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Immunotherapeutics & Vaccine Adjuvants for Cancer

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B.Sc.(Hons), Post.Grad.Dip., M.Sc.(Distinction)

A thesis submitted to the University of Dublin
Trinity College
For the degree of Doctor of Philosophy

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Immune Regulation Research Group
School of Biochemistry and Immunology
Trinity College Dublin

May 2013
This thesis is dedicated to my mother Rosemarie

with all my love and deepest respect.

You are an unforgettable mother, sister, friend and ooooh wise-one.

You truly are my inspiration in life.
DECLARATION OF AUTHORSHIP

This thesis is the sole work of the author and has not been submitted in part or in whole to this or any other university for any other degree. I, the author, agree to deposit this thesis in the University's open access institutional repository or allow the library to do so on my behalf, subject to Irish Copyright legislation and Trinity College Library conditions of use and acknowledgement.

Anna-Maria Corcoran, 16 May 2013
ABSTRACT

It is well established that innate immune responses not only mediate immunity to infection, but also promotes adaptive immunity to pathogens and tumours. Dendritic cells (DC) play a critical role as antigen presenting cells for naïve T cells and also in directing adaptive immune responses, and thus have considerable potential for use in cell based vaccines to prime anti-tumour effector T cells. Indeed, the recently approved DC-based cancer vaccine, Provenge®, has modest therapeutic efficacy against prostate cancer. Pathogen-associated molecular patterns (PAMPs) such as nucleotide-binding oligomerization domain (NOD) agonists and toll-like receptor (TLR) agonists or recombinant cytokines are powerful mediators of innate and consequently adaptive immunity and are thus attractive candidates for use as immunotherapeutics or adjuvants for cancer vaccines. Indeed, the TLR7/8 agonist, imiquimod, is already in clinical use as a topical application for basal cell carcinoma. The aim of this project was to examine novel immunotherapeutic/vaccine approaches that enhance innate and adaptive immunity against murine tumours. This study also set out to identify novel CD4+ T cell epitopes derived from a melanocytic differentiation antigen, tyrosinase-related protein 2 (TRP-2) for use as vaccine antigens. Therapeutic administration of TriDAP (a NOD1 agonist) as a monotherapy significantly delayed the growth of B16.F10 tumour cells in a murine melanoma model, however, it did not induce regression of murine tumours. TLR agonists are potent immune potentiators but have had limited success as adjuvants and monotherapies against cancer in humans. The results of this study demonstrated that the TLR4 agonist, monophosphoryl lipid A (MPL) when used as an adjuvant with a model antigen, keyhole limpet hemocyanin (KLH) promoted antigen-specific T\textsubscript{H}1 response but failed to generate T\textsubscript{H}17 responses. IL-1 is known to play a critical role in driving T\textsubscript{H}17 cell differentiation and expansion \textit{in vivo}. This study found that immunization of mice with KLH in combination with MPL and the recombinant
cytokine IL-1α as adjuvants promoted the induction of antigen-specific T<sub>H17</sub> as well as T<sub>H1</sub> cells. The next step was to examine this combination in a tumour model. Two novel murine TRP-2-derived T helper (T<sub>H</sub>) cell epitopes, TRP-2<sub>58-78</sub> and TRP-2<sub>148-165</sub>, were first identified using a threading approach for predicting peptide-MHC binding, and confirmed by testing ex vivo response to the peptides in mice immunized with the peptides or injected with the B16.F10 tumour cells. Immunization of mice with the newly identified murine TRP-2-derived T<sub>H</sub> epitopes (TRP-2<sub>58-78</sub> and TRP-2<sub>148-165</sub>) and a well known murine TRP-2-derived cytotoxic T lymphocyte (CTL) epitope (TRP-2<sub>180-188</sub>) in combination with MPL and IL-1α as adjuvants delayed tumour growth and increased survival, with some mice exhibiting complete tumour rejection. This study demonstrated that MPL and IL-1α were effective adjuvants to enhance anti-tumour immunity induced by a TRP-2-polyepitope cancer vaccine. These findings have considerable significance for the design of peptide-based therapeutic vaccines for use in human cancer patients.
PUBLICATIONS

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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ATP</td>
<td>Adenosine 5’-triphosphate</td>
</tr>
<tr>
<td>ATCC</td>
<td>American type culture collection</td>
</tr>
<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
</tr>
<tr>
<td>AIBD</td>
<td>Autoimmune inflammatory bowel disease</td>
</tr>
<tr>
<td>APECED</td>
<td>Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone marrow-derived dendritic cell</td>
</tr>
<tr>
<td>BMMΦ</td>
<td>Bone marrow-derived macrophage</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CRB</td>
<td>Cambridge Research Biochemicals</td>
</tr>
<tr>
<td>CARD</td>
<td>Caspase-recruitment domain</td>
</tr>
<tr>
<td>CCR</td>
<td>C-C chemokine receptor</td>
</tr>
<tr>
<td>CXCR</td>
<td>C-X-C chemokine receptor</td>
</tr>
<tr>
<td>CCL</td>
<td>C-C chemokine ligand</td>
</tr>
<tr>
<td>CXCL</td>
<td>C-X-C chemokine ligand</td>
</tr>
<tr>
<td>CD40L</td>
<td>CD40 ligand</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclo-oxygenase-2</td>
</tr>
<tr>
<td>CSC</td>
<td>Cancer stem cell</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T lymphocyte</td>
</tr>
<tr>
<td>CTLA-4</td>
<td>Cytotoxic T lymphocyte antigen-4</td>
</tr>
<tr>
<td>DAP</td>
<td>Diaminopimelic acid</td>
</tr>
<tr>
<td>DC</td>
<td>Dendritic cell</td>
</tr>
<tr>
<td>DHI</td>
<td>DOPAchrome to 5,6-dihydroxindole</td>
</tr>
<tr>
<td>DHICA</td>
<td>DOPAchrome to 5,6-dihydroxindole-2-carboxylic acid</td>
</tr>
<tr>
<td>DISC</td>
<td>Death-induced silencing complex</td>
</tr>
<tr>
<td>DTH</td>
<td>Delayed-type hypersensitivity</td>
</tr>
<tr>
<td>dH₂O</td>
<td>Distilled water</td>
</tr>
<tr>
<td>EAE</td>
<td>Experimental autoimmune encephalomyelitis</td>
</tr>
<tr>
<td>EBD</td>
<td>Effector binding domain</td>
</tr>
</tbody>
</table>
ELISA  Enzyme-linked immunosorbant assay
FACS  Fluorescence-activated cell sorter
FDA  Food and Drug Administration
GATA-3  GATA-binding protein 3
GM-CSF  Granulocyte-Macrophage Colony-Stimulating Factor
HPLC  High performance liquid chromatography
HSP  Heat shock protein
Hs/irr  Heat-shocked and irradiated
HPV  Human Papillomavirus
iDCs  Immature DCs
iBMDM  Immortalized mouse bone marrow-derived macrophage cell line
IKK complex  Inhibitor of nuclear factor-κB (IκB)-Kinase complex
IL  Interleukin
IL-1Ra  Interleukin (IL)-1 receptor antagonist
IC_{50}  Inhibition concentration
IFNGR  IFN-γ receptor
IRAKs  IL-1-receptor-associated kinases
IRF  Interferon (IFN)-regulatory factor
KLH  Keyhole limpet hemocyanin
LPS  Lipopolysaccharide
LCMS  Liquid chromatography mass spectrometry
LN  Lymph node
LRD  Ligand-recognition domain
LRR  Leucine rich repeat
MΦ  Macrophage
MALDI  Matrix-assisted laser desorption ionization
MALDI-TOF  Matrix-assisted laser desorption ionization-time of flight
M-CSF  Macrophage colony-stimulating factor
MHC  Major histocompatibility complex
MPL  Monophosphoryl lipid A
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>MS</td>
<td>Multiple sclerosis</td>
</tr>
<tr>
<td>Mal</td>
<td>MyD88-adaptor-like protein</td>
</tr>
<tr>
<td>MyD88</td>
<td>Myeloid differentiation primary-response protein 88</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-small-cell lung cancer</td>
</tr>
<tr>
<td>NOD</td>
<td>Nucleotide-binding oligomerization domain</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>iNOS</td>
<td>Inducible NOS</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
</tr>
<tr>
<td>NLRP3</td>
<td>NACHT, LRR, and PYD domain-containing protein 3</td>
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<tr>
<td>NY-ESO-1</td>
<td>New York esophageal squamous cell carcinoma 1</td>
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<tr>
<td>OVA</td>
<td>Ovalbumin</td>
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<tr>
<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PAP</td>
<td>Prostatic acid phosphatase</td>
</tr>
<tr>
<td>PND</td>
<td>Paraneoplastic neurological degenerations</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PDB</td>
<td>Protein Data Bank</td>
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<td>PD-1</td>
<td>Programmed cell death-1</td>
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<td>Prostaglandin E2</td>
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<tr>
<td>PGN</td>
<td>Peptidoglycan</td>
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<tr>
<td>PRR</td>
<td>Pathogen recognition receptor</td>
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<td>RAG-2</td>
<td>Recombination activating Gene-2</td>
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<td>RPE</td>
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</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell park memorial institute-1640 medium</td>
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<tr>
<td>SCID</td>
<td>Severe combined immunodeficient</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Transforming growth factor β</td>
</tr>
<tr>
<td>TAKs</td>
<td>Transforming growth factor β-activated kinases</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>TBK1</td>
<td>TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1</td>
</tr>
<tr>
<td>TAA</td>
<td>Tumour-associated antigen</td>
</tr>
<tr>
<td>TSA</td>
<td>Tumour-specific antigen</td>
</tr>
<tr>
<td>TIL</td>
<td>Tumour infiltrating lymphocytes</td>
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<tr>
<td>T&lt;sub&gt;H&lt;/sub&gt;</td>
<td>T helper cell</td>
</tr>
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<td>T helper type 1 cell</td>
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<td>T&lt;sub&gt;reg&lt;/sub&gt;</td>
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<tr>
<td>T-bet</td>
<td>T-box-containing protein expressed in T cells</td>
</tr>
<tr>
<td>TCR</td>
<td>T cell receptor</td>
</tr>
<tr>
<td>TIRAP</td>
<td>TIR-domain-containing adaptor-like protein; also known as Mal</td>
</tr>
<tr>
<td>TRAF6</td>
<td>Tumour-necrosis-factor-receptor-associated factor 6</td>
</tr>
<tr>
<td>TRAM</td>
<td>TRIF-related adaptor molecule</td>
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<td>TRIF</td>
<td>Toll/IL-1R-related domain-containing adaptor-inducing IFN</td>
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Chapter 1
Introduction
1.1 Overview of the Immune System

The immune system is an intricate defence system that has evolved to protect animals from infectious disease. It is composed of a number of different organs, tissues, cell types and molecules that act together in a dynamic network, recognising and eliminating foreign pathogenic organisms, such as bacteria, viruses and parasitic worms. The innate immune system exists in all multicellular organisms; only vertebrates have, in addition, the adaptive immune system. The innate and adaptive systems work together through direct cell contact and through interactions involving chemical mediators, cytokines and chemokines. Moreover, many of the cells and molecules of the innate immune system are also utilised by the adaptive immune system.

The innate immune system, which is present at birth and changes little throughout life, detects the presence and the nature of infection providing the first line of host defence. Innate immune mechanisms act immediately giving rise to the acute inflammatory response, which can be activated by infection, but do not generate immunological memory. Cells of the innate immune system continuously survey peripheral tissues for the presence of aberrant or infected cells, recognising specialised molecules, called pathogen associated molecular patterns (PAMPs) common to many pathogens rather than specific to a particular organism (Janeway and Medzhitov, 2002). An essential function of these innate cells is to alert the adaptive immune system to the presence of infection and to control the initiation and determination of the effector class of the adaptive immune response. Adaptive immune responses generate antigen-specific effector lymphocytes that target the specific pathogen, and memory T and B
lymphocytes that can prevent re-infection with the same organism. Thus, the power of an adaptive immune response resides in its antigen specificity and ability to respond faster to a second encounter with the same antigen, so called immunological memory. However, the generation of antigen specific immune responses is delayed as adaptive immunity generates a random and highly diverse repertoire of antigen receptors encoded by rearranging gene segments to recognise a huge variety of antigens, whereas innate immunity depend upon germline-encoded receptors to recognise features that are common to many pathogens (i.e. PAMPs). Therefore, another essential role of the innate immune system is to control the infection during the interim period between pathogenic challenge and the induction of adaptive immune responses.

Adaptive immunity is mediated by two major types of lymphocytes, B cells and T cells which bear antigen receptors of a single specificity on their surface. The lymphocyte receptor repertoire of mammals is determined by a unique genetic mechanism that operates during lymphocyte development in the thymus and the bone marrow. T cells mature under the influence of the thymus and on stimulation with antigen-processed peptides in association with major histocompatibility complex (MHC), give rise to cellular immunity. B cells mature mainly under the influence of bone marrow and give rise to humoral immunity, which involves production of soluble molecules called immunoglobulins. Only lymphocytes that encounter antigen to which their receptor specifically bind will be activated to proliferate and differentiate into effector cells. This selective mechanism called the clonal selection theory of acquired immunity was first proposed in the 1950s by Macfarlane Burnet (reviewed in (Ada, 2008)). Development of specific immunity is critically dependent on interactions between T cells and antigen presenting cells (APCs) as well as between T and B cells.
1.2 Antigen presenting cells (APC)

Initiation of an adaptive immune response involves phagocytosis or receptor-mediated endocytosis of the invading pathogen followed by processing and presentation of short antigenic peptides derived from the pathogen in association with MHC molecules. This is carried out by APCs of the innate immune system, which provide a primary interface between innate and adaptive immunity. Activation of naïve T cells require recognition of a foreign peptide bound to a self MHC molecule via the T cell receptor (TCR), and simultaneous delivery of a co-stimulatory signal by an APC in association with polarizing cytokine(s) produced by cells of the innate immune system. This is the three signal requirement of T cell activation. Activated T cells then proceed to migrate to the site of infection or tissue injury and either release cytokines which recruit T helper (Th) lymphocytes or activate cytotoxic T lymphocytes (CTLs) that mediate direct cytotoxicity of target cells.

