).

The antigen-sensitivity of T cells generated *in vitro* has been shown to depend on the quantity of antigen used for stimulation, with low peptide densities resulting in the generation of highly responsive cells (Alexander-Miller, Leggatt et al. 1996; Walter, Herrgen et al. 2003). Similarly, the antigen sensitivity of memory T cells in mice has been shown to correlate inversely with the peptide density used for dendritic cell labelling and *in vivo* priming (Bullock, Mullins et al. 2003).

CD8$^{(+)}$ T cells with high levels of antigen sensitivity can effectively respond to low densities of cognate peptide/MHC I on the target cell surface. Consequently, effector functions are triggered more readily, which corresponds with rapid and effective target cell elimination (Bennett, Ng et al. 2007). Highly sensitive CD8$^{(+)}$ T cells are associated with superior control of persistent viruses (Belyakov, Kuznetsov et al. 2006) and viral replication (Almeida, Price et al. 2007), they exert greater selection pressure on variable viruses such as the simian-human immunodeficiency virus (O'Connor, Allen et al. 2002), and they shown to be potent suppressors of the viral infection cycle in HIV infected individuals (Saez-Cirion, Lacabaratz et al. 2007). Moreover, T cells with high levels of antigen sensitivity have the capacity to efficiently eradicate tumour cells and the generation of such CTLs has been implemented in tumour immunotherapy (Dutoit, Rubio-Godoy et al. 2001) with the aim to break tolerance.

The selection of potent CTLs has been considered as a novel immunization approach. T cells of high 'functional avidity' can be selected from naturally occurring lymphocyte populations and expanded *in vitro,* or

they can be engineered by retroviral transduction of high affinity TCR genes, before they are adoptively transferred into the host.

High antigen sensitivity and polyfunctional outcomes are the basis of T cell efficacy and permit the successful control of tumours and viruses. However, the increased turnover of CD8$^{(+)}$ T cells that is associated with these properties can result in an irreversible exhaustion of vaccine-induced effective cytotoxic T cells through the loss of replicative capacity and induction of apoptosis (Monsurro, Wang et al. 2004; Narayan, Choyce et al. 2007). Successful vaccination must therefore seek to maintain effective T cell responses, while T cell proliferative capacity and survival is preserved.

3.20 Modification of immune stimulatory environment to aid immunity.

As well as expressing immunogenic epitopes, pathogens can also trigger the innate immune system (through Toll-like receptors), which alerts the body to danger and, in a specific cytokine environment, helps guide adaptive immune responses. Inducing artificially a specific immune stimulatory environment through targeted incorporation of cytokines, chemokines, and co-stimulatory molecules into synthetic vaccines, potentiates the immune response both quantitatively and qualitatively and enhances effective memory responses.

The most broadly applicable individual cytokine seems to be GM-CSF, which recruits dendritic cells and, subsequently, can induce a range of T cell responses, including differentiation of T_H1, T_H2 cells and CTLs, without skewing the response in favour of one type or another (Berzofsky, Ahlers et al. 2001). Other studies have shown that expression of IL-15 in vaccine vectors can selectively induce longer-lived memory CTLs (Oh, Berzofsky et al. 2003), while IL-12 has the capacity to steer the T helper cell population towards a T_H1 response. IL-2 was also found to improve the clinical efficacy of a peptide-based immunotherapeutic vaccine (Rosenberg, Yang et al. 1998). In addition to individual cytokines, the incorporation of multiple cytokines

such as GM-CSF and IL-12 or the two combined with TNFα has also been shown to result in more potent immune responses.

Combinations of cytokines with co-stimulatory molecules can also synergistically improve CTL responses and antiviral protection (Ahlers, Belyakov et al. 2002). For example, a triple combination of co-stimulatory molecules- B7, intercellular adhesion molecule 1 (ICAM-1) and lymphocyte function associated protein 3 (LFA-3)- expressed in recombinant viral vectors has been found to greatly augment CTL responses in antitumour immunity (Hodge, Sabzevari et al. 1999). Other costimulatory ligands, such as CD40L, have been shown to activate and induce maturation of dendritic cells recruited by GM-CSF, thus, amplifying the immune response. Moreover, chemokine-antigen fusions encoded in a DNA vaccine against HIV have also been shown to enhance immunogenicity of the antigen by inducing both systemic and mucosal immune responses (Biragyn, Belyakov et al. 2002). Similarly, the inclusion of receptors that provide signalling of B7 and TNFR co-stimulatory signals into chimeric antigen receptors (TCRzeta) could trigger self-sufficient clonal expansion and improved the induction of effector functions in resting human T cells (Finney, Akbar et al. 2004).

3.21 Relief of negative regulatory mechanisms for the recovery of anti-viral and anti-tumour immune responses.

Negative regulation of the immune system may contribute to the failure of various immune mediators to eradicate viral infections and tumour. Hence, it has been suggested that relief of negative regulatory mechanisms may maximize vaccine-induced immunogenicity.

Regulatory mechanisms of the immune system include $CD4^{(+)}CD25^{(+)}$ regulatory T cells, IL-13 producing NK cells, $\gamma\delta$ cells and NKT cells. Indeed, targeted deletion of $CD4^{(+)}CD25^{(+)}$ regulatory T cells can improve immune responsiveness (Sutmuller, van Duivenvoorde et al. 2001), while elimination of $CD4^{(+)}$ NKT cells can amplify vaccine-induced CTL responses and induce protection against an HIV-surrogate virus in a murine model (Ahlers,

Belyakov et al. 2002). Agents that block factors secreted or induced by regulatory T cells, such as IL-13 and TGFβ, can also synergize with other strategies to allow the CTL response to reach its full potential. Finally, blockade of the inhibitory co-stimulatory T cell receptor CTLA4 with antibodies can prevent inhibition of T cell activation and, subsequently, improve vaccine responses (Egen, Kuhns et al. 2002).

4. Dendritic cells and the quest for novel Immunotherapies.

Part I-The role of dendritic cells in immune responses.

The analysis of DC function in T cell-mediated immunity involves three paths of research: the uptake, processing and presentation of antigens, cytokine production and surface molecules that control the quality and quantity of the T cell response, and the properties required for the distribution and mobilization of DCs *in vivo*.

4.1 The biology of the dendritic cell.

Dendritic cells, the most potent antigen presenting cells of the immune system, possess a specialised ability to capture antigens, to process them to presentable peptides and to present these processed peptides in the peptide binding grooves of major histocompatibility complex (MHC) molecules for recognition by T cells.

4.1.1 Morphology.

Dendritic cells demonstrate a great plasticity in their morphology, which complements the corresponding task that the cell undertakes at certain stages of its development. DCs are dendritic when immature, morphology that allows enhanced antigen uptake. They become veiled when traveling to enhance movement, and they are dendritic in the lymph nodes to provide increased presentation surface.

4.1.2 Antigen capture.

Dendritic cells contain an effective endocytic system, specialized for antigen capture, especially at the immature stage of their life. They can take up antigens in the form of solutes during fluid phase phagocytosis, as ligands for specific endocytic receptors, and as particles. The efficiency of dendritic

cell antigen uptake is increased by the utilization of uptake receptors that deliver antigen to processing compartments.

4.1.3 Antigen processing.

Antigen presenting cells and especially dendritic cells are unusually effective, compared to other cells, in processing a wide range of complex entities, such as infectious agents and dying cells, to peptides that can be subsequently presented on both MHC class I and MHC class II.

When a dendritic cell becomes infected, viral proteins synthesized in the cytosol are source of peptides that, associated with MHC class I molecules, are presented to $CD8^{(+)}$ T cells. Degradation of cellular proteins occurs in large multi-subunit protease complexes, the proteasomes, by ubiquitin-mediated proteolysis. Professional antigen presenting cells, of which dendritic cells are known to be the most potent, are equipped with a modified proteasome, namely the immunoproteasome. Conversely to the proteasome of normal cells, in immunoproteasome certain subunits are substituted by more active ones to favour optimal recognition and unfolding of ubiquinated proteins so as to promote the generation of peptides presented by MHC class I molecules. Following protein degradation, peptides with a size of 8-10 amino acids are translocated from the immunoproteasome into the endoplasmic reticulum on a specialized transporter associated with antigen processing (TAP), entering a multi-protein assembly, the peptide loading complex (Figure 15). Tapasin, a central component of the loading complex required for MHC I loading, links TAP to MHC class I heavy-chain/β2-microglobulin dimers to facilitate peptide loading onto MHC-I (Li, Paulsson et al. 2000). Other constituents of the loading complex, such as chaperones calreticulin and ERp57, also aid the peptide-loading process by ensuring the correct folding and assembly of the MHC-I with the peptide.

ER lumen

MHC I heavy chain

peptide/ MHC I

ERp57

Crt

TAP

calnexin

β2 microglobulin

tapasin

peptides 8-10 amino acids

endogenously synthesized protein
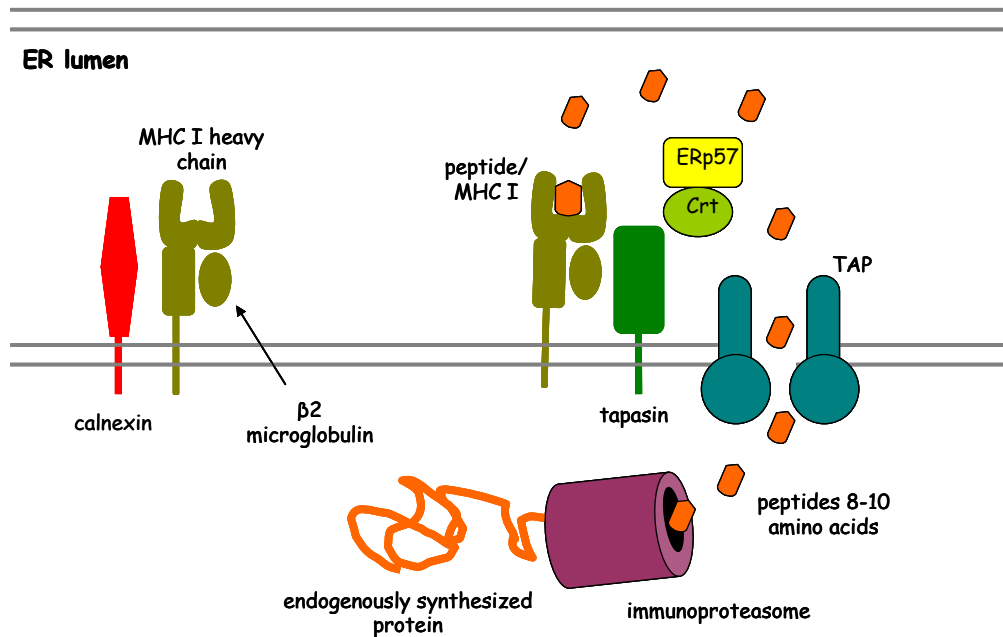
immunoproteasome

Figure 15   **Peptide/MHC class I loading in APCs.**

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-I. Other components of the loading complex, such as calreticulin (Crt) and ERp57, ensure correct folding and assembly of the MHC-I with the peptide. *Image inspired by Dr. D.Pennington's lectures.*

Endocytosed protein antigens are proteolytically processed for the generation of peptides that are presented to CD4$^{(+)}$ T cells in the context of MHC class II. MHC-II molecules are also formed in the ER however they remain protected from peptide loading by the invariant chain and cytosolic adapter molecules until recruited in the endocytotic pathway (Hammerling and Moreno 1990). Once they are exposed to the lysosomal acidic environment, the invariant chain is cleaved leaving a small fragment called CLIP (class II-associated invariant chain peptide) that is associated with the binding groove of the class II a:β dimer. The pool of conventional lysosomal proteases, supplemented by a specific protease called cathepsin S, generates peptides that replace CLIP and bind to the class II molecules. The selection of optimal peptides with maximum binding affinities is catalyzed by the heterodimeric transmembrane glycoprotein HLA-DM in humans, a specialized component of the MHC-II loading complex.

In addition to classical pathways of antigen presentation, dendritic cells also use an alternative mechanism, which involves cross-presenting of exogenous antigens to elicit CD8$^{(+)}$ CTLs. During cross-presentation, protein antigens are internalized and gain access to peptide processing pathways for presentation on MHC class I (Bevan 2006), allowing dendritic cells to induce CD8$^{(+)}$ T cell responses to immune complexes, non-replicating forms of microbes and vaccines, and dying cells. This unusual capacity is critical for priming CD8$^{(+)}$ T cell responses to viruses and other pathogenic organisms that do not directly infect antigen presenting cells. Furthermore, it is thought to be a way of preserving useful information from ingested particles that otherwise would be destroyed and, hence, constituting a delicate bridge from innate immunity to a specific adaptive immune response (Savina and Amigorena 2007).

It appears that different types of dendritic cells favour different pathways of antigen processing to compliment their specialized function.

4.1.4 Secretory products.

Dendritic cells secrete a number of mediators that depend on environmental signals and the maturation state of the cells. They produce various cytokines and chemokines, which influence the quality of the immune response. For example immature dendritic cells contribute to T cell and NKT cell responses by IL-2 production, IL-12 producing dendritic cells have the capacity to elicit T_H1/T_H2 helper differentiation and IL-10 production elicits antigen-specific regulatory T cells.

Furthermore, dendritic cells produce non-cytokine growth and suppressive factors, such as thiols and indoleamine 2, 3 dioxygenase (IDO), respectively. IDO is a tryptophan-degrading intracellular enzyme which is implicated in the suppression of T cell responses and the induction of tolerance. It is expressed by some DCs, although it is not clear which signals determine the differentiation of IDO-expressing DC subtypes. However, if the DC expresses IDO this expression can be upregulated by tolerogenic signals
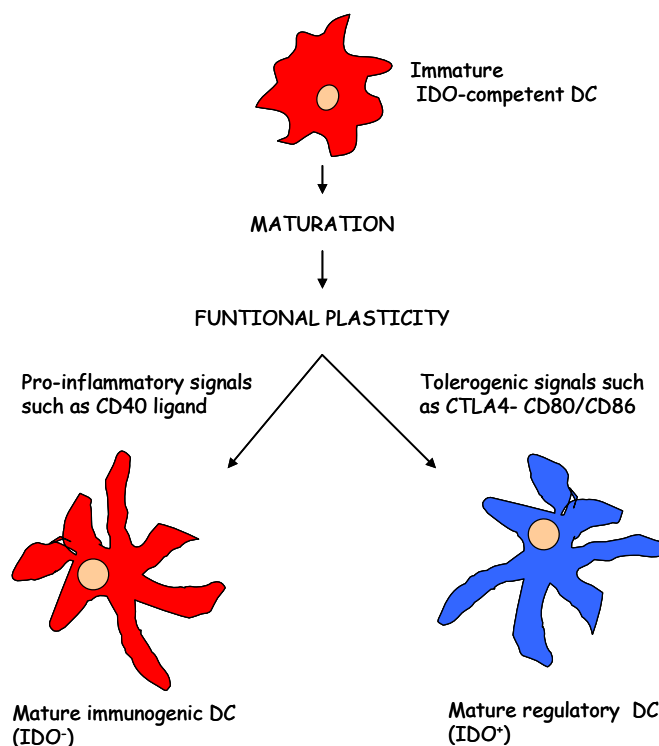
Figure 16   **Functional plasticity of IDO competent DCs.**

In the presence of pro-inflammatory maturation signals, such as CD40 ligation, functional IDO expression by DCs is downregulated. By contrast, tolerogenic signals, such as ligation of CD80/CD86 by cytotoxic T lymphocyte antigen 4 (CTLA4) on regulatory T cells result in differentiation of mature IDO$^{(+)}$ regulatory DCs that actively suppress T-cell responses. *Image reproduced from (Mellor and Munn 2004).*

such as binding of B7 (CD80/CD86) by CTLA-4 on regulatory cells, or downregulated by pro-inflammatory maturation signals such as CD40 ligation (Mellor and Munn 2004) (Figure 16). IDO-dependant T-cell immunosuppression is crucial in regulating T cell responses during a variety of chronic infections such as AIDS-HIV, malaria and hepatitis C, and in tumours. The mechanisms of IDO-dependent DC-suppression of T cells are still under investigation, but generally they are thought to involve both direct inhibition of T cell responses through tryptophan deletion and toxic metabolites, and indirect inhibition by altering the APC function (Mellor and Munn 2004).

Of particular interest is the ability of DCs to secrete exosomes, small vesicles that carry MHC and co-stimulatory molecules. Exosomes have been

used extensively in dendritic cell based but cell-free vaccination, as described later.

4.2 Dendritic cells have the ability to move about the body in a directed way.

The ability of dendritic cells (DCs) to initiate and orchestrate immune responses is a consequence of their initial localization within tissues and their specialized capacity for mobilization. The inherent migratory capacity and specialization for homing efficiently to the T cell zones of lymphoid organs for optimal interactions with T lymphocytes distinguishes dendritic cells from the other antigen presenting cells, such as macrophages (Randolph, Ochando et al. 2008). The pathways and mechanisms that govern DC migration to lymphoid and non-lymphoid tissues shape the immune response and the notably different migratory routes that distinct dendritic cell subsets undertake affects their specialized role in immune responses.

Dendritic cell mobility is apparent from the early stages of their differentiation when DC precursors travel from the bone marrow via the blood stream to reside in peripheral tissues. In the absence of apparent microbial stimuli dendritic cells in the steady state emigrate from the periphery to the lymph nodes through afferent lymphatics constitutively (Randolph, Sanchez-Schmitz et al. 2005) carrying self-antigens which they present to T cells to establish and maintain peripheral tolerance to self (Hawiger, Inaba et al. 2001). Microbial or inflammatory stimuli trigger dendritic cell maturation that is accompanied by upregulation of the chemokine receptor required for DC trafficking to lymph nodes, CCR7 (Sallusto, Palermo et al. 1999). Migration further induces maturation so that the DCs arriving within the LN express upregulated surface ligands, for example MHC and co-stimulatory molecules, necessary for optimal communication with naive T cells. Recruitment of monocytes and DC precursors from the blood to the site of infection or vaccination is also augmented by a chemokine-driven process; within the inflammatory environment dendritic cell precursors rapidly differentiate into immature DCs and replenish the depletion of mature DCs from that site

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(Banchereau, Briere et al. 2000). Finally, circulating DC precursors may also be capable of travelling into secondary lymphoid organs directly from the blood stream to form part of a resident network of cells (Bonasio and von Andrian 2006).

Trafficking via afferent lymph allows DCs to encounter and acquire antigen within peripheral organs that would otherwise be unable to travel freely through lymph to reach the T cell zone of lymph nodes for optimal interactions with T cells (Randolph, Ochando et al. 2008). Pioneering static imaging studies revealed that the delivery of soluble antigen from the periphery via afferent lymphatic vessels into the lymph node occurs in at least two successive waves (Itano, McSorley et al. 2003). First, soluble antigen arrives and is rapidly conveyed within reticular conduits (Sixt, Kanazawa et al. 2005), before being taken up, processed, and displayed as peptide/MHC complexes by resident DCs. A second wave of antigen, borne by DCs in the periphery, subsequently arrives a few hours later, while during the ongoing immune response, more waves of antigen-bearing dendritic cells also arrive in the lymph nodes. Because T cells have the capacity to re-interact with antigen-bearing DCs *in vivo*, and subsequently, integrate sequential signals from multiple APC encounters even at late stages in their differentiation program, the number of antigen-bearing DCs that reaches the draining lymph node may act as an important parameter by dictating the number of antigen-specific T cells that is recruited into the immune response, and by qualitatively modulating the activation program of T cells through APC re-encounter (Celli, Garcia et al. 2005).

4.3 Dendritic cell biogenesis and subtypes.

The several dendritic cell subtypes that have been identified appear to be distinct entities of separate origin. They generally differ in their surface phenotype, their genetic programme, their anatomical location, in their expression of receptors for pathogen-associated-molecular patterns, and their ability to influence T cell fate (Naik 2008). Generally, dendritic cells can be divided into two main categories; the conventional DCs (cDCs) that develop

from a common myeloid progenitor and the plasmacytoid DCs (pDCs) that develop from a lymphoid progenitor. Conventional DCs include migratory DCs, resident DCs and monocyte-derived DCs (Masson, Mount et al. 2008).

4.3.1 Migratory DCs.

Migratory DCs develop in peripheral tissues, such as skin and mucosa, and migrate into the regional lymph nodes, where they transport the antigens they encounter in the periphery. Their origin, function and properties are not clear. Langerhan cells are an example of migratory dendritic cells, found in the epidermis. In mice, Langerhan cells were shown to derive from blood (Ginhoux, Tacke et al. 2006), as well as from skin-resident precursors. They are long-lived in the epidermis, but can constitutively migrate from the skin to the draining lymph nodes. Once they encounter and take up antigen at peripheral tissues, migratory DCs travel to the draining lymph nodes via the lymphatics. In the lymph nodes, they accumulate at the paracortex in the vicinity of high endothelial venules (HEV) (Katakai, Hara et al. 2004) and present the processed antigens that they carry. Depending on the antigen they have encountered at the site of infection, migratory DCs mediate appropriate T_H responses by direct presentation of the antigen in the context of MHC-II, but, also, contribute to CD8$^{(+)}$ CTL cross-priming by transferring their antigen for presentation by lymphoid-tissue-resident DCs (Diebold 2008). Studies have shown that, in the absence of infection, peripheral dendritic cells (e.g. in the epidermis) are important for the induction of peripheral tolerance to skin antigens and commensal bacteria (Steinman, Hawiger et al. 2003; Waithman, Allan et al. 2007). Therefore, migratory dendritic cells provide flow of antigenic information from the periphery, both during immune homeostasis and infectious episodes (Masson, Mount et al. 2008).

4.3.2 Resident DCs.

Resident DCs live their entire life within the lymphoid organs. They originate from bone marrow precursors (Naik, Sathe et al. 2007) that enter lymphoid organs via the blood and they reside near the LN conduits where

they can access, take up and present soluble antigen that arrives via the lymph (Sixt, Kanazawa et al. 2005). All the lymphoid organs contain resident DCs, which represent half of the dendritic population in the lymph nodes and almost the entire dendritic cell population of the thymus and the spleen (Shortman and Naik 2007). By contrast to migratory DCs, which arrive to the lymph nodes in a mature state, resident DCs are phenotypically immature and active in antigen uptake and processing (Wilson, El-Sukkari et al. 2003). In mice, lymphoid-resident DCs can be divided in two categories based on their expression of the CD8 and CD4 surface markers. These are the CD8α DCs (CD8$^{(+)}$ CD4$^{(-)}$) and the non-CD8α DCs (CD8$^{(-)}$ CD4$^{(+)}$ and CD8$^{(-)}$ CD4$^{(-)}$), which differ in their cytokine production and their presentation of antigens on MHC molecules.

4.3.3 Monocyte-derived DCs.

Monocyte-derived DCs belong to the myeloid lineage and develop from the same myeloid precursor that gives rise to conventional DCs, but following a different differentiation pathway that includes a monocyte intermediate. Monocytes are found in the bone marrow, blood and the spleen. They conventionally give rise to macrophages, however, under inflammatory conditions they can differentiate into different subtypes of myeloid dendritic cells which differ in terms of their location, the expression of surface markers and their functions. There are two main classes of monocytes; the CD115$^{(+)}$ Gr1$^{(+)}$ and the CD115$^{(+)}$ Gr1$^{(-)}$. Dendritic cells develop from CD115$^{(+)}$ Gr1$^{(+)}$ monocytes upon inflammation, and they are shown to have immunostimulatory function in infected mice or immunosuppressive role in tumour-bearing mice (Geissmann, Auffray et al. 2008). The best defined monocyte-derived inflammatory-DC subset are the stimulatory TipDCs, which are characterised by their tumour necrosis factor-a (TNFa) and nitric oxide (NO) production (Diebold 2008). Inflammatory DCs migrate from peripheral tissues to lymphoid organs transferring antigen, but also act as replacements for migrated DCs, such as Langerhans, in peripheral tissues during inflammation (Ginhoux, Tacke et al. 2006).

4.3.4 Plasmacytoid DCs.

Plasmacytoid dendritic cells, named after their similarities to antibody-producing plasma cells, are dendritic cell precursors found in the blood, thymus, bone marrow, liver and the secondary lymphoid organs (Liu, Waskow et al. 2007). They are recognised by their ability to produce type I IFNs in response to pathogen recognition (Masson, Mount et al. 2008). Plasmacytoid DCs constantly screen for viruses, bacteria, certain types of TLR agonists and for other danger signals, such as inflammatory chemokines and altered concentrations of cytokines. Upon viral infection, the plasmocytoid precursors are recruited to the inflamed draining lymph nodes where they develop to dendritic cells with an immature phenotype (Liu 2005); they express low levels of MHC and co-stimulatory molecules and are weak stimulators of naïve T cells (Hochrein and O'Keeffe 2008). Plasmacytoid DCs are thought to contribute to antiviral immune responses by secreting large amounts of type I IFNs (Masson, Mount et al. 2008), which supports T_H1 cell activation and promotes cross-priming of CTLs (Diebold 2008).

The existence of multiple subsets of DCs reflects their functional specialisation to promote distinct immune responses (Pulendran, Tang et al. 2008) that aid tolerance maintenance or the generation of immunity. For example, in mice the balance between $CD8\alpha^{(+)}$ and $CD8\alpha^{(-)}$ DC subsets can differentially influence the T_H1/T_H2 balance of differentiated primed effector T cells. Stimulation of naive $CD8^{(+)}$ T cells depends on $CD8\alpha$ DCs and lymphoid-tissue-resident-$CD8\alpha^{(+)}$ DC subsets are particularly efficient for the cross-presentation of antigens on MHC-I (Lin, Zhan et al. 2008), whereas $CD8^{(-)}$ DCs are more efficient in processing antigens for presentation on MHC-II (Dudziak, Kamphorst et al. 2007). Conversely, a specialized mucosal $CD103^{(+)}$ DC subset can induce differentiation of $Foxp3^{(+)}$ regulatory T cells (Coombes, Siddiqui et al. 2007). Collectively, the different DC populations seem to be coordinated and probably operate in combination to provide a net response of tolerance or immunity as required.

4.4 Dendritic cell maturation and differentiation.

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. 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), 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 (Steinman, 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 (Garrett, 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 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.

Part II- Dendritic cell-based Immunotherapies.

Dendritic cells orchestrate a repertoire of immune responses that result in either resistance to infection or tolerance to self; thus, they play an important role in medicine, both in understanding how disease develops and in designing new treatments (Steinman and Banchereau 2007). Indeed, in the effort to combat various diseases, dendritic cells have attracted significant interest and they are now considered a promising target/tool in immunotherapy; particularly, when designing new-generation vaccines.

One approach for the control of chronic infections and cancer is a strategy that could stimulate a patient's own immune responses against a persisting virus or tumour that the immune system is already fighting; that is described as therapeutic vaccination. However, during chronic viral infections potentially useful anti-viral T cells often become functionally exhausted and do not respond properly to therapeutic vaccination (Ha, West et al. 2008). Thus, dendritic cell vaccines aimed at clearing a persistent infection have to either reverse this functional deficiency or induce new anti-viral effector T cells, in addition to inducing a humoral response. Unlike infectious pathogens, tumours do not induce a robust innate inflammatory response and, as a result, the ensuing adaptive response is often weak and ineffective. In this case, the purpose of vaccinating individuals with cancer is to prime naïve T cells to generate functional tumour-specific effector T cells, which may be achieved by channelling tumour antigens into DCs.

4.5 Mediating the immune response by dendritic cell based vaccination.

An improved understanding of the underlying mechanisms that dendritic cells employ in antigen processing, immune presentation and regulation of immune responses, has allowed the development of novel methods of dendritic-cell immunotherapy. Dendritic-cell based vaccination strategies are designed to overcome the mechanisms by which viruses and cancers evade the immune response and aim to induce antigen-specific

effector and memory cells. What makes dendritic cells a particularly useful tool in new-generation vaccines is that as well as being able to process antigen in the MHC class I pathway and to induce CTLs, they can also present antigens via the MHC class II pathway to stimulate T-helper cells, which regulate B cell responses; thus, mediating both the cellular and humoral arm of the immune response.

Dendritic cells were originally used in immunization strategies as adjuvants. Since then, various approaches have been reported including pulsing the cells with peptides, loading them with whole proteins and transfecting them with RNA encoding specific antigens. Furthermore, DC-derived exosomes have also been used for vaccination purposes.

In more recent dendritic cell vaccination studies, induction of an antigen-specific immune response was achieved either by direct targeting of antigen to DC surface receptors *in vivo* or by inducing maturation of dendritic cells that can then be loaded *ex vivo* with tumour or microbial antigens. Indeed, the identification of suitable DC-expressed cell-surface receptors that mediate endocytosis of bound antibodies has allowed the development of more direct strategies, where professional APCs are selectively targeted and loaded with antigen that is delivered associated with the endocytosed antibody. For example, targeting of a DC NK C-type lectin group receptor-1 (DNGR-1) that is selectively expressed in mouse $CD8\alpha^{(+)}$ cDCs allows antigen delivery *in vivo* to $CD8\alpha^{(+)}$ DCs that results in cross-priming of $CD8^{(+)}$ T cells and, together with an adjuvant, in the induction of potent CTL responses that can cure mice of a transplantable tumour (Sancho, Mourao-Sa et al. 2008).

The ability of DCs to cross-present antigens has provided a major opportunity for *in vivo* targeting strategies aimed at generating effective cellular responses against tumours or pathogens that are inefficiently cleared by the humoral immune system. Although the mechanism is still not clear, cross-presentation efficacy has largely been attributed to phagosome–ER fusion during or soon after phagosome formation, allowing endosomal

proteins to escape proteolysis and gain access to the ER, from where they may be transported into the cytosol (Ackerman, Kyritsis et al. 2005). However, cross-presentation is not an efficient process as endocytosed soluble antigens seem less efficiently cross-presented when compared with phagocytosed particulate antigens (Carbone and Bevan 1990; Reis e Sousa and Germain 1995; Schulz, Pennington et al. 2002). Therefore, much effort has been focused on enhancing endosomal escape to improve cross-presentation of antigens; several substances that facilitate endosomal escape and amplify cytoplasmic delivery have been introduced, such as biodegradable polymeric nanoparticles (Shen, Ackerman et al. 2006), fusogenic peptides (Laus, Graddis et al. 2000), cell-penetrating peptides (CPPs) and receptor-specific antibodies (Tacken, Joosten et al. 2008). However, most of these studies fail to conclude whether endosomal escape is substantially achieved.

Professional antigen-presenting cells such as DCs are thought to be very important in regulating the immune response in DNA-based vaccination strategies, as they can both phagocytose antigen released from any transfected cell and can be transfected directly by the plasmids. The use of gene-based dendritic cell vaccines circumvents the need for cross-presentation, since immunogens encoded by DNA are endogenously synthesized and access the classical MHC-I pathway like natural antigens. In addition, antigen-encoding DNA can access dendritic cells by various delivery systems, such as live attenuated viruses, bacteria, liposomes, polymer microparticles, bacterial ghosts or virosomes (Tacken, de Vries et al. 2007). A particularly promising approach is targeted-gene delivery to dendritic cells using adenoviral vectors which has the advantage that the viral DNA does not integrate into host chromosomal DNA (in which case it could become a latent viral infection) and is therefore transiently expressed. In other approaches to improve vaccines for tumour immunotherapy dendritic cells were transfected with tumour-derived RNA or fused with tumour cells to generate DC–tumour hybrids, achieving a wider range of tumour specific antigens for presentation. Finally, RNA interference (RNAi), a potent method for gene silencing that has developed rapidly over the last few years (Mao, Lin et al. 2007), has been

used to improve vaccine potency by modulating various functions within dendritic cells. For example, there are reports of RNAi-directed prolongation of DC life through the silencing of propaptotic genes (Peng, Kim et al. 2005) or RNAi-directed attenuation of immunosuppressive signals (Song, Evel-Kabler et al. 2006). These studies have demonstrated that the use of RNAi technology in developing new-generation DC-based vaccines results in more potent antiviral and antitumour immune responses (Mao, Lin et al. 2007). Alternatively, RNAi has also been used to modify DC maturation, in order to select for a particular type of immune response to infection; for example, knockdown of IL-10 expression in DCs resulted in an enhanced T_H1 response and a suppressed T_H2 response (Liu, Ng et al. 2004), while RNAi-induced silencing of IL-12 in DCs induced the opposite effect (Hill, Ichim et al. 2003).

Dendritic cell vaccines that are currently being investigated in clinical trials consist of autologous DCs that are matured and antigen-loaded *ex vivo* (Figure 17). In these studies, dendritic cells derive from monocytes or CD34$^{(+)}$ precursors that 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. These mature antigen-loaded autologous DCs are administered to patients with the intention of inducing antigen-specific T and B cell responses.

Figure 17
Vaccination studies using autologous DCs.

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.

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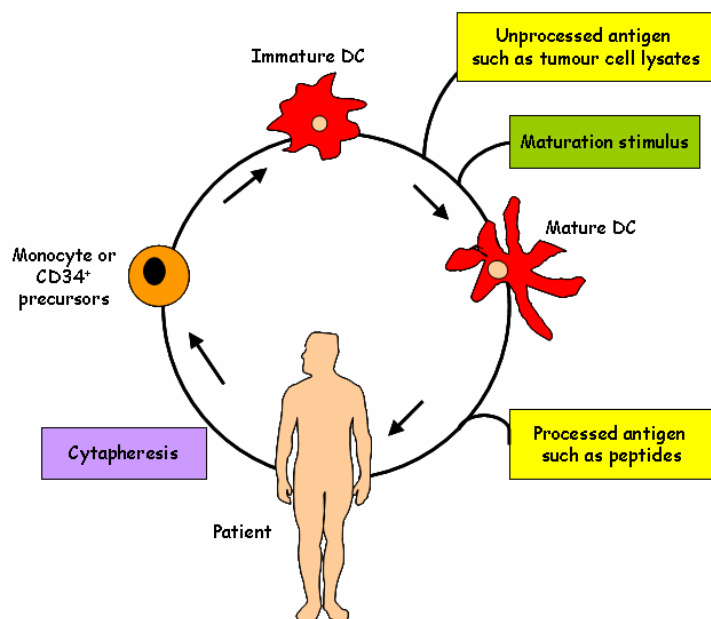
In addition, several clinical trials tested combinations of a DC-based therapy with other therapies, such as depletion of regulatory T cells (Dannull, Su et al. 2005), chemotherapy (Melief 2008) or administration of cytokines (Redman, Chang et al. 2008). Other studies have evaluated the use of TLR-ligand-activated DCs, the use of various DC subsets, and the use of DC-based therapy in combination with strategies that target co-stimulatory molecules, such as CTLA4, OX40, 4-1BB or PD1 (Tacken, de Vries et al. 2007).

4.6 Challenges and limitations in dendritic cell Immunotherapies.

To date, numerous clinical trials have demonstrated that DC vaccination can induce immunological responses in many of patients. Importantly, they have proved that these therapies are safe and well-tolerated with only minor side effects constrained to induration of the skin at the injection site and mild fever. However, dendritic cell vaccines have generally been evaluated in patients with late-stage cancer with a poor prognosis. These patients are likely to be less immunocompetent as a result of a large tumour burden and prior radiation therapy or chemotherapy. This might explain why, to date, clinical responses have only been observed in a minority of patients.

Immunotherapies based on strategies that target antigens to dendritic cells *in vivo* have the major advantage that the targeted DCs are naturally-occurring DC subsets and that the vaccines can be produced in bulk. However, compelling evidence from these targeting studies suggest that their efficacy depends on numerous factors, such as the expression pattern and biological properties of the targeting receptor and the maturation or activation status of the DC. Indeed, differences in the expression pattern of receptors on separate DC subsets, the intracellular signalling cascades they induce, and the intracellular processing pathways that the antigens follow can affect the immunological outcome of *in vivo* DC therapy. Furthermore, most of the receptors employed for *in vivo* DC-targeting strategies are also expressed by other cells, which may also affect targeting efficiency. On the other hand, *ex vivo* culture conditions can be controlled more carefully compared to the *in*

vivo environment and the dendritic cell quality can be better assessed before the cells are administered to the patient. Nevertheless, therapies based on *ex vivo* antigen loading require vaccines to be tailor-made for each individual and involve artificial maturation of cells in culture which can lead to heterogeneity in their immunological properties. Additional difficulties in both strategies include the duration of antigen presentation and the stability of the vaccine following administration.

Research in dendritic cell therapies has emphasized the importance of dendritic cell quality in achieving the desired immunological outcome. Maturation status, and the timing and route of maturation stimuli (in relation to antigen loading) can both influence antigen capture, processing and presentation. This is reflected in early DC-based studies that observed tolerance instead of immunity, resulting in a switch to the use of mature DCs. Still, the maturation protocols have a major effect on the type of differentiation that occurs; for example, applying stimuli too long before or too long after the antigen can impair antigen cross-presentation (Wilson, Behrens et al. 2006), while certain DC receptors, such as CD205, seem to lose their endocytic capacity on full DC maturation (Butler, Morel et al. 2007), abolishing uptake of targeted antigens. The migratory capacities of dendritic cells can also influence their ability to induce effective T cell responses as highlighted in clinical trials where only a small percentage of injected dendritic cells actually migrate form the injection site to the draining lymph node to present antigens to T cells. Furthermore, the ways antigens are handled, processed and presented by distinct dendritic cells may have an impact on the resulting immune response.

Part III- Dendritic cell-based, but cell-free vaccination approaches.

To overcome the difficulties of DC therapy, cell free antigen-presenting systems have been reported, including membrane vesicles derived from APCs, such as exosomes, which are secreted from endosomal compartments of APCs, and microvesicles derived from plasma membranes of APCs after sonication (Kim, Latouche et al. 2004; Kovar, Boyman et al. 2006).

4.7 Dendritic cell-derived exosomes can stimulate immunity.

Exosomes are small membrane vesicles, between 30 and 100 nm in diameter, originating from late endosomes and secreted by most cells in culture. Exosomal vesicles can be purified from cell culture supernatants subjected through a series of high speed centrifugations, following separation by sucrose gradient floatation; the resulting exosome pellet is relatively free of dead cells, protein aggregates or nucleosomal fragments. Interest in exosomes intensified after their description in antigen-presenting cells and the observation that they can stimulate immune responses *in vivo*.

4.7.1 Exosome biogenesis.

Protein composition analysis of exosomes has shown that they are secreted by living cells and they are distinct from microvesicles produced by apoptotic cells. All of the exosomal proteins that have been identified are found in the cytosol, in the membrane of endocytic compartments or at the plasma membrane (Thery, Zitvogel et al. 2002). However, they are not simply fragments of the plasma membrane, as they lack some abundant cell-surface proteins, such as Fc receptors in DC derived exosomes, while they contain other endocytic or cytosolic protein markers absent from the plasma membrane.

4.7.2 The molecular profile of exosomes.

Analysis of dendritic cell-derived exosome preparations has revealed the presence of known cellular proteins, which are potentially involved in exosome biogenesis, exosome targeting/docking/fusing with other cells and exosome putative immunological function (Thery, Regnault et al. 1999; Thery, Boussac et al. 2001). Examples include cytoskeletal components such as actin, actin-binding proteins and tubulin, as well as other cytosolic proteins involved in intracellular membrane transport and fusion (e.g. annexins, small GTPase family members or related proteins: rab7, rab11, rap1B, and rab GDP dissociation inhibitor) (Thery et al, 2001). In DC-derived exosomes, cytosolic proteins involved in signal transduction (e.g. $G_{i2}\alpha$, syntenin, and 14-3-3) are also present, as well as metabolic enzymes (e.g. thioredoxine peroxidase) (Thery, Boussac et al. 2001), and heat-shock proteins (such as constitutive isoforms of HSP70, HSP73 and HSP90) (Thery, Regnault et al. 1999). Heat-shock proteins (HSPs) are ubiquitous proteins that are involved in antigen presentation, as they interact with antigenic peptides and contribute to peptide loading onto MHC molecules (Srivastava 2002). Exosomes are highly enriched for a family of proteins called tetraspanins (e.g. CD9 and CD63), which are thought to be involved in the organization of large molecular complexes and membrane sub-domains as they interact with many protein partners, including MHC molecules and integrins (Thery, Zitvogel et al. 2002). MHC class I molecules are present in exosomes derived from most cell types. Several proteins are exposed at the surface of exosomes and bind ligands on other membranes. Some of these proteins are believed to have a role in 'addressing' exosomes to their cellular targets *in vivo*, for example MFG-E8/lactadherin, which is a major exosomal component that binds integrins expressed at the surface of DCs and macrophages, is thought to be involved in targeting DC-derived exosomes to other APCs (Thery, Regnault et al. 1999). Indeed, exosomes from antigen presenting cells have been extensively analysed and several proteins that may be involved in the biological function of exosomes have been identified. For example, exosomes from cells rich in MHC class II molecules have abundant MHC- II (Zitvogel,

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Regnault et al. 1998). Furthermore, DC-derived exosomes contain T cell co-stimulatory molecules, such as CD86, as well as a series of other transmembrane proteins, such as integrins (e.g. αMβ2) (Thery, Regnault et al. 1999) and immunoglobulin-family members, such as intra-cellular adhesion molecule 1 (ICAM-1) (Clayton, Court et al. 2001).