Most nucleated cells have the ability to process and present antigenic peptides in association with MHC class I molecules for recognition by CTLs. This is critical for recognition and eradication of cells infected with intracellular pathogens or transformed cells. In contrast, MHC class II expression is mainly confined to APCs which include dendritic cells (DCs), macrophages and B cells. However, the most potent activators of naïve T cells are mature DCs (Janeway et al., 2005), thought to predominately initiate most T cell responses in the homeostatic state. APCs express a multitude of pattern recognition receptors (PRRs) and cytokine receptors, which enable them to respond appropriately to an inflammatory environment by upregulating their expression of MHC and co-stimulatory molecules and thereby facilitating optimum antigen presentation.
1.2.1 Organization of MHC genes

MHC is a cell surface molecule encoded by a large gene family in all vertebrates. The genes encoding MHC molecules are located on chromosome 6 in humans and chromosome 17 in mice. The human MHC genomic region termed human leukocyte antigen (HLA) comprises several thousand allelic variants (Robinson et al., 2001). The mouse MHC genomic region is termed H2. Diversity of antigen presentation, mediated by MHC class I and II molecules, is attained by the fact that MHC genes are highly polymorphic and have many variants and also several MHC genes are expressed from both inherited alleles.

The MHC gene family is divided into three subgroups—class I, class II, and class III. The genetic organization of the human and mouse MHC is essentially similar in both species (Figure 1.1). In humans, three genes (HLA-A, -B, -C) encode MHC class I heavy chains. An individual can express up to six different MHC class I molecules, as both alleles of each locus are expressed. The murine class I region genes are termed H2-K, -D, -L. Three pairs of MHC class II α and β chains are found in humans, HLA-DR, -DP, and -DQ whereas two sets of genes encode murine class II molecules, H2-IA and H2-IE. MHC class III region encodes a variety of proteins including components of the complement system (such as C2, C4, and B factor) and cytokines such as TNF-α.
Figure 1.1 Maps of the human and mouse MHC loci

The genes encoding MHC molecules are located on chromosome 6 in humans and chromosome 17 in mice. The basic organization of the genes in the MHC locus is similar in humans and mice. Separate regions of the complex code for MHC class I and class II molecules. Several genes within these regions encode each chain. Class II loci are shown as single blocks but each locus consists of several genes. Class III MHC locus refers to genes that encode molecules other than peptide-display molecules. Sizes of genes and intervening DNA segments are not shown to scale.

The structure of class I MHC molecule is formed by an α chain composed of three domains (α1, α2, α3) and the non-MHC molecule β2 microglobulin (encoded on human chromosome 15). The α3 subunit is transmembrane and thus anchors the MHC class I molecule to the cell membrane. The antigenic peptide is bound within the peptide-binding groove, in the central region of the α1/α2 heterodimer (Figure 1.2). The
structure of class II MHC molecule is formed by α and β chains, each having two domains — α1 and α2 and β1 and β2 — each chain having a transmembrane domain (α2 and β2 respectively), anchoring the MHC class II molecule to the cell membrane. The antigenic peptide is bound within the peptide-binding groove, in the central region of the α1/β1 heterodimer (Figure 1.2).

![Figure 1.2 Structure of MHC class I and II molecules](image)

Adapted from (Porcelli, 2005)

Figure 1.2 Structure of MHC class I and II molecules
1.2.2 Dendritic cells

DCs are professional APCs and are unique in their ability to stimulate naïve T cell responses (Janeway et al., 2005) and therefore play a central role in directing and regulating the activation of the adaptive immune response. In addition to their role in inducing protective immunity, DCs in the thymus and peripheral lymphoid organs play a critical role in the induction and maintenance of self tolerance in the steady state (Rossi and Young, 2005). DCs are derived from haematopoietic stem cells in the bone marrow and are present in all mucosal tissues and lymphoid tissues, including bone marrow, the blood, skin, and internal organs. Immature DCs (iDCs) patrol and sample peripheral tissue microenvironments where they capture and process antigens. iDC are characterised by expression of low levels of MHC and co-stimulatory molecules, such as CD80, CD86 and CD40, and consequently are poor at presenting antigenic peptides to T cells. However, they do constitutively express high levels of PRRs associated with antigen capture, for example Toll like receptors (TLRs) and nucleotide-binding oligomerization domain-containing protein-like receptor (NLR) proteins (Akira et al., 2001, Martinon and Tschopp, 2005). Stimuli such as local tissue damage or detection of PAMPs via their expressed PRRs can induce maturation of iDC, typically associated with increased expression of MHC and co-stimulatory molecules and reduced endocytic capacity via down-regulated expression of antigen-uptake receptors (Banchereau and Steinman, 1998). Maturation also initiates migration of DC to peripheral lymphoid organs, where they present antigenic peptides in association with MHC molecules to naïve T cells (Villadangos and Schnorrer, 2007).
internalization, an up-regulation of the costimulatory molecules CD80, CD86 and CD40, morphological changes (e.g. formation of dendrites), cytoskeleton reorganization, secretion of chemokines, cytokines and proteases, and cell surface expression of adhesion molecules and chemokine receptors (Hammer and Ma, 2013). Mature DCs are unresponsive to C-C chemokine ligand 20 (CCL20), CCL5 and CCL3 and become sensitive to CCL19 (Dieu et al., 1998, Sato et al., 2001). Mature DCs also down-regulate cell surface expression of the chemokine receptors Cys-Cys chemokine receptor 1 (CCR1), CCR5, CCR6, and CXCR1 and up-regulate expression of CXCR4 and CCR7 (Dieu et al., 1998, Lin et al., 1998, Sallusto et al., 1998, Sozzani et al., 1998, Yanagihara et al., 1998, Sallusto et al., 1999). The up-regulation of CCR7 promotes responsiveness to CCL19 and CCL21 (Dieu-Nosjean et al., 1999, Caux et al., 2000). Maturation of DCs also induces the production of CCL22, CCL17 (chemokines that attract CCR4-expressing T cells), and CCL18 (Sallusto et al., 1999, Vulcano et al., 2001).

1.2.3 Sensors of infection
The innate immune response is not completely non-specific, but rather is able to discriminate between self and a variety of pathogens. Innate immune recognition is dependent on germ-line encoded proteins termed PRRs (Matzinger, 2002) which recognise highly conserved structural motifs termed PAMPs. These common molecular patterns are highly conserved and invariant among microorganisms of a given class. Escape mutants of these PAMPs are not generated because mutations or loss of PAMPs are either lethal or they greatly reduce the microbes adaptive fitness. Evidently, PAMPs are ideal targets for innate immune recognition and discrimination between self and non-self microbial infection.
The principal functions of PRRs include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signalling pathways and induction of apoptosis (Medzhitov and Janeway, 1997). Secreted PRRs include mannose binding lectin and C-reactive protein. In addition to soluble PRRs, the innate immune system also uses various PRRs that are expressed either on the cell surface, such as the mannose receptor and TLRs, or within intracellular compartments, such as NLR proteins.

1.2.3.1 Toll-like receptors

Originally identified as a component of the developmental pathway in Drosophila, members of the Toll family have emerged as signalling receptors for PAMPs (Lemaitre et al., 1996). TLRs belong to the type I transmembrane glycoprotein receptor family, containing 16-28 extracellular leucine rich repeat (LRR) domains (Matsushima et al., 2007) and a cytosolic Toll/IL-1 receptor (TIR) domain (Rock et al., 1998). Eleven TLRs have been identified in humans while thirteen can be found in the mouse genome, with TLRs 1-9 being conserved between both species (Akira et al., 2006). The expression pattern of TLRs differs: TLR1, TLR2, TLR4, TLR5 and TLR6 are all expressed on the plasma membrane, whereas TLR3, TLR7, TLR8 and TLR9 are expressed in the endolysosomal compartment. Differences are now beginning to emerge in the signalling pathways utilised by different TLRs. Some of these differences arise due to the recruitment of different adaptor proteins to intracellular TIR domains. These adaptor molecules also possess the signature TIR domain which serves as a point of contact between the adaptors and the receptors. They are so called because they function to couple TLRs to downstream signalling cascades and the activation of
transcription factors, which ultimately lead to target gene(s) expression and consequently, the biological outcome. Currently, four positive adaptors have been identified – MyD88 (myeloid differentiation primary-response protein), Mal (MyD88-adaptor-like protein), TRIF (Toll/IL-1R-related domain-containing adaptor-inducing IFN) and TRAM (TRIF-related adaptor molecule) – and one negative adaptor – SARM (O’Neill and Bowie, 2007). SARM is a negative regulator of NF-κB and IRF activation (Carty et al., 2006). The adaptor protein, MyD88, which is recruited by the TIR-domain-containing adaptor-like protein (TIRAP) adaptor protein, is utilized by all TLR receptors, with the exception of TLR3, which transduces its signals mainly through the MyD88-independent pathway. TLR4 is unique in that it also signals through the MyD88-independent pathway. TIR domain-containing adaptors and TLR signalling pathways is shown in Figure 1.3.
Figure 1.3 Involvement of TIR-domain containing adaptors in TLR-signal transduction pathways.

Binding of ligand to the extracellular LRR domains of TLRs causes rearrangement of the receptor complexes and triggers the recruitment of specific adaptor proteins to the intracellular TIR domain. These adaptors, which all have TIR domains, include MyD88, Mal (TIRAP), TRIF, and TRAM. MyD88 mediates the TLR signalling pathway that activates IRAKs (IL-1-receptor-associated kinases) and TRAF6 (tumour-necrosis-factor-receptor-associated factor 6). TRAF6 triggers the activation of TAKs (transforming growth factor β-activated kinases), which initiate a kinase cascade involved in the activation of the IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex), which consists of IKK-α, IKK-β and IKK-γ, and phosphorylation of inhibitory protein IκB. Phosphorylated IκB then dissociates from the complex and is rapidly targeted for ubiquitination and degradation by proteasomes. This pathway is used by TLR1, TLR2, TLR4, TLR5-7, and TLR9 and releases NF-κB from its inhibitor allowing it to translocate to the nucleus and induce expression of inflammatory cytokines. In addition, TAK1 activates the IKK complex and MAP kinases such as ERK1/2, JNK and p38MAPK, leading to AP-1, c-Jun and c-fos activation to trigger inflammatory cytokine gene transcriptions (not depicted in diagram). Mal is essential for MyD88-dependent signalling through TLR2 and TLR4. By contrast, TRIF is essential for the TLR3- and TLR4-mediated activation of the MyD88-independent pathway, with IKK-ε and TBK1 (TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1) mediating activation of interferon (IFN)-regulatory factor 3 (IRF3) downstream of TRIF, which subsequently lead to the production of IFN-β. The fourth TIR-domain-containing adaptor, TRAM, is specific to the TLR4-mediated, MyD88-independent/TRIF-dependent signalling pathway.
1.2.3.2 Nod-like Receptors

Nucleotide-binding oligomerization (NOD) proteins are a family of cytosolic proteins, which comprise: a variable amino-terminal effector binding domain involved in signalling, a centrally located nucleotide-binding oligomerization domain that mediates self-oligomerization, and carboxyl-terminal LRRs that serve as a ligand-recognition domain (LRD). The structural diversity of the effector domains allows NOD proteins to interact with a wide variety of signalling partners and to activate multiple signalling pathways. Recent studies on two members of the NOD family of proteins, NOD1 and NOD2, have shed light on the mechanism of detection of pathogenic bacteria that can avoid TLR-mediated recognition. Peptidoglycan (PGN) is the major constituent of the cell wall of Gram-positive bacteria, while in Gram-negative bacteria, it is found in the periplasmic space. An important difference between Gram-positive and Gram-negative PGN residues is the nature of the third amino acid of the stem peptides. Whereas a lysine residue is found in the third position in most Gram-positive bacteria, *meso*-diaminopimelic acid (DAP) replaces lysine in Gram-negative bacteria (Schleifer and Kandler, 1972). Interestingly, these differences are sensed by NOD1 and NOD2. The minimal structure detected by NOD1 is dipeptide γ-D-glutamyl-*meso*-DAP (iE-DAP) (Chamaillard et al., 2003). In contrast, muramyl dipeptide MurNAc-γ-Ala-D-isoGln (MDP) is the minimal motif sensed by NOD2 (Girardin et al., 2003b). During the bacterial life cycle, PGN is remodelled for cell division and growth which generates small PGN fragments containing these motifs. In addition, lysozyme present in the lysosomes of host phagocytes cleaves the glycan chain of PGN generating muropeptides containing the iE-DAP and MDP structures. However, the exact mechanism of delivery of these muropeptides to the cytosolic receptors NOD1 and NOD2 is still limited. NOD1 and NOD2 physically associate with a common
downstream adaptor protein, RIP2 through homophilic caspase-recruitment domain (CARD)-CARD interactions (Hasegawa et al., 2008). Kobayashi et al. demonstrated that NF-κB activation by NOD1/NOD2 expression was completely abolished in fibroblasts of RIP2"/" mice, and complementation of RIP2"/" fibroblasts with a RIP2 expression vector restored these defects, indicating that RIP2 is essential for NF-κB activation by both NOD1 and NOD2 (Kobayashi et al., 2002). An intermediate region located between its CARD and its kinase domain mediates RIP2 interaction with the regulatory subunit IKKκ, linking NOD1 and NOD2 to the phosphorylation and degradation of IkB-α and activation of NF-κB (Inohara et al., 2000a).