The presence of these molecules indicates that exosomes may represent a way of communication, i.e., exchange of antigenic information, between cells of the immune system. More recent research has shown that exosomes contain both mRNAs and microRNAs that can be delivered to other cells and can be functional at this new location (Valadi, Ekstrom et al. 2007). This indicates that exosomes may also play a role in intercellular genetic exchange.

4.7.3 Exosome function.

Once released, exosomes can fuse with membranes of neighbouring cells, establishing membrane, protein, and genetic exchange, in the absence of direct cell-cell contact. Although the properties of exosomes can be characterized through their molecular composition, which usually reflects the specialized function of their original cells, the physiological relevance of exosomes remains unclear. Early studies suggested that exosomes may function as an alternative to lysosomal degradation (Pan and Johnstone 1983), while others proposed that exosomes are involved in antigen presentation through transfer of peptide/MHC complexes, antigens or HSP-associated peptides. One hypothesis is that exosomes could be produced in peripheral tissues by immature dendritic cells that have encountered antigen and before their migration to lymph nodes in order to sensitize other DCs in the periphery, which have not encountered antigens themselves for T cell stimulation (Thery, Regnault et al. 1999). Certainly, exosome-mediated information exchange may occur in numerous physiological events. For example, exosomes secreted from CD8$^{(+)}$ CTLs may assist the delivery of lytic substances (e.g. perforin and granzymes) to the target cell, while other proposed functions of exosomes include their participation in the induction of

immunological tolerance, enhancement of sperm motility and in tissue development (Stoorvogel, Kleijmeer et al. 2002). Nonetheless, despite these studies it is still unclear to what extent, and for what purpose, exosomes are produced physiologically *in vivo*.

4.7.4 Exosomes as therapeutic tools.

Even though the physiological purpose of exosomes remains unclear, the presence of molecules involved in antigen presentation has made APC-derived exosomes an appealing tool for use in immunotherapy. Indeed, DC-derived exosomes express MHC class I and MHC class II, as well as co-stimulatory molecules, and have been shown to promote T cell–dependent anti-tumour immune responses *in vivo* (Zitvogel, Regnault et al. 1998). In the presence of APCs, exosomes efficiently mediate the induction of MHC class I-restricted CD8$^{(+)}$ T cell expansion and differentiation *in vitro* and *in vivo*, leading to tumour rejection. Exosome-mediated elimination of tumour was tumour-peptide specific, and the observed long-term protection was also tumour specific. Moreover, other studies demonstrated that melanoma-cell-line-derived exosomes were a source of 'shared' tumour-rejection antigens and they could mediate protection against allogeneic tumours in mice, possibly by stimulating protective CTLs via cross-priming (Wolfers, Lozier et al. 2001).

Exosome vaccine preparations are preferable to those of whole cell cultures for several reasons. For example, quality control parameters are easier to define as MHC content can be measured and calibrated. In addition, exosome production and purification procedures are more reliable and reproducible, while exosomal membranes are more stable than DC cultures, and can be stored for long periods.

As well as a therapeutical vaccine vector, exosomes released from tumour cells have been used in immunotherapy for the identification of the immunogenic properties of the tumour. Tumour cells secrete exosomes that carry tumour antigens associated with MHC-I, as well as cytosolic candidate

tumour antigens (Thery, Zitvogel et al. 2002). Tumour-derived exosomes bear antigens specific to the individual's cancer and can be loaded on DCs that are then used to induce T cell responses, allowing the generation of tumour-specific MHC class I-restricted CTL clones *in vitro.* This could be particularly helpful in cancer immunotherapy because tumour derived exosomes are also produced *in vivo* and can be isolated from malignant effusions of cancer patients who have non-immunogenic tumours (Andre, Schartz et al. 2002).

4.7.5 Limitations of exosome immunotherapy.

Exosome production is a feature of immature dendritic cells, when multivesicular bodies still form in the cytosol. However, immature dendritic cells express low levels of MHC and co-stimulatory molecules and consequently exosomes derived from these cells do not efficiently activate naïve T cells. For this reason, exosome vaccination requires antigen/exosome uptake and exosome-derived antigen-presentation by mature host dendritic cells for efficient T cell activation *in vitro* and *in vivo*. Consequently, an efficient exosome-based vaccine necessitates an adjuvant that activates dendritic cells *in vivo*.

When designing an exosomal vaccine it is also important to address the possible risks of spreading infectious particles that may reside in exosome-producing cells. This is best described in the 'Trojan exosome' hypothesis, which, based on the fact that retroviral particles and exosomes contain a similar array of host cell lipids and proteins, and use the same proteins for cell targeting and vesicle biogenesis, predicts that exosomes may support transmission of retroviruses. Indeed it is reported that HIV *trans* infection may also occur by viral association with dendritic cell-derived exosomes (Hladik, Lentz et al. 1999), which possibly increase the infectivity of the virions that are coupled to them (Hladik and McElrath 2008). In addition to viruses, exosomes may act as vehicles for prion spread between cells (Fevrier, Vilette et al. 2004).

Safe exosome-based vaccines should be of autologous origin, being derived from dendritic cells from a patient's blood. However, blood from a single individual doesn't yield enough exosomes for an individual vaccine (Chaput, Taieb et al. 2005). Thus, methods for improving exosome isolation need to be improved if this strategy is to become a realistic vaccine option.

Thus far, exosomes have been described in experimental settings that have resulted in T-cell priming. However, care must be taken in selecting the most appropriate cells for exosome isolation, as it is possible that T-cell stimulation by exosomes might also induce tolerance (Karlsson, Lundin et al. 2001). Moreover, it is also possible that exosomes may have an immunosuppressive nature. For example, melanoma cells secrete Fas-Ligand-bearing exosome-like vesicles that have pro-apoptotic activity on T cells (Andreola, Rivoltini et al. 2002), thus the use of exosomes may have counter-productive effects when trying to induce potent effector T cell responses.

4.8 Dendritic cell-derived plasma membrane fragments can induce immune responses.

As an alternative to injecting intact dendritic cells or exosomal vaccines, dendritic cell-derived plasma membrane fragments have been investigated (Kovar, Boyman et al. 2006). This material is prepared by ultracentrifugation after sonication of IFNγ-matured dendritic cell lines. These vesicles closely resemble exosomes and activate naïve T cells well both *in vitro* and *in vivo* (Kovar, Boyman et al. 2006). The sonicated membrane vesicles were shown to express functional MHC-I and T cell co-stimulatory molecules, such as ICAM-1 (CD54), B7.1 (CD80) and B7.2 (CD86), which presumably explains their direct immunogenicity in the absence of antigen presenting cells *in vitro*. Their ability to activate naïve T cells, like exosomes, is strictly peptide-specific, but requires the presence of soluble peptide at high concentrations during T cell stimulation. Nevertheless, a great advantage of sonicated membranes is that they are obtainable in much greater quantities than exosomes.

5. Proposed system and thesis aims.

This study attempts to reconstitute the optimal antigen presentation properties of dendritic cells using endoplasmic reticulum derived membranes, known as 'microsomes'. The aim is to overcome the limitations of current vaccination approaches and create a vaccine that will promote protective immune responses targeted to particular pathogens, especially those against which the more basic approaches of vaccination have so far failed.

The dendritic cell-based but cell-free systems described to date, and reviewed above, contain MHC-I, MHC-II and co-stimulatory molecules, and can induce naïve T cell responses *in vivo* when mixed with or pre-loaded with defined peptides (Zitvogel, Regnault et al. 1998; Kovar, Boyman et al. 2006). However, because it is not known how peptide/MHC is processed in these membrane vesicles, both the quality and the quantity of peptide/MHC are difficult to control in the preparation. The MHC molecules on the surface of APCs are pre-processed; therefore the desired vaccine peptides must be added in competition with endogenous pre-bound peptides. Thus obtaining a high concentration of the required peptide/MHC may be problematic, especially when these peptides are of medium or low affinity (Yewdell and Haeryfar 2005).

Here, a new form of APC-based, but cell-free vaccine is described using ER-enriched microsomes derived from mature DCs or other APCs. All eukaryotic cells have an endoplasmic reticulum (ER) (Porter 1953), which is a highly complex single membrane network that extends throughout the cytoplasm. The ER plays a central role in cell biosynthesis, as it is the starting point for the synthesis of transmembrane and secreted proteins, including the components of the immunological synapse; MHC-I and MHC-II, along with co-stimulatory molecules such as B7. Although the ER is interleaved extensively with other intracellular elements, it is possible to isolate ER membranes from other components of the cell (Dallner 1974). Upon cell homogenization the ER is fragmented into small (~100nm diameter) closed vesicles, known as microsomes, which can be easily purified.

Microsomes have been used extensively as a tool for studies of the functional and biochemical properties of the endoplasmic reticulum. These include studies of the interaction between peptides and the different components of the peptide-loading complex (e.g. TAP, tapasin and MHC-I) during the peptide-loading process (Wang, Gyllner et al. 1996; Li, Paulsson et al. 2000). This research revealed the presence in the microsomes of peptide-receptive MHC-I molecules that bind to peptides in a highly peptide-specific process.

In this thesis I investigate the hypothesis that microsomes derived from APCs, and filled with defined peptides, directly interact with peptide-specific T cells to stimulate their functional activation *in vitro* and *in vivo* (Figure 18).

To test this hypothesis the antigen presenting properties of antigen presenting cells are first established. Microsomes are extracted from antigen presenting cells and they are characterized for components of the immune synapse and their ability to bind peptides. Subsequently, induction of T cell responses by peptide-loaded microsomes is analysed *in vitro* and *in vivo*. Finally, the ability of the microsomal vaccine to induce protective immunity against an acute viral infection and established murine tumours is investigated.

When these microsomal vaccines were injected intravenously into mice they induced peptide-specific immune responses, which were able to protect these animals from acute viral infection and eradicated specific-peptide-carrying tumours.

We propose that peptide/MHC-armed microsomes from DCs can be an important alternative to DC-based vaccines both for protection from viral infection and for the treatment of cancer.
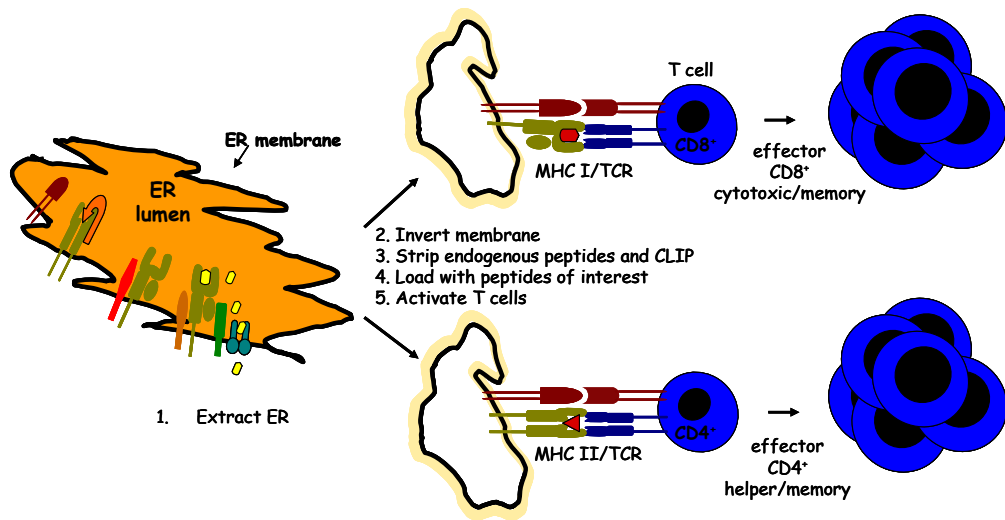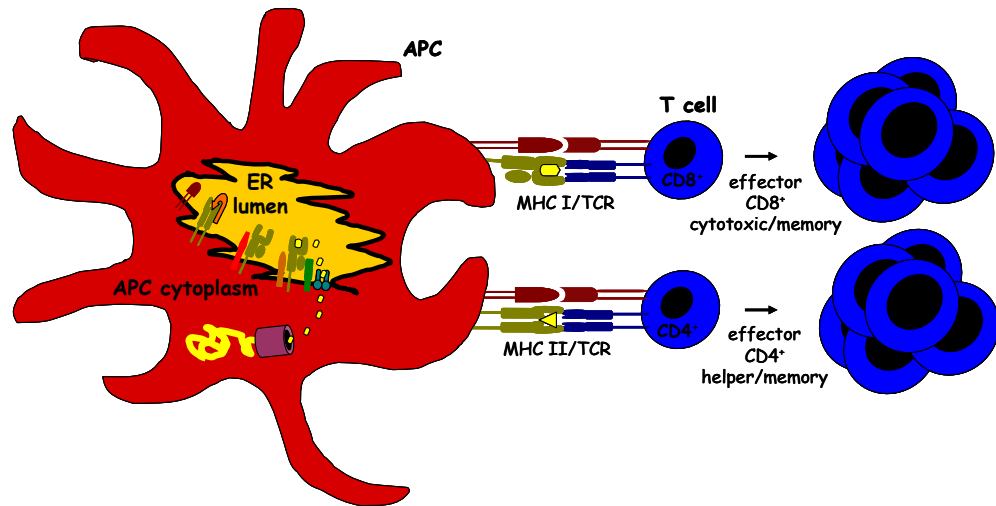
Figure 18 **Thesis proposal.**

In this thesis I investigate the hypothesis that microsomes derived from APCs, and filled with defined peptides, directly interact with peptide-specific T cells to stimulate their functional activation *in vitro* and *in vivo*.

ER- enriched microsomes were extracted from antigen presenting cells (1); they were inverted so as the luminal surface is exposed (2); endogenous peptides and CLIP were removed by acid stripping (3); antigenic peptides of interest were loaded on peptide-receptive microsome-associated MHC molecules (4); and, microsomes presented peptide/MHC and co-stimulatory signals to T cells for the induction of effector T cell responses (5).

MATERIALS AND METHODS

1. Experimental tools and conditions.

1.1 Animals.

C57Bl/6 mice (H-2K^b) were purchased from Harlan UK Ltd, (Oxon, England). OT-I transgenic mice on the C57Bl/6 background expressing a TCR specific to the H2-K^b-SIINFEKL peptide complex were kindly provided by Professor Dimitris Kioussis, MRC National Institute for Medical Research, London. All animals were maintained in specific pathogen-free facilities at Queen Mary and Brunel University.

1.2 Cell lines.

Jaws-II, RMA-S and RAW309Cr.1 cell lines were obtained from American Type Culture Collection (ATCC). LC 721.221/HLA-A2 cell lines were kindly provided by T. Elliott. DC2.4 line was kindly provided by Dr. Mann D. Southampton, UK.

Jaws-II were cultured in Alpha DMEM supplemented with 20% FBS, 1% P/S and 5ng/ml GM-CSF. Cultures were maintained by transferring floating cells to a centrifuge tube. Attached cells were sub-cultured using 0.25% trypsin-0.03% EDTA. Adherent and cells in suspension were pooled and centrifuged at 250 x *g* for 10min; the pellet was then resuspended in fresh medium and dispensed into new flasks. The growth medium was changed once per week. All other cell lines were cultured in RPMI 1640 or DMEM supplemented with 10% FBS and 1% P/S. They were prepared by gentle scraping. 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.

Mouse recombinant granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4) were purchased from PeproTech, Inc. (Rocky Hill, NJ). Chicken egg albumin (ovalbumin; OVA) and LPS were obtained from Sigma (St Louis, MO). All cell culture reagents were from Invitrogen Ltd. (Gibco-BRL, Rockville, MD). Ficoll-Paque was from Amersham (Amersham Biosciences UK Limited, Little Chalfont). ELISA (OptEIA, mouse IL-2 ELISA Set) and ELISpot (BD ELISPOT set mIFNγ) were obtained from BD Biosciences.

Fluorescein isothiocyanate (FITC)-conjugated antibodies to CD54, CD80, CD86, 25-D1.16 and FITC-conjugated Streptavidin; phycoerythrin (PE)-conjugated antibodies to H2-I-A and Y3 were from BD Biosciences. The anti-mouse tapasin antiserum was generated by immunization with a peptide (CATAASLTIPRNSKKSQ) derived from the C terminus of mouse tapasin, as described before (Li et al, 1999). K^b-SIINFEKL specific Pentamer was from Proimmune Ltd. CD4$^{(+)}$ and CD8$^{(+)}$ microbeads (Miltenyi Biotec) were used for isolation of CD4$^{(+)}$ or CD8$^{(+)}$ T cells from spleen according to manufacturer's protocol.

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, OVA$_{257-264}$ (SIINFEKL) and OVA$_{324-340}$ were reconstituted in 1% DMSO in PBS under sterile conditions. Stock concentrations of matrix influenza MP peptide (GILGFVFTL), HIV GAG peptide (ATLYGVHQKI) and nuclear influenza KTR peptide (SAYWAIRTK) had been previously reconstituted in PBS in the lab. HCMV pp65 peptide (NLVPMVATV) was diluted in cell culture medium at high stock concentration. Working concentrations of all peptides were prepared in the appropriate cell culture medium.

2. Peptide modifications.

2.1 Peptide biotinylation.

OVA$_{324\text{-}340}$ was biotinylated using the NHS-LC-Biotin reagent (Pierce Chemical). 1mg of Sulfo-NHS-LC-Biotin was dissolved in 90μl of pure H$_2$O to give a concentration of 10mM Biotin solution in 100mM sodium phosphate buffer, pH 7.5, 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. When the reaction was completed 1.8μl of 1M TRIS-HCL pH 7.4 was added to a final concentration of 10mM. The mixture was incubated at room temperature for 5min to utilize the remaining free NHS-LC-biotin and prevent the reagent to react with other proteins on the microsomes. Biotinylated peptides were aliquoted at 1000μg/ml and stored at -80°C until use.

2.2 Peptide iodination.

Modification: The ε-amino group of lysine in the H2-K^b-specific SIINFEKL peptide was covalently modified by a photo reactive cross-linker and labelled by iodination as previously described (Wang, Raynoschek et al. 1996). Specifically, The H-2K^b-binding OVA peptide (residues 257–264, SIINFEKL) was modified by coupling a phenyl azide with a nitro group to the e-amino group of lysine (position 7) to allow for photo-activation and by substitution of the isoleucine at position 3 with tyrosine to allow for iodination. Modification of the OVA peptide by ANB-NOS was performed by mixing 0.5mg of ANB-NOS dissolved in 200ml of dimethyl sulfoxide, 100mg of peptide dissolved in 100ml of phosphate-buffered saline, and 50ml of 0.5M CAPS (pH 10). The reaction was allowed to proceed for 30min on ice. To remove excess ANB-NOS and ions, the mixture was purified by gel filtration on a Sephadex G-10 column and subsequently by HPLC. An aliquot (1μg) of the peptide was labelled by chloramine T-catalyzed iodination (^{125}I) and used for measuring the peptide binding of H2-K^b.

Labelling: All steps were performed in a well-ventilated hood. A Chloramine-T (CAT) solution and a Sodium Metabisulfite (SMBS) solution were prepared in PBS, each at concentration of 1mg/ml of the respective chemical. 10µl of 1mg/ml SIINFEKL peptide solution was added in the reaction vial containing ^{125}I solution. The labelling reaction was initiated by the addition of 10µl CAT solution to the reaction vial. After 1min of thorough mixing, the labelling reaction was terminated with the addition of 20µl of SMBS solution. At this stage, the radioactivity of the reaction vial was measured using a NaI detector and the distance corresponding to 1000cps was marked. The prepared sample was diluted with PBS to a volume of 200µl, before applied on a NAP-5 (G10) column. The first fraction was collected, more PBS was added (200µl) and subsequent fraction was collected; the process was repeated so as to collect a total of 15 fractions. The relative radioactivity of each fraction was measured using the NaI-detector before the iodinated peptide was pooled in one vial.

Cross-linking: The cross-linking of the peptide on microsomal membranes was performed in low lighting conditions. ^{125}I-labeled and ANB-NOS-modified peptide was mixed with 10µl of microsomes (concentration of OD 60 A280/ml) to a final concentration of 100nM in RM buffer and incubated for 15min at room temperature. The microsomal suspension was processed by repeated freeze-thaw cycles, before subjected under irradiation UV at 366 nm for 5min at room temperature. Microsomes were washed with PBS and centrifuged at 20 000 x g for 10min at 4°C. The resulting pellet was spontaneously frozen in liquid nitrogen and stored at -80°C overnight. Cross-linked microsomal proteins were analyzed by SDS–PAGE.

3. Protein analysis.

3.1 Protein purification.

Total cell or microsome protein extracts were generated using RIPA buffer with protease cocktail tablets (Complete [TM] Roche Diagnostics) or 1% NP40, 5% PMSF lysis buffer. The samples were placed on rotator for 1 hour at 4ºC. Whole cell or microsome lysates were clarified by centrifugation for 15min at 20 000 x g, 4ºC and the supernatants were collected and stored in -20ºC until assayed for protein.

3.2 Protein quantization.

Protein determination was performed using the Bicinchoninic Acid Protein Assay Kit (SIGMA) adjusted to 96 Well Plate Assay. Standard protein concentrations of 0, 20, 40, 60, 80 and 100μg/ml were prepared using a 2mg/ml stock of non acetylated BSA in lysis buffer. Briefly, 5μl of standard, sample or blank (corresponding lysis buffer in RM buffer) were added per well. 8 parts of the BCA working reagent were mixed well with 1 part of the protein sample. The protein assay containers were sealed and the samples were incubated for 30min at 37 ºC, before colour absorbance was measured at 570nm using a plate reader equipped with the appropriate filters. A standard curve of net absorbance versus protein sample concentration was produced from the assay data. The actual concentration of the protein present in the unknown sample was calculated as follows:

(μg of unknown protein samples) times (dilution factor)

A separate standard curve was determined for each assay.

Alternatively, for rough estimation of protein content in the microsome preparations following their extraction, protein was measured on spectrophotometer at a wavelength of OD280nm. Microsome preparations were normalized and stored in RM buffer at OD280nm: 60, which was about 4mg/ml.

3.3 Western blot analysis of protein.

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 comb 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. The protein samples and the molecular weight markers are diluted (1:4) in 4 x Western sample buffer, boiled for 5 minutes, pulse centrifuged and chilled on ice. 2-20μg of protein is then run per lane. The gel is run at 200V/cm until the bottom dye reaches the bottom of the gel. After running the top plate is removed and the stacking gel is cut away. The gel is then submerged in Western transfer buffer, onto a piece of pre-wet Hybond C Exta. The transfer apparatus is then assembled. A pre-wet scotch-brite pad is covered with 2 sheets of 3MM paper, then the membrane and gel, 2 more sheets of 3MM paper and a second pre-wet scotch-brite pad. The sandwich is then placed into the transfer tank with approximately 4 litres of Western transfer buffer. The transfer of protein to the membrane for 16 hours at 20mA was then performed at 4°C, with continuous circulation of the buffer.

3.4 Western blot hybridisation.

Membranes were blocked with 50ml of Western block buffer consisting of 5% w/v non-fat dry milk, 2% w/v bovine serum albumin (BSA), 0.1% v/v Tween-20 in PBS, for 2-3 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 1 hour at room temperature. After 5 washes for 10min with 5ml of wash buffer (0.1% milk, 0.1% Tween-20 in PBS), the secondary antibodies (HRP-

conjugated) were diluted according to manufacture's instructions and added in 5ml block solution. After 1 hour at 37°C, 5 washes for 10min in wash buffer were performed, with the membrane stored under 0.1% milk, 0.1% Tween-20 in PBS at 4°C until ECL detection.

3.5 ECL detection.

The ECL detection system (Amersham) was used to visualise antibody binding to Western blots via horseradish peroxidase-linked secondary antibodies following the manufacturer's protocol. 2ml of ECL^{TM} solution 1 and 2 were mixed in a small container immediately before use. The membrane was placed in this solution for 5min and then blotted with 3MM paper to remove excess liquid. It wass then covered with cling film (saran wrap) and placed in Kodak cassette. The membrane was exposed to Kodak XARS5 film for 10 seconds upwards until optimal exposure is found.

Rainbow molecular weight markers (range 10 000- 250 000, Amersham) were used to define protein size.

4. Preparation of microsomes from antigen presenting cells.

4.1 Fractionation of cell contents.

Microsomes from cell lines or bone-marrow derived DCs were prepared and purified according to previously described protocols (Wang, Raynoschek et al. 1996). Specifically, cell cultures were grown to a minimal number of 10^9 cells. One day before collection the culture medium was renewed and IFNγ was added when indicated. All the steps during the microsome preparation are performed on ice or at 4°C.

Cells were collected, centrifuged at 250 x *g* for 5min and washed once with cold PBS. The collected cell pellet was re-suspended in STKMM buffer supplemented with 3µl/ml 100mM PMSF (STKMM homogenisation buffer). The cell suspension was further centrifuged at 250 x *g* for 5min and the supernatant was removed. The resulting pellet was re-suspended in a small volume of purified H_2O supplemented with 3µl/ml 100mM PMSF. Cells were homogenized using a fine glass cell Douncer. Following 40 strokes of homogenization, STKMM homogenization buffer was added and the suspension was mixed well. The homogenate was centrifuged at 10 000 x *g* for 10min at 4°C in JK-18 tubes. Supernatants were collected into new tubes and further centrifuged at 100 000 x *g* for 60min at 4°C. The resulting pellets were carefully washed once and resuspended in STKMM homogenization buffer using a small glass homogeniser. The homogenate was further centrifuged at 100 000 x *g* for 60min at 4°C. The resulting pellet of the total microsomal cellular fractions was re-suspended in RM buffer, simultaneously frozen in liquid nitrogen and stored in -80°C or the vapour phase of liquid nitrogen until further use.

4.2 Purification of ER-enriched microsomal membranes.

Total microsomes were sub-fractionated by flotation in discontinuous sucrose gradient for fractionation of microsomal membranes at 0-4°C. Total

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 2.5M sucrose. Centrifugation in a TH-641 rotor for 60min at $110,000 \times g$ yielded a microsome band on top of the 2M sucrose cushion. The microsome layer was collected by careful aspiration and resuspended in RM buffer. At this stage the protein concentration of the preparation was measured. An RM blank was inserted into the spectrophotometer and the absorbance at 280nm was set to zero. The absorbance of the microsome preparation was then measured at 280nm and recorded. Microsomes were preferably diluted to a concentration of 4mg/ml at OD280nm: 60. Microsomes suspensions in RM buffer were aliquoted in sterile plastic vials; the microsome aliquots were spontaneously frozen in liquid nitrogen and stored in -80°C until use.

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

5.1 Flow cytometry.

Flow cytometry analysis was used to detect cell surface markers on cells and microsomes. Single cell suspensions were obtained from homogenised spleens and lymph nodes, or from PBMCs, or from cell cultures. Microsome preparations were re-suspended using fine surgical needles before flow cytometric analysis. Cells or microsomes were incubated in 200μl FACS media for 30min at 4°C, washed in 1ml FACS media and collected by centrifugation. Primary antibodies were diluted according to manufacturer's instructions in FACS media and added to the cell or microsome pellet, mixed gently and incubated at 4°C for 1 hour. The suspension was washed once with 1ml of cold FACS media, and pellets were collected by centrifugation. Secondary antibodies were diluted according to manufacturer's instructions and added to the pellets, mixed gently and incubated at 4°C for 30min. The suspensions were washed twice with 1ml FACS media and filtered through nylon mesh, resuspended in 500μl FACS media and transferred to FACS tubes for FACS analysis. Stained cells and microsomes were analysed by flow cytometry.

A PE -Pro5^{TM} was used for the analysis of fluorescently labelled cells and the mean fluorescent intensity from three experiments was presented. The isotype Ig was used as background controls for all the staining of both cells and microsomes. The setting of side-scatter and forward-scatter was same between cells and microsomes. Due to the uneven sizes of microsomal vesicles, the entire events detected in side-scatter and forward scatter were used for analysis on fluorescent channels. The medium fluorescent intensity was then compared.

5.2 Detection of T cell-specific release of IL-2 by ELISA

The detection of recombinant mouse interleukin-2 (IL-2) in the cell culture supernatants was achieved with the development of enzyme-linked immunosorbent assay (ELISA) using them OptEIA™ Set (BD Biosciences). This immunoassay is calibrated against purified Baculovirus-expressed recombinant human IL-2.

Briefly, the wells of a 96-well micro plate were coated with 100μl of capture antibody, diluted according to manufacturer's instructions in coating buffer. The plates were sealed and incubated overnight at 4°C. Next day the wells were aspirated and washed 3 times with >300μl/well wash buffer. After the last wash the plates were inverted and blotted on absorbent paper to remove any residual buffer. Plates were then blocked with 200μl/well assay diluent and incubated at room temperature for 1 hour. The assay diluent was then aspirated and plates were washed as before. Standard and sample dilutions were prepared in assay diluent, and 100μl/well of each standard, sample and control were added in the appropriate well. The plates were sealed and incubated at room temperature for two hours. Subsequently, the wells were aspirated and washed as before, but with 5 total washes. Working detector was prepared according to manufacturer's instructions (Detection antibody and SAv-HRP reagent) and added at 100μl/well. Plates were sealed and incubated at room temperature for 1 hour. Wells were aspirated and washed as before, but with 7 total washes. In this final step wells were soaked in wash buffer for at least 30sec for each wash. Substrate solution was then added at 100μl/well and the plates were incubated at room temperature in the dark for 30min. To stop the reaction, 50μl of STOP solution were added in each well and the absorbance was read at 450nm and 570nm immediately after stopping the reaction. For wavelength correction absorbance at 570nm was subtracted from absorbance at 450nm.

The mean absorbance for each standard, sample and control was calculated and the mean zero standard absorbance was subtracted from each. To determine the IL-2 concentrations of the samples, a standard curve was

plotted based on the absorbance of the known standards. For samples that were diluted, IL-2 concentration was multiplied by the dilution factor.

5. 3 Microsome labelling with chemical fluorescence.

For the detection of microsomes by fluorescence microscopy microsomes 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 microsomes were used. Microsomes 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. Microsome suspension was centrifuged at 20 000 x g 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 microsome 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 20 000 x g for 5min at 4°C and the supernatatnt was removed. Labelled microsomes were re-suspended in 50μl of RM buffer and stored in -80°C and protected from dark until further use.

5.4 Internalization assay for the detection of microsome phagocytosis by dendritic cells.

Internalization assays were performed using Jaws-II cells to detect if microsomes are being phagocytosed by dendritic cells. The microsomes used for this assay had been previously labelled with chemical fluorescent.

Jaws-II cells sub-cultured for 3-4 days in advance of performing the assay on coverslips in 24 well-microplates. For the assay the cells were incubated with FITC-labelled microsomes or with FITC-labelled dextran

beads (3pm in diameter) suspended in culture medium for 5 hours at 37°C. Free-microsomes or dextran beads were removed by vacuum aspiration. Trypan blue (Invitrogen pH 5.0) was added for 1min to quench extracellular FITC-conjugated beads or fluorescent microsomes. The cells were washed briefly with PBS and fixed with 1% paraformaldehyde. Fixed cell preparations were analysed by confocal microscopy.

6. Isolation of primary cells.

6.1 Bone-marrow derived primary dendritic cells.

Immature DCs were generated from mouse bone marrow progenitors by culturing them in the presence of GM-CSF. Bone marrow cells were prepared from 10 week-old C57Bl/6 mice and red blood cells were lysed by RBC lysis buffer (Invitrogen). 10^6 cells/ml were cultured in RPMI medium, supplemented with 10% FBS and 100IU/ml of GM-CSF, for six days. To generate mature DC, the DC culture was transferred on day 6 in 1μg/ml LPS and cells were incubated in 5% CO_2 at 37°C for 24 hours.

6.2 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 250 x g for 10min at 4°C and the supernatant was discarded by aspiration. The pellets were re-suspended in 5ml of RBC lysis buffer per spleen and incubated at room temperature for 4-5min with occasional shaking. The lysis reaction was stopped by diluting the lysis buffer with 20-30ml of 1 x PBS. The cell suspension was centrifuged at 250 x g for 10min at 4°C, the supernatant was discarded by aspiration and the resulting cell pellet was re-suspended in the appropriate buffer for use in the next step of subsequent experimental procedures. At this stage, the cells were counted.

6.3 Isolation of mononuclear cells from human peripheral blood.

Mononuclear cells were isolated from human peripheral venous blood, which was obtained by the blood bank, by density gradient centrifugation over Ficoll-Histopaque. Non-adherent cells (NAC) and monocytes were separated by adherence to plastic. 40-70ml of blood bag/Buffy coat represents approximately 400-500ml whole blood with plasma removed. 25ml of Ficoll-

hypaque were aliquoted per 50ml test tube in sterile conditions and allowed to equilibrate to room temperature. A dilution of 1/5 of Buffy coat in PBS 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 PBS (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 complete culture medium. Erythrocytes were lysed by incubation with 15ml red blood cell lysing buffer per pellet for 20min at room temperature. The cell suspension was washed with complete medium and centrifuged at 250 x g centrifuge force for 10min at room temperature. The resulting pellet was resuspended in a small volume of serum free medium and the cells were counted.

7. Activation assays.

7.1 Peptide loading of microsomes.

Microsomes suspended in RM buffer were first processed by freeze–thaw (30sec in liquid nitrogen and 5min at 37°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 3) and incubation for three minutes on ice. The microsomal membranes were recovered by centrifugation through a 0.5M sucrose cushion on a microcentrifuge and re-suspended in RM buffer.

For MHC-I loading, 20μg/ml human β2-microglobin and SIINFEKL peptide at indicated doses were pulsed onto 1-4μg of microsomal membranes by incubation for one hour in 5% CO_2 at 37°C. After loading the excess peptides were removed by centrifugation through a 0.5M sucrose cushion on a microcentrifuge and the loaded microsomes were resuspended as homogenous population in culture medium.

For MHC-II, after the freeze-thaw process, microsomes in RM buffer were mixed with equal volume of stripping buffer and 500μg/ml OVA, or as indicated, for 5min at 37°C. After loading excess peptides were removed by centrifugation through a 0.5M sucrose cushion on a microcentrifuge and the loaded microsomes were resuspended as homogenous population in culture medium.

7.2 Peptide loading of cells.

For antigen presentation assays 10^5, or as indicated, DCs were pulsed (loaded) with the relevant peptides at the indicated concentrations for 1-6 hours in 5% CO_2 at 37°C. Excess peptides were washed by centrifugation. For antigen presentation assays using metabolically inactive cells, following peptide loading APCs were fixed in suspension with ice cold filtered and sterile 1% Paraformaldehyde in PBS or were irradiated.

7.3 Activation of T cells *in vitro*.

The OT-I T cells were isolated from spleens of OT-I transgenic mice by positive selection using a MACS system (Miltenyi Biotec).

Unless otherwise indicated, a constant number 10^6 of the corresponding T cells were incubated with 10^5 of peptide-loaded DCs or 1 to 4µg of peptide-loaded microsomes in 5% CO_2 at 37°C for 24-72 hours. Antigen presentation efficacy was evaluated by quantifying IL-2 release from T cells in the supernatants after 24-48 hours and by T cell proliferation by measuring tritium incorporation after three days of culture.

8. Characterization of lymphocyte specificity, frequency and function.

8.1 Positive isolation of CD4$^{(+)}$ T cells from primary mononuclear mouse cells of lymphoid organs.

The cells were washed with PBS, centrifuged at 250 x g for 10min at 4°C and the supernatant was discarded by aspiration. The cell pellet was resuspended in 900µl of buffer (0.5% BSA in PBS) per 10^8 cells. 100µl MACS CD4$^{(+)}$ microbeads were added to 10^8 cells, the cell and bead suspension was mixed well and incubated for 15-30min at 4°C. Cells were washed with an amount of buffer 20x the volume of the labelling solution and centrifuged at 250 x g for 10min 4°C. The supernatant was removed by aspiration and the cell pellet was resuspended in 500µl buffer per 10^8 cells. A positive selection column was placed in the magnetic field and washed with 1-2ml of buffer. The cell suspension was then added and the negative cells were allowed to pass through. The column was washed with 7ml of buffer, removed and placed in a container. The positive fraction of the cells was then washed all through the column with 7ml of buffer.

8.2 Detection of IFNγ producing cells by ELISpot.

The enzyme-linked immunospot (ELISPOT) assay (BD Biosciences) was used for the detection and enumeration of individual cells that secrete IFNγ *in vitro*. The plates were coated overnight at 4°C with 100µl IFNγ - specific capture Ab diluted according to manufacturer's instructions. The wells were washed once with blocking solution (complete culture medium) and the plates were then blocked with 200µl blocking solution for 2 hours at room temperature. The blocking solution was discarded and 100µl of known number of total splenocytes or PBMCs co-cultured with known amount of peptide or peptide loaded microsomes was added. The lids were replaced and the cultures were incubated in ELISPOT plates at 37ºC, in a 5% CO_2 and humidified incubator for 24 hours. The cell and microsome suspension was

aspirated and the wells were washed twice with de-ionized water allowing 3-5 minutes soaking at each wash step. This was followed by washing the wells 3 times with wash buffer I. The wash buffer was aspirated, the plates blotted onto paper tissue and 100μl of detection antibody diluted in dilution buffer according to manufacturer's instructions was added. The lids were replaced and the plates were incubated at room temperature for 2 hours. The detection antibody solution was discarded and the wells were washed 3 times with wash buffer I, allowing soaking for 1-2 minutes at each wash step. The enzyme conjugate (strepavidin-HRP) was diluted in dilution buffer according to manufacturer's instructions immediately before use and 100μl were added to the wells for 1 hour at room temperature. The enzyme conjugate solution was discarded and wells were washed 4 times with wash buffer I, allowing soaking for 1-2 minutes in each step. Subsequently, wells were washed with wash buffer II twice. The plates were blotted on paper tissue and 50μl of the final substrate solution was added to each well. The spot development was monitored closely over a period of 5-60 minutes and the colorimetric substrate reaction was stopped at appropriate time by washing wells with de-ionized water. The plastic tray under the plates was removed to facilitate even drying. The wells were allowed to air-dry at room temperature overnight. The plates were then stored in sealed plastic bag in the dark, until they were analyzed. Spots were enumerated manually under dissecting microscope.

8.3 Proliferation assays.

To determine T cell proliferation 1μCi/ml [3H] Thymidine was added after three days of culture with peptide loaded DCs or peptide-loaded microsomes. The cells were harvested after 8 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 and the medium could be kept as a stock solution at 4°C for one month. Culture plates were transferred to the

radiation culture area after three 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°C for 8 hours. At this stage the working surfaces were tested for radiation levels. Following 8 hours of incubation, the labelled cultures were harvested in a cell harvester or stored at -20°C 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.