1.3 Antigen processing

Antigen processing is the generation of peptides from an intact antigen involving modification of the native protein by a cell for presentation of the peptide at the cell surface in association with MHC. Endogenous antigens are processed via the proteasome in the cytosol and translocated to the endoplasmic reticulum for peptide loading onto MHC class I molecules and presentation to CD8" T cells, whilst exogenous antigens are processed by proteases in the endosomal pathway, leading to peptide presentation to CD4" T cells in association with MHC class II. However, there is evidence of cross-over of exogenous antigens into the MHC class I-restricted antigen presentation pathway, allowing exogenous antigens to be taken up by APCs and presented on MHC class I (cross-presentation) for priming of CD8" T cells (cross-priming) (Bevan, 1976) to generate CD8" T cell immunity to viral infections and cancer. Signals mediated by type I IFNs (Le Bon et al., 2003), CD4" T cells (Bennett et al., 1997) and TLR-mediated signals (Datta et al., 2003, Schulz et al., 2005b) augment
cross-priming of antigen-specific CD8+ T cells in vitro and in vivo. Three models of cross-priming are currently proposed (reviewed in (Kasturi and Pulendran, 2008)). The canonical model proposes that endocytosed or phagocytosed antigen is exported into the cytosol for degradation into antigenic peptides in the proteasome. The peptides are then transported into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP) complex and loaded on to MHC class I molecules for cell surface presentation. The ER-phagosome fusion model, a revised version of the canonical model, is based on the evidence that ER-associated proteins (e.g. Sec61, TAP, and calnexin) are present in phagosomes. The third model, the early endosome model proposes that cross-presentation occurs in early endosomes in a TAP-dependent (Burgdorf et al., 2008) or TAP-independent manner (Shen et al., 2004). It is now known that cross-presentation of the tumour-specific response by APC is a major mechanism of the developing endogenous immune response; therefore, exogenous proteins can be presented in the context of MHC class I.

1.4 Effector cells of the immune system

1.4.1 T cells

T lymphocytes are bone-marrow derived cells of the adaptive immune system and their site of maturation is the thymus. The definite T cell lineage marker is the TCR, a membrane receptor which recognises peptide sequences presented to the T cell in association with MHC. There are two defined types of TCR: one is a heterodimer of two disulphide-linked polypeptides (α and β); the other consists of γ and δ polypeptides. Most T cells utilize αβ heterodimers, while only a small minority (less than 5%) use γδ heterodimers. The signalling CD3 complex is associated with the TCR on the cell
surface. It is composed of a collection of five polypeptides (γ, δ, ε and ζ). Binding of
the TCR to antigen-derived peptides in association with MHC on APCs triggers CD3
interaction with cytoplasmic tyrosine kinase enzymes and subsequent intracellular
signalling leading to gene transcription. T cells can be subdivided into two
predominant sub-types, based on expression of the accessory molecules CD4 and CD8,
which also contribute to the MHC restriction of the T cell. CD4 molecules interact with
the β2 domain of MHC class II, thus T cells expressing CD4 always recognise antigens
in association with MHC class II molecules and are referred to as helper T (Th) cells.
Conversely, CD8 expressing T cells recognise antigens in association with MHC class I
molecules (i.e. MHC class I α3 domain interaction with CD8 molecules) and are known
as CTLs. Although relatively rare in the circulation (2-4%), γδ T cells are relatively
frequent in mucosal epithelia, where they may play a role in protecting the mucosal
surfaces of the body. In the mouse, different tissues have their own specific subsets of
γδ T cells, each displaying limited TCR diversity (Hayday, 2000). However, in
humans, specific γδ T cell subsets are not as prominent or as restricted in their diversity,
reflecting variability between human and mouse γδ T cell populations. γδ T cells are
not MHC restricted and are thought to respond to a variety of antigens, including non-
peptide antigens, phosphoantigens, heat shock proteins and alkylamines (Hayday,
2000). γδ T cells are thought to function largely like their αβ counterparts, however,
there is evidence to suggest that γδ T cells do not rely on APC for antigen recognition
and additionally they have been demonstrated to efficiently process and present peptide
antigen to αβ T cells (Brandes et al., 2005).
1.4.1.1 Activation of T cells

The necessary requirement for the generation of primary immune responses is the activation of naïve T cells in lymph nodes. A naïve T cell is a T cell that has differentiated in the bone marrow, and successfully undergone the positive and negative processes of central selection in the thymus. A naïve T cell is considered mature, but is distinguished from effector and memory T cells, as it is thought not to have yet encountered cognate antigen in the periphery. The activation lead to a differentiation of naïve T cells, which are characterised by the surface expression of L-selectin (CD62L); the absence of the activation markers CD25, CD44, and CD69; and high levels of the CD45RA isoform of the molecule CD45, into effector and memory T cells, which are responsible for destroying the targets and initiating rapid secondary responses, respectively. Several studies have shown that naïve T cells are more difficult to activate than effector and memory T cells. For instance, naïve T cells require higher concentrations of antigen and are more dependent on co-stimulation than effector/memory cells (Sagerstrom et al., 1993, Dubey et al., 1996, Pihlgren et al., 1996, Croft and Dubey, 1997).

Activation of a naïve T cell requires at least two contact-dependent signals for effective activation. The first signal is received via the interaction of the TCR with an antigenic peptide presented by the appropriate MHC molecule on the cell surface of an APC (Janeway et al., 2005). The second signal is a co-stimulatory signal provided by the interaction of non-polymorphic surface molecules expressed on the T cell and the APC. Signal 2 is transmitted when a concurrent interaction occurs between the CD28 receptor on the T cell and its ligand expressed by the APC, CD80 and CD86 (Linsley et al.,
The combination of these two signals lead to production of a mitogenic cytokine, IL-2, which acts in an autocrine and paracrine manner to promote T cell proliferation (Linsley et al., 1991). TCR occupancy in the absence of adequate co-stimulation fails to induce IL-2 production and lead to clonal anergy. In addition to the requirement for signal 2, further T cell differentiation is directed through reciprocal interactions with other co-stimulatory molecules expressed by both the APC and the T cell, which can also affect the function of the APC itself. For example, activation of T cells results in the upregulated expression of CD40 ligand (CD40L). Ligation of CD40 on the APC by CD40L on the T cell further activates the APC to upregulate the expression of other co-stimulatory molecules, such as 4-1BB ligand (4-1BBL) and OX40 ligand (OX40L) which in turn feed back to the T cell via ligation with 4-1BB and OX40 respectively (Croft, 2003). In addition to providing co-stimulation, APCs may also determine the type of immune response by the expression of chemokines and by the release of pro- or anti-inflammatory cytokines that further direct the differentiation of the effector T cell response (polarising signal 3). This is particularly important for the skewing of T helper responses into a T\(_{H1}\), T\(_{H2}\), T\(_{H17}\) or regulatory T (T\(_{reg}\)) cell response (de Jong et al., 2005).

Antigen-specific T cell responses have primarily been considered in terms of activation signals delivered through the TCR and the co-stimulatory molecule CD28. However, recent studies have demonstrated the importance of inhibitory signals for regulating lymphocyte activation (Tivol et al., 1995). CD28 and its homologue cytotoxic T lymphocyte antigen-4 (CTLA-4) share the same counter-receptors on APCs (CD80 and CD86). However, CD28 and CTLA-4 have opposite effects on T cell activation. While
CD28 is critically important for sustaining T cell responses, CTLA-4 functions to inhibit T cell responses (Krummer and Alison, 1995). The presence of two co-stimulatory receptors which transduce signals resulting in opposing responses allows immunotherapeutic regiments to be designed to either suppress or augment the immune response. For example, one strategy to augment anti-tumor T cell responses is to block CTLA-4-CD80/CD86 interactions. It has been demonstrated that CTLA-4 blockade can enhance T cell responses to tumors in vivo (Leach et al., 1996, Quezada et al., 2006).

1.4.2 The T<sub>H1</sub>/T<sub>H2</sub> paradigm

CD<sup>4</sup><sup>+</sup> T helper cells are so called because of their integral role in facilitating the adaptive immune response in particular in providing help for B cell antibody production. CD<sup>4</sup><sup>+</sup> T cells activated via the TCR secrete a number of different cytokines, which serve to drive and direct other immune cells of both the innate and adaptive immune systems. Classically, effector CD<sup>4</sup><sup>+</sup> T cells have been divided into the T<sub>H1</sub> or T<sub>H2</sub> lineage based on their cytokine profiles (Mosmann and Coffman, 1989). T<sub>H1</sub> cells are defined on the basis of their production of interferon-γ (IFNγ) and are involved in cell-mediated immunity against intracellular pathogens and the activation of CTL responses (Trinchieri, 2003). IFNγ is known to act on many cell types and lead to the upregulation of MHC class I and MHC class II, increasing antigen expression, enhancing macrophage killing of intracellular bacteria and improving recognition and potential killing by CTLs. T<sub>H2</sub> cells are critical for the control of certain parasitic infections by driving humoral immunity through the secretion of IL-4, IL-5 and IL-13, which culminates in the production of antibodies (Coffman, 2006).
IL-12 is the dominant polarizing factor in directing the differentiation of naïve T cells into Th1 cells (Trinchieri, 2003). Th1 differentiation begins with the activation of signal transducer and activator of transcription (STAT)-1 by IFNγ produced by innate cells, including NK cells or by mature Th1 cells. IL-27 has also been demonstrated to activate STAT-1 (Lucas et al., 2003). STAT-1 signalling lead to an upregulation in expression of the transcription factor T-box-containing protein expressed in T cells (T-bet) (Szabo et al., 2000), which potentiates the expression of IFNγ through chromatin remodelling of the IFNγ locus and the IL-12 receptor, whilst simultaneously suppressing Th2 associated factors. This step enables the activation of STAT-4 by IL-12, further enhancing the production of IFNγ and conferring IL-18 responsiveness to cells by inducing the expression of the IL-18 receptor. STAT-4 activation by IL-12 is an important determinant of Th1 development in CD4+ T cells (Jacobson et al., 1995), as STAT-4-deficient mice have defective Th1 development (Kaplan et al., 1996) and IL-12 receptor-deficient mice have defective IFNγ production (Wu et al., 1997). Conversely, activation of T cells in the presence of IL-4 signals through STAT-6 to promote the expression of the transcription factor GATA-binding protein 3 (GATA-3). GATA-3 and c-MAF are required for the chromatin remodelling and direct activation of the Th2 cytokines IL-4, IL-5, and IL-13 for Th2 development (Ho et al., 1998, Ouyang et al., 1998). GATA-3 also negatively regulates expression of factors associated with the Th1 skewing pathway, such as expression of the IL-12 receptor.
1.4.3 Additional T helper subsets

Additional $T_h$ subsets have now been described (Figure 1.4) that display effector functions that are quite distinct from the canonical $T_h1/T_h2$ paradigm and thus, there has been a shift from the $T_h1/T_h2$ paradigm to the $T_h1/T_h2/ T_h17/T_{reg}$ hypothesis, a multi-lineage commitment from the same $T_h$ precursor cells.

1.4.3.1 Regulatory T cells

Regulatory T cells ($T_{reg}$) are unique populations of lymphocytes capable of regulating the immune response (Mills, 2004). The existence of suppressor T cells and their role in the maintenance of immune homeostasis was first proposed in 1971 (Gershon and Kondo, 1971). However, their existence was discredited for sometime due the lack of specific techniques to identify the cells and also because of scientific scepticism regarding the existence of suppression. In the mid 1990s, Sakaguchi et al resurrected interest in suppressor cells by demonstrating that the transfer of lymphocytes depleted of $CD4^+CD25^+$ T cells into athymic mice caused the development of various autoimmune diseases in the recipient mice and that reconstitution with $CD4^+CD25^+$ T cells prevented autoimmune reactions in these mice (Sakaguchi et al., 1995). Several subsets of suppressor, reborn as $T_{reg}$ cells with distinct phenotypes and mechanisms of action have since been discovered. It is now firmly established that there are both natural and diverse populations of inducible (or adaptive) $T_{reg}$ cells (Shevach, 2006). Natural $T_{reg}$ cells develop in the thymus by strong TCR interaction with self peptide (Jordan et al., 2001) and then enter peripheral tissues, where they suppress the activation of other self-reactive T cells. Natural $T_{reg}$ cells constitutively express CD25, however, as this marker is not unique to $T_{reg}$ cells, other markers have been proposed,
including CTLA-4 (Read et al., 2000), and glucocorticoid-induced TNF receptor (GITR) (Shimizu et al., 2002). The transcription factor forkhead box P3 (Foxp3) is critical for the development and function of natural Treg cells (Hori et al., 2003). By contrast, induced Treg cells develop from non-regulatory CD25⁺ T cells in the periphery, independently of the thymus. This extrathymic conversion requires special immunological conditions such as continuous exposure to low dose antigen, exposure to a systemic peripheral antigen or exposure to cytokines, such as transforming growth factor β (TGFβ) or IL-10 (Shevach, 2006). TGF-β- or IL-10-secreting adaptive Treg, known as T helper 3 (Th3) or T regulatory 1 (Tr1) cells respectively, are generated from naïve T cells in the periphery following encounter with antigen presented by immature/semi-immature DCs that have distinct activation status from DCs that promote differentiation of other T helper subtypes. However, suppressor activity is not confined to these well-defined populations of CD4⁺ Treg cells, as CD8⁺, natural killer T (NKT) and γδ T cells can also have Treg cell function. Antigen-activated CD8⁺ γδ T cells has been shown to prevent insulin-dependent diabetes in mice (Harrison et al., 1996) and TGF-β- and IL-10-secreting γδ Treg cells can suppress the anti-tumour activity of CTLs and NK cells (Seo et al., 1999). In addition, NKT cells can secrete regulatory cytokines, including IL-10 (Sonoda et al., 2001).

It is now well characterised that CD4⁺CD25⁺ T cells require stimulation via the TCR in order to exhibit their suppressive effects (Takahashi et al., 1998). Furthermore, much lower concentrations of antigen can stimulate CD4⁺CD25⁺ T cells to exert their suppressive activity than the antigen concentration required for the activation and proliferation of CD4⁺CD25⁺ T cells (Takahashi et al., 1998). Following activation, however, CD4⁺CD25⁺ Treg cells do not require further TCR ligation and inhibit T cell
responses in an antigen non-specific manner (Thorton and Shevach, 2000a). The precise mechanism of the suppressive function of natural and inducible T\textsubscript{reg} cells has not been fully elucidated, but in different experimental models, suppressive activity has been shown to be mediated either through secretion of immunosuppressive cytokines or through cell-cell contact. Inducible populations of T\textsubscript{reg} cells, which include T\textsubscript{R1} cells, T\textsubscript{H3} cells and CD8\textsuperscript{+} T\textsubscript{reg} cells, secrete immunosuppressive cytokines IL-10 and/or TGF\beta. These immunosuppressive cytokines inhibit the cytokine production and proliferation of effector T cells, including T\textsubscript{H1} cells, T\textsubscript{H2} cells and CD8\textsuperscript{+} CTLs. Cytokine-mediated suppression might also operate at the level of the APC, by modulating APC function toward a tolerogenic rather than immunogenic phenotype.

The suppressive mechanisms of natural T\textsubscript{reg} cells is still debated, but there is evidence that cell-cell contact is required (Shevach, 2002). Recent evidence supports a pivotal role of CTLA-4 (CD152) in Foxp3\textsuperscript{+} T\textsubscript{reg} cells for their suppressive activity (Wing et al., 2008, Kolar et al., 2009a). There is also evidence for IL-10 and secreted or cell-surface TGF\beta (Nakamura et al., 2001, Belkaid et al., 2002). Co-transfer of CD4\textsuperscript{+}CD25\textsuperscript{+} T cells can inhibit the development of autoimmune inflammatory bowel disease (AIBD) that is induced by the transfer of CD4\textsuperscript{+}CD25\textsuperscript{-} T cells into immunodeficient mice (Asseman et al., 1999). However, administration of a neutralising IL-10 antibody at the time of transfer, inhibited the protective effects. Furthermore, CD4\textsuperscript{+}CD25\textsuperscript{+} T cells from IL-10-deficient mice failed to prevent AIBD in this model (Asseman et al., 1999). Finally, it has been proposed that T\textsubscript{reg} cells might inhibit pathogenic effector T cell responses by competing for shared resources (Barthlott et al., 2003).
1.4.3.2 T helper type 17 (Th17) cells

More recently, an effector T helper cell population, distinct from Th1, Th2, and Treg cells, that produces IL-17, has been described (Harrington et al., 2005, Park et al., 2005). This new subset of Th cells has been designated Th17 cells. Analogous to STAT4-mediated T-bet in Th1 cells and STAT6-dependent GATA-3 in Th2 cells, Th17 cell development is controlled by transcription factors RORγt (the human ortholog is RORC), RORα (which have synergistic and partially redundant functions), STAT3 and interferon regulatory factor 4 (IRF4) (Ivanov et al., 2006, Brüstle et al., 2007, Harris et al., 2007, Yang et al., 2007, Yang et al., 2008b). Recent studies in humans and mice have identified a critical role for IL-1 in promoting the differentiation of Th17 cells (Acosta-Rodriguez et al., 2007, Wilson et al., 2007, Ben-Sasson et al., 2009, Chung et al., 2009, Ghoreschi et al., 2010, Gulen et al., 2010) but also in the expansion and maintenance of polarized effector Th17 cells (Sutton et al., 2006b, Chung et al., 2009). Importantly, it has been shown that IL-1 signalling induces the expression of RORγt and IRF4, transcription factors identified as critical for Th17 differentiation (Ivanov et al., 2006, Brüstle et al., 2007, Chung et al., 2009). In line with these studies, IL-1RI expression has been reported on naïve and memory CD4+ T cells (Lee et al., 2010). Moreover, both naïve and memory CD4+ T cells up-regulate IL-1RI expression following activation with anti-CD3 and anti-CD28, which mimics TCR engagement and costimulation (Rao et al., 2007, Chung et al., 2009). Furthermore, it has recently been reported that IL-1RI is selectively expressed on Th17 cells (Chung et al., 2009, Guo et al., 2009). Expansion of memory Th17 cells is promoted by IL-1 and IL-23 (Langrish et al., 2005a, Higgins et al., 2006, Sutton et al., 2006a).
Initially, it was unclear whether Th17 cells represented a separate lineage or an IL-17 producing population that diverged from the Th1 lineage at some point during development. However, it has been confirmed that Th17 cells are a unique lineage of Th cells, following the observation that T-bet-deficient mice had no defect in IL-17 production (Park et al., 2005). Moreover, neither T-bet nor GATA-3 were required for the generation of Th17 cells in vivo (Reinhardt et al., 2006). In addition, Park et al demonstrated that addition of anti-IFNγ or anti-IL-4 increased the production of IL-17 by Th17 cells (Park et al., 2005). Furthermore, it has been shown that IFNγ, IL-27, and IL-4 all act directly on CD4+ T cells to suppress Th17 differentiation, but once differentiated, mature Th17 cells acquire resistance to the suppressive effects of these cytokines (Harrington et al., 2005, Batten et al., 2006, Diveu et al., 2009), suggesting that suppression of Th17 cell development by IFNγ, IL-27 and IL-4 is limited to an early stage of the Th17 differentiation process. Collectively, these studies provide firm evidence that Th17 cells are an independent lineage of CD4+ T cells.

Thus, the differentiation of T helper responses can no longer be confined to Th1 and Th2 subsets. Additional subsets have now been described with diverse immunological functions (Figure 1.4) and the potential remains for the identification of further Th differentiation lineages.
Naïve T cells primed by PAMP-activated APC such as DC, can differentiate into T\textsubscript{H}1, T\textsubscript{H}2, T\textsubscript{H}17 or T\textsubscript{R}1/ T\textsubscript{R}3 Treg cells. IL-4 is known to drive the differentiation of T\textsubscript{H}2 cells, which mediate their effects via the anti-inflammatory cytokines IL-4, IL-5, and IL-13. T\textsubscript{H}1 cells differentiate in response to IL-12, and produce IFN\textgamma and IL-2. IL-1 and IL-23 promote the differentiation of T\textsubscript{H}17 cells, which are negatively regulated by IFN\textgamma, IL-4, and IL-27. IL-10 and TGF\textbeta are involved in the differentiation of T\textsubscript{reg} cells, which also secrete IL-10 and TGF\textbeta and can suppress effector T cell responses.
1.4.3.3 T\textsubscript{H}1 cells in anti-tumour immunity

The role of T\textsubscript{H}1 cells in promoting protective anti-tumour immune responses is well established (Nastala et al., 1994, Zitvogel et al., 1996, Aruga et al., 1997, Tsung et al., 1997, Nishimura et al., 1999, Ikeda et al., 2004, Chamoto et al., 2006, Zhang et al., 2007, Wakita et al., 2009). Studies have shown that induction of T\textsubscript{H}1 cells mediate effective anti-tumour immunity following immunization with antigen and certain adjuvants (that are T\textsubscript{H}1 inducers) (Ikeda et al., 2004). Tumour-specific T\textsubscript{H}1 cells can overcome strong immunosuppression in tumour-bearing hosts and induce tumour regression in mice (Nishimura et al., 1999, Ikeda et al., 2004, Chamoto et al., 2006, Zhang et al., 2007, Wakita et al., 2009). IFN\textgamma\ is the signature cytokine produced by T\textsubscript{H}1 cells. IFN\textgamma\ has potent anti-tumour activity against various experimental tumours (Giovarelli et al., 1986, Maekawa et al., 1988, Gansbacher et al., 1990), and it induces MHC class I protein expression on tumour cells, thereby enhancing tumour cell immunogenicity and facilitating tumour recognition and elimination by tumour-specific CTLs (Dighe et al., 1994, Kaplan et al., 1998, Brandacher et al., 2006). Solid tumours require the development of an adequate blood supply (through angiogenesis; formation of new capillaries from pre-existing blood vessels) in order to grow (Folkman, 1990). T\textsubscript{H}1 cells can impair tumour angiogenesis directly by inhibiting endothelial cell proliferation via IFN\textgamma\ or indirectly through the induction of anti-angiogenic chemokines such as chemokine (C-X-C motif) ligand (CXCL)\textsubscript{9} and CXCL\textsubscript{10} (Brandacher et al., 2006). IFN\textgamma\ may also play a direct role in tumoricidal activity by generating inducible nitric oxide synthase (Brandacher et al., 2006).
1.4.3.4 Th17 cells in tumour immunity

While much of the focus in recent years has been on the role of Th17 cells in the pathogenesis of many inflammatory and autoimmune disorders (reviewed in (Iwakura et al., 2008, Tesmer et al., 2008)), there is increasing evidence that Th17 cells can promote protective anti-tumour immune responses. The frequency of IL-17+ T cells infiltrating the tumour was shown to be inversely correlated with the Gleason score in prostate cancer patients, suggesting that Th17 cells mediate an anti-tumour effect in the development of prostate cancer (Sfanos et al., 2008). In addition, increased tumour ascites IL-17 and/or Th17 cells positively predict patient survival in ovarian cancer (Kryczek et al., 2009a). In gastric adenocarcinoma patients, high levels of intratumoural IL-17 expression correlate with increased survival over those with lower IL-17 expression levels (Chen et al., 2011). IL-17-deficient mice show accelerated tumour growth and metastasis in different tumour models, and forced expression of IL-17 in tumour cells was shown to suppress tumour progression (Hirahara et al., 2001, Benchetrit et al., 2002, Kryczek et al., 2009b, Martin-Orozco et al., 2009). Moreover, adoptive transfer of TCR transgenic CD4+ T cells specific for a melanoma differentiation antigen polarized to a Th17 cell phenotype in vitro, was shown to trigger B16.F10 melanoma eradication in immuno-component mice (Muranski et al., 2008). Taken together, these studies provide strong evidence that Th17 cells are involved in tumour immunity and suggest that Th17 cells may be a target cell population for cancer immunotherapy. However, it is well appreciated that Th17 cells and/or IL-17 have also been shown to have pro-tumorigenic functions. Studies in mice showed that the growth of B16.F10 melanoma and MB49 bladder carcinoma is reduced in IL-17−/− mice (Wang et al., 2009) whereas decreased growth of mouse tumor cells (EL4 lymphoma, Tramp-C2 prostate cancer and B16.F10 melanoma) was observed in IL-17R-deficient mice (He
et al., 2010). In hepatocellular carcinoma and non-small-cell lung cancer (NSCLC) patients, the frequency of intratumoral IL-17-positive cells was shown to inversely correlate with patient prognosis (Zhang et al., 2009, Chen et al., 2010). Furthermore, patients with gastric cancer were shown to have a higher proportion of $T_h$17 cells in peripheral blood which was associated with clinical stage (Zhang et al., 2008). Evidence suggests that IL-17 acts as an angiogenic factor (Numasaki et al., 2003, Takahashi et al., 2005). Early studies showed that exogenous IL-17 could promote tumour growth by inducing tumour vascularization, particularly in severe combined immunodeficient (SCID) mice and immune-deficient nude mice (Tartour et al., 1999, Numasaki et al., 2003, Numasaki et al., 2005). Thus, undoubtedly the role of $T_h$17 cells in cancer is highly complex, and it remains controversial whether these cells promote tumour growth or mediate protective anti-tumour immune responses.
1.4.4 Cytotoxic T Lymphocytes

Naïve CD8⁺ T cells differentiate into cytotoxic cells termed CTLs. However, naïve CD8⁺ T cells require more co-stimulatory activity to induce their differentiation into effector cells than do naïve CD4⁺ T cells. This requirement can be met via activation by mature DCs which have high intrinsic co-stimulatory activity and also by the presence of CD4⁺ T cells during the priming of the CD8⁺ T cell, so called CD4 help. CTLs mediate the direct killing of infected host cells expressing peptides derived from pathogens in association with MHC class I molecules. CTLs can also recognise cells expressing altered self-antigens, facilitating the eradication of transformed cells that could potentially be pathogenic, such as tumour cells.