8.4 Determination of T cell cytotoxicity against tumour cells.

Cytotoxicity assay was used for evaluation of T cell specific cytotoxic activity against tumour. The target cells used for this assay were the OVA-producing thymoma cell line E.G7. Target cells (10^6) in 600μl were labelled with 300 μCi "Cr sodium chromate for 45min. After washing, 10^4 labelled targets and serial dilutions of effector cells (CD8$^{(+)}$ T cells from immunized mice) were incubated in 200μl of RP10 in round-bottomed 96-well plates. After 4 hours of incubation in 5% CO_2, at 37°C, supernatants were collected and specific lysis was determined as: percent specific lysis = 100 x [(release by CTL – spontaneous release)/(maximum release – spontaneous release)].

9. Microscopy.

9.1 Confocal fluorescent microscopy.

For the detection of microsome binding to T cells, microsomes were prepared, loaded with peptides and co-cultured with T cells as described above, but on poly-D-lysine-coated multiwell slides for 1 hour. Unbound microsomes were washed with PBS and the slides were fixed with 1% paraformaldehyde for 10min before mounting.

9.2 Electron microscopy

Electron micrographs were prepared from Jaws-II cells for microscopic analysis of the cytoplasm and its examination for the presence of ER membrane structures.

A solution of 4% low melting point agarose was prepared in distilled water at 42°C and maintained in liquid phase in a test tube immersed in a water bath at the same temperature. Jaws-II cells were washed once with PBS and resuspended in cell culture medium. The cell suspension was fixed in a solution of 0.5% glutaraldehyde in 0.1M sodium cacodylate adjusted to pH 7.4. Fixation was allowed over a period of 45min on mild shaker. Fixed cells were then centrifuged at 250 x g for 5min, the fixative was removed and the cell pellet was rinsed twice with 0.1M sodium cacodylate buffer. A small amount of the warm agarose was added to the pellet of fixed cells and gently mixed. The gel-embedded cell pellet was allowed to cool before 70% ethanol was added. The sample was sealed and stored at 4°C until further processing for EM observations. Immediately before the analysis with transmission electron microscopy the embedded in agarose cell preparations were cut in sections and placed in the appropriate chambers.

10. Detection of immunity *in vivo*.

10.1 Immunization.

Microsomes from Jaws-II cells or bone-marrow derived DCs or Jaws-II cells or bone-marrow DCs were loaded either with SIINFEKL or $OVA_{324-340}$ as immunogens, or with a HLA-A2 restricted epitope of the influenza matrix protein (GILGFVFTL) as an irrelevant control peptide. $1x10^6$ cells or 20μg microsomes in 100μl of physiologic saline were injected into the dorsal tail vein of each mouse; after seven days mice were immunized with the same doses for boosting.

10.2 Viral infection.

Mice were infected intranasally with the recombinant vaccinia virus encoded with chicken OVA (32-13) or matrix influenza MP in 50μl at the indicated doses. The mice were weighed and observed for illness daily. *In vivo* replication of vaccinia virus was examined by plaque assay on lung tissues which were removed, weighed and grounded with a mortar and pestle. Serial 10-fold dilutions of clarified supernatants were used to infect sub-confluent monolayers of BSC40 cells in triplicate in 24-well plates. After 1 hour, the plates were covered in 0.75% methylcellulose in 10% MEM and incubated at 37^oC. The cells were fixed with formalin 2 days after infection and stained with 2% crystal violet in 40% methanol, and plaques were counted under a dissecting microscope. Data are presented as geometric mean log10 PFU per gram of lung at dilutions that produced more than five plaques per well.

10.3 Tumour induction and measurements.

Tumours were established by subcutaneous injection of $2x10^5$ EG7 cells in 100μl of physiologic saline. Tumour cells were 100% viable as measured by trypan blue staining. The initial growth of tumour was evaluated by palpation. Tumour growth was measured at 48-hour intervals using a

vernier caliper. All animals were killed at a tumour size of approximately $1.5cm^3$.

10.4 *Ex vivo* analysis of antigen specific T cells.

For CD8$^{(+)}$ T cells, splenocytes were stained with FITC-conjugated anti-CD8 (BD Biosciences). After washing, cells were either stained with PE-conjugated K^b-SIINFEKL Pentamer (Proimmune Ltd.) or isotype matched controls, and analyzed by flow cytometry.

For evaluation of CD4 T cell responses, CD4$^{(+)}$ T cells were purified from spleen and lymph nodes by CD4-coated magnetic beads (Miltenyi Biotec) to more than 90% as measured by PE-CD4 antibody by flow cytometry. $2x10^5$ CD4$^{(+)}$ T cells were cultured with either 2μg of OVA$_{324-340}$ peptide-loaded microsomes from bone-marrow derived DCs or $1x10^5$ DC cells pulsed with the same peptide for 48 hours before measuring IL-2 production by ELISA (BD Bioscience) or three days before proliferation assay with ^{3}H-TdR (Amersham) incorporation.

11. 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.

12. List of Buffers.

Alpha growth medium: -4 mM L-glutamine, 1 mM sodium pyruvate, 5ng/ml murine GM-CSF, ribonucleosides, deoxyribonucleosides, 20% FBS, 1% Penicillin-Streptomycin–L-Glutamine.

BCA: -50 parts of Reagent A (a solution containing bicinchoninic acid, sodium carbonate, sodium tartrate and sodium bicarbonate in 0.1N NaOH, pH 11.25) with 1 part of Reagent B (a 4% (w/v) copper (II) sulphate pentahydrate).

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

ELISA:

Coating buffer: -0.1M Sodium Carbonate, pH 9.5, 7.13g NaHCO3, 1.59g Na2CO3; q.s. to 1L; pH 9.5 with 10N NaOH.

Assay diluent: -PBS, 10% Heat Inactivated FBS, pH 7.0.

Wash buffer: -PBS, 0.05% Tween-20.

Substrate: -Tetramethylbenzidine (TMB), Hydrogen Peroxide. BD Pharmingen™ TMB Substrate Reagent Set.

Stop solution: -2N H_2SO_4.

ELISPOT

Coating buffer: -1× PBS.

Blocking solution: -complete cell culture medium.

Wash buffer I:	-1× PBS, 0.05% Tween-20.
Wash Buffer II:	-1× PBS.
Dilution Buffer:	-1× PBS, 10% FBS.
Substrate Solution:	-BD™ AEC Substrate Reagent Set.
Freeze medium:	-95% complete growth medium; 5% DMSO.
FACS media:	-5% BSA in PBS.
Lysis Buffer:	-1% NP40, 5% PMSF or protein tablets.
Paraformaldehyde 1%:	-25g Paraformaldehyde powder, 250ml PBS, drops of 1M NaOH.
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.
STKMM:	-250mM Sucrose, 50mM TEA-HCl pH 7.5, 50mM KOAc, 5mM Mg(OAc)2, 0.1% mercaptoethanol.
Stripping buffer:	-0.26M citric acid, 132mM Na_2HPO_4, 2% BSA, pH 3.

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. Antigen presentation analysis *in vitro.*

The demonstration that different cell types can process and present antigens to T cells at vastly different efficiencies has led investigators to consider certain cells as "professional" antigen presenting cells (APCs), a group that typically includes B lymphocytes, macrophages (MØ), and especially dendritic cells (DCs) (Trombetta and Mellman 2005). Professional APCs engage T cells in an antigen-specific manner to induce their functional activation during an immune response.

The purpose of this thesis is to investigate the reconstitution of optimal presentation properties of professional APCs using ER-derived microsomes, for the stimulation of T cell functional activation *in vitro* and *in vivo*. Before we can investigate their reconstitution using microsomes, the presentation properties of APCs need to be established.

The best described professional APCs are the dendritic cells (DCs). The majority of DCs that naturally occur *in vivo* derive from bone marrow progenitors; therefore, bone-marrow-derived-DCs (BMDCs) would be an ideal tool in studies of antigen presentation. However, fresh preparations of BMDCs from mouse bone marrow progenitors *ex vivo* result in a heterogeneous population that is contaminated with other bone marrow-derived cells, such as macrophages. In addition, the differentiation and maturation of BMDCs *in vitro* often results in the generation of various DC-subtypes, including tolerogenic DCs. Furthermore, the experiments described in this work would require a large number of animals to be sacrificed in order to yield enough bone marrow progenitors for the generation of sufficient numbers of DCs for antigen presentation analysis and for extraction of microsomes for antigen presentation studies. By contrast, immortal DC lines that would have stable characteristics and would grow at high density could overcome these difficulties, and provide a system that offers consistent conditions and satisfies the quantitative requirements of this investigation.

Therefore, my results begin with the analysis of the efficiency of antigen presentation using antigen presenting cell lines *in vitro*. Defined antigen presenting cell lines are loaded with antigenic peptides (Figure 19), which they are then allowed to present to restricted peptide/MHC-recognising T cell clones (Figure 20) for the induction of T cell responses. Antigen presentation is evaluated based on the T cell responses induced.

Peptide	Sequence	Protein	MHC
SIINFEKL (OVA$_{257-264}$)	SIINFEKL	Chicken Ovalbumin	H-2K^b
SIYNFEKL (OVA$_{257-264}$)	SIYNFEKL	Chicken Ovalbumin	H-2K^b
OVA$_{324-340}$	ISQAVHAAHAEINE AGR	Chicken Ovalbumin	H2-I-A
MP	GILGFVFTL	Influenza-A matrix protein	HLA-A2
KTR	SAYWAIRTK	Influenza-A nuclear protein NP383-391AA	HLA-A2
HCMV	NLVPMVATV	HCMV	HLA-A2
GAG	ATLYGVHQKI	HIV GAG	HLA-A2

Figure 19   **Antigenic peptides used in this study.**

APCs	Adherent or Suspension	Cell type	Antigen expression	Reference
Jaws-II	Mixed A&S	Monocyte; bone marrow immature dendritic cell line derived from C57.BL/6, p53-deficient mouse;	$H-2K^b$ $H-2D^b$ $I-A^b$	MacKay VL, Moore EE . Immortalized dendritic cells. US Patent 5,648,219 dated Jul 15 1997
RMA-S	S	TAP2-deficient tumour cell line, derived from the Rauscher leukemia virus-induced mouse T cell lymphoma RBL-5 of C57.BL/6 origin	$H-2K^b$ $H-2D^b$ $I-A^b$	Kärre, K., et al, 1986
RAW309Cr.1	A	Monocyte; macrophage derived from mouse spleen lymphoma (RAW 309 tumour)	$H-2^d$ $H-2^b$	Raschke WC, et al. 1978.
DC2.4	S	Mature dendritic cell line obtained from C57.BL/6 bone marrow cells infected with a retrovirus encoding *myc* and *raf* by using supernatant from NIH J2 Leuk cells	$H-2K^b$ $H-2D^b$ $I-A^b$	Shen Z et al, 1997
LCL 721.221	S	Epstein-Barr virus (EBV)- transformed human Lymphoblastoid B-cell line from a normal female donor	HLA-A, -B and -C null	Kavathas et al, 1980

T cells	Adherent or Suspension	Cell type	MHC restriction	Reference
B3Z	S	IL-2 secreting, lacZ-Inducible T cell Hybridoma	OVA-specific $H-2K^b$ restriction	Karttunen J et al, 1992
MF2.2D9	S	T-T cell Hybridoma	OVA-specific, $I-A^b$ restriction	Yu Y et al, 2002
E.G7-OVA [derivative of EL4]	S	Chicken egg ovalbumin (OVA) transfected clone of EL4; T lymphocyte derived from C57.BL/6 mouse lymphoma	$H-2^b$	Engelhard et al, 1984 Moore MW, et al, 1988

Figure 20   Cell lines used in this study.

1.1 Analysis of the efficiency of antigen presentation using antigen presenting cell lines *in vitro*.

A C57.Bl/6mouse-derived immortal dendritic cell line called Jaws-II that has been used for studies of pathogen-specific (Otsu, Gotoh et al. 2006; Jiang, Shen et al. 2008), antiviral (Indrova, Reinis et al. 2004) and antitumour (Pajtasz-Piasecka, Rossowska et al. 2007) immunity were the APCs used initially. The Jaws-II line is a granulocyte-macrophage colony stimulating factor (GM-CSF)-dependent cell line of immature dendritic cells that mature in culture in the presence of GM-CSF and high concentrations (20%) of serum. Jaws-II are established from bone marrow cells of C57.Bl/6 origin, which has the 'b' haplotype expressing the MHC class I H-2 alleles K (H-$2K^b$) and D (H-$2D^b$) and the MHC class II allele I-A (I-A^b) *(JAX Mice Database, The Jackson Laboratory)*. Antigen presentation was examined in the context of MHC class I or MHC class II using two well defined chicken ovalbumin (OVA) derived peptides; the first peptide is the ovalbumin fragment from amino acid 257 to amino acid 264 with amino acid sequence 'SIINFEKL' (Khilko, Corr et al. 1993) which binds the mouse MHC molecules H2-K^b (MHC-I), and the second peptide is the ovalbumin fragment from amino acid 324 to amino acid 340 (OVA$_{324-340}$) with amino acid sequence 'ISQAVHAAHAEINEAGR', which binds the mouse MHC molecules H2-I-A (MHC-II). Peptides were loaded to APCs by incubation of the cells with peptides (peptide-pulsing) in culture conditions for 6 hours. The efficiency of the antigen presentation was assessed using T cells with TCRs that recognise the complexes of peptide plus MHC. T cell activation was quantified based on the amount of soluble IL-2 that T cells produced following *in vitro* stimulation with peptide-pulsed APCs for a period of 24 or 48 hours.

Figure 21A shows that Jaws-II cells loaded with the H-2K^b restricted peptide SIINFEKL induced antigen-dependent T cell responses when co-cultured with the B3Z T cell line recognizing SIINFEKL-K^b complex. Similarly, when Jaws-II cells were loaded with an I-A restricted peptide

A. MHC-I (SIINFEKL-Specific)
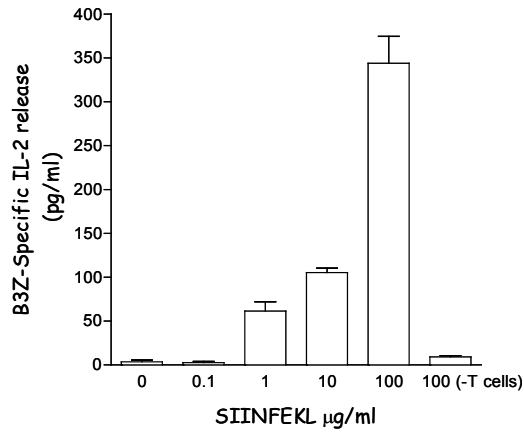
B. MHC-II (OVA$_{324-340}$-Specific)
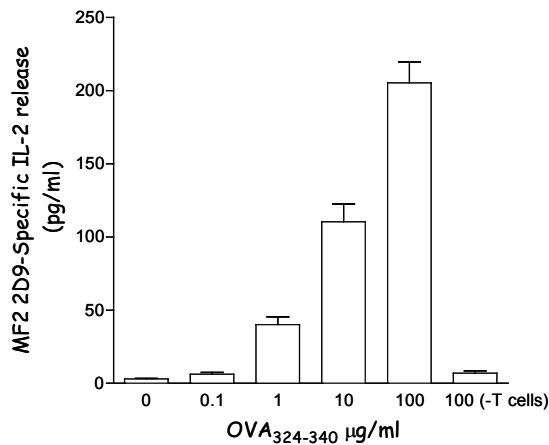
Figure 21   **APCs induce peptide-specific T cell responses.**

10^5 Jaws-II cells were pulsed with different concentrations of SIINFEKL or OVA$_{324-340}$ peptides for 6 hours. Excess peptides were washed before cells were co-cultured with either

A. the SIINFEKL-K^b recognising B3Z T cell line or with

B. the OVA$_{324-340}$-I-A recognising MF2.2D9 T cell line.

T cell activation was determined by IL-2 release 24 hours later, measured by ELISA. 100(-) bars represent negative control of peptide-pulsed APCs cultured without T cells. The data are presented as mean of quadruplicate cultures ±SEM and are representative of three experiments.

ISQAVHAAHAEINEAGR they induced the MF2.2D9 T cell line recognizing ISQAVHAAHAEINEAGR in the context of H2-I-A (Figure 21B). By contrast, Jaws-II cells that hadn't been loaded with peptide failed to induce B3Z or MF2.2D9 T cell responses. T cell responses were evaluated by

measuring IL-2 production by ELISA following 24 hours of stimulation. In both cases, the response was dose-dependent with respect to the peptide, as increasing dose of peptide concentration at loading was associated with increasing production of IL-2 (Figure 21).

To examine whether the responses were also peptide/MHC-dependent, the B3Z T cell line was co-cultured with increasing numbers of Jaws-II cells that had been loaded with a constant concentration of SIINFEKL ($100\mu g/ml$). Figure 22 shows that the responses were dependent on the number of antigen-presenting cells, however declined when the number of APCs was higher than the number of T cells. It was observed that the optimal responses were induced with 1x APC: 10x T cell ratio.

Number of APCs	$5x10^4$	$1x10^4$	$5x10^4$	$1x10^5$	$5x10^5$	$5x10^4$ (-T cells)
Ratio APCs:T cells	1:20	1:100	1:20	1:10	1:2	-
SIINFEKL concetration at loading	$0\mu g/ml$			$100\mu g/ml$		
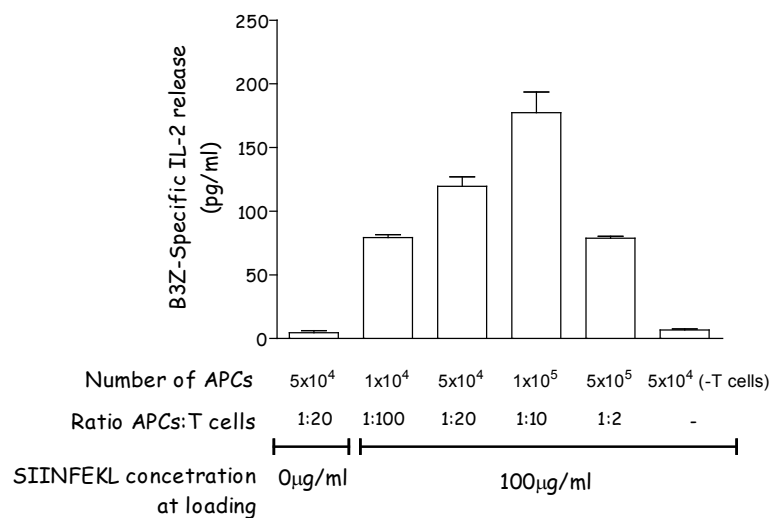
Figure 22   **The level of T cell activation depends on the ratio of APCs/T cells.**

Different numbers of Jaws-II cells were pulsed with $100\mu g/ml$ SIINFEKL peptide for 6 hours. Excess peptides were removed by washing before cells were co-cultured with $1x10^6$ B3Z T cells. After 24 hours, IL-2 production was measured by ELISA. Bar to the far right ($5x10^4$–T cells) is a negative control to represent basal IL-2 release from $5x10^4$ peptide-pulsed Jaws-II cells cultured without T cells. The data are presented as mean of quadruplicate cultures ±SEM and are representative of three experiments.

1.2 Antigen presentation does not require metabolically active cells.

It has been previously demonstrated that metabolically inactive APCs can present exogenously loaded antigen and induce T cell responses (Falo, Sullivan et al. 1985).

To examine whether antigen presenting cells can still present peptides efficiently during an inactive metabolic state, Jaws-II were pulsed with SIINFEKL peptide previous to their irradiation or their treatment with either 1% paraformaldehyde, or 1% formaldehyde, and then allowed to present SIINFEKL to B3Z T cells (Figure 23). Following treatment with 1% paraformaldehyde (Figure 24A) or irradiation (Figure 24B), cells could induce T cell responses similar to those triggered by untreated SIINFEKL-loaded APCs. However, their presenting capacity was reduced to the basal levels seen with APCs that hadn't been loaded with peptides, when the cell membranes were mechanically disrupted (i.e. loosely adherent cells were removed by scraping rather than with cold PBS wash) or treated with formaldehyde (Figure 24A).[1] These findings suggest that the presentation of pre-processed antigen does not depend on metabolically active cells; however it is crucially dependent on the integrity of the structure of the antigen presenting cell membrane.

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[1]Paraformaldehyde (PFA), a polymerized form of formaldehyde, is an insoluble white powder that cannot be used as a fixative in this form. Formaledehyde, which has the same chemical properties with paraformaldehyde, is a gas that in aqueous solutions spontaneously polymerizes. However, to be useful as a fixative, a solution must contain monomeric formaldehyde as its major solute. For that reason, most of the commercially available formaldehyde solutions are in fact 37% formaldehyde in water, containing 10-15 % methanol as a preservative. Methanol slows down the polymerization that leads eventually to precipitation of paraformaldehyde. However, methanol is known to rapidly precipitate proteins and permeabilize cells by distorting the structure of the cell membrane. Methanol-free formaledehyde can be obtained by hydrolysis of paraformaldehyde, by extensive heating of the paraformalehyde powder to 60°C in water containing the salts required to buffer the solution to pH 7.2 to 7.6. Fixation of the cell, using either solution, blocks the cellular metabolism.
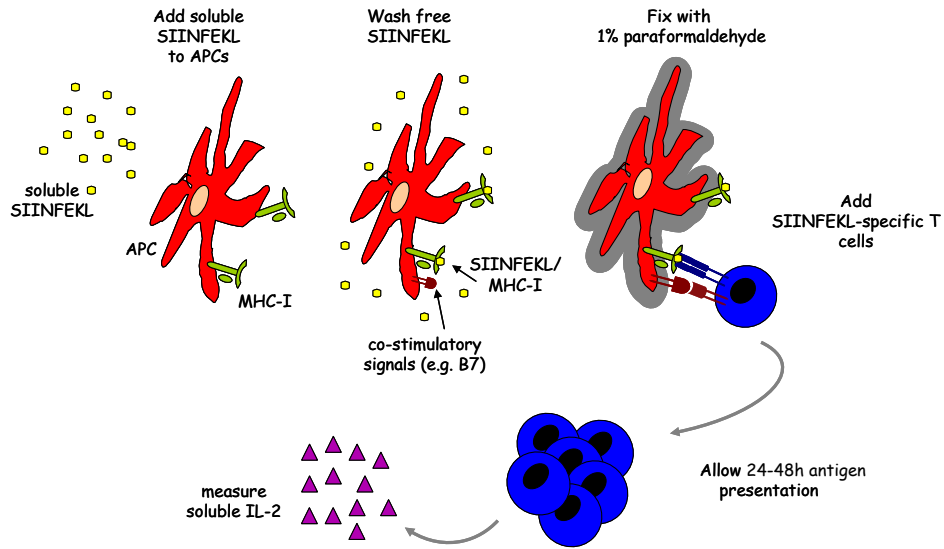
Figure 23   **Experimental design for antigen presentation analysis in metabolically inactive cells.**

Jaws-II cells were pulsed with SIINFEKL peptide for 6 hours. Excess peptide was washed away and the peptide-loaded cells were fixed with 1% Paraformaldehyde prior to co-culture with SIINFEKL-K^b recognising T cells. T cell activation was evaluated by IL-2 production.
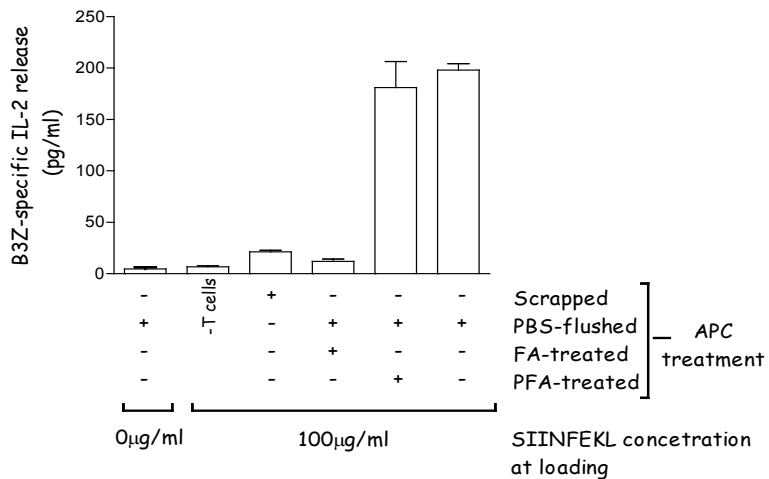
Figure 24A   **Antigen presentation does not require metabolically active APCs.**

10^5 adherent Jaws-II cells were pulsed with 100μg/ml SIINFEKL peptide for 6 hours and excess peptide was then washed away. Adherent cells were either scraped from plastic surface or removed by rinsing with cold PBS. Peptide-loaded cell suspensions were either fixed with 1% Paraformaldehyde or 1% Formaldehyde prior to co-culture with B3Z T cell line. T cell activation was evaluated by IL-2 release measured with ELISA after 24 hours. The data are presented as mean of quadruplicate cultures ±SEM and are representative of three experiments.
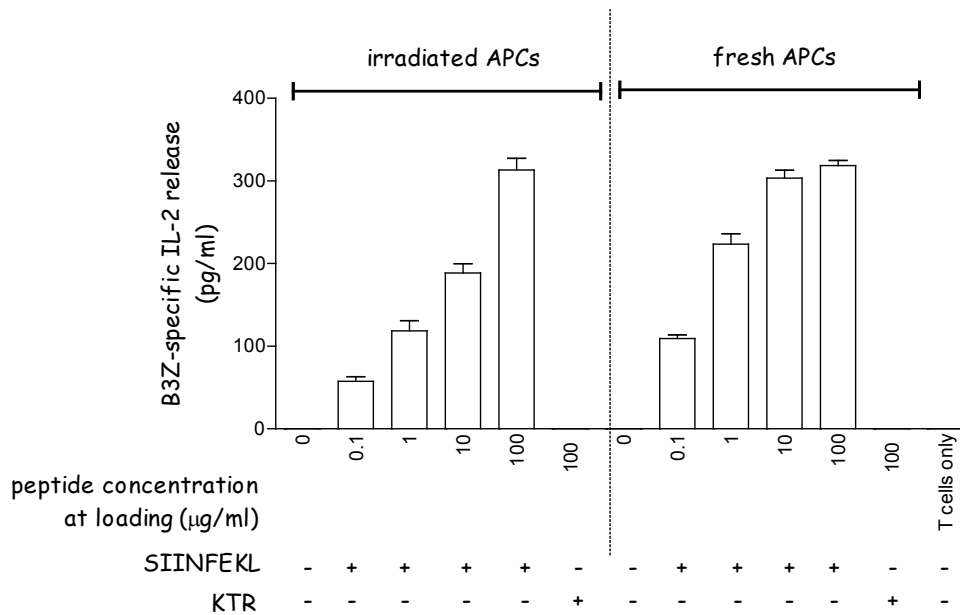
Figure 24B   **Antigen presentation does not require metabolically active APCs.**

10^5 Jaws-II were pulsed with serial dilutions of SIINFEKL peptide for 6 hours before excess peptide was washed away. Cells were then either irradiated or remained untreated prior to co-culture with SIINFEKL-K^b recognising B3Z T cell line. Cells loaded with the irrelevant KTR peptide were used as a negative control. 24 hours later T cell activation was evaluated by IL-2 release measured with ELISA. The data are presented as mean of quadruplicate cultures ±SEM and are representative of three experiments.

Summary of Section 1

The results presented here show that antigen presenting cells (APCs) can induce activation of T cells *in vitro*, in the context of MHC class I and MHC class II. T cell responses are peptide-dependant and relative to the antigen concentration and the ratio of APC/T cells. Antigen presentation does not require metabolically active APCs, but does depend on the intact structure of the antigen presenting cell membrane.

2. Microsomes- molecular characterization.

This chapter focuses on the molecular characterization of the ER-enriched microsomes that can be isolated from APCs. First, the cytoplasm of the APC is observed and the morphology of internal membrane structures is examined in an effort to identify the presence of endoplasmic reticulum. Following the description of microsome extraction and purification from APCs, a series of biochemical experiments are presented, which aim to characterise the molecular profile of the isolated microsomes.

2.1 The cytoplasm of APCs is rich in endoplasmic reticulum.

All eukaryotic cells have an endoplasmic reticulum (ER) (Porter 1953). It's highly complex single membrane, which represents more than half of the total membrane of the single cell, is organised into a network that extends throughout the cytoplasm. It forms a continuous sheet enclosing the ER lumen, a single internal space which often occupies more than 10% of the total cell volume. The ER is part of the endomembrane system, which consists of internal membranes that divide the cells into functional and structural compartments, or organelles. Collectively, the membrane network provides not only an intracellular transport system, but also surfaces for lipid and protein synthesis.

Observation of the antigen presenting Jaws-II cells with electron microscopy revealed an abundance of endomembrane structures in their cytoplasm (Figure 25).
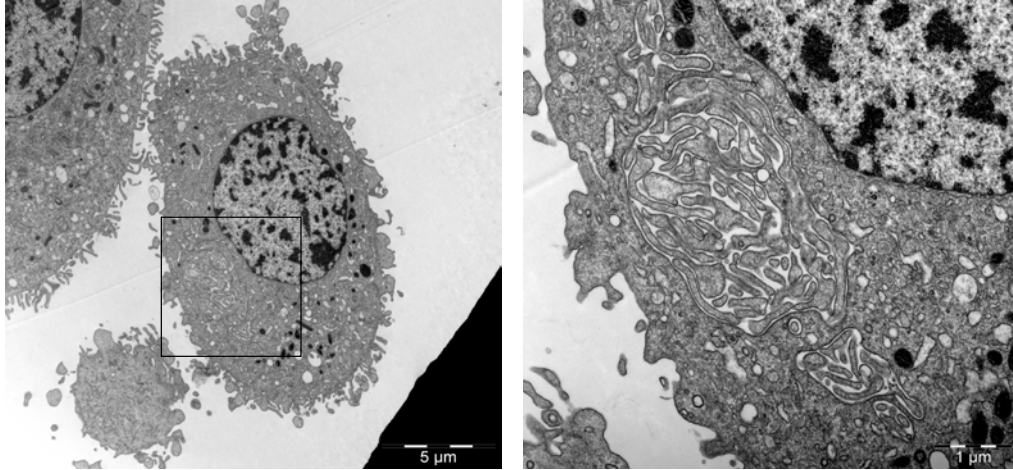
Figure 25   **The cytoplasm of APCs is rich in endoplasmic reticulum as observed with Electron Microscopy.**

Electron micrographs prepared from Jaws-II cells before microsome extraction. Right panel represents the area marked on the image of the left panel magnified to a scale 1:5.

2.2 Microsome isolation.

Although the ER is interleaved extensively with other intracellular elements, it is possible to isolate the ER membranes from other components of the cell. Upon cell homogenization the ER is fragmented into small (~100nm diameter) closed vesicles, known as the microsomes. Deriving from the Greek words *μικρό-σώμα,* which means small-body, microsomes is a word used to define the small vesicle-like particles of fragmented ER that are not normally found in living cells. Examination of extracted microsomes with electron microscopy (Figure 26) reveals a mixture of small vesicles with adherent ribosomes (rough microsomes) that are thought to derive
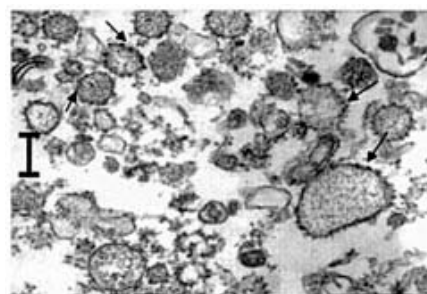
Figure 26   **ER-enriched microsomes**.

Electron micrograph of ER-enriched microsomes prepared from RAW309Cr.1 cells (Scale bars: 100nm). Arrows indicate ribosomes.

from fragmented rough ER, and small vesicles that lack ribosomes (smooth microsomes) and are thought to derive from smooth ER. Although the presence of ribosomes indicates that rough microsomes are fragments of rough ER, the origin of smooth microsomes is more difficult to define as they could also include fragments of various parts of the endomembrane system (i.e. Golgi apparatus) (Dallner 1974). Electron micrographs of microsomes purified from an APC line (RAW309Cr.1) reveal that ribosomes are found on the outside of the microsome surface, whereas the interior enclosed area is biochemically equivalent to the ER lumen (Kvist and Hamann 1990).

To prepare microsomes from APCs (Figure 27A), cells were disrupted manually with a Dounce homogenizer, which is an apparatus consisting of a glass tube with a tight-fitting glass pestle. The nuclei, mitochondria and larger cell debris were removed by centrifugation at 10,000 x g. The total microsomes were recovered by centrifugation at 100,000 x g and sub-fractionated by flotation in discontinuous sucrose gradients. The generated microsome layer on top of the 2M sucrose cushion was collected and resuspended in RM buffer. ER-enriched microsomes can be further purified by flotation in discontinuous sucrose gradients following an additional high-speed centrifugation step (Figure 27B). The yield of microsomes is usually about 3-5% of the total APC cell weight (Li, Paulsson et al. 1999).
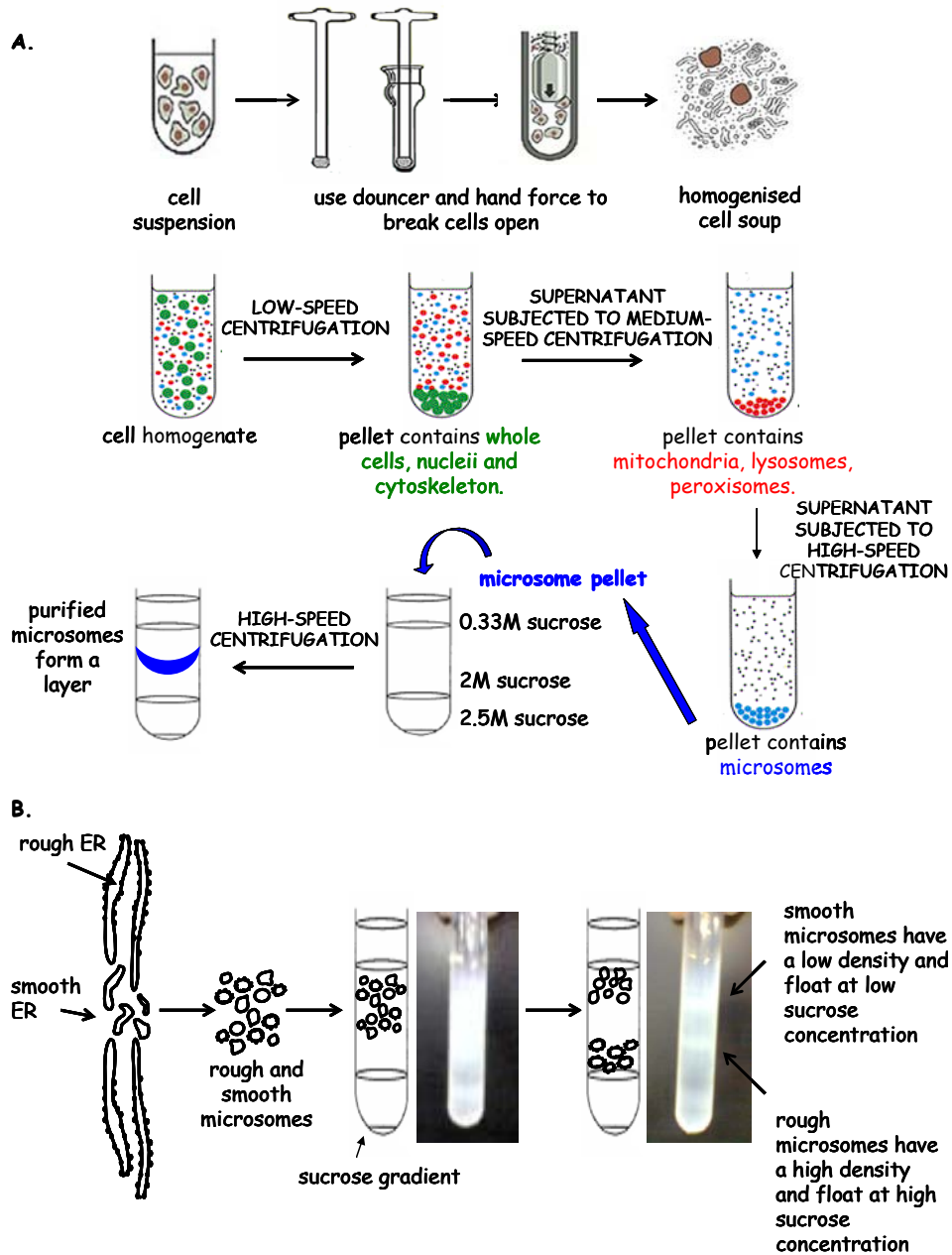
Figure 27 **Microsome preparations.**

A. To prepare microsomes from APCs, an APC cell suspension in a hypertonic solution was placed in a douncer. Cells were sheered between a close fitting rotating plunger and the thick walls of a glass vessel on ice. Carefully applied 40 strokes resulted in homogenization that left most membrane bound organelles intact. The nuclear, mitochondrial and larger cell debris was removed from the cell homogenate by centrifugation at 10,000 x *g*. The total microsomes were recovered by centrifugation at 100,000 x *g* and further subfractioned by flotation in discontinuous sucrose gradients.

B. ER-enriched microsomes form a layer on top of 2M sucrose layer and can be further purified following flotation in discontinuous sucrose gradients. All procedures were carried out on ice or at 4°C.

2.3 Microsomes express MHC.

The ER plays a central role in cell biosynthesis as it is the starting point for the synthesis of all secreted and membrane proteins. There are two classes of ER proteins; transmembrane proteins that are only partly translocated across the ER membrane and become embedded in it, and membrane-free soluble proteins that are fully translocated across the ER membrane and are released into the lumen of the ER. The synthesis and assembly of both MHC-I and MHC-II occurs in the ER (Kvist and Hamann 1990).

To confirm that microsomes are derived from enriched ER membranes we used western blot analysis of microsomes from Jaws-II cells to detect the presence of the ER-resident protein, tapasin (Figure 28). This analysis also revealed the presence of MHC-I molecules in the microsomes and their parental Jaws-II cells, and that 2μg of total microsomal protein measured by protein assay (BCA protein assay SIGMA) contained similar levels of MHC-I to that present in 10^5 APCs (Figure 29).

Figure 28 **Western blot analysis of ER-associated tapasin in Jaws-II cells and Jaws-II-derived microsomes.**

Immunoblotting analysis of 10μg lysed proteins from Jaws-II cells or Jaws-II derived microsomal membranes with antibodies against tapasin.
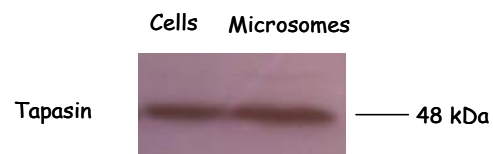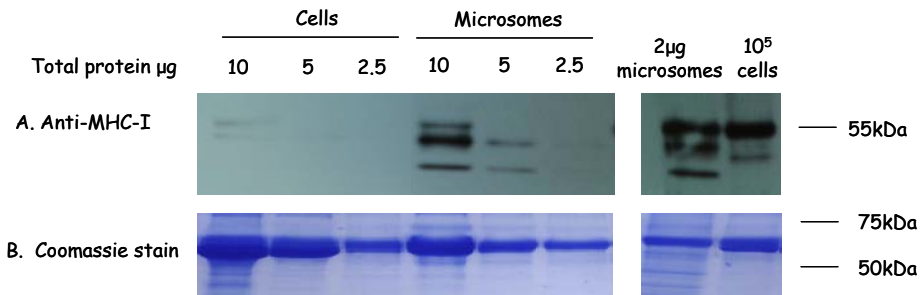
Figure 29   **Analysis of cellular and microsomal fractions for total protein and MHC.**

Lysates prepared from whole Jaws-II cells or Jaws-II-derived microsomes were analyzed

A. by MHC class I-specific western blot and

B. by coomassie blue staining.

2.4 Microsomes express co-stimulatory molecules.

In addition to antigen recognition, activation of T cells depends on co-stimulation for initiating adaptive immune responses. Antigen presentation in the absence of co-stimulation leads to T cell anergy. Effective T cell responses require both the efficient presentation of peptide/MHC and co-stimulatory signals on DCs, as expression of co-stimulatory molecules signifies that antigen was likely to have been acquired in an environment that contained pathogens or 'danger'.