After exposure to antigen in vivo, CD8⁺ T-cell responses proceed through a sequence of developmental events including an initial expansion phase in which a significant number of cytotoxic effectors are generated, and a subsequent contraction phase in which about 90% of these cytotoxic effectors die, leaving a stable population of memory cells capable of mounting a rapid secondary response to antigen (Sprent and Surh, 2002). CTLs use two major mechanisms to kill their targets (i.e. infected host cells/tumour cells): the granule exocytosis pathway and the death receptor pathway (Shresta et al., 1998). In the calcium-dependent granule exocytosis pathway, CTLs secrete perforin and granzymes, in close proximity to the target cell. In the presence of calcium, perforin polymerizes and initiates changes in the target cell membrane, which allows granzymes to pass into the cell (Lowin et al., 1994). Granzymes are serine proteases that can activate caspases, thereby initiating apoptosis in the target cell (Medema et al., 1997). IL-12 and IFNγ act on CTLs to augment their cytotoxicity by inducing expression of perforin and granzymes and by upregulating expression of
adhesion molecules (Trinchieri, 2003). In the death receptor pathway, CTLs express the death ligand CD95L on the cell surface, triggering apoptosis via binding to the death receptor CD95 on the target cell (Li et al., 1998). Engagement of CD95 with CD95L attracts intracellular adaptor proteins which in turn lead to the recruitment of the death-induced silencing complex (DISC) (Kischkel et al., 1995). At the DISC, procaspases 8 and 10 are cleaved to yield the active initiator caspase-8 and -10 (Kischkel et al., 1995). Caspases are specific proteases that cleave intracellular proteins at aspartate residues. Activated initiator caspases then cleave and activate "executioner" caspases, mainly caspase-3, -6 and -7 which initiates an amplifying proteolytic cascade of caspase activation leading to cleavage of death substrates such as lamin A, lamin B, lamin B2, PARP, and DNAPK (Rathmell and Thompson, 1999). This ultimately lead to the biochemical and morphological changes characteristic of apoptosis. There is also cross-talk between the extrinsic apoptosis pathway, which is mediated by membrane death-receptors and the intrinsic apoptosis pathway, with both pathways converging at the activation of downstream caspases. In some cells, mitochondria (which mediate the intrinsic pathway of apoptosis) are used as "amplifiers" of the apoptotic signal. Caspase-8 proteolytically cleaves and activates Bid, a pro-apoptotic Bcl-2 family member. Bid then facilitates the release of cytochrome c and other apoptogenic factors from the intermembrane space of the mitochondria into the cytosol (Zamzami and Kroemer, 2001). Concomitantly, the mitochondrial transmembrane potential drops. In the cytosol, cytochrome c forms a complex with adenosine 5'-triphosphate (ATP), Apaf-1, and procaspase-9, to form the apoptosome, within which the initiator caspase-9 is activated. This activated initiator caspase then induces the subsequent activation of downstream executioner caspases.
1.4.5 Natural Killer (NK) cells

NK cells were initially identified in 1975 as lymphocytes of the innate immune system with the ability to kill tumour cells (Herberman et al., 1975, Kiessling et al., 1975). Since then, NK cells have been shown to recognize and destroy an array of stressed cells (transformed/infected/injured/ antibody-coated cells) and also to secrete cytokines (such as IFNγ) that participate in shaping adaptive immune responses. NK cells, which express the transcription factor E4BP4, are phenotypically defined in humans as CD3−CD56+ lymphocytes, further subdivided into CD56dim CD16bright capable of enhanced proliferation and cytokine production and CD56bright CD16− cells which display more potent cytotoxicity (Caligiuri, 2008). In mice, NK cells are defined as CD3−NK1.1+CD122+. NK cell effector responses to target cells are determined by an integration of signals from cell-surface MHC class I-specific inhibitory receptors and several types of activating receptors; the net balance of activating and inhibitory signals dictating whether an NK cell becomes activated or not. The inhibitory receptors often have immunoreceptor-tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic domains while most activating receptors are associated with immunoreceptor-tyrosine-based activation motif (ITAM) containing adaptors. NK cells use inhibitory receptors to recognize MHC class I molecules (HLA molecules in humans, H-2 molecules in mice) on susceptible target cells, the absence of which triggers their elimination by cytotoxic NK cells, the so called hypothesis of missing self proposed by Kärre (Kärre, 2008). NK cell receptors can be structurally divided into: the killer cell lectin-like receptors (KLR) which includes NKR-P1, CD94-NKG2, and NKG2D receptor families; and immunoglobulin (Ig) superfamily receptors which includes the killer cell Ig-like receptors (KIR), natural cytotoxicity receptors (NCR), and FcγRIII (CD16) (reviewed in (Shegarfi et al., 2012)). Cytotoxicity of NK cells is mediated through
granule exocytosis involving perforin/granzyme target cell lysis; Fas (CD95)/FasL (CD178)-mediated apoptosis; and antibody dependent cell-mediated cytotoxicity (ADCC) via expression of low-affinity Fc receptor, CD16.

The current dogma is that immunological memory is present only in the adaptive immune system. However, this view has been challenged recently as “adaptive” memory-like NK cells have been described (Cooper et al., 2009, Sun et al., 2009, Paust et al., 2010). Lanier and colleagues showed that NK cells bearing the virus-specific receptor Ly49H selectively proliferate and persist in mice for several months after cytomegalovirus infection (Sun et al., 2009). Adoptive transfer of these Ly49H+ NK cells into naïve mice followed by viral challenge resulted in a robust secondary expansion and protective immunity.

1.4.6 Natural killer T (NKT) cells

NKT cells are a subset of T lymphocytes that express a limited array of αβ TCRs and NK cell surface markers. They have been classified into four groups (Kronenberg and Gapin, 2002). The most widely studied group – known as type 1, or invariant NKT (iNKT) cells and type II NKT cells respond to CD1d-expressing cells (i.e. are CD1d-restricted). CD1d, an MHC-like molecule that associates with β2-microglobulin, presents host-derived (self-) and pathogen-derived lipid antigens. iNKT cells are defined by their expression of an invariant TCR-α chain (Vα14-Jα18 in mice; Vα24-Jα18 in humans) paired with TCR-β chains limited in their Vβ usage (Vβ2, 7, or 8 in mice; Vβ11 in humans), and by their ability to recognize α-galactosylceramide (α-GalCer) presented by CD1d (reviewed in (Vivier et al., 2012)). Their developmental
programmes are controlled by transcription factor PLZF (Vivier et al., 2012). iNKT cells release high levels of cytokines and a vast array of chemokines upon activation including IFNγ, TNFα, TGFβ, IL-2, IL-4, IL-10, IL-13, IL-17; allowing these cells to recruit or influence other innate and adaptive immune cells, such as CD4⁺ and CD8⁺ T cells, B cells, NK cells, neutrophils and DCs (reviewed in (Matsuda et al., 2008)). Other iNKT effector functions include perforin/granzyme-mediated cytotoxicity (Matsuda et al., 2008) and CD95/CD178-mediated cytotoxicity (Wingender et al., 2010). There is evidence to suggest that iNKT cells have a role in tumour immunosurveillance in mice (Smyth et al., 2000b). Furthermore, iNKT frequency is decreased in a variety of solid tumours in humans and increased iNKT cell numbers have been associated with a better prognosis (reviewed in (Vivier et al., 2012)).

1.5 Tolerance

The TCR is antigen specific and clonally restricted, generated by random gene rearrangement within the TCR germ-line DNA. This can generate an enormous TCR repertoire, each recognising a distinct peptide motif. The immune system must select and maintain a mature T cell repertoire sufficiently diverse to respond with great sensitivity and specificity to a wide range of foreign antigens, whilst maintaining immunological self-tolerance, which is a state of unresponsiveness to self-antigens. Tolerance in higher vertebrates is induced by exposure to self-antigen and operates largely at the level of T lymphocytes. T cell tolerance is induced in the thymus during early T cell differentiation (central tolerance) and in the secondary lymphoid tissues after export of mature T cells from the thymus (peripheral tolerance) (Goldrath and Bevan, 1999).
1.5.1 Central tolerance and thymic selection

All developing T cells (thymocytes) mature in the thymus, following differentiation pathways that result in the generation of functionally distinct populations of mature T cells that have survived the process of thymic selection. The outcome of thymic selection i.e. the TCR repertoire available for MHC peptide recognition, is governed by the strength of the interaction between the TCR on the developing T cell and self-MHC molecules expressed by thymic epithelial cells presenting peptides derived from self-antigens. Initially, interaction of double positive (CD4⁺CD8⁺) thymocyte TCR and self-peptide-MHC, expressed by cortical thymic epithelial cells (cTECs), provides a survival signal that promotes further progression down the differentiation pathway, thereby positively selecting T cell progenitors that are MHC restricted (Sprent and Kishimoto, 2001, Hogquist et al., 2005). The lack of interaction or weak interaction with self-peptide-MHC lead to thymocyte death by neglect. Positive selection in the thymic cortex, however, also enriches for self-reactive progenitors which must be made self-tolerant to avoid the induction of autoimmunity. Thus, TCR triggering in the cortex, lead to maturation into single positive thymocytes and to upregulated expression of CC-chemokine receptor 7 (CCR7) and other molecules which direct the MHC-restricted progenitors toward the thymic medulla (Hogquist et al., 2005). In the medulla, thymocytes interact with medullary thymic epithelial cells (mTECs), which express self-peptide-MHC. Thymocytes with high affinity TCRs for self-peptide-MHC undergo negative selection via clonal deletion, thereby eliminating potential pathogenic self-reactive T cells. However, there are also mechanisms in the medulla that select for high affinity self-reactive cells and result in differentiation into a regulatory cell phenotype. This positive selection of T_{reg} cell populations in the thymus enables T cells to actively restrain immune responses to motifs that are recognised as self. These
mechanisms exist to prevent autoimmunity, which would arise should self reactive T cells be allowed to populate the periphery. However, given that many tumour associated antigens are derived from aberrantly expressed self antigens, this can have a negative effect on the recognition of transformed cells. More recently, a role in both negative selection and T<sub>reg</sub> cell differentiation has been attributed to thymus-resident DCs and also to DCs that have migrated from the periphery into the thymus (Zanoni and Granucci, 2011).

The capacity of medullary epithelial cells for negative selection and the generation of T<sub>reg</sub> cells has been attributed to their ability to express tissue-specific genes under the control of the transcription factor AIRE (Mathis and Benoist, 2009). AIRE-deficient mice develop organ-specific autoimmunity (Mathis and Benoist, 2009), and mutations in the AIRE gene on chromosome 21p22.3 in humans cause a rare autoimmune disease called autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (Fierabracci, 2010). AIRE functions to induce the ectopic expression of a large set of proteins that are considered to be restricted to peripheral tissues, such as preproinsulin II that is normally restricted to the pancreatic islet beta cells (Anderson et al., 2002). The role of AIRE in the clonal deletion of self-reactive T cells has been confirmed with the use of TCR transgenic mice (Mathis and Benoist, 2009). The role of AIRE as a transcriptional regulator has been confirmed in vivo, through monitoring expression changes in the genes with promoter sequences bound by AIRE protein (Ruan et al., 2007).
1.5.2 Mechanisms of peripheral tolerance

Although many tissue-specific antigens are expressed in the thymus under the control of AIRE and can induce negative selection of self-reactive T cells, some T cells may escape negative selection, including low-affinity self-reactive T cells and T cells specific for tissue-sequestered antigens that do not reach the thymus for presentation. In addition, antigens encountered through diet/environment could elicit pathogenic immune responses if they were not limited in the periphery. Mechanisms of peripheral tolerance have thus evolved to control the activity of the autoreactive T cell repertoire. Clonal deletion of self-reactive T cells has also been reported to occur extrathymically in peripheral lymphoid organs (Hernandez et al., 2001, Liu and Lefrancois, 2004). Furthermore, autoreactive T cells in the periphery chronically engaged by self-peptide-MHC complexes die by apoptosis via Fas receptor engagement by FasL and Bim-dependent triggering of a Bcl-2 and Bcl-xL-regulated mitochondrial death pathway (Mueller, 2010). Thus, for some self-peptide-MHC complexes, peripheral deletion of autoreactive T cells is essential for maintaining tolerance. Evidence is now accumulating that an incomplete form of maturation generates a tolerogenic DC which induces T cell anergy (i.e. functional unresponsiveness) (reviewed in (Mueller, 2010)) and thus represents an important mechanism in the maintenance of peripheral tolerance to self antigens.

Peripherally induced T\(_{\text{reg}}\) cells also participate in, controlling autoreactive T cells (expressing low-affinity TCRs that have escaped thymic negative selection), and limiting immune responses to foreign antigens. Peripherally-induced T\(_{\text{reg}}\) cells can exhibit a long lifespan as resting cells (in an intermitotic stage) independent of the antigen that induced their formation, thus, permitting the induction of cells with the
prospect to suppress anticipated immune responses. Immunosuppression occurs by specific co-recruitment of activated regulatory cells and CD4/CD8 effector cells in a local milieu. It has been shown that in subimmunogenic antigen presentation conditions, antigen-specific T preg cells can be generated from naïve T cells (reviewed in (Daniel et al., 2009)). Conversion into T preg cells has been shown to require an intact TGF-βRII on naïve T cells and depended on IL-2 production by the converting cells (Daniel et al., 2009). Indeed, IL-2 is crucial to maintain functional integrity of T preg cells, since IL-2- or IL-2 receptor-deficient mice lack T preg cells and suffer from autoimmune diseases. Moreover, adoptive transfer of peripheral T preg cells into IL-2-deficient hosts or IL-2 depletion experiments established a nonredundant role of IL-2 signalling for T preg homeostasis in the periphery and lymphoid tissue (DO'Cruz and Klein, 2005, Setoguchi et al., 2005). However, it remains controversial whether IL-2 signalling plays an essential role in T preg generation or only in survival and expansion of T preg cells. In vitro, conversion of naïve peripheral CD4^ T cells into T preg cells can be achieved through TCR engagement in the presence of TGFβ, suggesting that TGF-βRII signalling may play a role in the induction of T preg cells in vivo (Daniel et al., 2009). Moreover, Hill et al provided evidence that retinoic acid indirectly enhances the expression of Foxp3 induced by TGFβ in naïve T cells by inhibiting the release of factors from bystander CD4^CD44^hi T cells (Hill et al., 2008). In addition, retinoic acid has been shown to induce histone acetylation at the Foxp3 promoter, thereby increasing Foxp3 expression stability (Daniel et al., 2009).

It is now realised that active regulation to maintain self-tolerance is not only a property of CD4^ T cells but also involves CD8^ T cells. However, CD8^ suppressor cells still remain less well characterised than Foxp3 T preg cells, because studies have been hindered
by the lack of reliable markers to identify these cells. The mechanisms by which CD8\(^+\) suppressor cells are induced are not clearly understood. Furthermore, it is not yet clear whether different subpopulations of CD8 \(\text{T}_{\text{reg}}\) cells rely on distinct suppressive mechanisms, depending on the context of the immune reaction (Lu and Cantor, 2008, Smith and Kumar, 2008).

1.6 Immunity to Cancer

One molecular hallmark of carcinogenesis is genetic instability, giving rise to multiple mutations and inherited changes in gene expression by epigenesis over a lifetime that allows cells to progress from normal tissue, to pre-malignant dysplastic growths, and eventually to fully malignant tumours. Over-activity of genes that promote cancer growth (oncogenes) or inactivation of genes that restrict tumour growth (tumour suppressors) ultimately lead to these mutations or epigenetic changes. The last few decades of research in cancer biology has focused on identification of mutations acquired in cancer, and determining their role in tumour progression, which are important for disease prevention and therapeutics.

Perhaps the most informative cancer progression model, described as a multistep chain of events, is the colorectal cancer model (Fearon and Vogelstein, 1990). Hanahan and Weinberg famously defined six cell-intrinsic characteristic changes (termed hallmarks) universally found in varying degrees in tumours: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and Weinberg, 2000) (Figure 1.5). However, recent work has shown that
three more hallmarks may also be essential for the development of cancer, namely the ability of cancer cells to acquire stem cell like characteristics to allow self-renewal (or for the inciting mutations to arise in a bona fide stem cell), the so called cancer stem cell model; the ability of cancer cells to evade elimination by the immune system; and inflammation, which has long been known to be a key characteristic of the microenvironment of most neoplastic tissues (Reya et al., 2001, Mantovani, 2009).

Two pathways link inflammation and cancer. In the intrinsic pathway, activation of different classes of oncogenes drives the expression of inflammation-related programmes that instruct the development of an inflammatory microenvironment. In the extrinsic pathway, inflammatory conditions promote cancer development, e.g. colitis-associated cancer of the intestine or gastric malignancy caused by Helicobacter pylori.

Figure 1.5 The hallmarks of Cancer

Taken from (Hanahan and Weinberg, 2000)
In 1909, Paul Ehrlich was the first to conceive the idea of immune system control of neoplastic disease (reviewed in (Dunn et al., 2004)). With the functional demonstration of mouse tumour-specific antigens supporting the ideas of Ehrlich, the “cancer immunosurveillance” hypothesis was proposed by Sir Macfarlane Burnet and Lewis Thomas, which stated that sentinel thymus-dependent cells (speculated to be lymphocytes) continuously recognize and eliminate nascent transformed cells (reviewed in (Dunn et al., 2004)). This concept was then challenged by studies in T cell deficient nude mice, which failed to show an increased incidence of cancer (reviewed in (Dunn et al., 2002)). However, these conclusions were flawed as nude mice retain NK cells, which are not thymus dependent, and γδ T cells, a subset of which may develop extrathymically (Dunn et al., 2002). A landmark study by Shankaran and colleagues invigorated interest in immunosurveillance by demonstrating important anti-tumour roles of IFNγ and lymphocytes (Dunn et al., 2002). In that study, about 50% of mice deficient for the recombination activating gene-2 (RAG-2), involved in T and B cell antigen receptor formation, spontaneously developed malignant tumours of the gastrointestinal tract. Furthermore, RAG-deficient mice also lacking the interferon-responsive signal transducer and activator of transcription-1 (STAT-1) (RkSk mice), not only developed these tumours more rapidly but also developed tumours of the mammary gland, suggesting that multiple non-overlapping mechanisms contribute to immunosurveillance. Additional studies using perforin knockout mice suggest that cytotoxic cells (CTLs, NK and NKT cells) are among the major lymphocyte contributors to immunosurveillance (Kim et al., 2007). Several other immune effector cells/molecules have been knocked out to demonstrate their respective roles in immunosurveillance in mice, including αβ T cells, NK cells, NKT cells, γδ T cells, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and IL-12 (Dunn et
Taken together, these studies confirm a role for both innate and adaptive immune mechanisms in preventing the development of cancer.

Direct evidence for immune surveillance in humans has come from studies of patients with paraneoplastic neurological degenerations (PND), which represents a model of tumour suppression with co-existent autoimmunity. These patients have tumours that are usually clinically undetectable and which ectopically express neuronal proteins, present with symptoms of neuronal degeneration, have high titer antibodies to neuronal proteins, and have circulating T cells in the blood capable of killing histo-compatible cells expressing the neuronal antigens. These processes contribute to autoimmune neuronal degeneration and suppress tumour growth (Albert et al., 1998). Indirect evidence for immune surveillance in humans has come from studies demonstrating infiltration of tumours with immune effector cells, with the extent of T cell infiltration correlating with disease outcomes in many cases, the generation of endogenous immunity to tumour antigens, and the observations that immunosuppressed patients have an elevated risk of developing cancer (reviewed in (Sharma and Browning, 2005, Reiman et al., 2007)). Collectively, the data obtained from both mouse and human studies provide strong support for the existence and physiological relevance of cancer immunosurveillance.

Recent studies have shown that tumours which form in the absence of an intact immune system are more immunogenic than tumours that arise in immunocompetent hosts (Svane et al., 1996, Engel et al., 1997, Shankaran et al., 2001), providing evidence that
the immune system may also promote the emergence of primary tumours with reduced immunogenicity that are capable of escaping immune recognition and destruction. Compelling evidence for this concept, now termed immunoediting, has come from key studies on adoptive T cell therapy in patients with melanoma and also active vaccinations in both murine models and humans (Reiman et al., 2007). In one study, 35 patients with metastatic melanoma were treated with non-myeloablative lymphodepletion followed by the infusion of *ex vivo* expanded autologous tumour-infiltrating lymphocytes (Sarrabayrouse et al.). However, of the 18 patients who demonstrated objective clinical response, five showed evidence of immunoediting with either loss of antigen expression (e.g. MART-1) or MHC class I expression (Dudley et al., 2005). In light of such findings, a broader hypothesis termed, “cancer immunoediting” was developed to encompass the potential host-protective and tumour-sculpting functions of the immune system throughout tumour development (Dunn et al., 2002, Dunn et al., 2004).

Cancer immunoediting is a dynamic process composed of three phases: elimination, equilibrium, and escape. The elimination process represents the original concept of cancer immnosurveillance. During the elimination phase, mechanisms of innate and adaptive immunity serve to patrol peripheral tissues, eradicating abnormal or transformed cells and when it is successful in combating tumour formation, it represents the complete editing process without progression to the subsequent phases. In the equilibrium phase, the host immune system and any tumour cell variants that have survived the elimination process enter into a dynamic equilibrium. This competition between tumour growth and the immune system is the longest of the three processes and can persist for many years. During this period of Darwinian selection, many of the
original tumour cell escape variants are destroyed by host effector mechanisms, but new tumour variants arise carrying a multitude of different mutations that provide them with increased resistance to immune attack. Furthermore, by destroying susceptible cells, the immune system actively selects for these resistant, poorly immunogenic, tumour cell variants. This ultimately leads to the escape phase whereby the immunologically sculpted tumour variants that have acquired insensitivity to immunological recognition and elimination through genetic or epigenetic changes expand in an uncontrolled manner, resulting in clinically observable malignant disease. Thus, cancer immunoediting is an ongoing battle between the host immune system and the tumour, whereby the immune system not only protects the host against tumour development but can also sculpt the immunogenic phenotype of a developing tumour.

1.7 Tumour induced immunosuppression & immune evasion

Growth of tumour cells and cancer progression is associated with the development of mechanisms of evading and inhibiting the immune system (Figure 1.6). Downregulation or abrogation in expression of MHC class I and associated molecules have been reported for a number of aggressive tumours (Chang et al., 2003). Among different molecular mechanisms that lead to altered MHC class I expression are loss of heterozygosity in human chromosomes 6 and/or 15 in which class I heavy chain or β2-microglobulin genes are located (Maleno et al., 2002, Rodriguez et al., 2005) or alternatively mutations effecting these genes (Perez et al., 1999); coordinated downregulation of HLA A, B, or C loci (Mendez et al., 2008); and downregulation of the antigen processing machinery, including TAP and LMP genes (Restifo et al., 1993, Seliger et al., 1997). Total or partial loss of MHC class I molecules in tumour cells
represents a mechanism of tumour escape from CTL recognition. However, as total loss of MHC class I antigens renders the tumour susceptible to attack by NK cells which kill MHC class I-deficient cells – a phenomenon that is part of the “missing self” hypothesis (Kärre, 2008), most tumours selectively downregulate only a single MHC allele (Chang et al., 2003).

In addition to direct evasion of the CTL response, tumour cells can produce immunomodulatory cytokines, such as IL-10 (Kim et al., 1995) and TGFβ (Van Belle et al., 1996). The presence of IL-10 in murine tumours has been reported to promote tumour progression by driving the differentiation of CD4+ T_{reg} cells (Seo et al., 2001). Blockade of the effects of TGFβ on T cells has recently been shown to enhance T cell-mediated anti-tumour immunity (Gorelik and Flavell, 2001, Bollard et al., 2002). In addition to the effects on T cells, studies have also demonstrated that TGFβ in the tumour microenvironment promotes the conversion of CD4+CD25+ T cells into CD4+CD25+ T_{reg} cells (Chen et al., 2003, Liu et al., 2007). Silencing TGFβ mRNA using small interfering RNA (siRNA) enhances anti-tumour immunity by reducing tumour-associated Foxp3+ T_{reg} cells (Conroy et al., 2012).

A further complication is the effects of the immunosuppressive environment created by the growing tumour on DCs infiltrating the tumour or in the draining lymph node. Tumour-associated cytokines, such as VEGF, IL-6 and IL-10 inhibit the functional maturation of DCs (Pinzon-Charry et al., 2005). Most tumour cells express elevated levels of cyclooxygenase-2 (Cox2), an enzyme involved in the synthesis of PGE2 from arachadonic acid. PGE2 interferes with DC function by downregulating the expression
of MHC class I and II molecules and co-stimulatory molecules, CD80 and CD86, thus inhibiting antigen presentation (Pinzon-Charry et al., 2005). PGE2 also inhibits IL-12 production by DCs while increasing IL-10 production (Pinzon-Charry et al., 2005). Zeytin et al. demonstrated in a Cox2 overexpression murine model of spontaneous intestinal neoplasia, that mice fed the Cox2 inhibitor celebrex had a 65% reduction in polyp formation, and in combination with a CEA-poxvirus-based vaccine induced a 95% reduction in polyp formation (Zeytin et al., 2004). Therapeutic administration of DC pulsed in vitro with Hsp70 in the presence of a Cox2 inhibitor NS-398 reduced progression of B16.F10 tumours in mice (Toomey et al., 2008). Moreover, treatment of tumour-bearing mice with an inhibitor of Cox2/PGE2 reduced the frequency of Foxp3+ Treg cells and decreased tumour burden (Sharma et al., 2005). Furthermore, celecoxib, a Cox2 inhibitor, enhanced the efficacy of bacillius Calmette-Guerin (BCG) immunotherapy in a mouse model of urothelial cell carcinoma (Dovedi et al., 2008).

Expression of indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes tryptophan degradation, is another mechanism exploited by tumours to evade the immune system. PGE2 can also upregulate IDO expression in DC. IDO metabolises tryptophan leading to tryptophan starvation and an accumulation of downstream metabolites, such as kynurenine and picolinic acid, which inhibits T-cell proliferation and activation, ultimately leading to T cell apoptosis (reviewed in (Liu and Wang, 2009)). Furthermore, it has been proposed that IDO may play an important role in proliferation and conversion of Treg cells in tumours (Munn and Mellor, 2004) as well as in the activation of pre-existing Treg cells (Sharma et al., 2007). Oral administration of the Cox2 inhibitor celecoxib was shown to enhance the efficacy of a DC vaccine against spontaneous mammary tumours in mice by decreasing IDO expression (Basu et al., 2006).
Tumour cells can develop resistance to immune cell-mediated death by becoming insensitive to apoptotic signals. One strategy tumours use to acquire apoptosis resistance is the overexpression of antiapoptotic molecules. Tumours with elevated expression of the antiapoptotic protein, FLIP\(_I\), were shown to escape from T cell-mediated immunity in a mouse model despite the presence of the perforin/granzyme pathway (reviewed in (Igney and Krammer, 2002)). Moreover, an increased ratio of FLIP\(_I\) to caspase-8 correlated with resistance to CD95-mediated apoptosis in Epstein-Barr virus-positive Burkitt’s lymphoma cell lines (Igney and Krammer, 2002). Tumours can also acquire apoptosis resistance by downregulation or inactivation of proapoptotic molecules. Engagement of CD95 (Fas) by its ligand, CD178 (FasL), normally induces cellular apoptosis. In comparison to their normal counterparts, some tumour cells show a decreased expression of the death receptor, CD95. This has been reported in a number of human tumours (Igney and Krammer, 2002, Durrant et al., 2010). Transcriptional mechanisms may account for the down-regulated expression of CD95 by tumour cells (Igney and Krammer, 2002). Oncogenic Ras appears to down-regulate CD95 (Igney and Krammer, 2002). In addition, loss of CD95 expression was accompanied by p53 aberrations in hepatocellular carcinomas (Igney and Krammer, 2002). In contrast, a variety of tumours have been reported to express CD178 (FasL), which has been proposed as a mechanism by which tumours may induce activation-induced cell death in infiltrating CD95 expressing T cells (Durrant et al., 2010). Deletions and mutations of TRAIL-R1 and TRAIL-R2 (death receptors), have also been observed in tumours (Igney and Krammer, 2002). Tumour cells have also been reported to directly interfere with the perforin/granzyme pathway, resisting CD8-mediated cytotoxicity via overexpression of a serine protease inhibitor that inactivates granzyme B (Medema et al., 2001).
Treg cells are specialised in controlling responses to self antigens and therefore play a significant role in suppression of anti-tumour immune responses. The numbers of natural Treg cells (CD4^CD25^{high}FoxP3^+) are increased in the peripheral blood and populate the tumour microenvironment and draining lymph nodes of patients with different cancers including solid tumours and haematological malignancies (Woo et al., 2001, Liyanage et al., 2002, Ichihara et al., 2003, Sasada et al., 2003, Wolf et al., 2003, Curiel et al., 2004, Marshall et al., 2004, Alvaro et al., 2005, Kawaida et al., 2005, Ormandy et al., 2005, Wang et al., 2005b, Beyer et al., 2006, Yang et al., 2006, Fu et al., 2007). Higher Treg cell frequencies are associated with poor prognosis and decreased survival rates in patients with gastric carcinoma (Ichihara et al., 2003, Sasada et al., 2003), ovarian cancer (Curiel et al., 2004) and hepatocellular carcinoma (Fu et al., 2007). Furthermore, inducible Treg cells, including T_{h3} and Tr1 cells, are generated in the periphery, driven by interactions with DC and other APCs (Durrant et al., 2010).