Similar to MHC molecules, the biosynthesis and maturation of co-stimulatory molecules occurs in the ER. Flow cytometry analysis showed that the surface expression of the co-stimulatory molecules B7-1 (CD80) and B7-2 (CD86), and the adhesion molecule ICAM-1 (CD54), could be detected among Jaws-II-derived microsomes (Figure 30A) and their parental cells (Figure 30B), suggesting that the ER-enriched microsomes have the capacity to present co-stimulatory signals to T cells. The findings were confirmed by western blot analysis of co-stimulatory expression in an IFNγ-activated (He, Tang et al. 2007) mature dendritic cell line DC2.4 and in microsomes purified from activated DC2.4 cells (Figure 30C).

A. Microsomes

B. Cells

C.
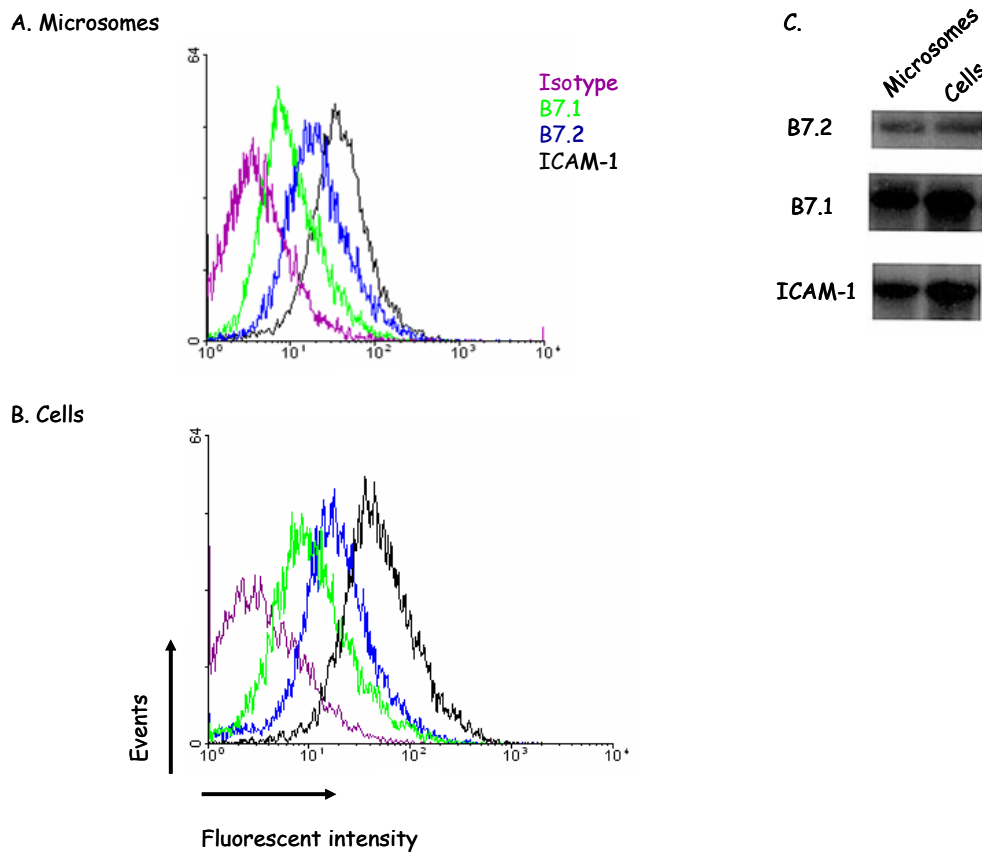
Figure 30   **Expression of co-stimulatory molecules in microsomal membranes and their parental cells.**

A. Microsomes from Jaws-II cells and

B. Jaws-II cells

were analysed for the co-stimulatory molecules B7.1, B7.2 and adhesion molecule ICAM-1 by flow cytometry. The isotype Ig was used as background control for all staining of both microsomes and cells.

C. Western blot analysis of co-stimulatory molecules in 20μg of lysates from whole DC2.4 cells and from DC2.4 cell-derived microsomes.

2.5 Microsome-associated MHC can bind to antigenic peptides.

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

Since assembly of MHC molecules occurs on the luminal side of the ER membrane, the assembly of the MHC molecules present in microsomes would occur on the interior surface of the microsome 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 microsomal membrane so as to expose the luminal side of microsomes, by a repeated freeze-thawing procedure.

Detection of peptide/MHC-I complexes was achieved with the use of specific antibodies, as well as with a modified iodinated OVA peptide that is described later. The H2-K^b molecules in the processed microsomes 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 31A). MHC-I assembly with antigenic peptides takes places in the ER, however MHC-II molecules are protected from peptide loading by invariant chain (Ii) and CLIP until they reach the endocytotic compartments; there, under acidic conditions the Ii is degraded and exchanged with antigenic peptides (Wilson and Villadangos 2005). To explore optimal ways of loading peptides onto microsomal MHC-II molecules, an I-A restricted OVA peptide [residues 324-340, amino acid sequence 'ISQAVHAAHAEINEAGR'] was labelled with biotin (using the NHS-LC-Biotin reagent from Pierce Chemical) to enable the detection of peptide/MHC-II assembly (Figure 31B) in 'inside-out' converted microsomes. Despite an efficient loading of MHC-I molecules under similar conditions (Figure 32A middle panel), the $OVA_{324-340}$ peptide could not be effectively loaded onto MHC-II molecules in the inside-out inverted microsomes at pH 7.0 (Figure 32B middle panel), possibly due to the association of Ii with MHC-II. However, in an acidic buffer of pH 5, peptide

A. MHC-I (SIINFEKL-K^B) B. MHC-II (Biotinylated-OVA-I-A)

Fluorescent intensity detected via streptavidin-FITC antibody
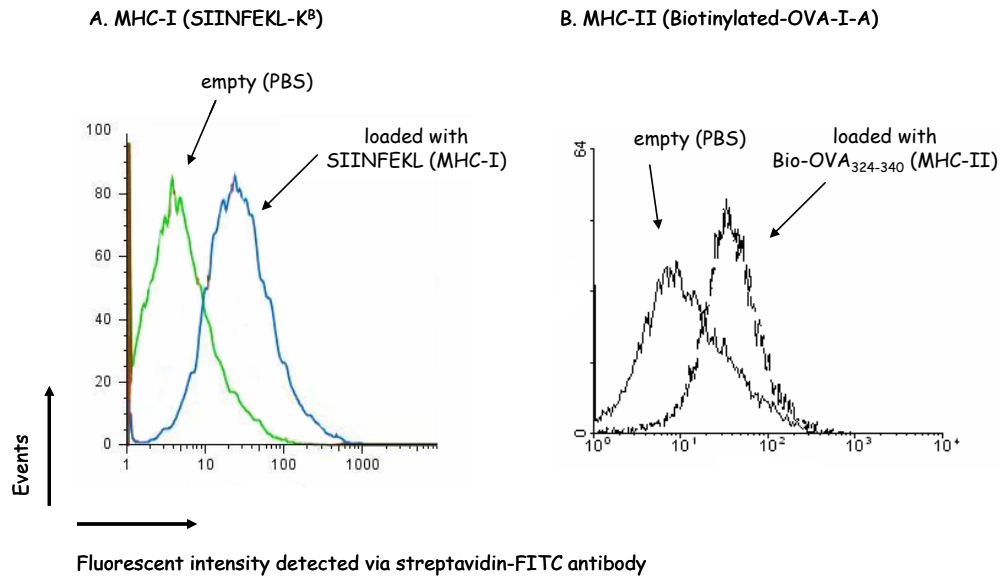
<u>Figure 31</u>   **Flow cytometric analysis of peptide loading onto MHC molecules in microsomal membranes.**

Microsomes from Jaws-II cells were pulsed with either SIINFEKL peptide, or biotinylated $OVA_{324-340}$ peptide, or PBS. Excess peptides were washed and peptide-loaded microsomes were analyzed in comparison to microsomes that had not been loaded with peptides.

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

B. Panel displays the mean fluorescence intensity of FITC-labeled streptavidin, specific for biotinylated $OVA_{324-340}$ peptide.

loading onto MHC-II was dramatically increased (Figure 32B lower panel). In addition to generating peptide receptive MHC-II molecules, the acid-stripping process also led to a significant increase in peptide-receptive MHC-I molecules (Figure 32A lower panel), which may be due to the dissociation of pre-processed peptides on MHC-I molecules.
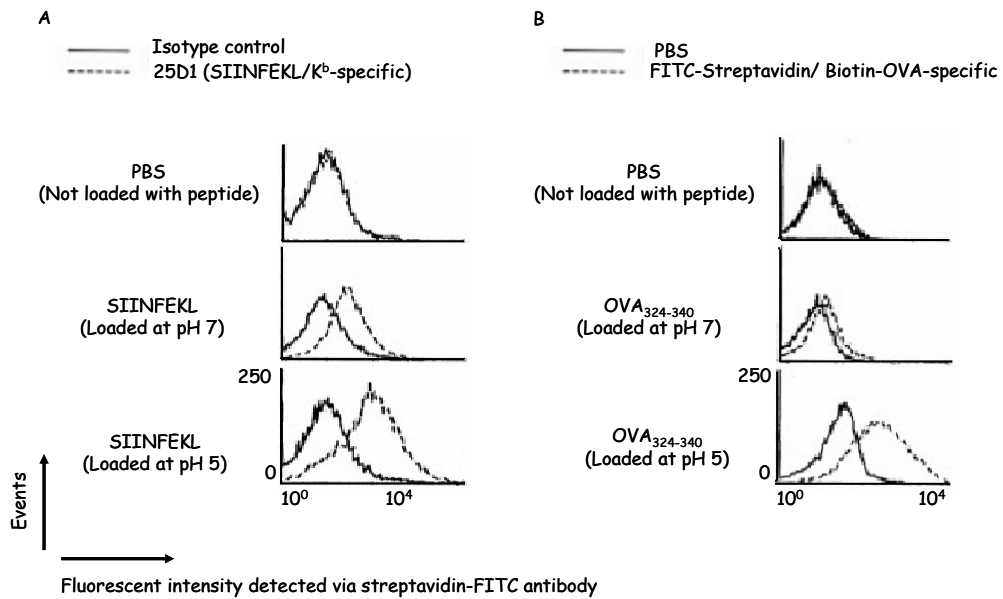
Figure 32   **Acidic treatment induces peptide-receptive MHC-II and enhanced peptide loading onto MHC-I molecules on microsomal membranes.**

Expression analysis by flow cytometry of

A. SIINFEKL-K^b complex detected by 25-D1.16 antibody on SIINFEKL-loaded microsomal membranes derived from Jaws-II with or without pre-treatment with acid-stripping and;

B. Biotin-OVA$_{324-340}$-Ia complexes detected by FITC-Streptavidin on peptide-loaded microsomal membranes from Jaws-II cells under pH 7.0 or pH 5.0 conditions. Microsomal membranes with PBS serve as a background control.

The loading of peptide onto H2-K^b molecules in the processed microsomes was confirmed by crosslinking K^b with crosslinker modified SIYNFEKL peptides (Figure 33). Specifically, a cross-linker (ANB-NOS) was conjugated to the e-amino group of the lysine residue of SIINFEKL and the isoleucine at position 3 was substituted with tyrosine to allow iodination of the peptide (Wang, Raynoschek et al. 1996). These modifications permit the photo-cross-linking of the peptide to MHC-I and thus the detection of the assembled peptide/MHC complexes. The increased level of peptide-receptive MHC molecules in ER membranes following acid stripping allowed them to bind more peptide compared with their parental cells (Figure 33).
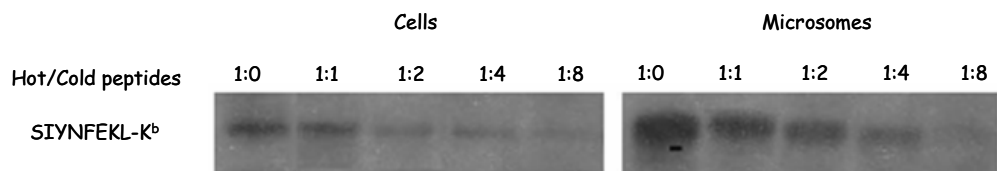
Figure 33   Visualization of crosslinked H2-K^b molecules with a radio-labeled modified peptide.

 $2x10^6$ RAW309Cr.1 cells or 20μg of RAW309Cr.1-derived microsomes were incubated with 100mM crosslinker-modified and ^{125}I-labeled SIYNFEKL peptides and exposed to UV irradiation before lysis. Total lysates were precipitated with the Y3 antibody, specific for K^b molecules, and the precipitates were separated on a SDS-gel. The crosslinked K^b molecules were visualized after exposure to X-ray film. The presented data are representative of two experiments.

The optimized protocol (Figure 34) for the preparation of microsomes with peptide-receptive MHC-I and MHC-II molecules allows effective loading of antigenic peptides *in vitro*. Furthermore, the use of crosslinker-modified MHC-I and biotinylated MHC-II reporter peptides allows monitoring the level of peptide/MHC on the microsomes, which is essential in regulating the level of T cell responses.

MHC-I loading

Newly synthesized peptide-receptive MHC-I and peptide/MHC I are found in the ER.
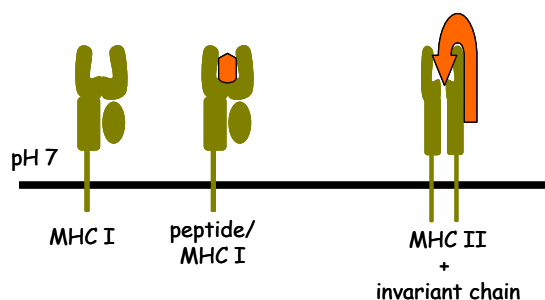
In acidic environment β2m dissociates from MHC-I heavy chain, resulting in the loss of bound endogenous peptide.
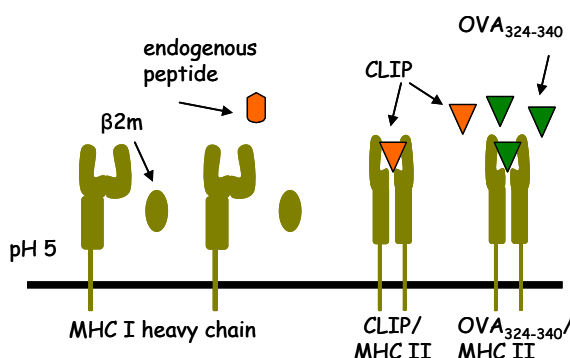
Addition of soluble β2m and suitable class I peptides (e.g. SIINFEKL) allows the formation of peptide/MHC I molecules.
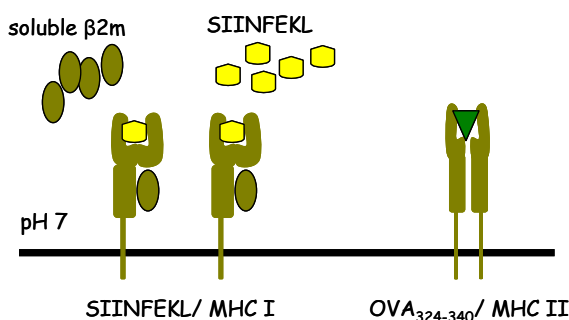
MHC-II loading

Newly synthesized MHC-II are found in the ER bound to an invariant chain that protects their peptide-binding site.

In acidic environment invariant chain dissociates from MHC-II, leaving a CLIP bound to the peptide-binding site. Suitable class II peptides (e.g. OVA$_{324-340}$) replace CLIP to form peptide/MHC II.

Figure 34   Experimental design 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 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-II molecules. In addition to generating peptide receptive MHC-II molecules, the acid stripping process also led to a significant increase in peptide-receptive MHC-I molecules, which may be due to dissociation of pre-processed peptides on MHC-I molecules.

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Summary of Section 2

The observation of APCs with Electron Microscopy revealed an abundance of endoplasmic reticulum within their cytoplasm. Microsomes are isolated and purified from APCs by cell fractionation after differential density gradient high speed centrifugations. Microsomes contain APC components of the immune synapse, such as MHC-I, MHC-II and the co-stimulatory molecules B7.1, B7.2 and ICAM-1, and they bind antigenic peptides. Acidic treatment of microsomal membranes resulted in peptide-receptive MHC-II and enhanced peptide-receptive MHC-I molecules.

3. Microsomes present peptide/MHC to murine T cells *in vitro*.

In this chapter it is investigated whether microsomes can effectively present peptides to mouse T cells *in vitro*. Antigen presentation by microsomes is analysed using defined peptides and the conditions for the induction of optimal T cell responses are evaluated. The interaction of microsomes with T cells is examined by microscopy using fluorescent-labelled microsomal membranes.

3.1 Inverted microsomes induce optimal T cell responses.

Although microsomes express plasma membrane components from APC that are associated with the immune synapse, and we had shown that MHC in microsomes can assemble with exogenous peptides, it was unclear whether peptide-loaded microsomes could induce naïve T cell responses.

As described above, the repeated freeze-thaw process is applied in order to disrupt the microsomal membrane, 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 (Rodriguez Boulan, Kreibich et al. 1978; Rodriguez Boulan, Sabatini et al. 1978), which is equivalent to the lumen of the microsomes (Kvist and Hamann 1990). Therefore, after 'inversion' of microsomes by repeated freeze-thaw to expose the luminal microsomal surface, the MHC glycoproteins should also be exposed, allowing better access of soluble peptides to the peptide-binding site of the MHC molecule. We hypothesize that added peptides can access and bind more MHC molecules on microsomes that have been processed by freeze-thaw, and that this results in the induction of optimal microsome-mediated T cell immune responses (Figure 35).
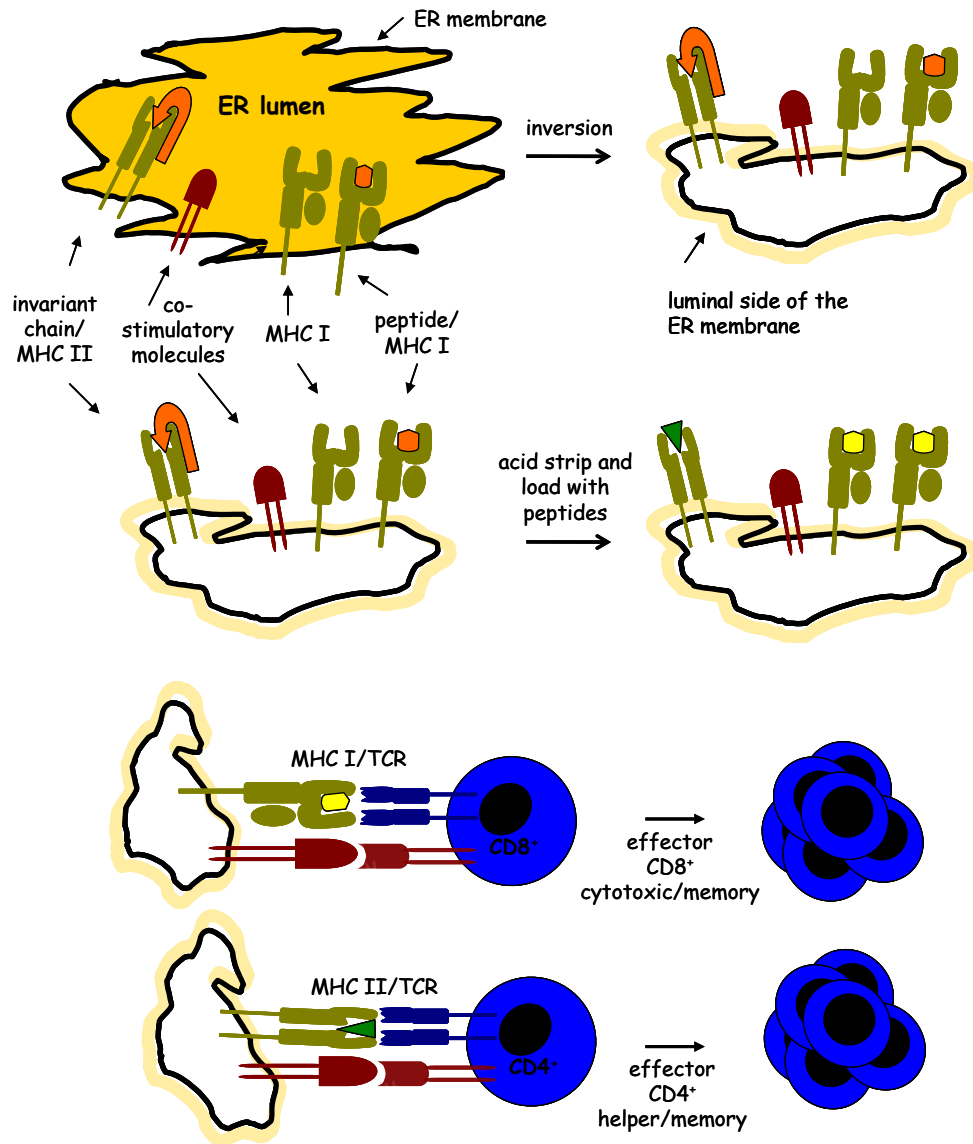
Figure 35 **Experimental design for the analysis of T cell responses induced by inverted microsomes**.

Microsomes are processed by repeated freeze-thaw so as the luminal side of the microsomal membrane is exposed. Endogenous peptides and CLIP are dissociated from MHC I and MHC II respectively, by acid stripping, and peptides of interest are loaded on microsomes. Peptide loaded microsomes induce effector T cell responses in the context of class I and class II.

To investigate this hypothesis, microsomes from Jaws-II cells were either processed by repeated freeze-thaw to facilitate inversion of the luminal side or used directly as purified, before loading with SIINFEKL peptides. Excess peptides were washed and peptide-loaded microsomes were incubated with splenic T cells isolated from OT-I TCR transgenic mice (Hogquist, Jameson et al. 1994), which express TCRs specific to SIINFEKL-K^b, similar to B3Z cells. T cells without peptide-loaded microsomes were used as a negative control. T cell responses were evaluated by IL-2 production measured with ELISA after 24 hours. Microsomes loaded with SIINFEKL peptides could induce OT-I T cell responses, whereas microsomes loaded with an irrelevant KTR peptide did not. The processing of microsomes so as to expose the microsomal luminal surface resulted in increased induction (3 fold) of T cell responses (Figure 36) compared to the responses induced by non-processed microsomes.
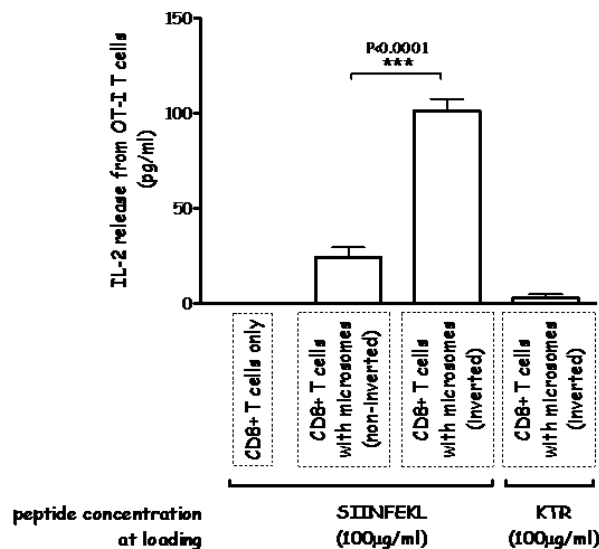
Figure 36   **The exposure of the microsomal luminal surface facilitates the induction of optimal T cell responses *in vitro*.**

Microsomes prepared from Jaws-II cells were either processed by repeated freeze-thaw to facilitate inversion of the luminal side or used directly following their preparation, and pulsed with SIINFEKL before co-cultured with OT-I T cells for 24 hours. T cell activation was assessed by IL-2 production using ELISA. The microsomes loaded with an irrelevant KTR peptide are a negative control. The data are presented as mean of quadruplicate cultures ± SEM and are representative of three experiments.

3.2 T cell activation by peptide-loaded microsomes depends on microsome concentration.

To verify that T cell responses were proportional to the surface of antigen-presenting membrane, serial dilutions of microsomes from Jaws-II cells were loaded with 100µg/ml SIINFEKL peptide. After removal of excess peptide, peptide-loaded microsomes were co-cultured with 10^6 OT-I T cells for 24 hours before measuring IL-2 production. T cells without peptide-loaded microsomes were used as a negative control. The data demonstrate that T-cell activation was relative to peptide-loaded microsomal membranes concentration (Figure 37). However, excess of peptide-loaded microsomes reduced T-cell activation, as induction of T cells with 4µg/ml of microsomal membranes resulted in less IL-2 production compared to that produced when T cells were induced with 2µg/ml of microsomal membranes.
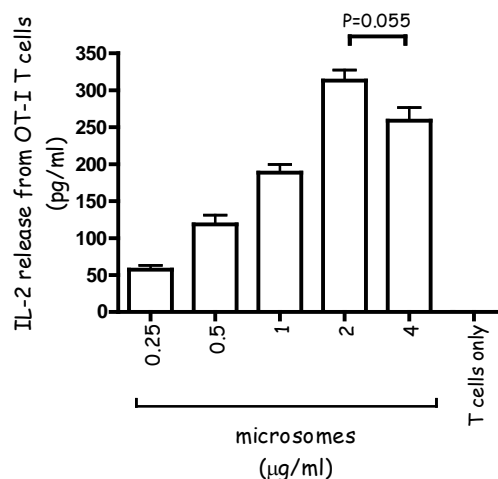
Figure 37 **Induction of T cell responses *in vitro* is relevant to microsomal vaccine concentrations.**

Microsomes from Jaws-II cells were serially diluted before pulsed with 100µg/ml SIINFEKL peptide. After removal of excess peptides, peptide-loaded microsomes were co-cultured with 1×10^6 OT-I T cells. IL-2 production was measured with ELISA after 24 hours. The data are presented as mean of quadruplicate cultures ± SEM and are representative of three experiments.

3.3 T cell activation by peptide-loaded microsomes depends on peptide concentration.

To examine whether microsomes activate T cells in a peptide-dependent manner, 2μg of microsomes (quantified by BCA protein assay SIGMA) from Jaws-II cells were loaded with increasing concentrations of SIINFEKL peptide (10μg/ml, 100μg/ml and 1000μg/ml SIINFEKL concentration at loading). After removal of excess peptide, SIINFEKL-loaded microsomes were co-cultured with 1×10^6 OT-I T cells for 24 hours prior to measuring IL-2 production. The resulting data revealed that the induced T cell responses were peptide-dose-dependent (Figure 38A). Similarly, the responses of the $OVA_{324-340}$-recognising in the context I-A MF2.2D9 T cells, induced by 2μg of Jaws-II-derived microsomes loaded with increasing concentrations of $OVA_{324-340}$ peptide (10μg/ml, 100μg/ml and 1000μg/ml $OVA_{324-340}$ concentration at loading), were also peptide- dose-dependent (Figure 38B). In both experimental settings, T cell responses were not only peptide-dose-dependent, but also peptide-specific, since microsomes loaded with the same concentrations of an irrelevant peptide (MPp) could not induce T cell responses (Figure 38A&B lower panels).
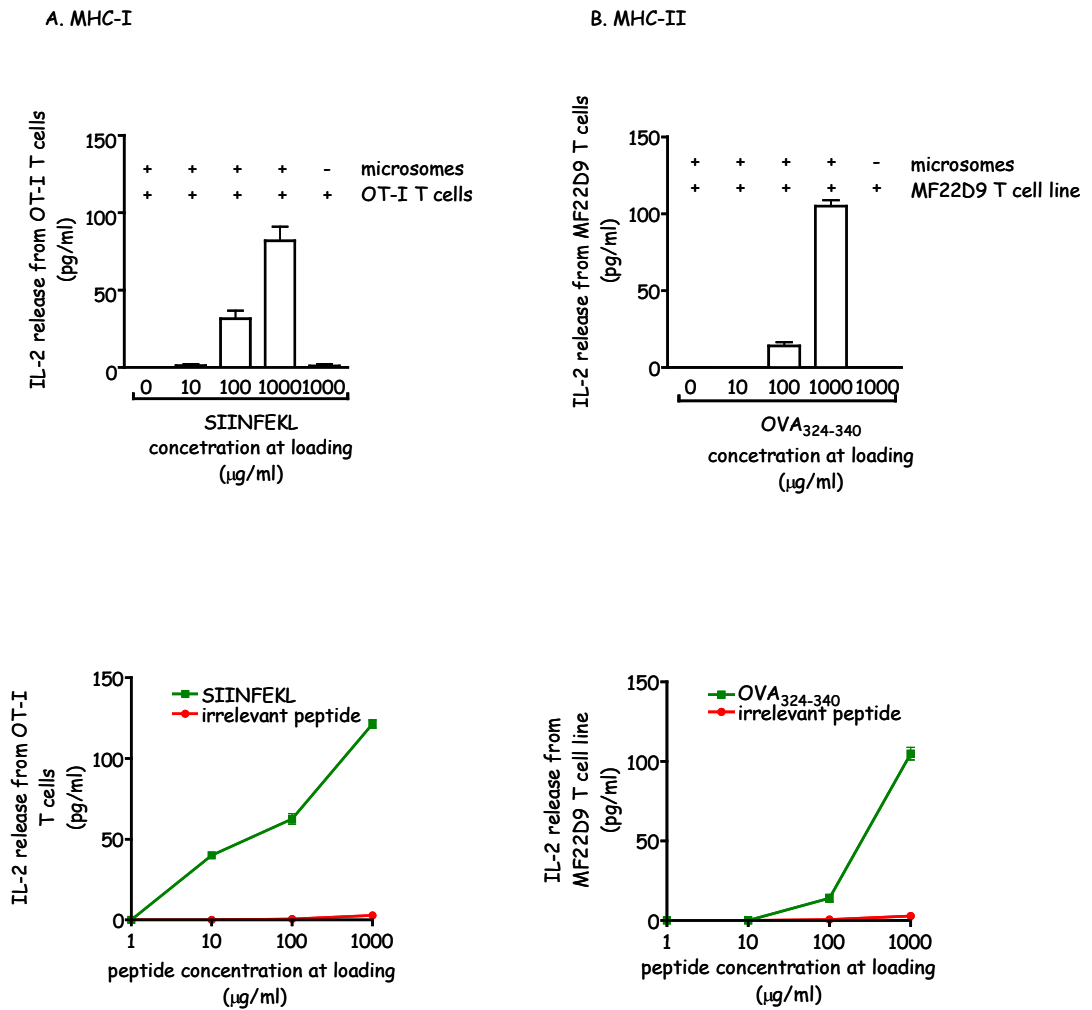
Figure 38   **Induction of T cell responses by peptide-loaded microsomes.**

2μg of microsomes from Jaws-II cells were pulsed with SIINFEKL or OVA$_{324-340}$ peptides at different concentrations as indicated for 6 hours. Excess peptides were washed before cells were co-cultured with either

A. OT-I T cells specific for SIINFEKL-K^b or with

B. MF2.2D9 T cell line specific to OVA$_{324-340}$-I-A.

The production of IL-2 was measured by ELISA after culture for 24 hours. Lower panel displays the findings in comparison to microsomes loaded with irrelevant MP peptides. The data are presented as mean of quadruplicate cultures ± SEM and are representative of three experiments.

3.4 T cell binding to microsomes results in T cell activation and depends on T cell-antigen recognition.

Although peptide-loaded microsomes could induce peptide-specific T cell responses it is unknown whether ER-derived microsomes could directly interact with, and bind to, T cells after assembly of MHC with exogenous peptides. This was examined in two separate experimental settings using fluorescent-labelled microsomes or microsomes engineered to express fluorescent proteins.

Microsomes were extracted from Jaws-II cells and labelled with FITC1 (using the Fluorotag FITC Conjugation Kit from SIGMA) prior to their loading with peptides. After removal of excess peptides, peptide-loaded microsomes were incubated with T cells and their interaction was observed with confocal microscopy. Fluorescent micrographs revealed that SIINFEKL-loaded fluorescent microsomes could interact and bind to the SIINFEKL–K^b recognising B3Z T cells (Figure 39A right panel). By contrast, binding to B3Z T cells was considerably less when fluorescent microsomes were loaded with an irrelevant influenza peptide MPp (Figure 39A left panel).

FITC1 is a fluorophore which interacts with free amino acids of proteins to form stable conjugates. One could question whether FITC1 binding to amino acids would interfere with peptide binding sites of MHC molecules in microsomal membranes, therefore influencing the interaction of microsomes with T cells. To confirm that this is not the case, the above experiment was repeated using microsomes extracted from an APC cell line, RMA-S, which was transfected to express green fluorescence protein (GFP) and a retention signal (E19K). The ER-retention signal is a C-terminal tetrapeptide KDEL (in mammals) (Pelham 1990), which is necessary for localization and maintenance of membrane proteins in the ER (Pelham 1989). Here, it is used to ensure the retention of the (transfected) fluorescent proteins in the ER and, subsequently, in the extracted microsomes. In this experiment, binding of microsomes to T cells was assessed following incubation of SIINFEKL-loaded microsomes with splenic OT-I T cells, which express

TCRs specific to SIINFEKL-K^b, similar to B3Z cells. As revealed by fluorescent microscopy images, fluorescent microsomes loaded with SIINFEKL peptides could interact and bind to SINFEKL-K^b-recognising OT-I T cells (Figure 39B right panel), while binding to OT-I T cells could not be detected when fluorescent microsomes were loaded with an irrelevant influenza peptide MPp (Figure 39B left panel).

To examine the functional consequence of this interaction, microsome-bound T cells were cultured in normal medium for 24 hours before measuring IL-2 production. Both classes of SIINFEKL-loaded fluorescent microsomes, FITC1-stained (Figure 40A) and GFP- expressing (Figure 40B), could induce T cell responses similar to those induced by SIINFEKL-loaded microsomes that hadn't been fluorescently labelled, suggesting that this type of membrane manipulation does not interfere with presentation of antigens to T cells. Furthermore, fluorescent microsomes that were loaded with an irrelevant peptide (MPp) could not induce T cell responses, indicating that peptide-loaded fluorescent microsomes retained their peptide-specific recognition by T cells. Thus, fluorescent microsomes can be used for the detection of antigen-specific T cells and for their subsequent activation *in vitro*.

A.
Confocal images
of FITC1 stained
microsomes
cultured with B3Z
T cells after
loading with
irrelevant (MPp)
(left panel) or
specific
(SIINFEKL)
(right panel)
peptides.

microsomes+ MPp   microsomes+ SIINFEKL

B.
Confocal images of
GFP expressing
microsomes
cultured with OT-I
T cells after
loading with
irrelevant (MPp)
(left panel) or
specific
(SIINFEKL) (right
panel) peptides.

microsomes+ MPp   microsomes+ SIINFEKL

Figure 39   **Peptide-loaded microsomes interact with specific T cells.**

A. Microsomes prepared from Jaws-II cells were labelled with FITC1 by conjugation before loading with SIINFEKL [microsomes+SIINFEKL] or irrelevant MP peptide [Microsomes +MPp]. Following wash of excess peptides peptide-loaded microsomes were allowed to interact with B3Z cells for 1 hour. Confocal images show that microsomes that had been loaded with SIINFEKL peptide could interact and bind better with B3Z cells.

B. RMA-S cells were transfected with a green fluorescence protein tagged with a retention signal, so that the GFP protein can be retained in the ER. The microsomes from these cells were pulsed with SIINFEKL [microsomes+SIINFEKL] or an irrelevant MP peptide [microsomes+MPp] before allowed to interact with OT-I cells. Fluorescence microscopy shows that only microsomes that had been loaded with SIINFEKL could interact and bind stably to OT-I T cells.
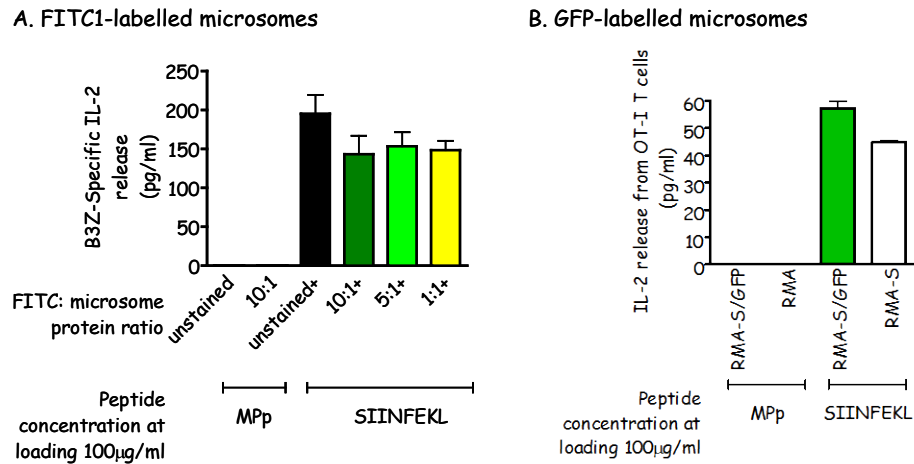
Figure 40 Analysis of fluorescence interference with antigen presentation on microsomal membranes.

A. Equal amounts of Jaws-II microsomes that had been labelled with FITC1 were pulsed with 100μg/ml of SIINFEKL peptide for 6 hours. Excess peptides were washed before cells were co-cultured with B3Z T cells. B3Z T cell activation was determined based on IL-2 production 24 hours later and measured by ELISA. Peptide-loaded (+) unstained microsomes were compared with peptide-loaded (+) microsomes that had been labelled with several dilutions of FITC1 (10:1, 5:1, and 1:1). Unstained microsomes and microsomes stained with the maximum FITC1 dilution that had been loaded with MP peptide serve as negative controls. The data are presented as mean of quadruplicate cultures ± SEM and are representative of three experiments.

B. The RMA-S cell line was transfected with a green fluorescence protein tagged with a retention signal, so that GFP protein could be retained in the ER. Equal amounts of fluorescent microsomes prepared from transfected cells were pulsed with 100μg/ml SIINFEKL or an irrelevant MP peptide for 6 hours. Excess peptides were washed before cells were co-cultured with SIINFEKL-K[b]-specific OT-I cells. T cell activation was determined based on IL-2 production 24 hours later and measured by ELISA. Peptide-loaded microsomes from non-transfected RMA-S cells served as positive controls. Microsomes from transfected or non-transfected cells loaded with irrelevant peptides served as negative controls. The data are presented as mean of quadruplicate cultures ± SEM.

Summary to Section 3

The interaction of microsomes with T cells was studied using fluorescent microscopy, which revealed that fluorescent microsomes could bind T cells in a peptide-specific manner. Fluorescent labelling did not interfere with T cell-antigen recognition on microsomes. T cell activation was dependent on microsome concentration, and processing of microsomes in an attempt to expose their luminal surface improved their ability to stimulate T cell responses *in vitro*. Finally, it was demonstrated that microsomes could induce peptide-specific T cell responses *in vitro* in the context of MHC I and MHC II antigen presentation.

4. Microsomes present peptides to mouse T cells *in vivo*.

In the previous chapters, the immunogenicity of microsomes was demonstrated and characterized *in vitro*. However, the only way of assessing a vaccine's ability to induce protective immune responses is by analysing immunity *in vivo*. This chapter begins with an experiment that aims to evaluate whether vaccination of healthy C57.Bl/6 mice with peptide-loaded microsomes from cell line (Jaws-II) or autologous DCs can elicit antigen-specific T cell responses. Subsequently, immunized animals, along with unimmunized controls are challenged with an infectious or lethal dose of OVA-expressing vaccinia virus. Animals challenged with 'infectious' dose become sick (e.g. weight loss), but typically can recover without treatment. By contrast, challenge with a 'lethal dose' of the virus results in an acute viral infection from which most animals fail to recover without treatment. The prevalence and severity of the infection in the immunized animals is assessed and compared to the course of disease in unimmunized control animals. At the end of the assays *in vivo*, the secondary lymphoid organs are analyzed *in vitro* to evaluate specific T cell immune responses. The efficacy of the microsomal vaccine in therapeutic context is also examined *in vivo* with an animal model of an antigen-bearing tumour. Finally, internalization assays which examine whether microsomes are being phagocytosed by antigen presenting cells are described, in an effort to elucidate the mechanism by which microsomes induce T cell responses *in vivo.*

4.1 Induction of CD8$^{(+)}$ T cell responses *in vivo* by microsomal vaccine.

The ability of peptide-loaded microsomes to induce T cell responses *in vivo* was examined in naïve C57.Bl/6 mice, which have an H2 haplotype b. In addition to easy breeding, this strain has a permissive background for expression of mutations; this allows a great availability of congenic strains

(e.g. OT-I mice) and of various congenic cell lines (e.g. Jaws-II cells), making them ideal controls and models for disease.