A number of studies have implicated the existence of antigen-specific Tr1 cells in cancer, secreting high levels of IL-10 but also inhibiting in a contact-dependent manner (reviewed in (Durrant et al., 2010)). In addition, several other cell types with suppressive capability are expanded in cancer patients or have been shown to suppress anti-tumour immunity, including IL-10-secreting CD8^+ T cells, invariant NKT cells and γδ T cells (Conroy et al., 2008). Treg cells contribute to down-regulation of immune activity of effector T cells by a variety of mechanisms including the production of anti-inflammatory cytokines, IL-10 and TGFβ; enzymatic degradation (via expression of ectoenzymes CD39 and CD73) of ATP to immunosuppressive adenosine; modulating DC function to promote the induction of tolerance rather than immunity; or engagement of the Fas/FasL and granzyme/perforin pathways (reviewed in (Vignali et al., 2008)). Treg cell depletion in mouse models of cancer has been shown to improve endogenous...
immune-mediated tumour rejection (Onizuka et al., 1999, Shimizu et al., 1999a, Golgher et al., 2002, Jarnicki et al., 2006) and antigen-specific anti-tumour immunity (Tanaka et al., 2002). CD25 depletion, combined with vaccination and local tumour radiation, led to the elimination of established tumours (Kudo-Saito et al., 2005). However, the promising results from animal models has not translated into clinical success, with the assessment of human anti-Tac (anti-CD25) and ONTAK (receptor-binding domain of IL-2 and diphtheria toxin fusion protein) in clinical trials for the treatment of human cancer demonstrating disappointing results (Foss, 2000, Frankel et al., 2002, Kreitman, 2004, Zou, 2006). A major concern is the lack of specificity with this approach, as CD25 depletion may eliminate both activated effector T cells (which express CD25) and Treg cells, potentially proving more damaging than beneficial. Furthermore, long-term removal of Treg cells raises the possibility of autoimmunity. Other approaches have been assessed for their ability to combat Treg cell-mediated suppression of anti-tumour immunity including anti-GITR- and anti-PD-1-antibody therapies (reviewed in (Conroy et al., 2008)). However, these non-targeted approaches may leave patients vulnerable to autoimmune manifestations, potentially limiting their clinical utility. Ipilimumab, a fully human monoclonal IgG1k antibody against CTLA-4 (an immune-inhibitory molecule expressed in activated T cells and in suppressor Treg cells), was approved by the Food and Drug Administration (FDA) in March 2011 and by the European Commission four months later, as monotherapy for the treatment of advanced (unresectable or metastatic) melanoma in previously-treated patients (FDA and EU approval) or in chemotherapy naïve patients (FDA approval only) (Graziani et al., 2012). The main drawback is the immune-related toxicity of ipilimumab which requires prompt diagnosis and treatment according to product-specific guidelines to
adequately manage the immune-related adverse effects which can be life-threatening (Graziani et al., 2012).

As well as T cells, myeloid cells can also develop into suppressor cells. Myeloid-derived suppressor cells (MDC) are a heterogenous population comprised of macrophages, neutrophils, and DCs. They cells are recruited and activated by various tumour-derived soluble factors such as TGFβ, IL-10, VEGF, GM-CSF, IL-6 and PGE2. They favour tumour growth by suppressing T cell responses via several mechanisms, including production of the enzyme, arginase 1, which lead to increased metabolism of L-arginine (Nagaraj and Gabrilovich, 2008).

![Figure 1.6 Mechanisms by which tumours avoid immune recognition](Taken from (Janeway, 2012))
1.8 Tumour Immunogenicity

Tumour immunogenicity, defined as the ability of a tumour to elicit adaptive immune responses that can prevent its growth, requires that tumour cells express antigens firstly at adequate levels and secondly in a form that leads to immune activation instead of immune tolerance. Identification of tumour antigens has been critically important over the past two decades in tumour immunology and has allowed considerable advances in the field of cancer vaccine. T. Boon and colleagues were the first to identify a tumour associated antigen, which was achieved by epitope analysis of TILs isolated from melanoma patients (van der Bruggen et al., 1991). CTL lines, derived by repetitive \textit{in vitro} stimulation of TIL with autologous tumour cells, were screened by using MHC-matched target cells transfected with a tumour-derived cDNA library. Subsequently, the increased release of cytokines in the supernatant due to the recognition by the tumour-specific CTL clone allowed selection of cells harbouring the antigen-coding cDNA. These were then subcloned and re-screened to finally isolate a single cDNA that was able to serve as a CTL target and the encoded gene was termed melanoma gene, MAGE. This approach was also used for the identification of melanoma antigens tyrosinase (Brichard et al., 1993, Kang et al., 1995), gp100 (Bakker et al., 1994, Kawakami et al., 1995), and Melan-A/MART-1 (Coulie et al., 1994, Kawakami et al., 1994). Most TIL-derived tumour peptides found in recent years are MHC class I-restricted; however, a melanoma antigen recognised by a HLA-DR1-restricted CD4\textsuperscript{+}TIL has been identified (Wang et al., 1999).
The pioneering work by Boon and colleagues has led to the identification of numerous tumour antigens recognized by T cells (Boon et al., 1994, Wang and Rosenberg, 1996), and the list continually accumulates (van der Bruggen et al., 2012). Some of these antigens are expressed exclusively by tumours as a product of mutations (mostly point mutations), which lead to a modified peptide sequence, and hence thus called tumour-specific antigens (Chen et al.). Cancer-germline genes, such as members of the melanoma antigen (MAGE) A, B or C families and LAGE families, encode tumour-specific antigens because, normally, only male germline cells (spermatocytes and spermatogonia) express cancer-germline genes, but these cells do not present the cancer germline gene products as antigens to T cells due to lack of expression of HLA molecules. Tumourigenic viruses (e.g. human papilloma virus and Epstein–Barr virus) can encode antigenic peptides presented by tumour cells. Finally, overexpressed (compared with normal cells) nonmutated proteins (e.g. p53, Her2/neu) and the expression of tissue-specific genes in tumour cells, notably in melanoma and prostate carcinoma, such as differentiation antigens present on normal melanocytes as well as melanomas (e.g. Tyrosine-related protein-2 (TRP-2), tyrosinase, gp100) may all contribute to the presence of tumour-associated antigenic (TAA) peptides.

1.9 Immunotherapy for Cancer

Immunotherapy for cancer is fast becoming the fourth therapeutic modality alongside surgery, radiotherapy and chemotherapy. Tumour immunotherapy can be classified as (a) active, represented by vaccines, aimed at eliciting a specific immune response against TSAs and TAAs; (b) passive, consisting of administration of anti-tumour antibodies to the patient; or c) adoptive, consisting of the collection of cells, such as
lymphocytes or APCs, from a patient (a process called leukapheresis), manipulation/stimulation of the cells *in vitro* followed by reinfusion into the patient. To date, six monoclonal antibodies, a prophylactic vaccine, and a therapeutic vaccine have been approved by the FDA for cancer therapy.

1.9.1 Cancer Vaccines

Active immunization, known as vaccination, is the most successful application of immunological principles to human health and has been widely applied to prevent many infectious diseases. Vaccination is based on the induction of long-term persistence of vaccine-induced immunity, namely immunological memory. The principle of vaccination rests on an initial artificial encounter of an antigen/immunogen with the immune system, which generates immunological memory in order to combat a second encounter with an invading pathogen harbouring the same antigen/immunogen. The concept of stimulating the immune system to combat cancer originated over a century ago when William Coley treated advanced sarcoma patients with streptococcal extracts which ultimately lead to a clinical immune response against the tumour and the success of the first cancer vaccine (reviewed in (Hoption Cann et al., 2003)). It is now known that the components of the bacterial extract stimulated the immune system in a non-specific way, explaining the success of Coley’s toxin. Significant progress in anti-tumour vaccination was made by Klein and colleagues in 1960, who demonstrated that mice could be immunized against a lethal dose of their own methylcholanthrene-induced carcinoma, which consequently lead to the rejection of the carcinoma cells in the immunized mice upon re-challenge (Klein et al., 1960).
Compared to all other standard modalities (surgery, chemotherapy, radiotherapy, and passive immunotherapy), an effective vaccine-based immune response against the tumour may be the only cancer treatment with the potential to last a lifetime. Vaccination strategies are either prophylactic, aimed at preventing disease or therapeutic, aimed at resolving established disease. Prophylactic vaccines have been successful in preventing virally-induced cancers (Paavonen et al., 2007), but have a very limited application as cancer is a multi-stage disease driven by genetic instability, generating a multitude of unpredictable mutations and resultant heterogeneous cell phenotypes. Therefore it is preferable to commence vaccination once the cancer has developed. The goal of active vaccination using TSAs or TAAs is to induce a strong T cell response, against the immunizing antigen and thereby against the tumour cells expressing the antigen. As demonstrated in murine models, antigen-specific CTLs play a pivotal role in anti-tumour immunity. However, tumour antigen-specific CD8$^+$ T cells in cancer patients and experimental animals have failed to eliminate antigen-expressing tumour cells, indicating that there mere existence in the tumour microenvironment is not sufficient for the rejection of established tumours (Wick et al., 1997, Prevost-Blondel et al., 1998). Tumour antigen-specific T cells must not only receive the first signal required for optimal T cell activation (i.e., TCR interaction with the MHC class I/class II-tumour-peptide complex) in situ, but they must also receive the appropriate co-stimulatory molecule interactions and the correct growth/effector polarizing cytokine molecules (e.g., IL-12 for $T_{h1}$ polarization, IL-1 plus IL-23 for $T_{h17}$ polarization, and not immunosuppressive IL-10 and TGFβ) to differentiate into functional cytotoxic/helper effector cells rather than anergic cells, which sustain a state of self tolerance to the tumour. Traditional vaccines often contain many components that can elicit additional T cell help for CTL responses or function as adjuvants e.g. bacterial
DNA or LPS in whole cell vaccines. However, these components have been eliminated from new generation tumour vaccines (which are based on the subunit and multi-epitope approach, with selection of a specific tumour-associated antigen target), which, therefore, require potent adjuvants. Thus, in the very recent past, research has largely focused on the development of immunological adjuvants which can be used in combination with a specific antigen to enhance the immune response to vaccine antigens and/or overcome immunosuppressive factors within the tumour environment.

1.9.1.1 Tumour-cell based vaccines

Whole tumour cells or tumour cell lysate represent the richest source of tumour rejection antigens for the development of versatile cancer immunotherapy. Theoretically, tumour-cell vaccines possess at least three advantages over a single-target approach: (a) the immune response is not HLA-restricted (i.e. they can be used for patients with any HLA-type); (b) many different and unknown tumour antigens can be targeted in the one vaccine; (c) the variety of both MHC class I and class II epitopes processed could potentially stimulate both an innate (NK cells, macrophages) and adaptive (CD8^+ and CD4^+ T cells) immune response. The first distinction is between vaccines using autologous (patient-specific) or allogeneic (non-patient specific) tumour cells. Many tumour antigens arise due to somatic mutations in normal gene products and are thus patient-specific. Immunizing with autologous tumour cells derived from patient tumour samples does not require identification and isolation of these mutant gene products for each individual patient. However, there is difficulty associated with obtaining sufficient tumour tissue for the preparation of vaccines for each individual patient (Copier and Dalgleish, 2006). Alternatively, vaccines can be derived from
allogeneic whole cells and generic immortalised cell lines, although these vaccines lack
the patient specific mutant gene products. These autologous or allogeneic tumour cells
may be unmodified, modified for expression of MHC molecules, co-stimulatory
molecules, or cytokines, or used in combination with adjuvants. Alternatively, these
cells can be used in the form of tumour-cell lysates (Copier and Dalgleish, 2006).

In the past two decades, several different vaccines derived from whole tumour cells or
tumour-cell lysates have been evaluated in preclinical models and clinical trials.
Vaccination with irradiated autologous tumour cells engineered to secrete GM-CSF
have been trialled in patients for the treatment of prostate cancer (Simons et al., 1999)
and non-small cell lung carcinoma (Salgia et al., 2003). Encouraging clinical and
immunological responses in phase I and II clinical trials involving an allogeneic vaccine
called GVAX (Cell Genesys), which is composed of two irradiated human prostate
cancer cell lines, LNCaP and PC-3, modified by \textit{ex vivo} transduction with an
adenoassociated viral vector encoding the human GM-CSF gene (Simons et al., 2006,
Small et al., 2007, Higano et al., 2008), lead to the initiation of a phase III trial in 626
patients with prostate cancer. However, in 2008, Cell Genesys terminated the trial
based on results by the independent data monitoring committee, which indicated a
\(<30\%\) chance that GVAX would meet the defined endpoint of improvement in overall
survival (Cell Genesys, 2008). Recently, a randomised phase III trial of an allogeneic
melanoma cell lysate vaccine with IFNa reported that there was no survival advantage
of the vaccine compared with IFNa treatment alone (Mitchell et al., 2007). The main
drawback of both autologous and allogeneic whole-cell tumour vaccines, therefore,
appears to be the limited ability to stimulate immune responses in patients (Ribas et al.,
2003).
1.9.1.2 Peptide vaccines

Epitope-based vaccines have two main advantages over the use of tumour cells or lysates: (a) production, storage, and distribution are faster and more cost-effective, and (b) the identification and administration of well defined antigens is preferable since tumour-cell preparations mostly contain self-proteins which are potentially capable of generating an autoimmune response. Furthermore, well defined antigens allow for post-vaccination immune response monitoring via techniques such as HLA/epitope tetramer assay, and ELISPOT assay. Tumour cells/lysates carry multiple antigens which make immune monitoring much more complicated than evaluating only one CTL type, which is specific for the epitope. Phase I clinical trials have been carried out using synthetic peptides corresponding to defined tumour antigens. Immunization of 39 chemoresistant stage IV melanoma patients with MAGE-3.AI peptide provided encouraging results with 7 out of 25 Immunized patients demonstrating an objective clinical response (Marchand et al., 1999). However, no evidence for CTL responses were detectable in the blood of the four patients analysed, including two who displayed complete tumour regression. In contrast, Rosenberg et al immunized 31 metastatic melanoma patients with the modified g209-2M peptide in incomplete Freund's adjuvant along with IL-2 and observed tumour regression in 13 of 31 patients (42% ) receiving the peptide vaccine plus IL-2 (Rosenberg et al., 1998). Peripheral blood mononuclear cells harvested after, but not before, immunization exhibited a high degree of reactivity against the native g209-217 peptide and against HLA-A2\(^{+}\) melanoma cells. These studies indicate that vaccination with synthetic peptides is well tolerated. However, the use of epitope-based vaccines has certain drawbacks: (a) there is poor immunogenicity of a single epitope; (b) peptides may be proteolytically degraded at the site of immunization in vivo; (c) tumours can easily escape immune recognition through
antigen mutation or loss; (d) their use is HLA-restricted and limited to the subset of patients matching the HLA allotype (usually HLA-A2+).