In this study, two groups of four C57.Bl/6 mice were immunized intravenously with either SIINFEKL peptide-loaded microsomes from Jaws-II cells at a dose of 20µg microsomes/ mouse, or microsomes only at the same dose. The mice were then boosted by repeating the same immunizations as before after seven days (Figure 41). One day after the boost the mice were sacrificed for analysis of T cell responses.
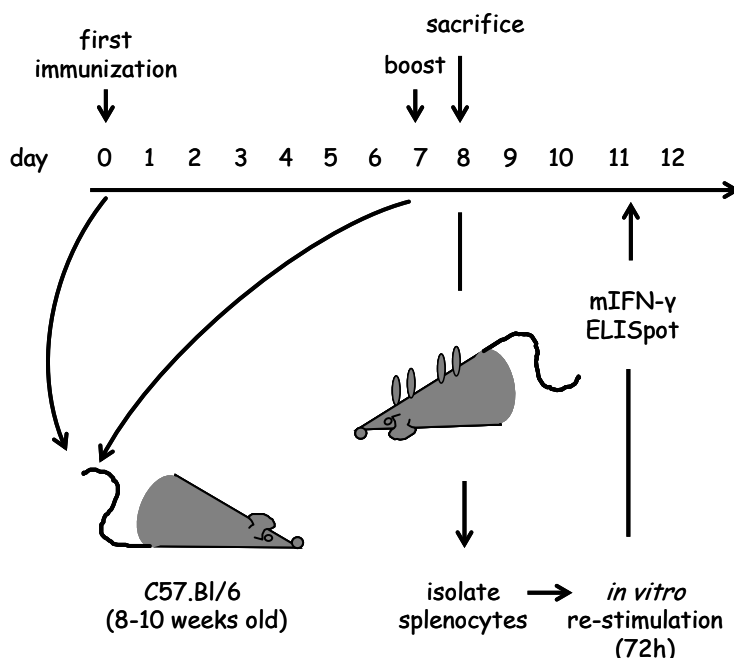
Figure 41 **Experimental design for the analysis of murine immune responses following vaccination with SIINFEKL-loaded microsomes.**

Healthy mice were vaccinated with SIINFEKL-loaded microsomes, and then boosted with same immunizations after seven days. One day after boost total splenocytes were reactivated *in vitro* with free SIINFEKL peptides and peptide-specific responses were evaluated by IFNγ production, measured with ELISpot.

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Mice vaccinated with

mouse

| | 1 | 2 | 3 | 4 |

Microsomes + SIINFEKL

Microsomes only

Re-stimulation *in vitro* with 100μg/ml SIINFEKL
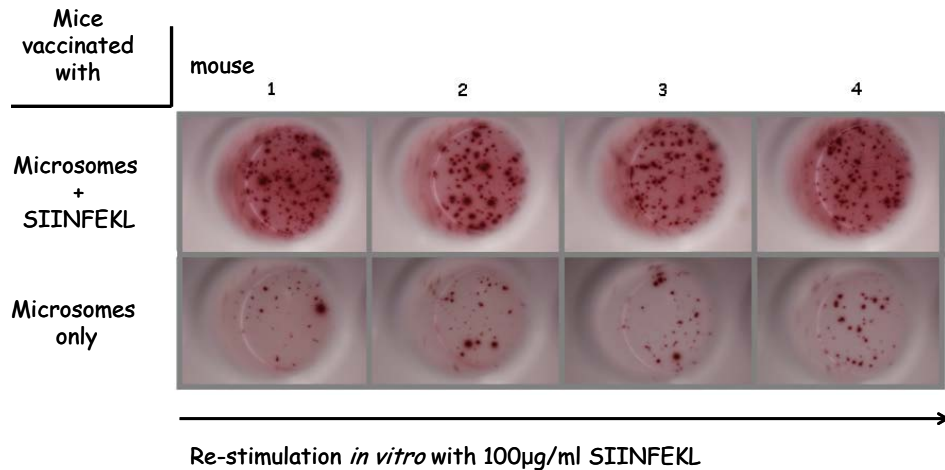
Figure 42 **SIINFEKL-loaded microsomes induce CD8[(+)] T cell responses in naïve mice.**

Four C57.Bl/6 mice were injected intravenously with a dose of 20μg/mouse SIINFEKL-loaded microsomes from Jaws-II cells and boosted once with the same dose after 7 days. The four mice injected with microsomes that hadn't been loaded with peptide served as controls. One day after boosting, total splenocytes were incubated with SIINFEKL peptide at 100μg/ml for three days. T cell responses were assessed by quantification of IFNγ producing cells by IFNγ ELISpot assay. Each well is representative of triplicate cultures for each mouse.

Total splenocytes were isolated and re-activated *in vitro* with 100μg/ml soluble SIINFEKL peptide for 3 days. Cells from the mice immunized with SIINFEKL-loaded microsomes produced more IFNγ than cells from the mice immunized with microsomes only, as illustrated by an IFNγ ELISpot assay (Figure 42). This is an assay adapted from the enzyme linked immunosorbent assay (ELISA), in which cells are cultured over antibodies attached to a plastic surface. Specific cell-secreted products (i.e. cytokines) are captured by the corresponding antibody and are then detected using an enzyme-coupled antibody that cleaves a colourless substrate to generate a localized coloured spot, each spot representing i.e. a cytokine-producing cell. In the experiment described here, total single cell-suspensions purified from homogenized spleens of the immunized mice were used to allow uptake and presentation of free SIINFEKL peptide, that was used as a stimulant for re-activation of SIINFEKL-specific CD8[(+)] T cells *in vitro*, by antigen presenting cells naturally found in the spleen. The greater number and

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higher intensity of the 'IFNγ-spots' generated by splenocytes from mice immunized with SIINFEKL-loaded microsomes compared to the 'IFNγ-spots' generated by splenocytes from mice immunized with microsomes only, following re-stimulation *in vitro* with SIINFEKL, indicate that immunization of mice with SIINFEKL-loaded microsomes could trigger the activation of SIINFEKL-specific CD8[(+)] T cells *in vivo*.

4.2 Protection against vaccinia virus infection *in vivo* by a microsomal vaccine that induces both CD4[(+)] and CD8[(+)] T cell responses.

The capacity of peptide-loaded microsomes to induce effective anti-viral immune responses *in vivo* was assessed in a vaccinia viral infection model in C57.Bl/6 mice (Figure 43). Vaccinia virus causes an acute viral infection in mice. Although neutralizing antibodies are crucial for clearing the virus, protection against vaccinia infection appears to be mediated by functions of cellular immunity (Novembre, Raska et al. 1989; Xu, Johnson et al. 2004). The recombinant vaccinia virus used here carries a gene encoding chicken ovalbumin (Restifo, Bacik et al. 1995).
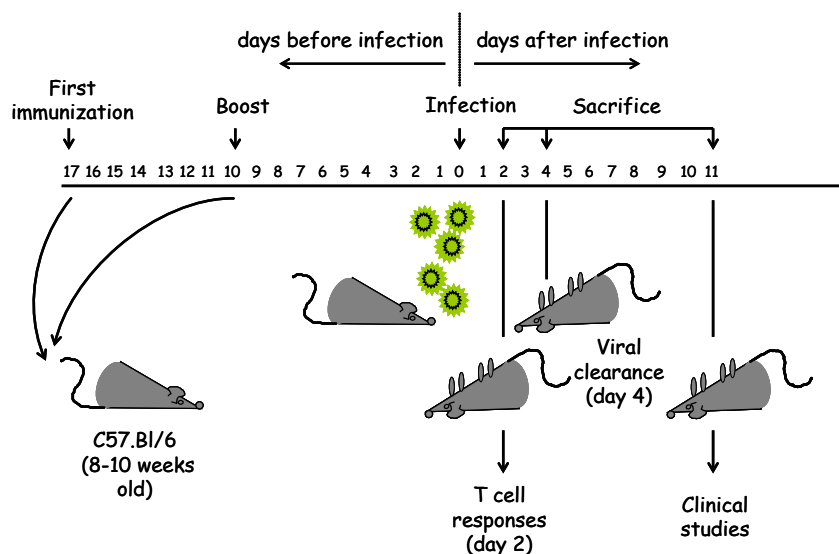
Figure 43 Experimental design for the evaluation of protection induced by microsomal vaccine against murine viral infection.

C57.BL/6 female mice were immunized twice intravenously with microsomes loaded with a mixture of SIINFEKL and OVA$_{324-340}$ peptides. Ten days after the last immunization mice were challenged with an infectious or lethal dose of recombinant vaccinia virus that carried the OVA-gene. T cell responses were analyzed in total splenocytes two days after infection; viral clearance was assessed in lung tissue 4 days after infection, while the clinical outcome was monitored over the eleven days post-infection.

4.2.1 Detection of protective immunity after vaccination with microsomes from dendritic cell lines.

Four groups of C57.Bl/6 mice were immunized intravenously with SIINFEKL peptide-loaded microsomes from Jaws-II cells at a dose of 20μg microsomes/mouse, or microsomes only at the same dose, or 100μg/mouse soluble SIINFEKL, or PBS. The mice were then boosted by repeating the same immunizations as before after seven days. Ten days after boosting, the mice were challenged with an infectious dose $2x10^5$ plaque forming units (PFU) of chicken ovalbumin-expressing vaccinia virus (OVA-VV) and observed for severity of infection and course of disease over eleven days. Mice in the control groups lost up to 20% of their weight, while the mice vaccinated with SIINFEKL-loaded microsomes didn't show any evidence of weight loss (Figure 44A). SIINFEKL-specific CD8$^{(+)}$ T cells were induced only in mice immunised with SIINFEKL peptide-loaded microsomes, but not in the other three groups as indicated by tetramer staining (Figure 44B).
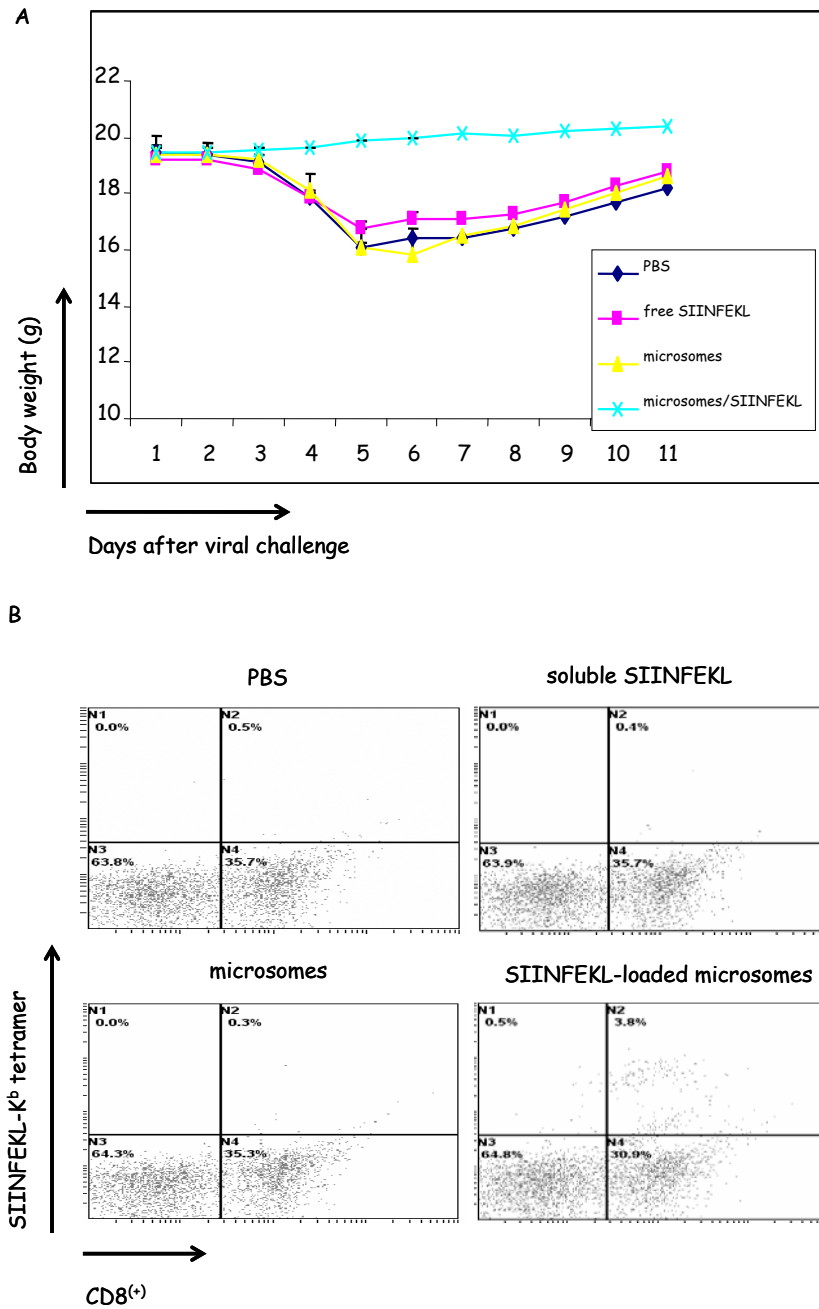
Figure 44 **Peptide loaded microsomes induce immune responses against acute viral infection.**

C57.Bl/6 mice were injected intravenously with a dose of 20μg/mouse SIINFEKL-loaded microsomes from Jaws-II cells [microsomes/ SIINFEKL]. The mice injected with a dose of 20μg/mouse peptide-free microsomes [microsomes], or 100μg/mouse SIINFEKL peptide [free SIINFEKL], or PBS [PBS], served as controls.

A. Body weight after challenge with infectious dose of VV-OVA virus.

B. Two days after challenging, total splenocytes were co-stained with tetramer specific to SIINFEKL-K^b and CD8 antibody.

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The above experiment was repeated using Jaws-II-derived microsomes that were loaded with a mixture of SIINFEKL and OVA$_{324-340}$ peptides. Mice were vaccinated with a dose of 20μg microsomes/mouse of microsomes loaded with SIINFEKL and OVA$_{324-340}$ peptides [M+OVAp], or with the same dose of microsomes loaded with an irrelevant influenza peptide (MPp) [M+MPp], or with 10^6 Jaws-II cells loaded with a mixture of SIINFEKL and OVA$_{324-340}$ peptides [DC+OVAp], or with 10^6 Jaws-II cells loaded with an irrelevant influenza peptide (MPp) [DC+MPp], or with a mixture of SIINFEKL and OVA$_{324-340}$ peptides only [OVAp]. Seven days after vaccination the mice were boosted by repeating the same immunizations. Ten days after boosting the mice were challenged with either a lethal dose 5x10^6 PFU of OVA-VV virus or with the same dose of a vaccinia virus expressing influenza matrix protein (MP-VV) (only mice that had been vaccinated with microsomes loaded with SIINFEKL and OVA$_{324-340}$ peptides were challenged with MP-VV). Forty-eight hours after challenge, three of eight mice were randomly chosen in each group and were sacrificed for analysis of T cell responses, while the remaining mice were used for clinical and viral clearance studies. Upon re-stimulation of CD4$^{(+)}$ T cells *in vitro* with the same treatments that had been used for the immunizations (Figure 45), the most potent T cell responses were observed in mice that were vaccinated with mixture of SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes, and subsequently challenged with OVA-VV virus (Figure 46 lower middle panel). SIINFEKL and OVA$_{324-340}$ peptide-loaded Jaws-II cells induced lower CD4$^{(+)}$ T cell responses (Figure 46 upper middle panel) compared to those induced by SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes. Mild T cell responses were also observed in mice vaccinated with mixture of the SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes that were subsequently challenged with MP-VV virus (Figure 46 lower right panel).

Clinical symptoms were closely associated with the observed immune responses induced by vaccination *in vivo* (Figure 47). Indeed, although mice immunized with SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes showed significant weight loss, they were protected from severe symptoms

and they recovered after the course of the disease (Figure 47 left panel). The vaccination efficacy of peptide-loaded microsomes from Jaws-II cells was compared with that of peptide-loaded Jaws-II cells. The results showed (Figure 47 left panel) that peptide-loaded DCs could also protect 75% of the mice from lethal dose of viral infection, but the recovery of the infected mice was slower than the group immunized with peptide-loaded microsomes. All the other mice died (due to legal regulations severe weight loss necessitated the sacrifice of these animals before natural death could be observed) (Figure 47 right panel).

These findings collectively suggest that immunization with microsomal vaccine is more efficient than with DCs, and demonstrate that peptide-loaded microsomes are highly immunogenic and could serve as protective vaccines for acute viral infections.
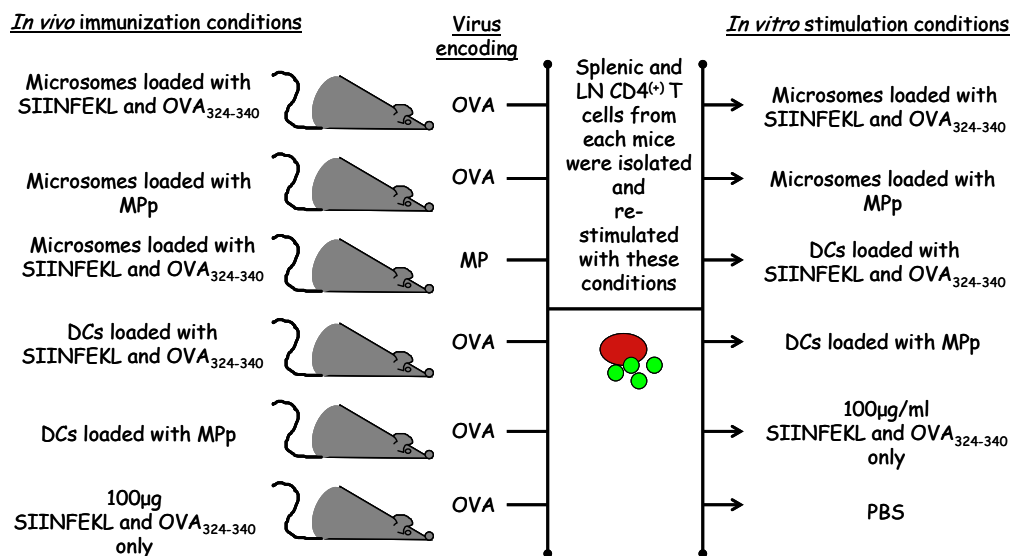
Figure 45 **Experimental design for analysis of CD4$^{(+)}$ T cell responses *in vitro* following viral infection in mice vaccinated with microsomal vaccine.**

Following infections of the immunized mice, CD4$^{(+)}$ T cells were isolated from total splenocytes and lymph nodes by positive selection. Purified CD4$^{(+)}$ T cells from each mice were activated with the described treatments *in vitro*. Two days later CD4$^{(+)}$ T cell-specific IL-2 release was measured by ELISA..
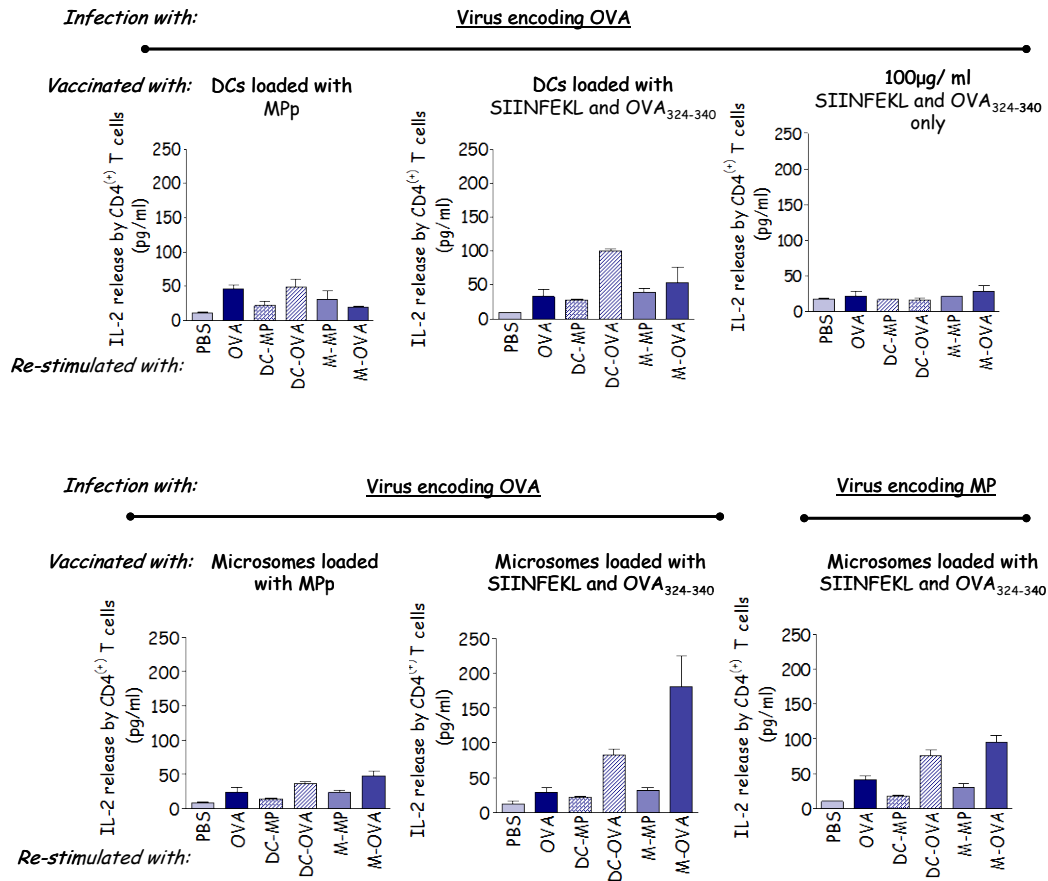
Figure 46 **Induction of CD4$^{(+)}$ T cell responses by peptide-loaded microsomes *in vivo*.**

C57.Bl/6 mice were injected intravenously with a mixture of SIINFEKL and OVA$_{324-340}$ loaded microsomes from Jaws-II [M+OVAp] at a dose of 20µg microsomes/mouse or 10^6 SIINFEKL and OVA$_{324-340}$-loaded Jaws-II cells (DCs) [DC+OVAp]. Mice injected with a dose of 20µg /mouse microsomes from Jaws-II loaded with irrelevant MP peptide [M+MPp], 10^6 Jaws-II cells loaded with irrelevant MP peptide [DC+MPp], mixture of 100µg SIINFEKL and 100µg OVA$_{324-340}$ peptides [OVAp], and PBS [PBS], served as controls. Two days after viral challenge, CD4$^{(+)}$ T cells were isolated from splenocytes and stimulated with the indicated stimuli at 2x10^5 CD4$^{(+)}$ T cells per well. Two days after stimulation, IL-2 production was measured by ELISA. Data are presented as mean of triplicate cultures ± SD.

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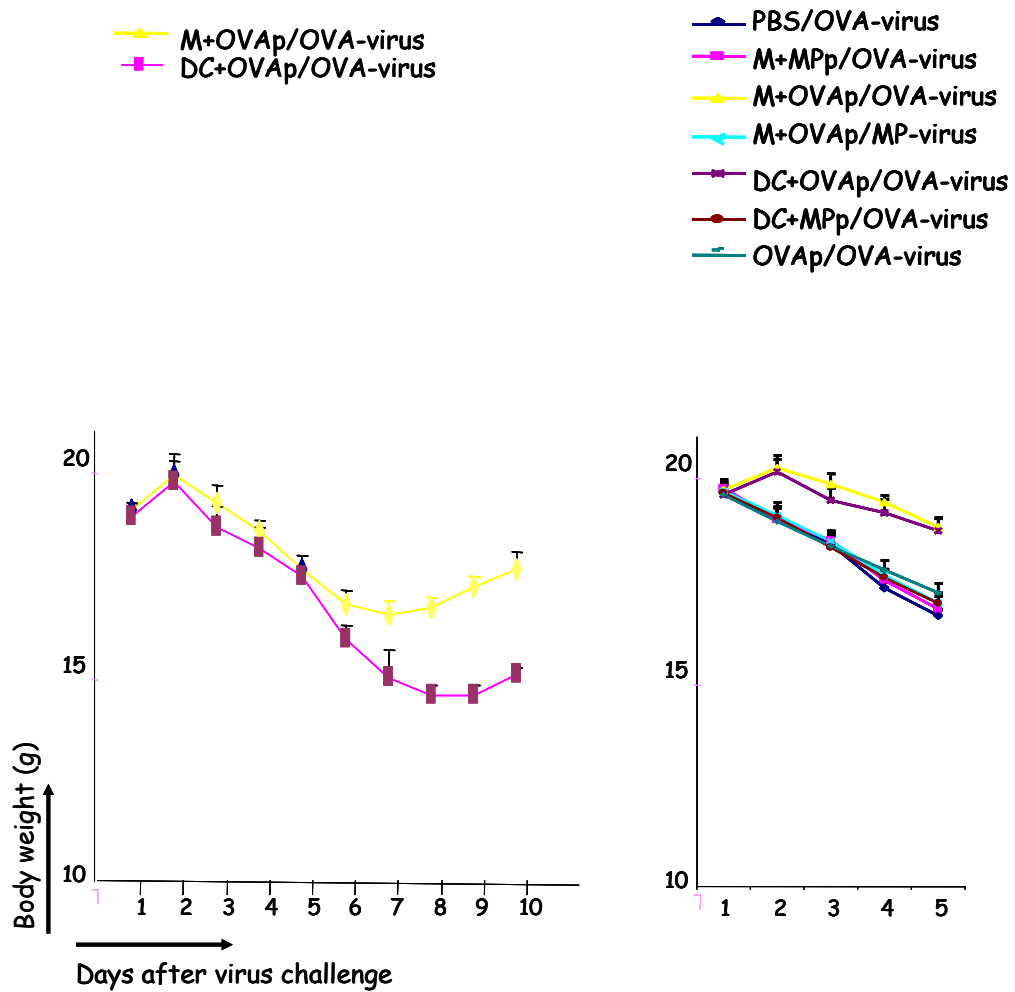
Figure 47 **Mouse weight analysis after lethal dose of viral infection.**

C57.Bl/6 mice were injected intravenously with Jaws-II-derived microsomes loaded with a mixture of SIINFEKL and $OVA_{324-340}$ [M+OVAp]at a dose of 20μg microsomes/mouse or 10^6 Jaws-II cells pulsed with a mixture of SIINFEKL and $OVA_{324-340}$ peptides [DC+OVAp], respectively and boosted once after 7 days. Microsomes loaded with irrelevant MP peptide [M+MPp], a mixture of free SIINFEKL and $OVA_{324-340}$ peptides [OVAp], or PBS [PBS], served as controls. Mice were infected with an OVA expressing vaccinia virus [OVA-virus], or with an influenza matrix protein MP expressing vaccinia virus [MP-virus]. After five days, all the mice had to be sacrificed due to dramatic loss of weight, except the mice that had received [DC+OVAp] and [M+OVAp]. Mice vaccinated with microsomes loaded with a mixture of SIINFEKL and $OVA_{324-340}$ [M+OVAp] recovered from lethal dose of infectious virus.

4.2.2 Evaluation of protective immunity after vaccination with bone marrow-DC-derived microsomes.

To confirm the efficacy of the microsomal vaccine in protection from viral infection the above experiments were repeated using microsomes from autologous bone marrow-derived dendritic cells from C57.Bl/6mice. The main advantage of using microsomes from autologous dendritic cells is the direct relation between the vaccine and the targeted immune response, and reduced risk of allogenic immune responses. Autologous dendritic cells were differentiated *in vitro* from bone marrow progenitors in the presence of IL-4 and GM-CSF. Prior to microsome extraction dendritic cell cultures were activated with LPS for the induction of maximum MHC and co-stimulatory expression. Microsomes were then extracted and loaded with peptides as described before.

Five groups of C57.Bl/6mice were immunized intravenously with either a mixture of SIINFEKL and $OVA_{324-340}$ peptide-loaded microsomes isolated from bone-marrow derived DCs of C57.Bl/6 at a d o ϵ of 20 µg microsomes/mouse [M+OVAp]; or microsomes loaded with irrelevant MP peptides at the same dose [M+MPp]; or with a mixture of SIINFEKL (100µg/mouse) and $OVA_{324-340}$ (100µg/mouse) [OVAp]; or with 10^6 DCs pulsed with these two peptides [DC+OVAp]; or with PBS [PBS]; as controls. The mice were then boosted by repeating the same immunizations after seven days. Ten days after boosting, twenty of the 30 mice in each group were challenged with an infectious dose $2x10^5$ PFU and the other ten with a lethal dose $5x10^6$ PFU of OVA-VV virus. Forty-eight hours after challenge, five of the twenty mice involved in the infectious dose group were sacrificed for analysis of T cell responses and, 4 days after, another five were sacrificed for viral clearance study, while the remaining mice were used for clinical study.

Flow cytometry analysis revealed that 4.3% of $CD8^{(+)}$ T cells in mice immunized with SIINFEKL and $OVA_{324-340}$ peptide-loaded microsomes were SIINFEKL-specific (Figure 48A middle panel), compared with 1.8% in mice immunized with peptide-pulsed DCs (Figure 48A bottom panel). SIINFEKL-

specific CD8$^{(+)}$ T cells were not detected in the other three groups as indicated by tetramer staining (Figure 48A). In conjunction with induction of antigen-specific CD8$^{(+)}$ T cells, the re-stimulation of CD4$^{(+)}$ T cells *in vitro* showed that SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes [M+OVAp] induced stronger IL-2 production and proliferative responses from mice immunized with a mixture of SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes (Figure 48B&C middle panels) than from mice immunized with peptide-pulsed DCs (Figure 48B&C bottom panels). These results indicate that an integrated immune response of both CD4$^{(+)}$ and CD8$^{(+)}$ T cells was induced in mice by peptide-loaded microsomes and demonstrates that, in the described experimental setting, the microsomes are more immunogenic than their parental DCs.

Clinical symptoms were closely correlated to the induction of immune responses *in vivo*. In mice receiving an infectious dose, a 'degree' of protection was observed in mice immunized with peptide-pulsed DCs [DC+OVAp]. However, only SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes completely protected against viral infection as these mice showed no weight loss (Figure 49A) and no viral particles were detected in lung tissue unlike the other groups (Figure 49B). With a lethal dose of virus, mice immunized with SIINFEKL and OVA$_{324-340}$ peptide-loaded microsomes showed weight loss but they were protected from death and recovered rapidly after infection (Figure 49C). Conversely, two out of ten mice immunized with peptide-pulsed DCs died after five days (Figure 49C). All mice in other groups died within six days post-infection (Figure 49C). Furthermore, the spleens were double the size in the protected mice immunized by OVA peptide-loaded microsomes (data not shown). These findings demonstrate that peptide-loaded microsomes are an effective vaccine in the protection against acute viral infection.
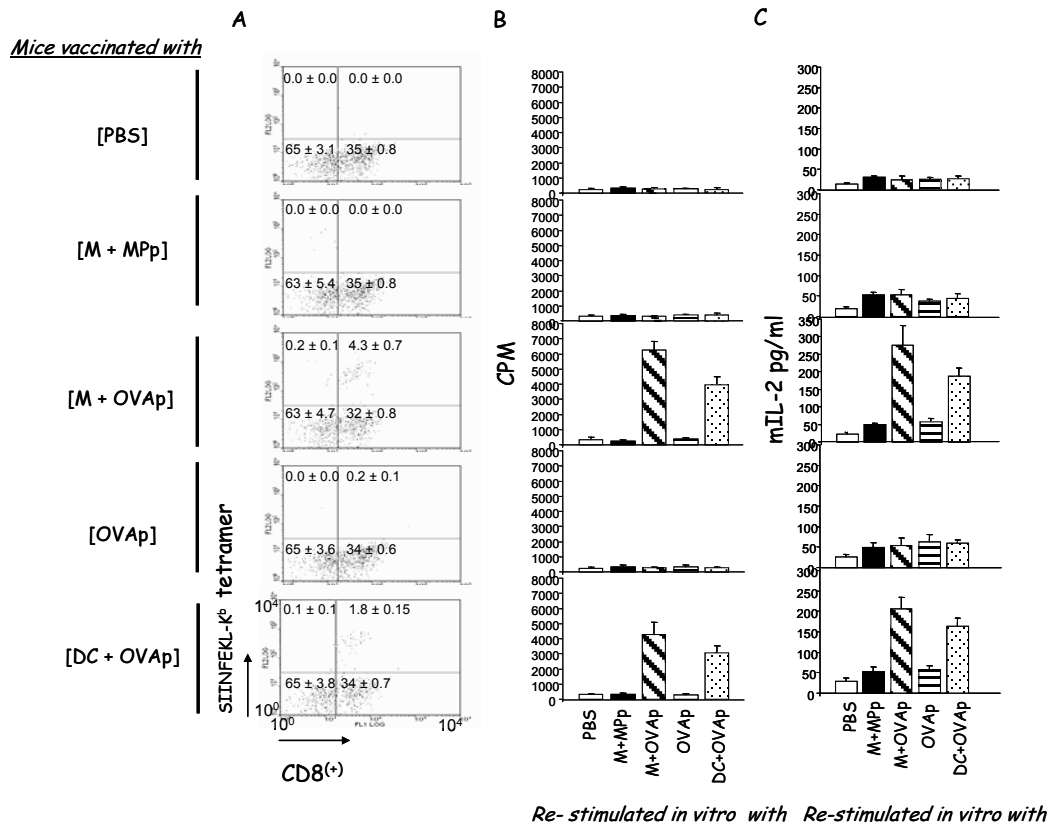
Figure 48 **Vaccination with OVAp loaded microsomes induces both CD8$^{(+)}$ and CD4$^{(+)}$ T cell responses in mice infected by a recombinant vaccinia virus with insertion of OVA-gene.**

CD57Bl/6 mice were injected intravenously with microsomes from bone-marrow-derived DCs loaded with a mixture of SIINFEKL and OVA$_{324-340}$ at a dose of 20μg/mouse [M+OVAp], or 10^6 bone marrow derived-DCs pulsed with SIINFEKL and OVA$_{324-340}$ [DC+OVAp], and boosted with the same immunizations once after 7-days. Mice vaccinated with microsomes loaded with irrelevant MPp peptide [M+MPp], mixture of free SIINFEKL and OVA$_{324-340}$ peptides [OVAp], and PBS [PBS], served as controls.

A. Two days after viral challenge, total splenocytes were co-stained with SIINFEKL-K^b specific tetramer and CD8 antibody.

For measuring CD4 T cell responses, CD4$^{(+)}$ T cells were isolated from splenocytes by positive selection and stimulated *in vitro* at $2x10^5$ CD4$^{(+)}$ T cells per well with the corresponding stimuli as explained above.

B. Following three days of re-stimulation, proliferation responses were detected by 3[H] incorporation (CPM) (mean of triplicate cultures ± SD) after exposure to 3[H]thymidine for 8 hours.

C. Following two days of re-stimulation, IL-2 production was measured by ELISA.

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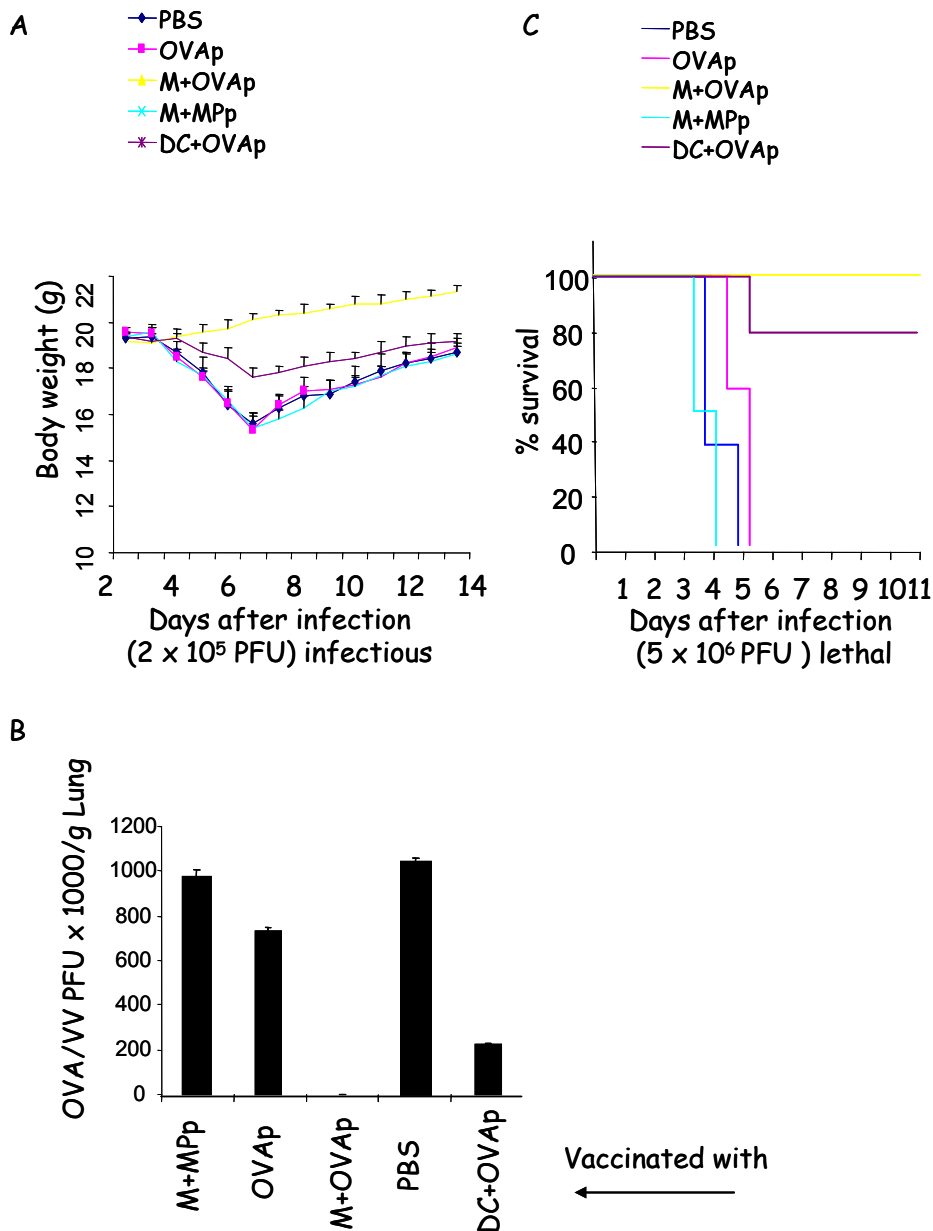
Figure 49 **Peptide-loaded microsomes induced immune responses against viral infection (VV-OVA).**

To study the anti-viral response, mice were immunized with microsomes from bone-marrow derived DCs loaded with a mixture of SIINFEKL and $OVA_{324-340}$ twice intravenously with an interval of 7-days. Mice injected i.v. with MPp loaded microsomal membranes, a mixture of SIINFEKL and $OVA_{324-340}$ peptides and PBS served as control groups, respectively. Ten days after the last immunization, all mice were challenged with VV-OVA virus at infectious or lethal doses as indicated. **A.** Body weight after challenge with infectious dose of VV-OVA virus and

B. Viral titers in the lungs on day 4 of mice challenged with an infectious dose of VV-OVA virus. Data show mean ± SD of log10 PFU per gram of lung tissues.

C. Survival of mice challenged with a lethal dose of VV-OVA virus.

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4.3 Vaccination with microsomal vaccine eliminates established tumours in mice.

Most cancers express tumour-specific and tumour-associated antigens that can elicit anti-tumour T cell immune responses, however these responses are often not sufficient to control tumour growth. Cancer immunotherapy aims to augment the efficient stimulation of a specific anti-tumour response. Cell-mediated immunity is thought to be crucial for the eradication of cancer cells and, subsequently, for the elimination of caner.

Having used the well characterised OVA model system to study T cell-priming by peptide-loaded microsomes in assays *in vitro* and in viral assays *in vivo*, the ability of tumour-antigen-loaded microsomes to induce an antigen-specific protective anti-tumour response was investigated in a similar system against an OVA-expressing tumour *in vivo* (Figure 50). The growth of OVA-bearing tumour cells EG.7, which are an MHC-II negative thymoma cell line (EL-4) (Carbone and Bevan 1989), of C57.Bl/6 origin, transfected with OVA cDNA, allows the analysis of the induction of OVA-specific CTL responses *in vivo*.

The efficiency of the microsomal vaccine for the eradication of established antigen-expressing tumours was investigated using Jaws-II-derived microsomes or autologous, bone-marrow dendritic cell-derived microsomes for immunizations. Four groups of C57.Bl/6 mice (six mice in each group) were injected with $2x10^5$ EG7 tumour cells subcutaneously. Initially, the presence of tumour was evaluated by palpation, thereafter tumour growth was measured at 48-hour intervals using a vernier calliper. When tumours reached $3mm^3$ in size, mice from one group were intravenously injected with 20μg of a mixture of SIINFEKL and $OVA_{324-340}$ peptide-loaded microsomes [Microsomes+OVAp]. In the control groups, mice were immunized with the same dose of microsomes loaded with irrelevant MP peptide [Microsomes+MPp], or with a mixture of OVA peptides at 100μg each peptide/mouse [OVAp], or with PBS [PBS]. All mice were boosted by repeating the same immunizations after seven days. Three weeks after

boosting, tumours in control groups grew to an average of 1.5cm^3 in size, while tumours in mice immunized with microsomes loaded with OVA peptides remained less than 4mm^3 (1 out of six) in size or were completely eradicated (Figure 51A), suggesting that the microsomal vaccine induced anti-OVA immune responses that effectively suppressed the growth of the OVA-expressing tumour. A cytotoxicity assay *in vitro* showed that CD8$^{(+)}$ T cells from mice immunized with OVA peptide-loaded microsomes, but not from control groups, raised potent cytotoxicity to EG7 cells (Figure 51B). These results demonstrate that immunization of mice with OVA peptide-loaded microsomes could induce OVA-specific immunity and prevent OVA-harbouring tumour development.
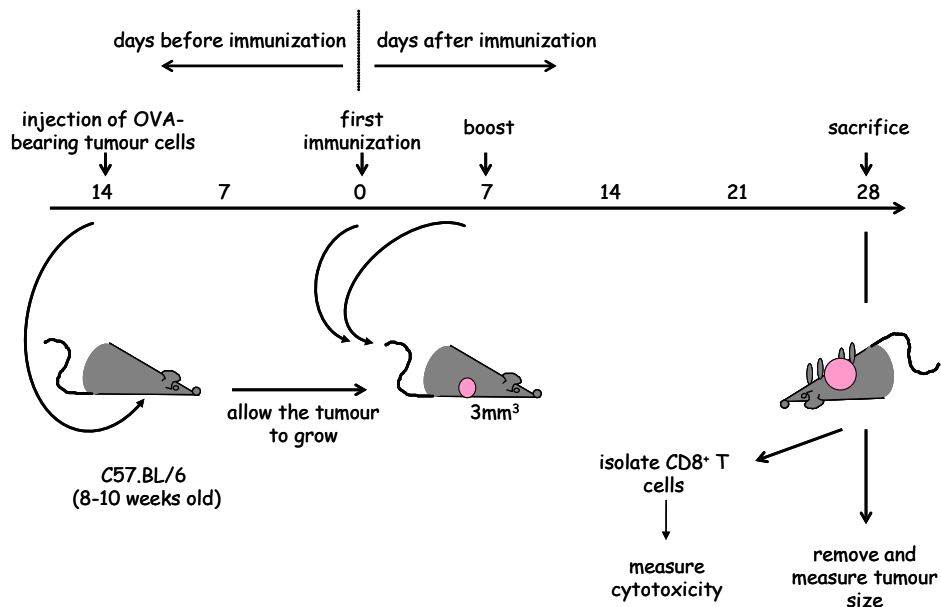
Figure 50   **Experimental design for the assessment of peptide-loaded microsomes as a therapeutic vaccine against tumours**.

For assessment of microsomal vaccine-induced immune responses against tumours, mice were injected subcutaneously with OVA-bearing tumour cells. When initial growth of tumours was detected by palpation after 14 days, mice were vaccinated with peptide-loaded microsomes twice with a 7-day interval. Tumour growth was monitored and 28-days after the first immunization mice were sacrificed. Tumours were removed for evaluation of their size. To detect specific anti-tumour CTL activity, CD8[(+)] T cells were isolated from lymph nodes and spleen and analyzed by cytotoxic assay *in vitro*.
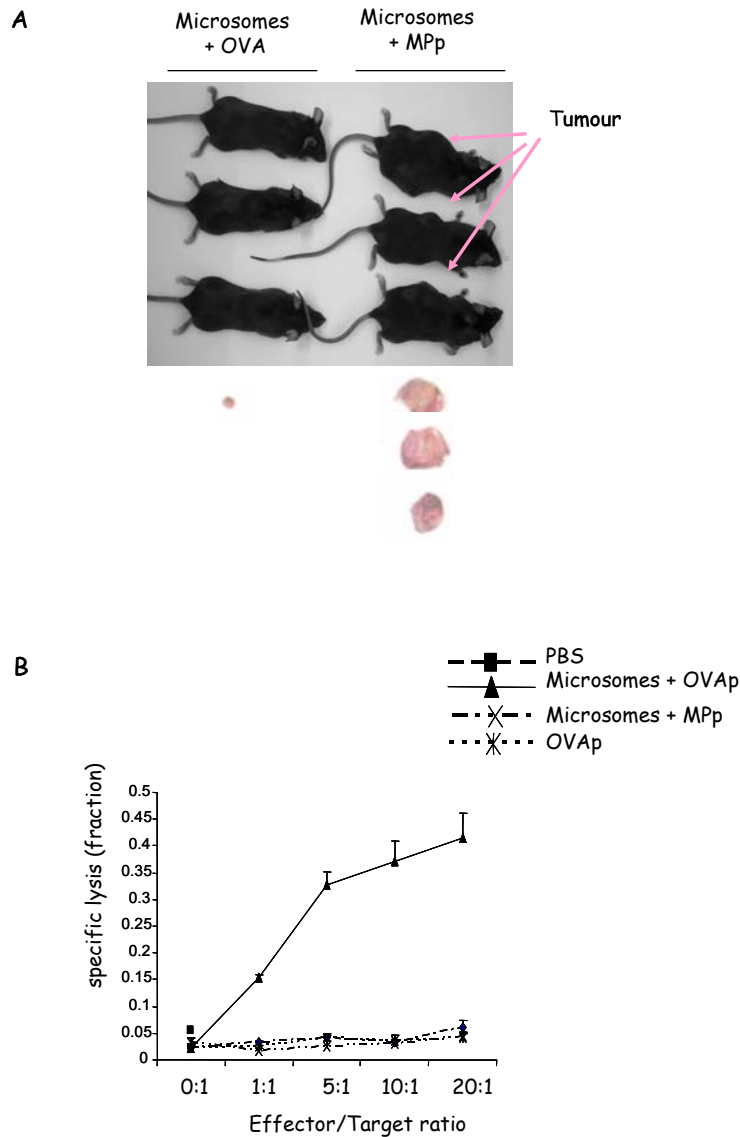
Figure 51 Microsomal vaccine-mediated immune responses against OVA-bearing tumours (EG7).

For assessment of microsomal vaccine-induced immune responses against tumours, mice were injected subcutaneously with $2x10^5$ EG7 tumour cells. When initial growth of tumours was detected by palpation after 14 days, mice were divided into four groups (five mice per group) and injected intravenously with microsomes from bone-marrow-derived DCs loaded with a mixture of SIINFEKL and $OVA_{324-340}$ [Microsomes + OVAp], or with MP peptides [Microsomes + MPp], or a mixture of SIINFEKL and $OVA_{324-340}$ free peptides [OVAp], or PBS only [PBS], twice with a 7-day interval. Tumour growth was monitored every second day. 21-days after the last immunization, mice were sacrificed and tumour size was compared across groups.

A. Only one mouse from the group immunized with microsomal membranes loaded with OVA peptides had a tumour of size 4mm^3, while all mice in the other groups had tumours with sizes about 1.5 cm^3.

B. CD8$^{(+)}$ T cells were isolated from spleens and used for a cytotoxicity assay *in vitro* against EG7 tumour cells.

4.4 Microsomes are not being phagocytosed by dendritic cells *in vitro*.

Immature DCs, both in peripheral tissues and in secondary lymphoid organs, express a large array of phagocytic receptors to efficiently phagocytose bacteria, cell debris, or even intact cells. By contrast to other professional phagocytes, dendritic cells have developed means to conserve antigenic peptides from the ingested particles, resulting in their increased presentation on MHC-I and MHC-II that serve to initiate adaptive immune responses (Savina and Amigorena 2007). It was therefore of interest to investigate the possibility that microsomes are endocytosed by APCs, in an effort to clarify whether the mechanism of microsome-induced T cell activation involved direct antigen presentation or cross-priming through resident DCs.

The potential microsome internalization by DCs was evaluated using assays *in vitro*. In physiological conditions, the most potent phagocytotic dendritic cells are those with an immature phenotype. Jaws-II is an immature DC line that matures in the presence of GM-CSF and high concentrations of bovine serum in culture. Consistent with previous experiments, the microsomes used here were extracted from mature Jaws-II (high culture passage, adherent cells of dendritic morphology). Internalization assays were performed using immature Jaws-II cells (low culture passage, non-adherent cells with round morphology). Microsomes from mature Jaws-II were labelled by conjugation to FITC1 (using Fluorotag FITC Conjugation Kit SIGMA) and incubated with immature Jaws-II cells for 6 hours. FITC-labelled dextran beads were incubated with immature Jaws-II cells as positive control. The cells were washed with PBS and incubated with trypan blue for 1 minute to quench extracellular derived fluorescent signals. Fluorescent micrographs revealed that although fluorescent dextran beads could be internalised and detected within the DCs (Figure 52A), fluorescent microsomes could not be detected (Figure 52B).

A. Jaws-II phagocytose fluorescent beads
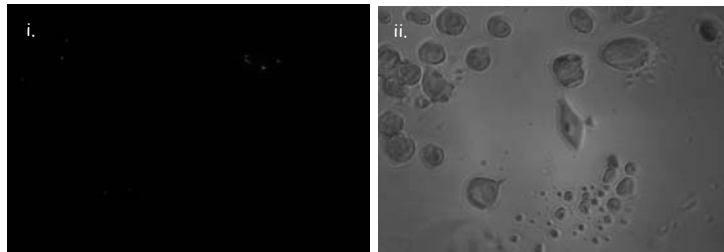
C. Fluorescent microsomes (control)
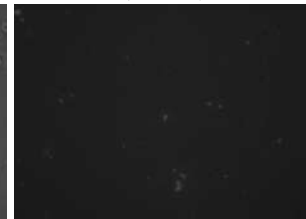
B. Jaws-II do not phagocytose fluorescent microsomes
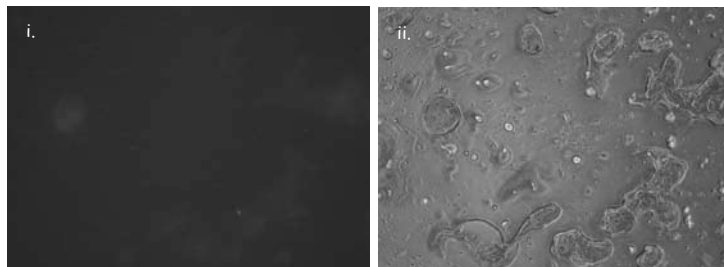
Figure 52 **Internalization assay for the detection of phagocytosis of microsomes by dendritic cells.**

A. Images of Jaws-II cells incubated with fluorescence beads observed with (i) confocal microscopy and (ii) light microscopy.

B. Images of Jaws-II cells incubated with FITC1 labelled peptide-loaded microsomes from Jaws-II cells observed with (i) confocal microscopy and (ii) light microscopy.

C. Confocal images of FITC1 labelled peptide-loaded microsomes from Jaws-II cells.
Data are representative of two experiments.

Summary to Section 4

The vaccination experiments presented here provide evidence that peptide-loaded microsomes can induce specific T cell responses *in vivo* that result in protection in mice from viral infection and eradication of established tumours. The findings show that protection from viral infection correlates with an integrated immune response that activates both $CD8^{(+)}$ and $CD4^{(+)}$ T cell responses. Internalization assays *in vitro* indicate that microsomes are not being internalized by dendritic cells as microsome-associated fluorescence could not be detected internally. However, microsomes are made of membranes that could become associated to dendritic cells without actually being internalized as an intact fluorescently-tagged vesicle. Therefore, the mechanism by which microsomes induce T cell responses *in vivo* cannot be ascertained, but the ability of microsomes to induce peptide-specific activation of T cells *in vitro* in the absence of DCs suggests that they can induce activation of T cells *in vivo* by direct priming.

5. Microsomes present peptides to human T cells *in vitro*.

An animal-tested/developed experimental vaccine will only be of use to humans if it induces similar immune responses in a human setting. Thus, to build upon the presented findings, which demonstrate that peptide-loaded microsomes protect mice from viral infection and eradicate established tumours, we attempted to examine the immunogenicity of microsomes in a human system. Microsomes from a human APC line were loaded with defined peptides of immunodominant cytomegalovirus epitopes and co-cultured with total peripheral blood mononuclear cells. T cell responses were evaluated by interferon γ production (Figure 53).

Human cytomegalovirus (HCMV), a member of the Herpesviridae, is widely found in humans around the world. HCMV infection is usually asymptomatic. Following primary infection, the virus persists in a latent state in cells of the myeloid lineage, with intermittent viral reactivation and shedding from mucosal surfaces, and containment by the host immune response. Several studies support important roles for B, T and NK cells in protection against HCMV disease, however CD8$^{(+)}$ T cells appear to be the most important effector cell for the control of the HCMV infection (Moss and Khan 2004). The virus exploits immune evasion strategies to prevent complete viral eradication by the immune system, establishing a life-long persistent infection within the host that can result in substantial morbidity and mortality, especially among immunosuppressed infected individuals. Increasing evidence suggests that HCMV evades the immune system by impairing DC function; as a result, T cell-recognition of the infected cells is compromised and DCs fail to prime and activate naïve T cells for the induction of immune responses (Rolle and Olweus 2009); thus, enabling viral spread.

Here we investigate whether microsomes can induce HCMV-specific T cell responses *in vitro*. T cell responses to HCMV can be detected by either

tetramer staining or ELISpot following stimulation *in vitro* with HCMV antigens. However, ELISpot is most commonly used due to that fact that tetramers are limited to a few well-defined peptide/MHC complexes. The ELISpot assay is a rapid and sensitive technique for the detection of IFNγ-producing T cells, representing the activation of antigen-specific T-cells (Keane, Price et al. 2000) even when those are of very low frequency.

The capacity of microsomes to present peptides to corresponding human T cells was evaluated using microsomes from an MHC-I-deficient human APC line, 721.221, which is transfected to express an HLA-A2 haplotype. Whole peripheral blood mononuclear cells (PBMCs), obtained from the blood bank, from HLA-A2 healthy individuals, who were tested negative for HIV and were serologically converted to HCMV, were co-cultured with HLA-A2-expressing microsomes (Figure 54) loaded with an HCMV peptide (amino acid sequence "NLVPMVATV"). Co-cultures with microsomes loaded with an irrelevant GAG peptide, or with 100μg/ml soluble HCMV or GAG peptide, or with PBS, served as controls. The presence of reactivated HCMV-specific CD8[(+)] T cells was indirectly detected by IFNγ ELIspot after reactivation *in vitro* with the described treatments for 24 hours. The findings (Figure 55) demonstrate that HCMV-specific IFNγ-secreting cells were detected in all individuals after stimulation with HCMV peptide-loaded microsomes [221/A2-derived microsomes + HCMV peptide]. Stimulation of PBMCs with soluble HCMV peptides [HCMV peptide only] could also result in the reactivation of HCMV-specific IFNγ-secreting cells, possibly due to uptake and presentation of the peptide by APCs naturally present in the PBMCs. However, HCMV-loaded microsomes induced better immune responses in terms of IFNγ secretion compared to the responses induced by HCMV peptide only (Figure 55B), while microsomes loaded with irrelevant peptides [221/A2-derived microsomes + GAG peptide] did not.

These data indicate that a microsomal vaccine can present peptides *in vitro* to induce specific reactivation of peripheral human cells from individuals who are carriers of latent viruses.
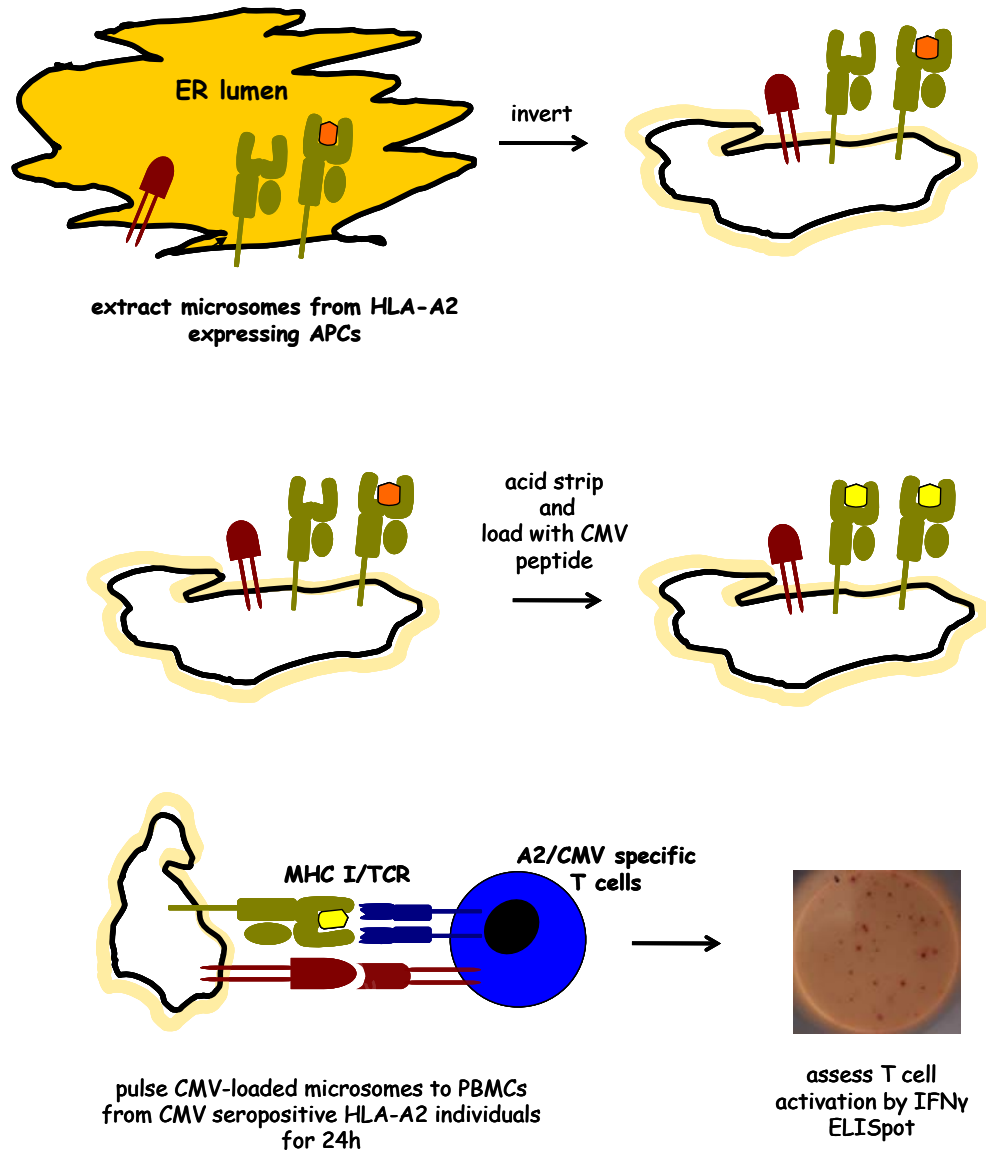
Figure 53 **Experimental design for detection of induced memory T cell responses *in vitro* in PBMCs from latently infected individuals by a microsomal vaccine.**

The MHC-deficient 721.221 B cell line was transfected to express the HLA-A2. Microsomes prepared from these transfected cells were inverted, stripped and loaded with an A2-specific CMV peptide that has amino acid sequence 'NLVPMVATV'. CMV peptide-loaded microsomes were co-cultured with PBMCs from HLA-A2 and CMV seropositive, but not symptomatic individuals. The presence of reactivated HCMV-specific CD8[(+)] T cells was analyzed by IFNγ ELISpot assay.
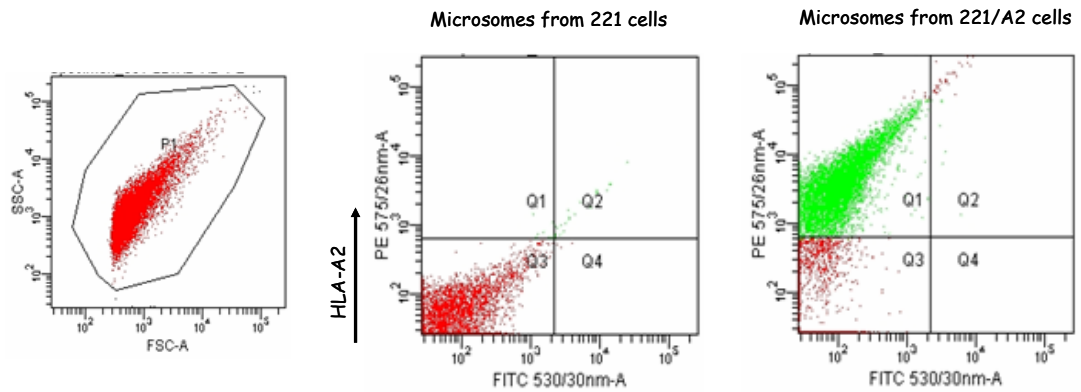
Figure 54 **Detection of HLA-A2 expression on microsomes from HLA-A2 transfected antigen presenting cells.**

4μl of microsomes from 721.221 or 721.221/A2 cells were stained with PE labeled anti-A2 antibody MA-2.1. The free-antibodies were washed off by centrifugation through a 0.5M sucrose cushion. Microsomes were collected and resuspended in FACS buffer for flow cytometric analysis of HLA-A2.
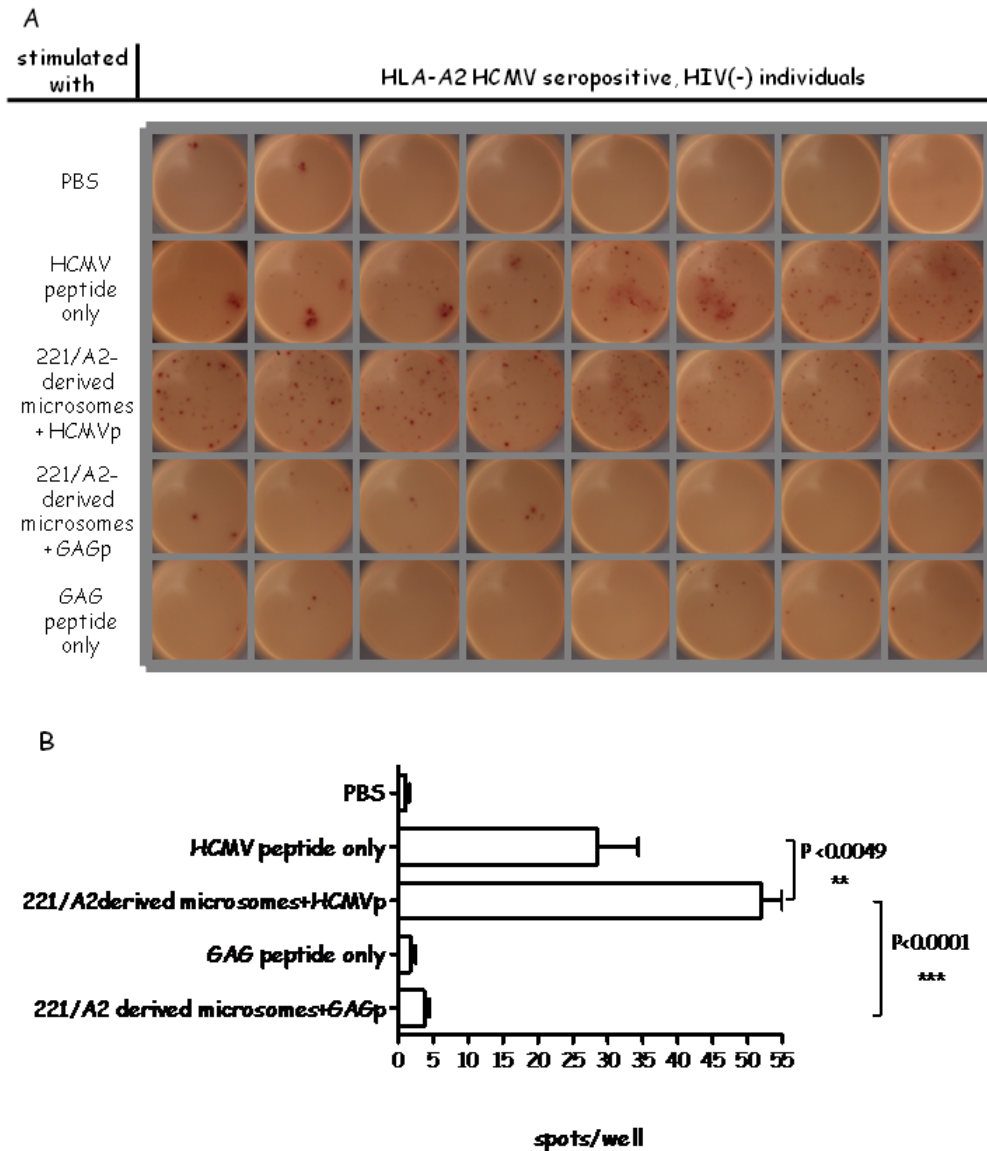
The capacity of HLA-A2 expressing microsomes to present specific peptides that re-activate anti-HCMV CD8$^{(+)}$ T cells *in vitro* was evaluated using HLA-A2$^{(+)}$, HCMV sero-converted individuals, by IFNγ ELISpot. Microsomes were prepared from MHC-I-deficient 721.221 cell line, which are professional APCs (lymphoblastoid B cells) that have been transfected to express HLA-A2. During the ELISpot assay, PBMCs were incubated for 24 hours with 2µg HLA-A2 expressing microsomes loaded with HCMV peptide [221/A2-derived microsomes + HCMV peptide], or with an irrelevant GAG peptide [221/A2-derived microsomes + GAG peptide], or with 100µg/ml HCMV peptide [CMV peptide only], or 100µg/ml GAG peptide [GAG peptide only], or PBS [PBS].

A. Each spot represents an IFNγ producing cell.
B. Each bar represents the mean spot number per treatment. The data is presented as mean of eight wells±SEM and is representative of eight individuals.

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 and HCV, other 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 now considered a promising target/tool when designing new-generation vaccines.

New-generation vaccines based on matured dendritic cells loaded with defined antigens appear very promising for both prophylactic vaccination and immune therapies (Nestle, Farkas et al. 2005; Osada, Clay et al. 2006). Although mature DCs have the capacity to induce effective primary and secondary immune responses *in vivo*, their use in vaccination strategies 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 (Steinman, Hawiger et al. 2003). Here, we have explored the hypothesis that the use of ER-enriched microsomes isolated from professional antigen presenting cells, such as DCs, can represent an alternative vaccination strategy to those using live DCs.

1. The microsomal vaccine: an overview.

Reconstitution of antigen presenting function in isolated endoplasmic reticultum membranes, the microsomes, is a novel approach in vaccine development. In this study, we show that endoplasmic reticulum-enriched microsomal membranes (microsomes) isolated from DCs contain high levels of peptide-receptive major histocompatibility complex (MHC-I) as well as similar levels of co-stimulatory molecules compared with their parental

dendritic cells. After loading with defined antigenic peptides, microsomal vaccines induce MHC class I- and MHC class II-restricted T cell responses that correlate with protection from acute viral infection and MHC class I-restricted T cell responses that correlate with eradication of established tumours in mice. Microsomal vaccines contain high levels of peptide/MHC, the quantity of which can be monitored in the preparation for the induction of optimal immune responses. The vaccine formulation is easy to make and is consistent across batches. The immunogenicity of microsomes can be maintained for more than 6 months if they are stored at -80°C or in liquid nitrogen making the microsomal vaccine preparation stable and cost effective.

This novel approach overcomes limitations of previously described systems using live-DCs as vaccines and raises expectations that microsomes may represent a new and more effective therapeutic vaccination strategy.

2. Studying antigen presentation.

In order to examine the reconstitution of antigen presenting function in isolated microsomal membranes, it was important to establish appropriate experimental systems that allowed us to validate the generation of peptide/MHC complexes and to test their ability to efficiently present peptides to T cells. In this study, two defined chicken ovalbumin (OVA) epitopes in context with H-2K^b and I-A were employed, allowing peptide/MHC-specific T cell clones or transgenic T cells to be used for validation of antigen-specific immune responses. The utilization of congenic cell lines for the study of antigen presentation *in vitro* allowed consistent analysis of the effects of the various vaccines on T cell responses both *in vitro* and *in vivo*. The irrelevant peptides that were used as controls are non-specific either for MHC binding or for the TCRs described.

Within these experimental systems, we investigated the properties of microsomal vaccines prepared from different types of professional antigen presenting cell.

3. Analysis of T cell responses.

Naïve T cells reside as small resting cells that can live for several months, being maintained in a process known as homeostasis. On recognition of their cognate peptide/MHC ligand on the surface of an antigen presenting cell, the T cell is activated to proliferate and differentiate as an effector cell. Activation of naïve T cells induces immediate expression of mitogenic cytokines, such as interleukin 2 (IL-2) that stimulates the expansion of activated T cells. For this reason, the level of IL-2 production has been a widely used parameter to assess vaccine-induced protective T cell responses *in vitro.* The majority of effector T cells that are induced following vaccination against viral infections are $CD4^{(+)} T_H1$ cells or $CD8^{(+)}$ cytotoxic T cells, which express interferon gamma (IFNγ), a chemokine involved in the clearance of various infections. Thus, the level of production of this cytokine is another important parameter in assessing the effector function of responding T cells.

Despite these useful biomarkers, the magnitude of a T cell response as defined by a single parameter is not a sufficient correlate of immune protection. A range of T cell functions together with observation of clinical symptoms can define more accurately the quality of a protective T cell response (Seder, Darrah et al. 2008). Indeed, immune responses that can be measured with immunology are not necessarily useful for immunity; a productive immune response must be appropriate to allow protection and survival (Zinkernagel 2007). Here, together with observations of clinical symptoms, we have used several indicators of a productive immune response to assess our vaccine candidate. These include analysis of proliferation, production of IL-2 measured by ELISA, antigen-specific T cell expansion *in vivo* by tetramer staining, T cell responses to re-call antigen *in vitro,* IFNγ production by ELISpot, and cytotoxicity tests for the evaluation of CTL responses. Collectively the combination of these factors aims to define a more detailed picture of vaccine-elicited antigen-specific T cell responses *in vivo,* and thus the protective efficacy of the vaccine in general.

4. Characterization of microsomal membranes.

The isolated microsomes are identified as enriched ER membranes as indicated by the attachment of ribosomes on the cytoplasmic side of the microsomal vesicles and the presence of the ER-resident protein, tapasin. The microsomes have limited contamination with endosomes as shown by immunoblotting with anti-LAMP2 antibody, an endosomal marker (Sofra, Mansour et al. 2009).

Electron microscopy and flow cytometry revealed microsomes to be small heterogeneous vesicles. Thus, it is not straightforward to compare them directly with their parental cells in flow cytometric analysis of specific markers. The levels of auto-fluorescence are bound to be different between microsomes and cells, as the size of microsomes is very variable and orders of magnitude smaller than that of the cells. The comparison was therefore made by flow cytometry using staining by specific antibody for a particular marker versus staining of the appropriate isotype control. The settings of side-scatter and forward-scatter were the same between cells and microsomes and the entire events detected were used for the analysis on fluorescent channels. The similar levels of MHC and co-stimulatory molecules in microsomes and their parental APCs were also confirmed with immunoblotting analysis.

5. Measurement of immunogenicity in microsomal and cellular antigen presenting systems.

An ideal comparison of the antigen presentation capacity of microsomes with that of the parental cells would be based on the number of peptide/MHC molecules on the same area of membrane surface exposed to T cells. However, this is technically challenging to achieve, because microsomes are very heterogeneous in size. To overcome this problem microsomes and cells were normalized on the basis of equal MHC-I levels.

6. Acid stripping allows MHC-II loading and enhances the levels of peptide-receptive MHC-I.

MHC class I heavy chain-β2 microglobulin dimers are loaded with high affinity peptides as the last step of their ER maturation process, before assembled peptide/MHC-I complexes are transported to the cell membrane and presented to T cells (Paulsson and Wang 2004). Therefore, most of the MHC molecules on the surface of APCs are pre-processed with high affinity peptides as peptide/MHC complexes. By contrast, ER enriched microsomes contain abundant peptide-receptive MHC-I molecules (Li, Paulsson et al. 1999). 'Empty' MHC class I molecules are retained until they form stable complexes with high affinity peptides by a loading quality control mechanism (Paulsson and Wang 2004), which serves to maintain a stable antigen presenting environment on the surface of the APC (Schoenhals, Krishna et al. 1999). To further confirm the retention of peptide-receptive MHC-I in the ER, the levels of SIINFEKL-K^b complexes were compared between microsomes purified from OVA-expressing APCs (OVA/RMA-S cells) and microsomes purified from APCs (RMA-S cells) and loaded with SIINFEKL. Indeed, microsomes that had been loaded with exogenous SIINFEKL peptides *in vitro* expressed higher levels of SIINFEKL-K^b compared to microsomes from OVA-expressing APC, suggesting that K^b molecules assembled with endogenous peptides are rapidly transported to the cell surface (Sofra, Mansour et al. 2009). Thus, the high efficacy of ER-enriched microsomes in inducing T cell responses *in vitro* is probably due to the abundant peptide-receptive MHC-I in ER.

Unlike abundant peptide-receptive MHC-I, MHC-II is protected from peptide loading in the ER by invariant chain (Ii). Ii provides a sorting signal for MHC-II to be transported to endosomal compartments where Ii is degraded to a small peptide called "clip" that is subsequently exchanged (by HLA-DM in humans) with antigenic peptides in low pH conditions (Hammerling and Moreno 1990). However, it is unknown whether the exchange of MHC II-Ii to MHC II-peptide can be achieved under acidic

condition *in vitro*. The presented data provide evidence suggesting that in acidic condition the MHC-II-associated Ii on microsomal membranes can dissociate and generate a peptide-receptive MHC-II molecule, allowing assembly with exogenous peptides. In addition, as demonstrated by the acid stripping and subsequent binding of biotinylated peptides to MHC-II on microsomes and the induction of effective CD4$^{(+)}$ T cell responses *in vitro*, the peptide/MHC-II complexes on microsomes appear stable and are able to fulfil the same physiological function as their counterparts in the endosomal compartments. Nonetheless, it is unknown whether the peptide-receptive MHC-II created by acid stripping can be receptive for all MHC-II-binding peptides or only for high affinity binding peptides, such as OVA$_{324\text{-}340}$. This remains to be investigated with the use of several MHC-II-specific peptides with different binding affinities.

The acid treatment protocol also stripped ribosomes from microsomes (Sofra, Mansour et al. 2009) and, importantly, it enhanced the generation of peptide-receptive MHC-I possibly by stripping the endogenous pre-processed peptides from the peptide/MHC complexes. Thus, acid treatment improves the efficiency of peptide loading onto MHC molecules and limits potential side effects, such as anti-ribosome induced immune responses.

7. Microsomes versus dendritic cells in vaccination strategies.

One of the major problems in human DC-based vaccines is the heterogeneity of DC maturation programmes. Different DC subsets can emerge from different induction protocols *in vitro* that will then include both immunogenic and tolerogenic cells. The ensuing diverse population of DC subsets will induce a mixture of inflammatory cytokines, such as IL-12, and inhibitory factors such as TGFβ, IDO and IL-10 (Szeberenyi, Rothe et al. 2000; Munn, Sharma et al. 2002; Smits, de Jong et al. 2005), which can severely hamper APC efficacy in inducing effective immune responses *in vivo*.

One strategy, employed to address this problem and enhance vaccine potency by modifying DC properties, is to target immunosuppressive proteins expressed by DCs using RNAi. Small interfering RNAs (siRNA) are a novel technology that selectively 'knocks-down' genes of interest. Indeed, recent studies have shown that siRNA-mediated silencing of the suppressor of cytokine signalling 1 (SOCS1) in DCs could attenuate immunosuppressive signals that allowed them to break a tolerogenic environment (Song, Evel-Kabler et al. 2006). As a result, treatment of DCs with SOCS1-specific RNA enhanced tumour- and virus-specific adaptive immune responses in mice (Mao, Lin et al. 2007). Although gene silencing *in vivo* may eventually have profound applications for vaccination and immunotherapy, there are several limitations that have to be addressed before the technology can be successfully administered to human patients as a medical treatment. For example, problems include inefficient delivery *in vivo*, incomplete silencing of target genes, non-specific immune responses, and off-target effects (Mao, Lin et al. 2007).

By contrast to DC preparations, microsomal membranes are free of potentially problematic soluble factors, whilst their preparation is biologically reproducible and consistent in terms of peptide loading and antigenicity. Indeed, by contrast to the heterogeneity of different APC subsets, it has been observed that peptide-loaded microsomes from three different APCs lines (with variable antigen presenting potency) show similar levels of immunogenicity (Li and Wang, unpublished data); these findings suggest that microsomal vaccines could overcome the negative factors that limit the presentation efficacy of some APC-based vaccines. In addition, ER-enriched microsomes do not contain cytosolic or nuclear proteins that could potentially trigger toxic side effects. In support of this, there were no observations of anti-microsome toxic responses *in vivo* (Li and Wang, unpublished data), indicating that ER-resident proteins are tolerated.

Since the ER is the site of synthesis of all transmembrane proteins of the cell, the presence of negative co-stimulatory molecules on microsomes like that seen on DCs, such as PD1 or CTLA4 ligands, cannot be excluded.

However, inhibition of these signals could be accomplished by treating microsomes with antagonistic antibodies specific for certain molecules before immunization. By contrast to live cells, the expression of molecules in microsomes is not regulated by active metabolism. Thus, treatment with specific antagonist antibodies may effectively inhibit negative co-stimulatory signals. For example, expression of a PD1 antibody in microsomes could bind to PD1 on T cells and, subsequently, inhibit PD1-mediated signalling; however this remains to be investigated.

MHC class I is a membrane complex that is processed in two steps; folding of native chain, followed by assembly with peptides in the ER. The peptide-assembled MHC complex is selectively regulated by export mechanisms, which allow it to be transported to the cell surface. By contrast, an 'empty' peptide-receptive MHC class I molecule exists in an unstable form and is retained in the ER by a quality control mechanism until it forms a stable peptide/MHC complex. The quality control of MHC class I antigen presentation also includes the restriction of export of unloaded MHC class I molecules or suboptimally loaded peptide/MHC complexes to the cell surface (Paulsson and Wang 2004). This mechanism provides microsomes with the advantage of carrying more peptide-receptive MHC molecules, compared to the antigen presenting cell plasma membrane; they can then be loaded *in vitro* with selected optimal peptides to form stable peptide/MHC complexes.

8. Microsomes versus other reported cell-based but cell-free vaccine formulations.

Both exosomes and plasma membrane-derived vesicles from APCs have been reported to induce potent immune responses *in vivo* (Zitvogel, Regnault et al. 1998; Kovar, Boyman et al. 2006). However, it is not clear how peptides are processed to form peptide/MHC complexes in these vesicles.

Exosomes can induce antigen-specific MHC class II-restricted T cell responses and also have been shown to activate CD8[(+)] T cells (Giri and Schorey 2008), suggesting a potential mechanism for antigen cross-priming.

Although their physiological role is not yet clear, available data suggest that exosome production during immune responses aids the increase in the number of dendritic cells that bear relevant peptide/MHC complexes (Thery, Zitvogel et al. 2002). Furthermore, exosomes secreted by DCs may mediate antigen presentation after peptide-bearing DCs are cleared from the system (Luketic, Delanghe et al. 2007). Thus, exosome-mediated antigen presentation may serve to amplify both the magnitude and longevity of an immune response.

Exosomes derive from a specialized late endocytic compartment found in antigen presenting cells which harbours newly synthesized MHC class II molecules in transit to the plasma membrane. The membranes of these compartments have their origin within the endocytic network and, thus, exosomes consist of proteins and lipids present within this network. Indeed, exosomes secreted from dendritic cells express high levels of functional MHC class I- and MHC class II- peptide complexes together with co-stimulatory molecules (Thery, Zitvogel et al. 2002). In addition, data suggesting that phagosome[2] formation involves partial fusion of the endoplasmic reticulum (ER) with lysosomal compartments (Ackerman, Kyritsis et al. 2003; Guermonprez, Saveanu et al. 2003; Houde, Bertholet et al. 2003) provides a framework to explain how antigens present on exosomes can be processed through the MHC-I presentation pathway. Other potential mechanisms of cross-presentation are also under study; the latest studies describe early endosomal compartments where internalised antigens are cross-presented (Burgdorf, Scholz et al. 2008; Di Pucchio, Chatterjee et al. 2008), a model that could also explain antigen presentation in the context of MHC class I by exosomes.

Although MHC class I is detected on exosomes, the peptide-receptive state and level of expression may not be comparable to that of ER-derived-microsomes, since endosomes are not the physiological compartments for MHC class I processing. Moreover, some viruses have the capacity to evade MHC class II-enriched compartments and can be released by exosomes

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[2] Phagosome is an intracellular vesicle that is formed around phagocytosed foreign material by inward folding of the plasma membrane.

(Stoorvogel, Kleijmeer et al. 2002), which could represent a limitation in applications of autologous exosomes as anti-viral vaccines. The potential advantage of exosomes is that the peptide/MHC-II complexes may be enriched from antigen pre-fed DCs, since the physiological processing of peptide loading on MHC-II occurs in endocytotic compartments. For technical reasons, it is not possible to biochemically compare the quality of peptide/MHC-II complexes in peptide-loaded microsomes *in vitro* with those in the endosomal compartments *in vivo*. Nevertheless, the immunogenic efficiency of peptide/MHC-II complexes in microsomes is promising as indicated by high peptide-loading efficiency and the induction of antigen-specific CD4[(+)] T cell responses *in vitro*. Moreover, an important advantage of a microsomal vaccine is the ability to present peptide/MHC and co-stimulatory signals directly to T cells. Although exosomes contain MHC-I and MHC-II as well as co-stimulatory molecules that are exposed on the surface of the exosome membrane (Thery, Regnault et al. 1999), efficient priming of T cells by DC-derived exosomes requires activated or mature DCs; presumably reflecting a possible role of exosomes as mediators of antigenic exchange between DCs or APCs to promote expansion of restricted T cell responses *in vivo* (Andre, Chaput et al. 2004). The possibility that microsomes are also indirectly presented, for example by being endocytosed and re-processed by DCs *in vivo,* cannot be excluded. Nevertheless, the observed ability of microsomes to directly activate antigen-specific T cells may be important in microsome vaccines against chronic viral infections or cancer: conditions during which endogenous DCs are less able to induce protective responses.

After pulsing with peptides, plasma membrane-derived vesicles could also interact directly with T cells and induce responses *in vivo* (Kovar, Boyman et al. 2006). However, the number of peptide-receptive MHC-I molecules on the surface of APC is severely limited due to the presence of endogenously pre-processed peptide/MHC I (Paulsson and Wang 2004). Therefore, it may be difficult to use these vesicles for the delivery of antigenic peptides with medium or low binding affinity, since they would not successfully compete with the pre-processed endogenous peptides bound on

MHC molecules. The nature of the sonication methods used in the preparation of plasma membrane-derived vesicles may also incorporate ER membrane fractions (Kovar, Boyman et al. 2006), which as shown in this work here, have abundant peptide-receptive MHC-I molecules. Importantly however, the preparation of a microsomal vaccine includes an 'inside-out' processing step that facilitates the exposure of the luminal side of the ER membrane, ensuring maximum peptide loading and efficient accessibility of co-stimulatory and MHC molecules to T cells.

9. *In vivo* distribution of antigen-carrying microsomes.

Effective T cell-targeting vaccines have to deliver antigens at a sufficient dose to peripheral lymphoid organs for optimal stimulation of naïve and/or memory T cells. Antigen-loaded DCs are better distributed to peripheral lymph nodes (LN) if administrated subcutaneously (Mullins, Sheasley et al. 2003). Although activated DCs can express homing receptors, which guide their migration from infected tissues to draining lymph nodes, it has been reported that intravenously injected DCs are predominantly distributed to major organs, such as lung, liver and spleen (Lappin, Weiss et al. 1999). The induction of homing receptors depends on the presence of inflammatory stimuli *in vivo*; however, persistent viruses, such as HIV and HCV, and many tumours induce the production of immunosuppressive cytokines, such as TGFβ and IL-10. Within such an immunosuppressive environment, it is possible that the administered DCs are negatively regulated and may even be induced to die.

By contrast, extracted microsomes are stable fragments that are not regulated metabolically. Thus, they are not able to actively migrate, meaning that they must be administered appropriately in order to effectively reach the secondary lymphoid organs. If microsomal membranes contain homing receptors, their expression should be stable but most probably non-functional, as microsomes do not have the ability to move or make new gene products. Examination of the tissue distribution of peptide-loaded microsomes from ^{35}S-methonine labelled bone-marrow-derived DCs, and their parental DCs, after

intravenous (i.v) administration showed that 24 hours after injection, 7.4% [3] of the bone-marrow-derived microsomes had accumulated in the spleen, and 0.7 % were found in LNs. Importantly, after seven days, 5.6 % and 0.8 % of bone-marrow-derived microsomes were still in the spleen and LN, respectively indicating that microsomes accumulate and are retained in major peripheral secondary lymphoid organs. By contrast, only 2.8 % and 0.2 % of peptide-pulsed bone marrow-derived DCs were distributed to spleen and LN 24 hours after i.v. injection and this reduced to 0.9 % and 0.2 % after 7 days. These observations (Sofra, Mansour et al. 2009) suggest that microsomes may have similar distribution properties compared with activated APCs and that they are retained in these sites as well, if not better, possibly because they are deficient in further migratory capacity.

Secondary lymphoid tissues such as the spleen, lymph nodes, and Peyer's patches are sites of immune induction. Naive T cells do not travel through all parts of the body; they re-circulate between the different secondary lymphoid compartments and blood, 'examining' antigen-presenting cells for the presence of their cognate ligand (Heath, Belz et al. 2004). Therefore, surveillance of the entire body by naive T cells depends on the trafficking of antigen, either in soluble form or associated with APCs, from peripheral tissues via the lymphatics or blood to the secondary lymphoid organs. The spleen monitors blood-borne antigens, while the lymph nodes screen their particular local regions. In the spleen, microsomes accumulate in the periarteriolar lymphoid sheath (PALS) (Sofra, Mansour et al. 2009), which mainly consists of T cells (Alvarez, Vollmann et al. 2008). This distribution pattern is consistent with the potent T cell responses induced *in vivo* by peptide-loaded microsomes, observed in this study.

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[3] The percentage of accumulated microsomes or DC was calculated as CPM/gram of the organ and divided by the total CPM of injected microsomes or DCs.

10. Mechanism of antigen presentation by microsomes.

Engulfment of apoptotic cells has been described as an important way of sampling pathogens during infection, especially for microbes that may not infect APCs. Thus, apoptotic cells are a source of many pathogenic antigens that are processed by APCs for presentation via the MHC class II pathway and, via cross-priming, via the MHC class I pathway (Savina and Amigorena 2007; Lev, Takeda et al. 2008). Indeed, although processing of endogenously synthesized antigens by the MHC class I pathway is more efficient, presentation of exogenously-derived antigens in the context of MHC class I is essential for cytotoxic T cell responses to viruses and tumours (Kasturi and Pulendran 2008).

In addition to internalizing dead cells, DCs may also 'sample' non-apoptotic cells, by "biting" pieces of live neighbouring cells (Harshyne, Watkins et al. 2001; Joly and Hudrisier 2003). Indeed, there is evidence that T cells (Hudrisier, Riond et al. 2001), B cells (Batista, Iber et al. 2001) and DCs (Valdez, Mah et al. 2002) 'nibble' live cells in this way. The transfer of cell fragments between live neighbouring cells has been described in several other systems, such as the transfer of melanin from melanocytes to keratinocytes in the skin (Seiberg 2001) or the capture of membrane-bound ligands in invertebrates (Cagan, Kramer et al. 1992; Trombetta and Mellman 2005).

Here we have shown that microsomes have the capacity to interact directly with T cells *in vitro* to 'deliver' antigenic peptides and co-stimulatory signals that trigger antigen-specific T cell responses. Direct interaction of microsomes with T cells is particularly important as administered microsomes accumulate in lymphoid organs (Sofra, Mansour et al. 2009), where resident DCs are found in a resting state (Carbone, Belz et al. 2004; Diebold 2008). However, if some microsomes are reprocessed by immature DCs, as shown for exosomes, these DCs may also present microsomal antigens to T cells. Thus, both direct and indirect antigen presentation mechanisms may be involved in the induction of T cell responses by microsomes *in vivo*. Although the data presented here seemed to suggest that was not the case and T cells

were directly primed by microsomes, analysis of T responses in allogenic mice following immunization with non-autologous microsomes could further elucidate this. Nevertheless, the stability and the prolonged existence of microsomes within the lymphoid organs could be important for amplifying the magnitude and sustaining the longevity of the immune response by booster vaccinations.

11. From mouse to human.

Vaccinia is an acute infectious virus (Xu, Johnson et al. 2004). Although the microsomal vaccine is being developed with the aim of potentially protecting from chronic infections that mainly require the induction of the cellular arm of the immune response, the described animal viral assay allows a first insight into the efficacy and characteristics of the microsomal vaccine. For obvious reasons, vaccination experiments have to be first evaluated in suitable animal models, before eventually a clinical trial is carried out in humans. However, one has to consider certain limitations when assessing the potential efficacy of human vaccination in animals, as described below.

The tests available for the evaluation of T cell behavior and T cell function in animal models are limited and not always accurate, since the presence of effector T cells not always correlates with protection (Rocha and Tanchot 2006; Zinkernagel 2007). Often, animal models of infection are too sensitive, and are usually associated with remarkable expansion of antigen-specific effector T cells that is not typical for the majority of naturally-occurring infections in humans (Steinman 2008). Re-activation of specific T cells *in vitro* often modifies their functionality making conclusions difficult to reach. Furthermore assays of infections, and models of antigen-bearing tumors, are testing T cell responses to a single specific immunodominant MHC-antigen complex. In addition, antigen delivery, including the dose and entry of the pathogen doesn't mimic the characteristics of natural infections. Moreover, key murine DC subtypes are absent from the human system, while peripheral dendritic cells that are not found in mice have also been described.

Finally, the human organism exists in a 'dirty' environment where the immune system continuously encounters a plethora of potentially harmful microorganisms, a situation that cannot be easily reproduced in the 'clean' environment of the laboratory animal house. This is an important issue in vaccine design, which may have started as an empirical activity but now relies on fundamental scientific challenges that immunology is attempting to address (Steinman 2008). These challenges include the discovery of disease-relevant antigens, adjuvants and protective mechanisms, and understanding how the various elements work and fit together to allow survival of both the individual and the species in natural environments.

Here, the immunogenicity of the microsomal preparation/vaccine is also tested on human cells. Stimulation of PBMCs with a soluble immunodominant HCMV peptide could induce re-activation of HCMV-specific IFNγ-secreting cells. This immune response to free peptides, which were used as a positive control, most probably occurs via presentation of the peptides by APCs naturally present in the PBMSs. HCMV peptide-loaded microsomes, however, induced better activation of CMV-specific IFNγ-secreting cells, while microsomes loaded with irrelevant peptides did not. The conclusion that peptide-loaded microsomes were better than free peptide in inducing IFNγ production was reached based on that the amount of the peptide that was used as free stimulant (100μg/ml) was the same with the amount of peptide that was used for the loading of microsomes. Furthermore, the amount of microsome-associated peptide that reached the PBMCs may had been even less, considering that after loading on microsomes excess peptides are washed away. This finding highlights the potential ability of microsomes to induce antigen-specific human T cell responses.

The microsomes used to activate human T cells were extracted from an MHC-I deficient, HLA-A2-transfected human antigen presenting cell line, the LCL 721.221. This is a mutant human lymphoblastoid B cell line, which is devoid of HLA-A, -B and –C expression, but in which the transfected HLA-A gene is expressed (Shimizu, Koller et al. 1986). Although MHC class I is expressed on almost all cells, MHC class II is expressed primarily on B

lymphocytes, macrophages and dendritic cells. The human B cell line LCL 721.221 expresses MHC-II (Drukker, Katz et al. 2002) and other genes involved in antigen presentation, including costimulatory molecules (LeMaoult, Caumartin et al. 2007). The HLA-A2 molecules in microsomes from LCL 721.221 cells could bind HLA-A2-binding peptides (Li, Paulsson et al. 2000), providing a reliable system for the investigation of microsome-mediated presentation of individual peptides to human cells.

In the system described here, the human T cell responses induced by microsomes were secondary re-call responses presumably of memory T cells. We would subsequently want to investigate if peptide-loaded microsomes can activate naïve human T cells. Nevertheless, the encouraging T cell response to non-autologous microsomes from cell lines observed here, without evidence of non-specific sensitivity is important for the manufacture of large scale vaccine preparations in advance, a factor crucial for the containment of viral spread in the population. Additionally, the potential of using cell lines transfected to express genes that represent optimal antigenic epitopes may provide greater flexibility for vaccine preparation, and will allow vaccines to be potentially tailored towards individual patients.

12. Microsomes provide an alternative to dendritic cell and exosome-based vaccination.

The ability of DCs to capture antigens in the periphery and traffic them from peripheral tissues to the T cell areas of lymphoid organs to initiate immunity has centred DC biology at the heart of modern vaccinology research. Although dendritic cell subsets share certain characteristics, such as a specific morphology, high expression of MHC class II and potent T cell-stimulating activity (Steinman 2008), certain markers divide DCs into distinct subsets (Villadangos and Young 2008). DC subsets vary in their anatomical location, the receptors they express and their ability to process antigen in certain ways. Given the importance of DC-subset diversity in determining the outcome of immunization, the limited knowledge and understanding of the intricacies of DC biology compromises the use of DCs for the control of

immunity (Steinman 2008). Indeed, methods for introducing appropriate antigens into MHC class I and II processing pathways, methods for isolating and activating DCs to guide desirable immune responses, and the routes of DC vaccine administration are still major challenges in the design and success of clinical trials with DC-based vaccine strategies.

The feasibility of dendritic cell-derived exosome production, especially in advanced cancer patients, together with lack of toxicity, and the interesting clinical observations of long-term stability, support further investigations into exosome-based Immunotherapies (Chaput, Taieb et al. 2005). However, the production of exosomal vaccines is also associated with significant limitations. These include difficulties imposed in the quality control of the exosome preparations and the restrictions related to autologous vaccine preparations. For example, the exosome yield from one individual is not sufficient for the production of the vaccine, and exosomes from infected individuals may act as carriers of the pathogen, thus enabling further viral spread. Perhaps more importantly, optimal exosome vaccines depend on the presence of properly activated host dendritic cells, which carry similar limitations to those mentioned for dendritic cell-based therapies, as detailed above.

By contrast, microsomes provide an alternative to dendritic cell and exosome-based vaccination, lacking many of the difficulties that are associated with these strategies. The microsomal vaccine is comparatively easy to prepare, stable and the quality of preparations can be checked at any step during the procedure.

Microsomes locate to anatomic sites of T cell induction and they efficiently deliver selected antigens and co-stimulatory molecules to both CD4$^{(+)}$ and CD8$^{(+)}$ T cells for the induction of antibody- and cell-mediated immune responses. Importantly, microsomes are metabolically inactive, endowing the vaccine with the stability and control that ensures a consistent immunological outcome.

13. Future considerations/ work.

At the time of the writing the analysis of vaccination with peptide-loaded microsomes is still ongoing. This should allow not only the full identification of the range of their effect in various infections, and in response to cancer, but should also provide insight into the underlying mechanisms that regulate their immune stimulatory functions. Ultimately, the aim is to evaluate the vaccine for use in humans. Briefly described below is a list of investigations that are to be conducted, using the results described above as a basis for experimental design.

The antigen presentation capacity of microsomes isolated from antigen pre-loaded-APCs will be evaluated. 'Feeding' whole proteins to APCs before extracting microsomes would allow antigens to be naturally processed and permit the physiological selection of optimal epitopes. Alternatively, the administration of multiple selected peptides to purified microsomes could serve to overcome restrictions associated with exhausted T cells that have become unresponsive to immunodominant epitopes.

Microsomal membranes will also be extracted from APCs that have been transfected to express high levels of selected cytokines or their receptors. The use of the E19K retention signal by genetic engineering facilitates retention of transmembrane proteins or receptors in the endoplasmic reticulum, so they are presented in the extracted microsomal membranes in higher concentrations than normal. This approach would be particularly useful for 'skewing' the immune response. For example, microsomes engineered with IL-12 would provide better activation of a T_H1 response, while microsomes carrying IL-4 could serve to bias towards a T_H2 response. Alternatively microsomes could also be engineered to express inhibitors that block suppressive molecules such as PD1 on the T cell membrane. This would be especially useful as it could contribute to breaking tolerance and/or reversing anergy, which are two of the major problems that the immune system faces during persistent infections.

In recent years the technological advances of imaging have allowed a better understanding of the interactions between APCs and T cells that result in induction of immune responses. In order to better understand the mechanism of T cell activation by peptide-loaded microsomes, it will be interesting to examine their interaction in more detail. For example, is an immune synapse formed between the microsomal membranes and the T cell membrane? Does the T cell 'scan' and bind the surface of microsomes? And how are microsome-associated signals integrated by T cells to trigger TCR activation? Answers to these questions will allow the development of microsomal vaccines that will offer a greater degree of flexibility and potency in their ability to promote protective T cell-mediated responses.

14. Conclusion.

The empirical observations of our ancestors led to the development of protective vaccines for a host of common and, at the time, devastating diseases- a major accomplishment of modern medicine. The discovery of vaccination was in turn the driving force behind the birth of Immunology, a discipline that investigates how the immune system deals with the myriad of disease-causing insults that affect us throughout our life. Immunology defines the basic principles that direct the immune response, and in this study, we have applied the knowledge of the immune system to develop an innovative vaccine strategy that can guide the immune system when nature fails to.

The findings of this research present a novel vaccine strategy based on cell-free microsomal membranes. This microsomal vaccine protects from a viral infection and was shown to regress an established murine tumour. Furthermore, it effectively delivers antigens to human T cells *in vitro*. Consistent with our hypothesis, peptide-loaded microsomes represent an exciting new alternative to currently-available vaccine strategies, that may offer an exciting way forward in our quest to provide effective vaccination against infectious disease and cancer.

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APPENDIX

Antigen-loaded ER microsomes from APC induce potent immune responses against viral infection

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Although matured DC are capable of inducing effective primary and secondary immune responses *in vivo*, it is difficult to control the maturation and antigen loading *in vitro*. In this study, we show that ER-enriched microsomal membranes (microsomes) isolated from DC contain more peptide-receptive MHC I and II molecules than, and a similar level of costimulatory molecules to, their parental DC. After loading with defined antigenic peptides, the microsomes deliver antigenic peptide–MHC complexes (pMHC) to both CD4 and CD8 T cells effectively *in vivo*. The peptide-loaded microsomes accumulate in peripheral lymphoid organs and induce stronger immune responses than peptide-pulsed DC. The microsomal vaccines protect against acute viral infection. Our data demonstrate that peptide–MHC complexes armed microsomes from DC can be an important alternative to DC-based vaccines for protection from viral infection.

Supporting Information available online

Introduction

Professional APC can induce powerful T-cell immune responses by capturing antigens, processing them into peptide–MHC complexes (pMHC) and presenting them to T cells [1]. Together with pMHC, APC also provide costimulatory signals to T cells, which control the magnitude, quality and memory of the induced immune responses [2]. The understanding of the function of APC led to exploration of novel vaccines with the use of antigen-armed APC against infectious diseases and cancer. In both these cases, T-cell-mediated immunity is essential for eradicating virally infected or malignantly transformed cells, particularly against many of the known infectious agents such as tuberculosis, malaria, herpes simplex, papilloma, HIV, Epstein–Barr and hepatitis C viruses [3]. DC, the most potent APC, have been used to elicit protective T-cell immune response to viral infections and cancer in mice and humans with defined antigens [4]. Although some initial promising results were reported, clinical applications have been limited due to difficulties in the quality control of DC matured *in vitro*, leading to immunogenic heterogeneity of matured DC in induction *versus* suppression of T-cell responses. The suppressive function of DC subsets largely results in the induction of T cells producing soluble negative regulators such as IL-10, TGF-β and IDO [5–7]. To overcome the

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difficulties of DC therapy, 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 [8, 9]. Exosomes and microvesicles contain MHC I and II and costimulatory molecules and could induce T-cell responses *in vivo* when mixed with or pre-loaded with defined peptides [9, 10]. However, because it is not known how the pMHCs are processed in these membrane vesicles, both the quality and the quantity of pMHC are difficult to control in the preparation. The MHC molecules on the surface of APC are pre-processed; therefore, the added peptide must compete with endogenous peptides, which may be difficult especially when these are of medium or low affinity [1].

We have now developed a new form of APC-based but cell-free vaccine by using the ER-enriched microsomes derived from mature DC or other APC. The microsomal membranes contained more peptide-receptive MHC molecules than their parental DC. The level of pMHC can be monitored and controlled quantitatively and qualitatively with reporter peptides. These pMHC I and II armed microsomes possess a high level of costimulatory molecules and can stimulate antigen-specific T cells. When these microsome vaccines were injected i.v. into mice they effectively distributed to peripheral lymphoid organs, induced potent CD4 and CD8 responses and protected from acute viral infection. Thus, the ER-derived microsomes isolated from APC or antigen-carrying cells appear to be an effective alternative to DC for both protective and therapeutic applications.

Results

Abundant peptide-receptive MHC I and II and costimulatory molecules in processed microsomes

Both MHC I and II molecules are synthesized and matured in the ER. MHC I assemble with antigenic peptides in the ER, while MHC II molecules in the ER are protected from peptide loading by the invariant chain (Ii) until they reach the endocytotic compartments, where the Ii is degraded and exchanged with antigenic peptides under acidic conditions [11]. Therefore, on the surface of APC, most of the MHC I or II molecules are pre-processed with high-affinity peptides as pMHC. In the previous study, we have shown that ER-enriched microsomes contain abundant peptide-receptive MHC I molecules [12]. The microsomes isolated were enriched ER membranes as indicated by the attachment of ribosomes on the cytoplasmic side of the microsomal vesicles and the presence of the ER-resident protein, tapasin (Fig. 1A and B) with limited contamination of endosomes as shown by an endosomal marker LAMP2 (Fig. 1B). To explore whether the peptide-receptive MHC molecules in ER-enriched microsomes from APC can present antigens to T cells, we first examined the ability of MHC molecules in isolated microsomal membranes to assemble with corresponding peptides after an

inside-out process consisting of repeated freeze-thawing. Three cycles of freeze-thawing could effectively open the microsomal vesicles, which became pleomorphic (Fig. 1C). The levels of MHC I and II molecules were similar on the microsomes and cell surface of APC (Fig. 1B and D, data for H2-Ia and -Kb). The H2-Kb molecules in the processed microsomes from the IFN-γ-treated DC lines, Jaws II and DC2.4, the macrophage line RAW 309 Cr.1 and the bone-marrow-derived DC could be loaded with the Kb-specific peptide SIINFEKL derived from chicken OVA residues 257–264 as shown by staining with the SIINFEKL–Kb-specific antibody 25-D1.16 (Fig. 1D, panel SIINFEKL Kb) and by crosslinking Kb with crosslinker-modified SIYNFEKL peptides (Fig. 1E), consistent with our previous reports [12]. However, only limited amount of MHC I molecules on the surface of APC were peptide receptive (Fig. 1D, panel SIINFEKL Kb and E). To further confirm the retention of peptide-receptive MHC I in the ER, we compared the amount of SIINFEKL–Kb complexes in the microsomes from OVA expressing mouse lymphoma (RMA) cells and the microsomes from RMA cells, which were loaded with exogenous SIINFEKL peptides *in vitro*. The levels of SIINFEKL–Kb complexes in the microsomes loaded with SIINFEKL were much higher than those in the microsomes from OVA expressing RMA cells (Supporting Information Fig. 1), indicating that Kb molecules assembled with endogenous peptides are rapidly transported to cell surface.

To explore the possibility of loading peptides onto microsomal MHC II molecules, a biotin-labeled, H2-Ia restricted peptide ISQAVHAAHAEINEAGR OVA 324–340 was used to detect pMHC II assembly in inside-out converted microsomes. Despite an efficient loading of MHC I molecules, the OVA 324–340 peptide could not be effectively loaded onto MHC II molecules in the inside-out converted microsomes at pH 7.0 (Fig. 2A), possibly due to the association of Ii with MHC II. We therefore processed peptide loading in an acidic buffer of pH 5, which dramatically increased the peptide receptiveness of MHC II molecules (Fig. 2A). In addition to generating peptide-receptive MHC II molecules, the acidic stripping process also led to the significant increase in peptide-receptive MHC I molecules (Fig. 2B), which may be due to the dissociation of pre-processed peptides on MHC I molecules. Moreover, acidic treatment released ribosomes from microsomes (Supporting Information Fig. 2), which may prevent the induction of antibodies against ribosomes. To compare the level of peptide-receptive MHC II molecules on the cell surface and in the microsomes of APC, the pMHC II on cells and in microsomes were analyzed after loading with a biotin-labeled OVA 324–340 peptide. Results showed that under acidic conditions the microsomes expressed much more of peptide-receptive MHC II molecules than the cell surface (Fig. 1D, panel Bio-OVA324–340). Thus, the developed protocol for the preparation of microsomes with inside-out orientation of peptide-receptive MHC I and II molecules allows effective assembly with antigenic peptides *in vitro*. Using crosslinker-modified MHC I and biotin-lyted MHC II reporter peptides, it was possible to control the level of pMHC in the microsomes, which is essential in controlling the level of T-cell responses.

In addition, analysis of the costimulatory molecules CD54, CD80 and CD86 molecules showed that IFN-γ-treated DC2.4 cells expressed well-matched levels on the microsomes and on cells (Fig. 2C), suggesting that the ER-enriched microsomes can present both pMHC and costimulatory signals to T cells.

Peptide-loaded microsomes induce T-cell responses *in vitro*

Based on the total protein content, on average 10^4 APC could produce 1–3 μg of microsomes. After the removal of excess
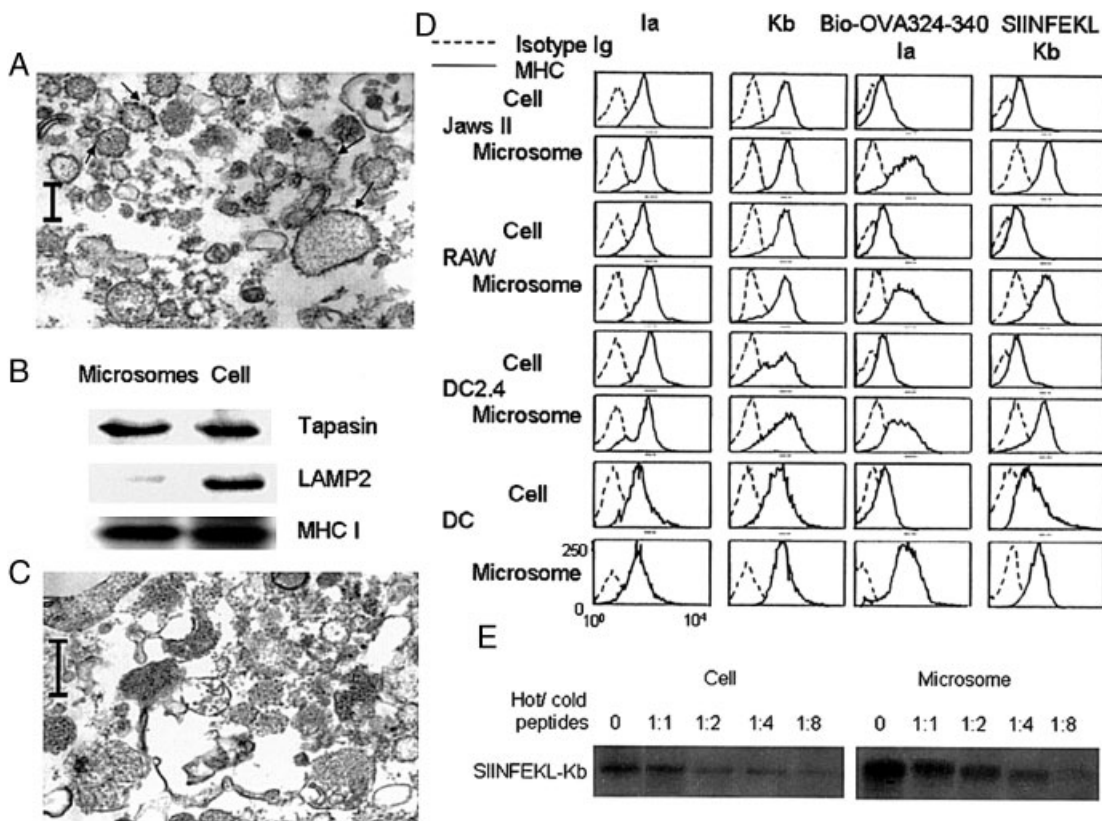

Figure 1. ER-enriched microsomes. EM images of ER-enriched microsomes prepared from RAW309Cr.1 cells before (A) and after (C) processing with freeze-thaw and acidic stripping (Scale bars: 100 nM). Arrows indicate ribosomes. (B) Immunoblotting analysis of 20 μg lysates from RAW309Cr.1 cells and microsomal membranes, respectively, with antibodies against tapasin, LAMP2 and MHC I, respectively. (D) Panel labeled Ia and Kb show the FACS analysis of the expression of these H2-Ia and H2-Kb molecules, respectively, in microsomal membranes and on the surface of three APC lines and bone-marrow-derived DC. Panels Bio-OVA-Ia and SIINFEKL–Kb display the mean fluorescence intensity of these two complexes in microsomes and on the cell surfaces. The loading of peptides onto microsomes and cells is described in "*the Materials and methods section*". (E) A total of 2×10^6 RAW309Cr.1 cells or 20 μg of microsomal membranes were incubated with 100 μM crosslinker modified and 125I labeled SIYNFEKL peptides and exposed to UV irradiation before lysis. The total lysates were submitted for precipitation with the Y3 antibody specific for Kb molecules and the precipitates were separated on a SDS-gel. The crosslinked Kb molecules were visualized after exposure to X-ray film. The presented data are representative of three experiments.
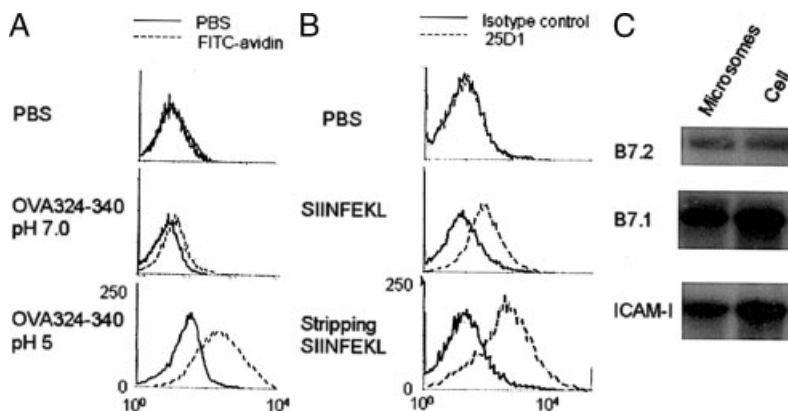

Figure 2. Acidic treatment induced expression of peptide-receptive MHC II and enhanced peptide loading onto MHC I molecules of microsomal membranes and expression of costimulatory molecules. (A) Displays biotin-OVA320–340-Ia complexes detected by FITC-Streptavidin under pH 7.0 or 5.0 and (B) shows FACS detection of the SIINFEKL–Kb complex by 25-D1.16 antibody in peptide-loaded microsomal membranes with or without pre-treatment with acidic stripping. Microsomal membranes with PBS serve as a background control. (C) Expression of costimulatory molecules in microsomal membranes and their parental DC2.4 cells was analyzed by immunoblotting with specific antibodies as indicated.
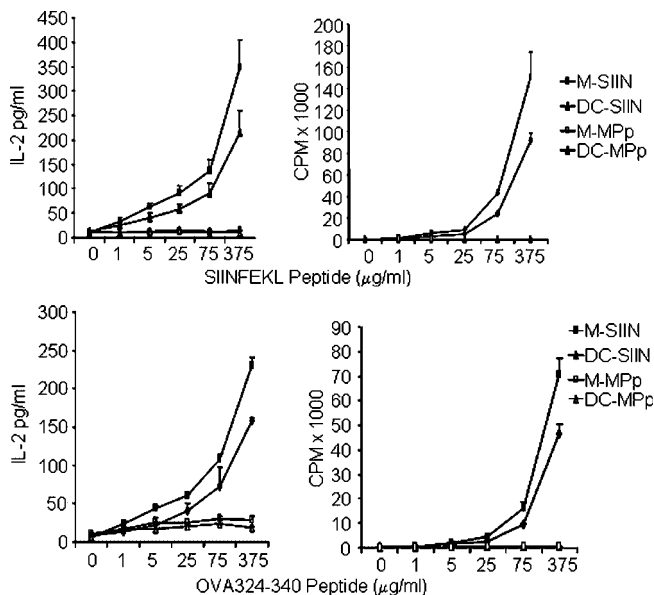
Figure 3. Peptide-loaded microsomes induce T-cell responses. Activation of OT1 (upper panel) or OTII (lower panel) T cells by SIINFEKL or OVA324–340 peptide-loaded microsomal membranes from bone-marrow-derived DC or peptide pulsed DC as shown by the production of IL-2 and proliferative responses. Microsomes or DC loaded with irrelevant MP peptide served as controls.

peptides, 1–2 μg of peptide-loaded microsomes from bone-marrow-derived DC or 1×10^5 peptide-pulsed DC were co-cultured with 1×10^5 OTI T cells for either 48 h prior to measuring IL-2 production or for 3 days before proliferation assay. Our results showed that SIINFEKL-loaded microsomes were more potent at inducing T-cell responses *in vitro* in a peptide-dose-dependent manner than peptide-pulsed DC (Fig. 3, upper panel). Similarly, OVA324–340 peptide-loaded microsomes stimulated IL-2 production in OTII cells more effectively than peptide-pulsed DC (Fig. 3, lower panel).

The immunogenicity of peptide-loaded microsomes could be maintained for more than 6 months if the microsomes were stored in −80°C (data not shown). Together, these results present the possible use of a reconstituted cell-free antigen-presenting system as an effective alternative to DC in stimulating both CD4 and CD8 T-cell responses.

Peptide-loaded microsomes are predominately distributed to peripheral lymphoid organs

Effective T-cell-based vaccines have to deliver antigens at a sufficient dose to peripheral lymphoid organs for optimal stimulation of effector T cells. It has been reported that antigen-armed DC are better distributed to peripheral LN, if administrated subcutaneously [13]. However, microsomes cannot actively migrate to lymphoid organs; we therefore examined the tissue distribution of peptide-loaded microsomes

from CFSE or ^{35}S-methonine-labeled bone-marrow-derived DC and their parental DC after intravenous administration. Twenty-four hours after injection, 7.4% bone-marrow-derived microsomes had accumulated in the spleen, and 0.66% in the LN. Importantly, after 7 days, 5.64 and 0.81% of bone-marrow-derived microsomes were still in the spleen and LN, respectively (Fig. 4A), indicating that microsomes accumulate and are retained in major peripheral lymphoid organs. In contrast, only 2.78 and 0.21% of peptide-pulsed and bone-marrow-derived DC were distributed to the spleen and LN 24 h after i.v. injection and this reduced to 0.93 and 0.18% after 7 days (Fig. 4A). Seven days after injection, microsomes in the spleen accumulated in the periarteriolar lymphoid sheath (PALS), which mainly consists of T cells (Fig. 4B). DC were also distributed to the PALS 2 days after injection (Supporting Information Fig. 3). However, 7 days after injection, very few DC were remained in the peripheral lymphoid tissues and distributed randomly in the follicles, suggesting that they are the dying DC taken up by phagocytes in the follicles (Supporting Information Fig. 3). The microsomes accumulated in T-cell areas as indicated by co-staining with anti-T- and B-cell markers (Fig. 4B). To investigate the possibility that microsomes are endocytozed by APC, the microsomes from CFSE-labeled DC2.4 cells were used for internalization assay with bone-marrow-derived DC. Results showed that FITC-labeled dextran were effectively endocytozed by DC, while endocytosis of microsomes was not detected (Fig. 4C). Although we cannot exclude the possibility that some of the microsomes were endocytozed by APC in peripheral lymphoid tissues *in vivo*, the potent T-cell responses suggest that such an accumulation is essential to deliver sufficient quantity of antigens to T cells in lymphoid system.

Microsome vaccine protects against vaccinia virus infection *in vivo* by induction of both CD4 and CD8 T-cell responses

The capacity of peptide-loaded microsomes in the induction of effective anti-viral immune responses *in vivo* was evaluated by an *in vivo* vaccinia viral infection model in C57BL/6 mice. The recombinant vaccinia virus used carries a gene encoding chicken OVA [14]. Five groups of C57BL/6 mice were immunized intravenously with; a mixture of SIINFEKL and OVA 324–340 peptide-loaded microsomes from bone-marrow-derived DC from C57BL/6 at a dose of 20 μg microsomes/mouse or same microsomes but loaded with irrelevant influenza matrix protein (MP) peptides at the same dose or a mixture of SIINFEKL (100 μg) and OVA324–340 (100 μg) or 10^6 DC pulsed with SIINFEKL and OVA 324–340 peptide or 10^6 DC pulsed with irrelevant MP peptides or PBS as the controls. The mice were then boosted after 7 days. Ten days after boosting, 20 of the 30 mice in each group were challenged with an infectious dose of 2×10^5 PFU and the other 10 with a sublethal dose of 5×10^6 PFU of OVA-VV virus. Forty-eight hours after challenge, 5 of the 20 mice in infectious dose group were sacrificed for analysis of

T-cell responses and 4 days after, another 5 were sacrificed for viral clearance study, while the remaining mice were used for clinical study.

About 4.3% of CD8 T cells in mice immunized with SIINFEKL and OVA324–340 peptide-loaded microsomes were SIINFEKL specific, while only 1.8% were in mice immunized with peptide-pulsed DC (Fig. 5A). SIINFEKL-specific CD8 T cells were not detected in the other three groups as indicated by tetramer staining (Fig. 5A). In conjunction with the induction of CD8-specific T cells, the *in vitro* restimulation of CD4 T cells showed that OVA324–340 peptide-loaded microsomes induced stronger IL-2 production and proliferative responses of CD4 T cells from the mice immunized with a mixture of SIINFEKL and OVA324–340 peptide-loaded microsomes than the mice with peptide-pulsed DC (Fig. 5B and C). These results indicate that an integrated immune response of both CD4 and

CD8 T cells were induced in mice by peptide-loaded microsomes and suggests that the microsomes are more immunogenic than their parental DC.

The clinical symptoms were closely related to the induction of immune responses *in vivo*. In the infectious dose groups, although a degree of protection was observed in mice immunized with peptide-pulsed DC, only SIINFEKL and OVA324–340 loaded microsomes completely protected against viral infection as these mice had shown no body weight loss and no viral particles detected in lung tissue unlike the other groups (Fig. 6A and C). With a sublethal dose of virus, although mice immunized with SIINFEKL and OVA324–340 loaded microsomes showed body weight loss, they were protected from death and recovered rapidly after infection (Fig. 6B), while two out of ten mice immunized with peptide-pulsed DC died after 5 days (Fig. 6B). All mice in other groups died
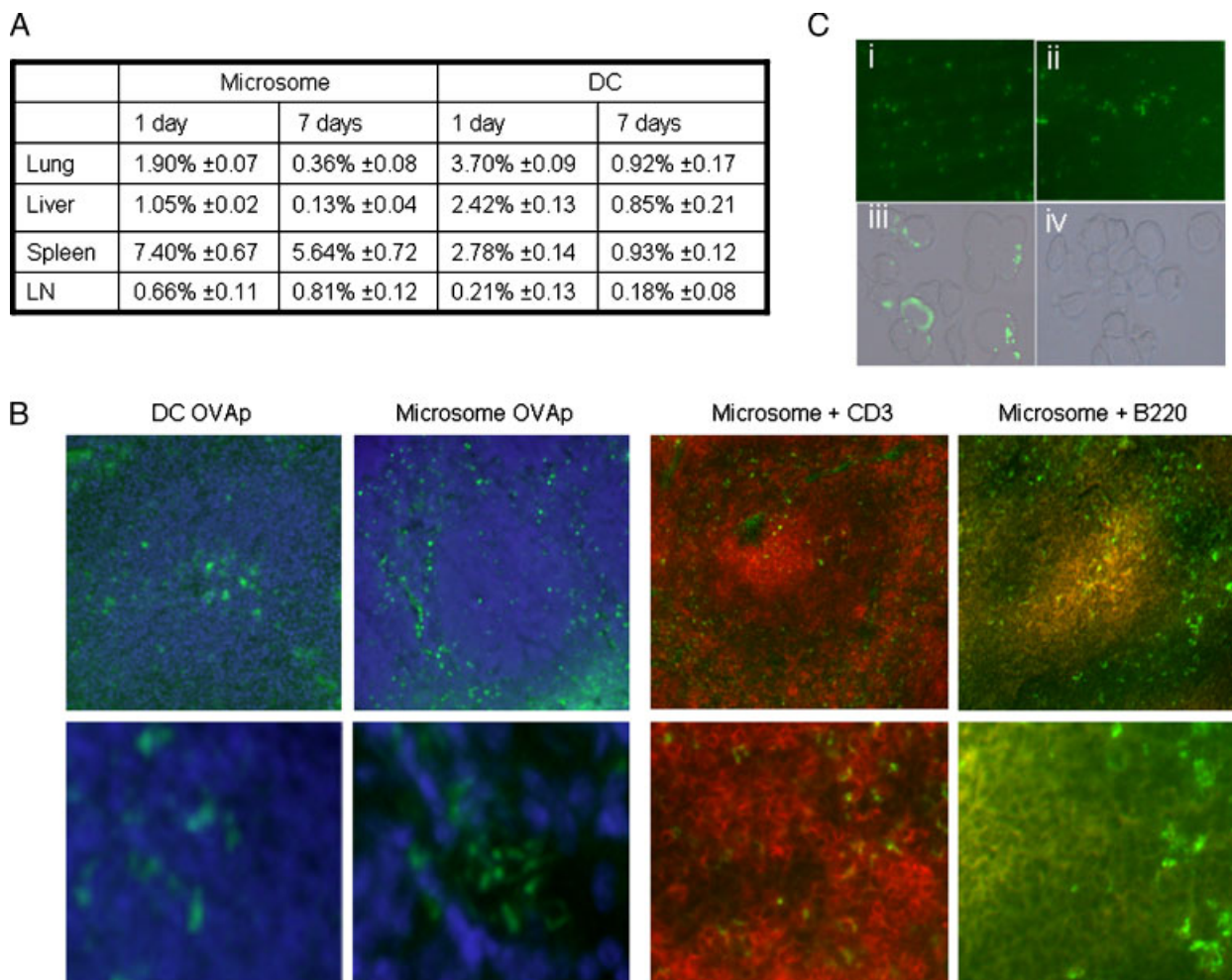

	Microsome		DC	
	1 day	7 days	1 day	7 days
Lung	1.90% ±0.07	0.36% ±0.08	3.70% ±0.09	0.92% ±0.17
Liver	1.05% ±0.02	0.13% ±0.04	2.42% ±0.13	0.85% ±0.21
Spleen	7.40% ±0.67	5.64% ±0.72	2.78% ±0.14	0.93% ±0.12
LN	0.66% ±0.11	0.81% ±0.12	0.21% ±0.13	0.18% ±0.08

Figure 4. Peptide-loaded microsomes accumulate in peripheral lymphoid organs, but not endocytozed by DC in vitro. (A) Distribution of SIINFEKL-peptide-loaded and ^{35}S-methionine-labeled microsomes from bone-marrow-derived DC or peptide pulsed DC in the indicated tissues from C57BL/6 mice at different times after i.v. injection. (B) Fluorescence microscopy showing distribution of peptide-loaded and CFSE-labeled microsomes in spleen 7 days after i.v. injection. Cell nuclei were visualized by DAPI counter-staining. The colocalization of microsomes (green) with T-cell areas as indicated by staining with anti-CD3 (bright red) or B220 (orange red) antibodies. Upper panel shows 10 × imaging and lower panel shows 40 × for DAPI staining and 20 × for antibody staining. (C) Fluorescence microscopy showing (i) FITC-labeled dextran; (ii) CFSE-labeled microsomes loaded with SIINFEKL peptides; (iii) DC incubated with FITC-dextran; and (iv) DC incubated with CFSE-labeled microsomes.

(Fig. 6B). Furthermore, the spleens were double the size in the protected mice immunized by OVA peptide-loaded microsomes indicating potent immune responses (data not shown) concordant with the observed accumulation of microsomes in peripheral lymphoid organs. These results demonstrate that peptide-loaded microsomes are highly immunogenic and better than live DC to serve as effective vaccines to protect against acute viral infection.

Discussion

Vaccines based on matured DC armed with defined antigens appear very promising for both prophylactic vaccines and immune therapies [15, 16]. However, the difficulties involved in the control of antigen loading, the preparation of matured DC *in vitro* and the complexities of DC subsets in the induction *versus* suppression of T-cell activation *in vivo* limit DC-based vaccine applications [17]. The present study describes an effective alternative to DC therapy using ER-enriched microsomes isolated from DC. The abundant peptide-receptive MHC and costimulatory molecules and accumulation in the peripheral lymphoid organs contribute to the high efficacy of the microsomal vaccine *in vivo*. The immunogenicity can be maintained for more than 6 months if the prepared microsomal vaccines are stored in −80°C or in liquid nitrogen (data not shown), making the prepared vaccine consistent and cost effective.

The sustained accumulation of antigen-carrying microsomes in the peripheral lymphoid organs explains the high efficacy of induced responses *in vivo* [18]. A possible explanation for such a high accumulation is that matured live DC may actively migrate in and out of the peripheral lymphoid organs, while microsomes from matured DC have the same homing potential, but could not actively migrate out from lymphoid organs. Recently, it has been shown that DC in the peripheral lymphoid organs are actively exchanged with DC in blood [19] in a dynamic equilibrium, which suggests that the low accumulation of injected DC into the lymphoid organs is largely due to the rapid equilibration with endogenous DC. Significantly, most of the injected microsomes are located in the PALS area, which could effectively present antigens to memory T cells [20–22].

One of the important findings is the loading of MHC II in the ER-enriched microsomes. Our results provide evidence suggesting that in acidic condition the Ii in microsomes can dissociate from MHC II molecules and generate a peptide-receptive status allowing assembly with exogenous peptides. Although we were not able to directly compare microsomal MHC II assembly with the assembly of MHC II in the endosomal compartments [22], the induction of CD4 T-cell responses *in vitro* and *in vivo* suggests that acidic processed microsomal membranes are able to deliver MHC II-restricted antigenic peptides to CD4 T cells. The acidic treatment also stripped ribosomes from microsomes as well as pre-processed peptides from

MHC I molecules, which increases the loading efficiency of pMHC molecules and reduces potential side effects such as anti-ribosome or anti-nuclear responses.

One of the major problems in human DC-based vaccines is the complexity of DC after *in vitro* maturation. The heterogenic populations of matured DC include both immunogenic and tolerogenic APC, which produce a mixture of inflammatory cytokines as IL-12 and inhibitory factors such as TGF-β, IDO and IL-10 [5, 23, 24], which hamper the efficacy of immune responses. The microsomes are free from soluble factors, and their preparation is biologically reproducible and consistent at the level of peptide loading and antigenicity when compared with live DC. In addition, the ER-enriched microsomes do not contain cytosolic or nuclear proteins, which reduces potential side effects. We have not found antimicrosome responses *in vitro* or *in vivo*, suggesting that the ER-resident proteins were tolerated.

Both exosomes and plasma-membrane-derived vesicles from APC have been reported to induce potent immune responses *in vivo* [9, 10, 25, 26]. It is however unknown how peptides are processed to pMHC in these vesicles. Some viruses can bud into MHC II-enriched compartments and be released by exosomes [27], which could limit applications for using autologous exosomes in anti-viral vaccines. The potential advantage of exosomes is that the pMHC class II may be enriched from antigen pre-fed DC due to the processing of pMHC II in endocytotic compartments. For technical reasons, we could not biochemically compare the pMHC II quality in peptide-loaded microsomes *in vitro* with that in endosomal compartments *in vivo*. Nevertheless, the observed high efficiency loading and the induction of CD4 T-cell responses demonstrate the immunogenic efficiency of pMHC II in microsomes. Furthermore an important advantage for a microsome vaccine is the ability to directly present pMHC and costimulatory signals to T cells. We demonstrated that microsomes were not endocytozed by DC *in vitro*. Although we could not exclude the possibility that some of the microsomes *in vivo* could be endocytosed by APC and the pMHC are indirectly presented by APC to T cells in lymphoid organs, the observed ability to directly activate antigen-specific T cells could be important in microsome vaccines to chronic viral infections or cancer: conditions where endogenous DC are less active *in vivo* [28, 29].

After mixing with peptides, the plasma-membrane-derived vesicles also showed the ability to directly interact with T cells and induce responses *in vivo* [9]. However, as we have shown, the number of peptide-receptive MHC I molecules on the surface of APC is limited due to the presence of pre-processed pMHC I. Therefore, it may be difficult to deliver antigenic peptides with medium or low affinity, which cannot compete with the preprocessed peptides on MHC molecules. Owing to the nature of the sonication methods used in the preparation, some of the ER membranes may also have been present in these preparations [9], which as we show have abundant peptide-receptive MHC I molecules. If ER-derived microsomes are used, the inside-out processing is required to load peptides effectively and to expose
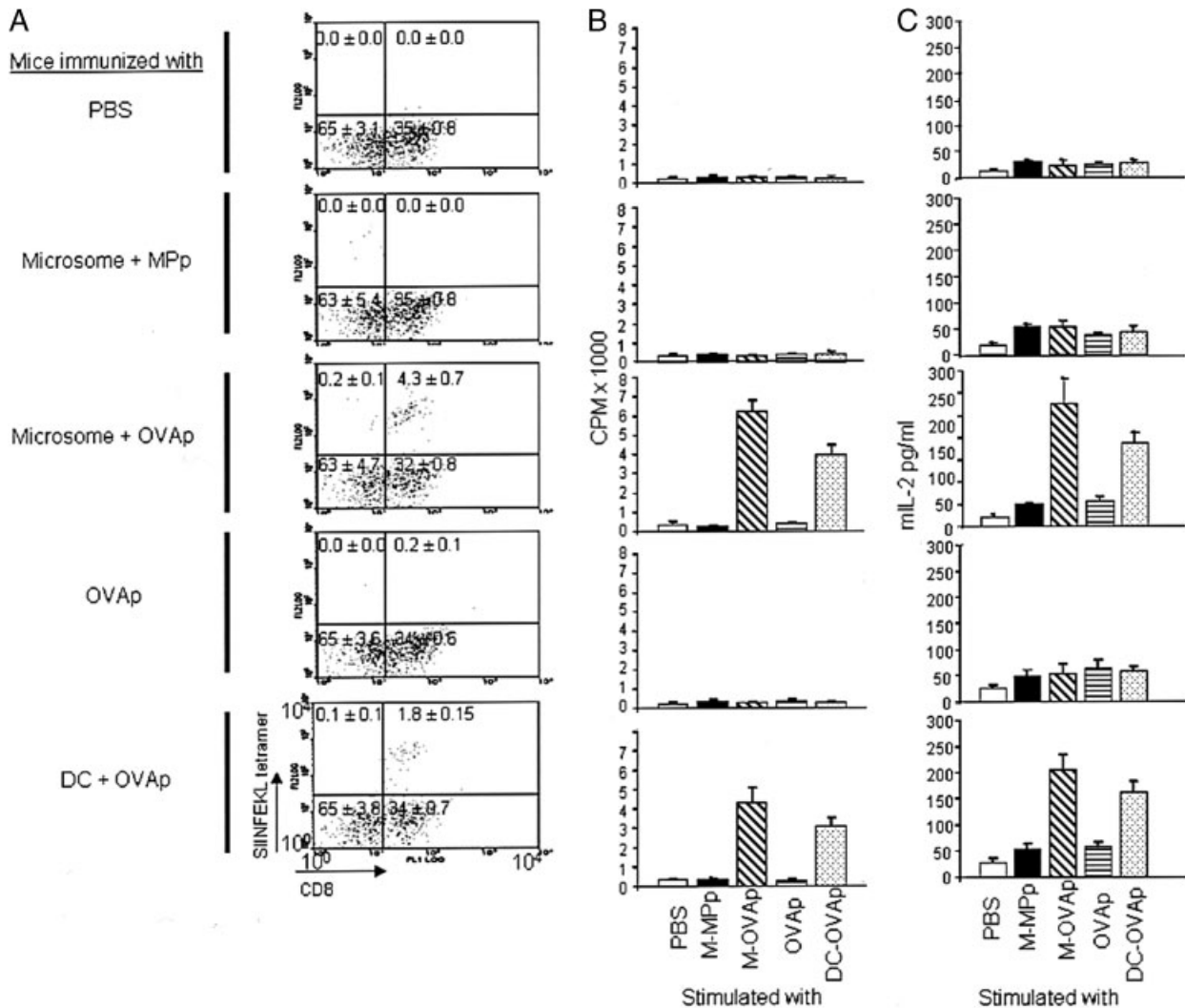
Figure 5. Induction of CD4 and CD8 T-cell responses by peptide-loaded microsomes *in vivo*. CD57BL/6 mice were injected i.v. with a mixture of SIINFEKL and OVA324–340 loaded microsomes (20 μg/mouse) from bone-marrow-derived DC and boosted once after 7 days. The mice injected with irrelevant MP peptide-loaded microsomes, mixture of SIINFEKL and OVA 324–340 peptides and PBS serve as controls. Seven days after boosting, total splenocytes were stained with SIINFEKL tetramer and CD8 (A). For CD4 T-cell responses, CD4 T cells were isolated from splenocytes and stimulated *in vitro* at 2×10^5 CD4 T cells *per* well with the corresponding stimuli as indicated. Two days after stimulation, IL-2 production was measured by ELISA (C) and 3 days after proliferation responses were detected by ^{3}[H] incorporation (CPM) (mean of triplicate cultures $\pm$ SD) after exposure to ^{3}[H]thymidine for 8 h (B).

the luminal side of costimulatory and MHC molecules to T cells (data not shown).

Compared with live DC, cell-free membrane vaccines do not include soluble inhibitory factors, but they also exclude inflammatory cytokines such as IL-12. However, the high efficacy of microsomal vaccines indicates that the inflammatory cytokines produced by mature DC are not essential for the induction of effective immune responses *in vivo*.

In summary, our results present a novel form of vaccine with pMHC I- and II-loaded microsomal membranes. This form of vaccine is comparatively easy to prepare, stable and the quality of preparations can be checked at any step during the procedure. We demonstrate that the microsomal vaccines are better than their parental DC for the induction of potent immune responses against viral infection *in vivo*.

Materials and methods

Mice and cell lines

OTI and OTII transgenic mice on the C57BL/6 background expressing a TCR specific to the H2-Kb-SIINFEKL and specific for H2-Ia-OVA324–340 peptide complex, respectively, 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. Jaws II, macrophage RAW309Cr.1 were obtained from American Type Culture Collection (ATCC). DC2.4 DC line was kindly provided by Dr. Mann D. Southampton, UK. All lines were cultured in RPMI 1640
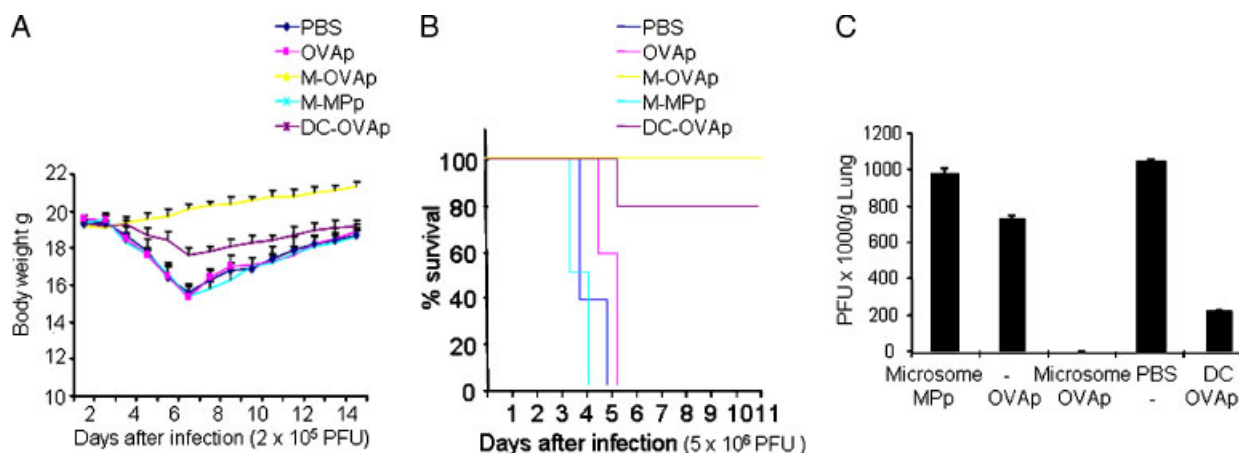
Figure 6. Immune responses against viral infection (VV-OVA). For anti-viral response, mice were immunized with a mixture of SIINFEKL and OVA324–340 loaded microsomal membranes from bone-marrow-derived DC twice i.v. with an interval of 7 days. Mice injected i.v. with MP-loaded microsomal membranes, a mixture of SIINFEKL and OVA324–340 peptides and PBS served as control groups. Ten days after the last immunization, all mice were challenged with VV-OVA virus at infectious or sublethal doses as indicated. (A) Body weight changes after challenge with infectious dose of VV-OVA virus and (B) survival of mice challenged with a sublethal dose of VV-OVA virus. (C) Viral titers in the lungs on day 4 of mice challenged with an infectious dose of VV-OVA virus. Data show mean±SD of log 10 PFU *per* gram of lung tissues.

or DMEM supplement with 10% FBS (Invitrogen). For Jaws II cells, the culture also contained GM-CSF (Invitrogen) at 5 ng/mL. For antigen presentation, the Jaws II, DC2.4 and RAW309Cr.1 cells were treated with IFN-γ at 100 IU/mL for 24 h.

Bone-marrow-derived DC

Immature DC were generated from bone marrow progenitors by culturing them in the presence of GM-CSF and maturated by LPS stimulation as described previously [11]. At the end of IFN-γ or LPS treatment, the dead cells were less than 5% in the cell lines and 15% in bone-marrow-derived DC.

Antibodies and flow cytometry

FITC-conjugated antibodies to CD54, CD80, CD86, 25-D1.16 and FITC-conjugated Streptavidin; PE-conjugated antibodies to H2-Ia and Y3 were from BD Biosciences. Antibodies against tapasin and H2 molecules were described previously [12]. Anti-LAMP2 antibody was from eBioscience. Kb-SIINFEKL Pentamer was from Proimmune. Antibodies used for immunoblotting of mouse CD54 (AF796), CD80 (AF740) and CD86 (AF-441-NA) were from R&D systems. A PE-Pro5TM was used for the analysis of fluorescently labeled cells and the medium fluorescent intensity from three experiments was presented. The isotype Ig was used as background controls for all the staining of both cells and microsomes. The setting of side-scatter and forward-scatter was the same between cells and microsomes. Owing to the uneven sizes of microsomal vesicles, the entire events detected in side-scatter and forward-scatter were used for the analysis on fluorescent channels. The medium fluorescent intensity was then compared. CD4 or CD8 microbeads (Miltenyi Biotec)

were used for isolation of CD4 or CD8 T cells from spleens of OTII or OTI mice, respectively, according to the manufacturer's protocol.

Peptides and peptide modification

Peptides were synthesized by Invitrogen and purified to more than 95% purity. The −amino group of lysine in the H2-Kb-specific SIYNFEKL peptide was covalently modified by a photo-reactive crosslinker and labeled with iodination (^{125}I) as described previously [30]. The N-terminus of peptide OVA324–340 was labeled and purified with a biotin-labeling kit (Pierce).

Preparation of ER-enriched microsomal membranes

Microsomes from cell lines or bone-marrow-derived DC were prepared and purified according to our previous publication [30]. Briefly, cells were washed and resuspended in homogenization buffer. After homogenization, tonicity was restored to 0.15 M NaCl. 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 5 mL of 0.33 M sucrose, layered in turn on top of a discontinuous sucrose gradient consisting of 2 mL of 2 M and 1 mL of 2.5 M sucrose. Centrifugation in a TH-641 rotor for 16 h at 110 000g at 4°C yielded a microsome band on top of the 2 M sucrose cushion, which was collected and resuspended in RM buffer (250 mM Sucrose, 50 mM triethanolamine-HCl, 50 mM KOAc, 2 mM MgOAc2, 1 mM DTT). Before preparation, cell lines and bone-marrow-derived DC were activated by treatment of 100 IU/mL of

IFN-γ or 1 μg/mL of LPS for 24 h, respectively. ^{35}S-methionine (Amersham) and the CFSE-labeled microsomal membranes were prepared from bone-marrow-derived DC after labeling with either 1mCi ^{35}S-methoinine for 6 h or 2.5 μM CFSE (Molecular Probe) for 10 min.

Processing and peptide loading of microsomes

The microsomes in RM buffer were first processed by freeze-thaw (30 s in liquid nitrogen and 5 min at 37°C) three times, followed by addition of an equal amount of stripping buffer (0.26 M citric acid, 132 mM Na_2HPO_4, 2% BSA, pH 3) and incubation for 3 min on ice. The microsomal membranes were recovered by centrifugation through a 0.5 M sucrose cushion on an airfuge and resuspended in RM buffer. For MHC I loading, peptides at 500 μg/mL or as indicated and human β2-microglobin (5 μg/mL) were loaded to the microsomal membranes by incubation for 1 h at 37°C. After loading the excess peptides were removed by centrifugation through a 0.5 M sucrose cushion on an airfuge and the loaded microsomes were resuspended as homogenous population in culture medium. For MHC II, after the freeze-thaw process, the microsomes in RM buffer were mixed with equal volume of stripping buffer and peptide at 500 μg/mL or as indicated for 5 min at 37°C. After loading excess peptides were removed by centrifugation through a 0.5 M sucrose cushion on an airfuge and the loaded microsomes were resuspended as homogenous population in culture medium.

In vitro stimulation of T cells

The OTI or OTII T cells were isolated from spleens of OTI or OTII transgenics by positive selection using a MACS system (Miltenyi Biotec), with purity consistently above 95% as defined by FACS analysis with anti-CD8 or anti-CD4 antibodies. A total of 1×10^5 T cells were incubated at 37°C with 1–4 μg of peptide-loaded microsomes for 48 h. IL-2 production was then measured by ELISA (R&D). For proliferation assay, [^{3}H]thymidine at 1 μCi/mL was added after 3 days of culture and the cells were harvested after 8 h. When APC were used for stimulation, IFN-γ-treated APC were pulsed with peptides at 500 μg/mL or as indicated for 1 h at 37°C in HBSS containing 5% FBS and for MHC I loading 5 μg/mL human β2-microglobulin was added. After peptide pulsing, APC were washed and irradiated at 4000 Rad. A total of 1×10^5 peptide-loaded APC were cultured in vitro with 1×10^5 OTI or OTII cells.

In vivo distribution of microsomal membranes or DC

Microsomes were purified from either CFSE or ^{35}S-methoinine-labeled bone-marrow-derived DC. An aliquot of 20 μg of peptide-loaded microsomes or 1×10^6 peptide-pulsed DC was injected into C57BL/6 mice i.v. At various times as indicated, spleen, lung, LN and liver were collected and CPM was measured. The percentage of accumulated microsomes or DC was calculated as CPM/gram of the organ and divided by the total CPM of injected microsomes or DC. The distribution of CFSE-labeled microsomes was morphologically analyzed on the frozen sections of the spleen 7 days after injection. The frozen sections were also used for staining with PE-labeled anti-B220 antibody (BD Biosciences) or rabbit anti-CD3 antibody (BD Biosciences) followed with Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Lab).

Internalization assay

Bone-marrow-derived DC were incubated with microsomes isolated from CFSE-labeled DC2.4 cells or with FTIC-labeled dextran for 5 h at 37°C. Free-microsomes or dextran were removed by low-speed centrifugation at 2500 rpm in PBS. Cells were then fixed and analyzed by fluorescent microscopy.

Immunization

Microsomes from bone-marrow-derived DC or bone-marrow DC were loaded either with OVA SIINFEKL or OVA OVA324–340 as immunogens, or with an HLA-A2-restricted epitope of the influenza matrix protein (GILGFVFTL) as an irrelevant control peptide. A total of 1×10^6 cells or 20 μg microsomes in 100 μL of physiologic saline were injected into the dorsal tail vein of each mouse after 7 days; the same dose was given again for boosting.

Viral infection

Mice were infected intranasally with the recombinant vaccinia virus encoded with chicken OVA [31] in 50 μL at the indicated doses. The mice were weighed and observed for illness daily, as described previously [31]. In vivo replication of vaccinia virus was examined by plaque assay on lung tissues, which were removed, weighed and ground with a mortar and pestle. Serial 10-fold dilutions of clarified supernatants were used to infect subconfluent monolayers of BSC40 cells in triplicate in 24 well plates. After 1 h, the plates were covered in 0.75% methylcellulose in 10% MEM and incubated at 37°C. The cells were fixed with formalin 2 days after infection and stained with 2% crystal violet in 40% methanol, and plaques were counted under a dissecting microscope. Data are presented as geometric mean log 10 PFU per gram of lung at dilutions that produced more than five plaques per well.

Ex vivo analysis of antigen specific T cells

For CD8 T cells, splenocytes were stained with FITC-conjugated anti-CD8 (BD Biosciences). After washing, cells were stained with

either PE-conjugated Kb-SIINFEKL Pentamer (Proimmune) or isotype-matched controls, and analyzed by FACS.

For evaluation of CD4 T-cell responses, CD4 T cells were purified from spleen and LN by CD4-coated magnetic beads (Miltenyi Biotec) to more than 90% as measured by PE-CD4 antibody on FACS. A total of 10^5 CD4 T cells were cultured with either 2 µg of OVA320–340 peptide-loaded microsomes from bone-marrow-derived DC or 1×10^5 DC pulsed with the same peptide for 48 h before measuring IL-2 production by ELISA (BD Bioscience) or 3 days before proliferation assay with ^{3}H-TdR (Amersham) incorporation.

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 $\pm$ SD of pooled data from four to six independent experiments.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

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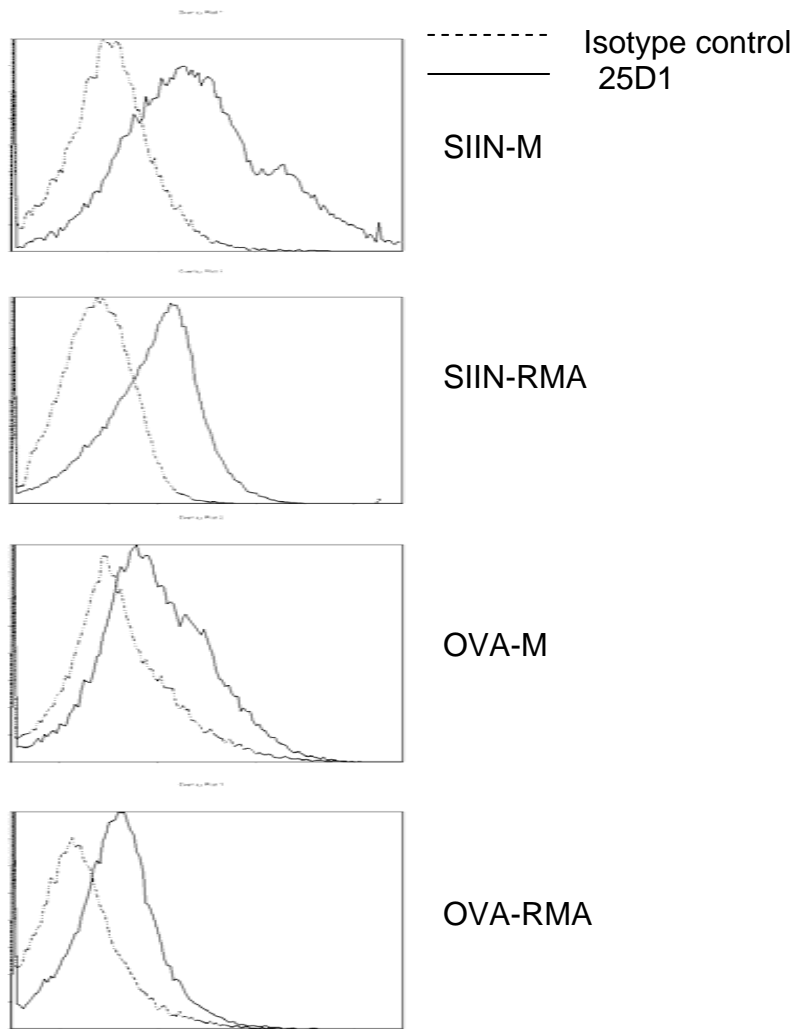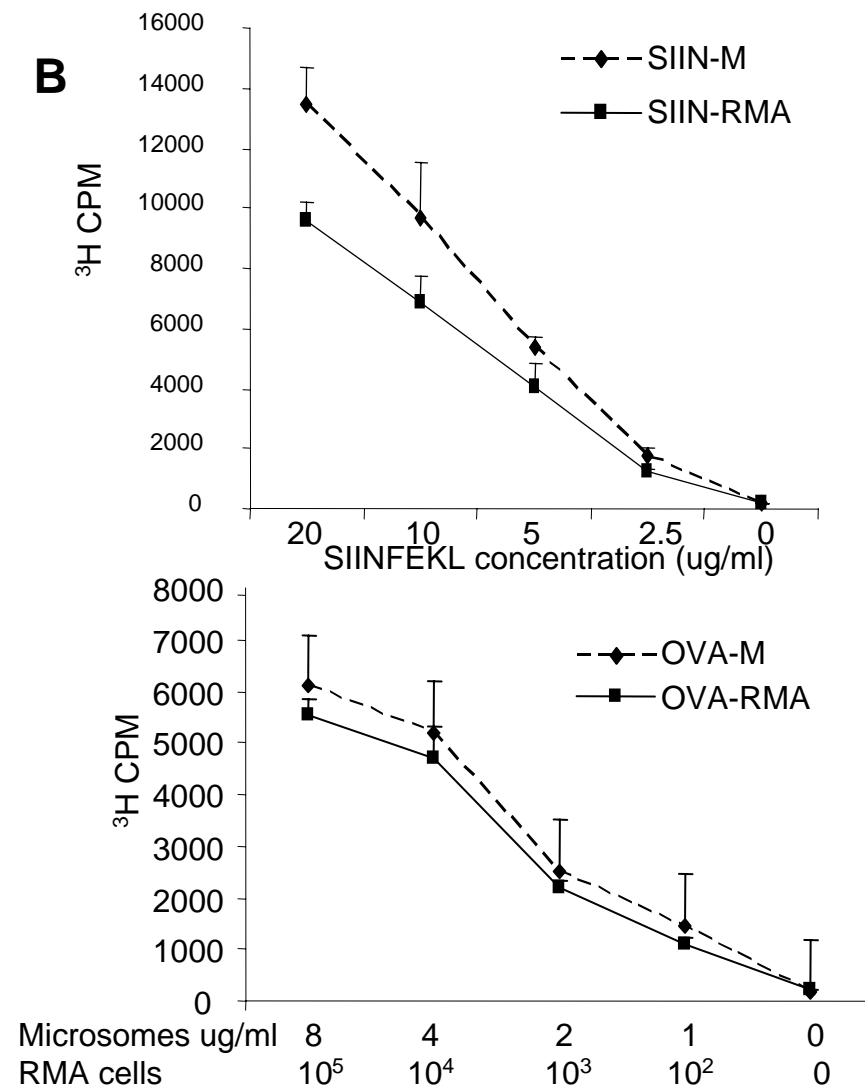e-mail: p.wang@qmul.ac.uk

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Supporting Information for this article is available at www.wiley-vch.de/contents/jc_2040/2009/38443_s.pdf

Supplementary 1. Antigen presentability of microsomes from antigen preloaded and unloaded APCs. Mouse RMA cell line transfected with OVA gene (OVA-RMA) was used as antigen-preloaded APCs, while native RMA cells were used as unloaded APCs. RMA cells and the microsomes from RMA cells were loaded with SIINFEKL in vitro as described in materials and methods, and indicated as SIIN-RMA and SIIN-M, respectively. The microsomes isolated from OVA-RMA cells were indicated as OVA-M. The SIINFEKL-Kb complexes in OVA-M, SIIN-M, and on OVA-RMA and SIIN-RMA were measured by 25D1 antibody (A). The antigenicity of SIIN-Kb complexes was analysed by stimulation of OTI cells (B). The loading, stimulation and 25D1 staining were described in materials and methods. The presented results are representative from three experiments.
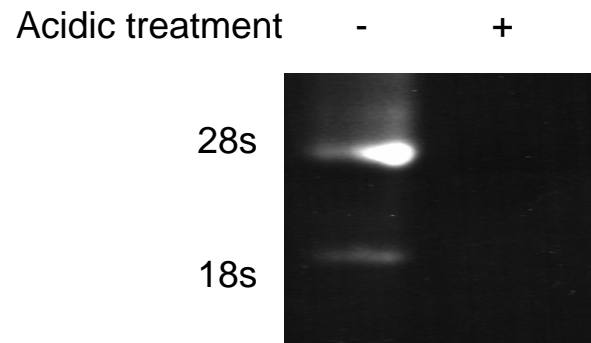
Supplementary data 2. Acidic treatment removes ribosomes from microsomes: Microsomes from DC2.4 cells were treated with or without acidic stripping. Ribosome RNAs were extracted by Trizol reagents (Invitrogen) according to manufacturer's protocol. RNAs were separated on 1% agrose gel. 18S and 28S rRNAs were indicated according to RNA standards (Invitrogen).
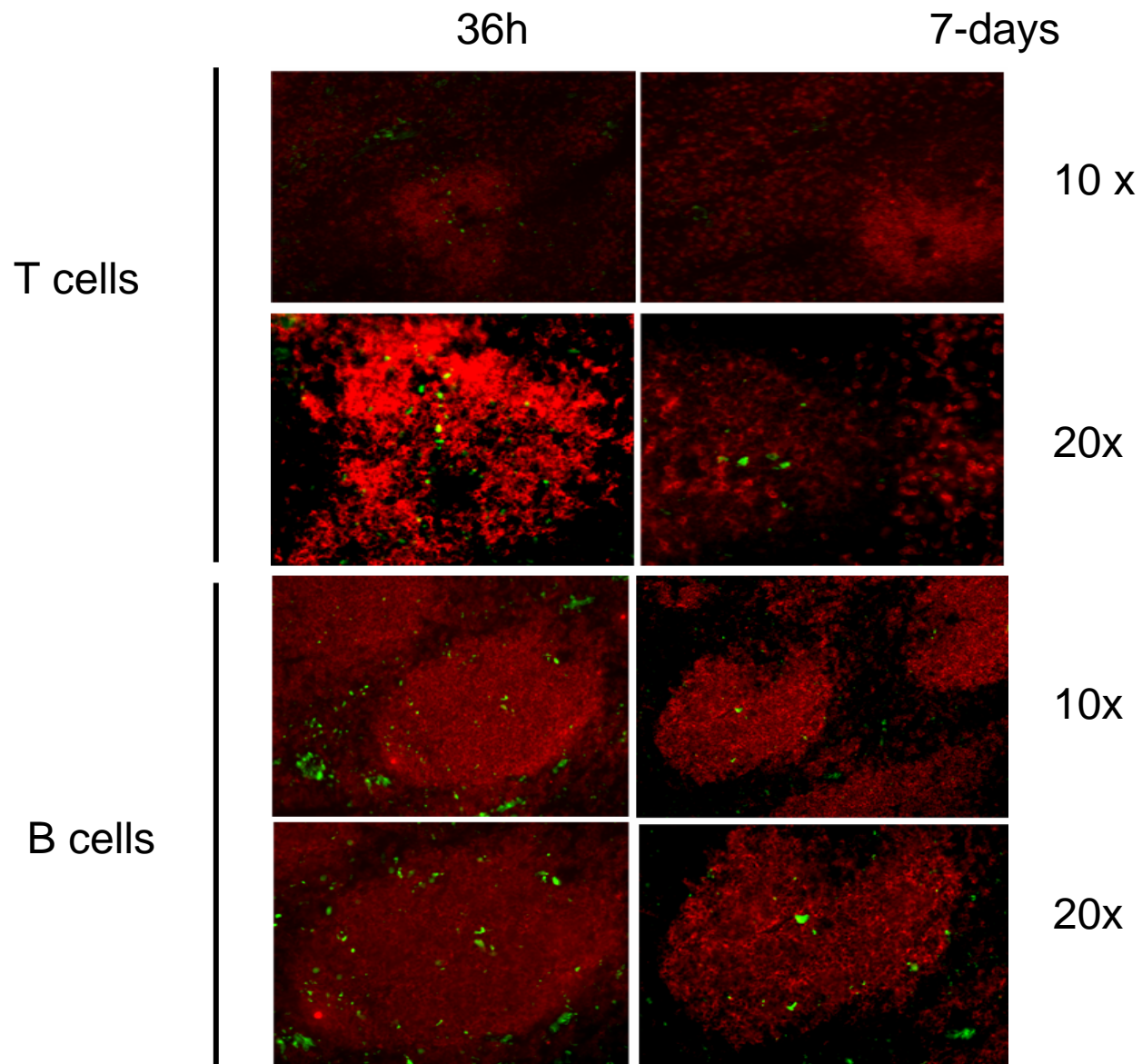
Supplementary 3. Distribution of DCs in spleen. Bone-marrow derived DCs were matured, activated and loaded with SIINFEKL peptide as described in the materials and methods. The loaded DCs were labeled with CFSE and injected i.v. into C57BL/6 mice. Spleens were isolated 36 hours or 7-days post-injection. The frozen sections of the spleens were stained first with anti-CD3 or B220 antibodies followed with Alexa Fluor 594-conjugated secondary antibodies as described in the materials and methods.