To date, most epitope-based vaccines induce HLA-A2-restricted responses that efficiently kill tumour cells, but the magnitude of responses are generally low and short lived in the absence of CD4+ helper T cells. Theoretically, the addition of CD4+ T cell help would allow the generation of lasting immunity. Knutson and colleagues illustrated a more successful vaccine strategy for generating peptide-specific CTLs capable of lysing tumour cells expressing HER-2/neu, involving immunizing patients with T-helper epitopes of HER-2/neu which had, embedded in the natural sequence, HLA-A2 binding motifs of HER-2/neu (Knutson et al., 2001). Thus, both CD4+ T cell help and CD8+ specific epitopes were encompassed in the same vaccine. Patients developed both HER-2/neu-specific CD4+ and CD8+ T cell responses, which were detectable for more than one year after the final vaccination in selected patients. In addition, the peptide-specific T cells were able to lyse tumour cells. These results demonstrated that HER-2/neu MHC class II epitopes containing encompassed MHC class I epitopes were able to induce long-lasting HER-2/neu-specific IFNγ-producing CD8 T cells. Thus, some of the drawbacks to this immunization strategy could be overcome by the use of longer peptides or the combination of several different epitopes in the same vaccine, while the relatively poor immunogenicity of peptides could necessitate that they be administered in combination with adjuvants (such as cytokines and/or TLR agonists) or alternatively loaded onto DCs or other peptide delivery systems such as liposomes.
1.9.1.3 Dendritic cell-based vaccines

DCs play an essential role in the initiation of the specific immune response, including antigen-specific CTL response and anti-tumour immunity, and hence vaccines targeting these cells should be effective (Nencioni and Brossart, 2004). Targeting mature DC has been attempted both ex vivo and in vivo. Ex vivo DC have been expanded and pulsed with a variety of antigens including peptides, proteins or tumour lysates (Celluzzi et al., 1996, Nestle et al., 1998, Liau et al., 1999). In mice, immunization with DCs pulsed with peptides derived from mutant p53, inhibited the growth of established tumours (Mayordomo et al., 1996). Protective immunity against tumour challenge has also been reported in mice vaccinated with DCs pulsed with whole tumour cell lysates (Nair et al., 1997). Induction of anti-tumour immunity with tumour RNA-transfected DC represents another approach for DC-based vaccines. RNA preparations for transfection of DC can be transcribed from cDNA (Boczkowski et al., 1996) or can be extracted from tumour cells (Liu, 1998). Vaccination of mice with MUC1 RNA-transfected DC was associated with induction of a MUC1-specific CTL response and resulted in the rejection of MUC1-positive tumours (Koido et al., 2000).

A number of clinical trials have been carried out in patients using DC based cancer vaccines. Nestle et al immunized 16 metastatic melanoma patients with peptide- or tumour lysate-pulsed DCs (Nestle et al., 1998). Four of the 16 patients whose HLA haplotype was inappropriate for peptide pulsing received DC pulsed with autologous tumour lysate. Immunological monitoring revealed delayed-type hypersensitivity skin reactions to peptides in eleven patients, and peptide-specific CTLs could be recovered from the skin biopsies of some patients. Regression of tumour was observed in five
patients, including two complete responses lasting over 15 months. Disappointingly, a more recent Phase III clinical trial in melanoma failed to show a significant survival advantage for patients immunized with peptide-pulsed DC over standard chemotherapy with Dacarbazine (Schadendorf et al., 2006). Clinical trials employing autologous PBMC pulsed with vaccine for the treatment of prostate cancer have provided more encouraging results. Sipuleucel-T (Provenge®) is an immunotherapy product consisting of autologous PBMC (isolated by leukapheresis) pulsed with a prostatic acid phosphatase (PAP)-GM-CSF construct which is designed to stimulate T cell immunity against PAP (Small et al., 2006, Schellhammer et al., 2009). An integrated analysis of 225 patients in two randomized, double-blind, placebo-controlled phase III clinical trials, D9901 and D9902A (147 patients in the vaccines arm and 78 patients in the placebo arm), demonstrated a statistically significant 4.3 month improvement in overall survival (23.2 months versus 18.9 months, respectively), with a 33% reduction in the risk of death (Higano et al., 2009). This was associated with an eightfold improvement in the induction of antigen-specific T cell responses following immunization with the PAP-loaded cell-based vaccine compared with the placebo control (Small et al., 2006). Provenge® has been approved by the FDA as the first therapeutic vaccine for the treatment of established tumours.

Several studies have demonstrated that the use of adjuvants in combination with DC expressing tumour antigens can improve the efficiency of DC-based vaccines (Pulendran, 2004, Jarnicki et al., 2008, Marshall et al., 2012). Mills group have recently demonstrated that the adjuvant combination of the TLR5 agonist, flagellin, and a PI3 kinase inhibitor significantly enhanced antigen-pulsed DC vaccine efficacy against B16.F10 melanoma tumours, with a subset of immunized mice rejecting the
tumour and resisting a re-challenge with the B16.F10 tumour cells (Marshall et al., 2012).

1.9.1.4 DNA based vaccines

Historically, Wolff and colleagues were the first to demonstrate that long-term gene expression in mouse skeletal muscle could be achieved with direct intramuscular injection of naked plasmid DNA (Wolff et al., 1990). This significant finding lead to the first vaccination studies, which utilize plasmid DNA to protect mice against challenge with influenza (Ulmer et al., 1993) and HIV-1 (Wang et al., 1993). Cell-mediated and humoral immune responses have been demonstrated following injection of naked plasmid DNA vaccines into the dermis or muscle tissue of mice (Ulmer et al., 1993, Raz et al., 1994), with such responses conferring protective immunity in a range of models of infectious disease (reviewed in (Donnelly et al., 1997)). Immunization of mice with a DNA vaccine encoding human CEA, which is overexpressed in human colon, breast and non-small-cell lung cancer, elicited CEA-specific T cell responses and protected mice from a subsequent challenge with a syngeneic CEA-expressing tumour cell line (Conry et al., 1995). More recently, immunization with plasmid DNA encoding the E7 tumour antigen from human papillomavirus (HPV) was found to delay tumour growth and significantly reduce metastasis of E7-positive tumours in a murine model (Cheng et al., 2002). It has been proposed that plasmid DNA-based vaccines possess intrinsic T<sub>H</sub>1-inducing adjuvant properties due to the presence of unmethylated CpG sequences (i.e. TLR9 ligand) (Krieg et al., 1998), which mediate production of T<sub>H</sub>1 inducing cytokines via interaction with TLR9 (Hemmi et al., 2000). However, the importance of TLR9 for activation by plasmid DNA has been diminished recently, by
the finding that TLR9-deficient mice are capable of responding to DNA vaccines (Spies et al., 2003), indicating the involvement of additional signalling pathways. Clearly, the mechanisms underlying DNA vaccine-induced immunity are complex and have yet to be fully elucidated.

Plasmid DNA vaccines are relatively easy to synthesise and administer compared with other vaccine modalities. In addition, multiple or multi-gene vectors encoding several antigens and immunomodulatory molecules can be delivered as single administration. Furthermore, studies with DNA vaccines have shown that even after multiple immunizations, anti-DNA antibodies are not produced (Smith, 2000), and there have been no reported adverse toxicities associated with transfection of cells in vivo (Stevenson et al., 2004). However, DNA vaccines have shown low immunogenicity when tested in human clinical trials, and they induce weak immune responses compared with traditional vaccines. This has prompted the development of strategies to increase the efficacy of DNA vaccines. One strategy to enhance the potency of DNA vaccines is the insertion of genes encoding biologically active molecules, such as cytokines, chemokines and co-stimulatory molecules into the DNA plasmid backbone. Co-immunization with cytokine adjuvants has been reported to improve the efficacy of DNA immunization in animal models (Barouch et al., 2004). An alternative strategy to improve the immunogenicity of DNA vaccines involves the use of heterologous prime boost vaccination regimens (McConkey et al., 2003). Enhancing the mode of DNA delivery has also been investigated as a method to improve the immunogenicity of DNA vaccines. Recently, electroporation of plasmid DNA to muscle has been tested in the clinical setting and may hold promise to enhance the immunogenicity of plasmid DNA (Low et al., 2009).
1.10 Vaccine Adjuvants

Traditional vaccines such as bacterial toxoid and attenuated viral vaccines often contain many PAMPs and, therefore, are sufficiently potent to induce protective immune responses. However, the trend in vaccine development away from traditional whole-cell or virus vaccines to new generation subunit vaccines has shown that isolated antigens are poorly immunogenic and thus require the addition of potent adjuvants or delivery systems to boost immune responses following immunization. Immunological adjuvants are substances used in combination with a specific antigen that produces a more robust immune response than the antigen alone. A key issue in adjuvant development is toxicity, which has undoubtedly restricted their clinical use in the past. However, the level of toxicity acceptable for an adjuvant to be used in a therapeutic vaccine, particularly if the vaccine is designed to treat established tumours, is likely to be higher than for a prophylactic vaccine designed for use in healthy individuals.

Adjuvants can be classified into different groups based on their principal modes of action, depending on whether or not they have direct immunostimulatory effects on APC/immune effector cells, or function as antigen delivery systems.

1.10.1 The use of TLR agonists and cytokines as cancer vaccine adjuvants

Patients with cancer, especially those with an established or advanced disease, are usually immunosuppressed and most tumour antigens are ‘self-antigens’, making it a significant challenge to vaccinate patients against a tumour antigen. Various immunostimulatory adjuvants are therefore under investigation in an effort to boost the immune system to overcome tolerance to tumour associated self-antigens and to counteract immune suppressing cytokines and regulatory cells within the tumour.
environment. TLR agonists have shown favourable potential for enhancing immune responses by their ability to activate maturation and promote inflammatory cytokines, including IL-12, from DCs, and thereby promote IFNγ-secreting T_{H1} cells, NK cells, and CD8^+ CTLs (Agrawal et al., 2003b, Liu et al., 2005, Jasani et al., 2009). Indeed, the TLR7/8 agonist, imiquimod, is already in clinical use as a topical application for basal cell carcinoma (Beutner et al., 1999). Moreover, BCG has been used to treat bladder cancer. Furthermore, the immunostimulatory activity of DNA vaccines has been associated with the prokaryotic-derived portion of the plasmid, which contains unmethylated CpG motifs that signal through TLR9 to promote the activation and maturation of DCs (Liu et al., 2005). An alternative to the use of cytokine inducing adjuvants such as TLR agonists, is the use of cytokines directly.

Cytokines are small proteins that act in an autocrine or paracrine manner. They bind to specific receptors, with individual cytokines having multiple functions. Several cytokines have the ability to enhance immune responses by either (a) promoting the differentiation, activation, or recruitment of APC, thereby enhancing antigen presentation and activation of antigen-specific T cell responses; or (b) by directly affecting the T cell compartment by promoting T cell proliferation, activation, and effector function. A specific cytokine can be incorporated into a vaccine either as a recombinant protein or as a gene. In DNA immunization, the antigen gene and the cytokine gene can be delivered within the same plasmid or alternatively two distinct plasmid coding the antigen and the cytokine molecule can be co-injected, whereas in protein vaccines, cytokines can be given as recombinant proteins in addition to the vaccine. Several cancer vaccines administered with cytokine adjuvants, particularly IL-12, GM-CSF and IL-2, have had modest clinical success (Rosenberg et al., 1998,
Bendandi et al., 1999, Osanto et al., 2000, Peterson et al., 2003) and thus it is important to identify and evaluate new cytokine adjuvants that can augment the immunogenicity and therapeutic efficacy of cancer vaccines.

1.10.1.1 Interleukin-1-alpha (IL-1α)

The IL-1 family consists of two agonistic proteins, IL-1α and IL-1β, and one antagonistic protein, the IL-1 receptor antagonist (IL-1Ra), which bind to the IL-1 receptor without transmitting an activation signal (reviewed in (Apte and Voronov, 2002)). Mononuclear cells are potent producers of IL-1α and IL-1β, whereas nonphagocytic cells secrete low levels of IL-1β. IL-1β is active in its secreted form (17.5 kDa), whereas IL-1α is mainly active as an intracellular precursor (31 kDa) or as a membrane-associated form (23 kDa), but is only marginally active as a secreted molecule (17.5 kDa). The precursor of IL-1α (proIL-1α) is processed by calpain, a Ca²⁺-dependent protease that cleaves the proIL-1α into the mature form, whereas the cysteine protease, caspase-1, cleaves the inactive precursor of IL-1β. IL-1α and IL-1β bind to the IL-1 receptors (IL-1R), which belong to the immunoglobulin superfamily. These receptors are abundantly expressed on many cell types. IL-1RI is a signalling receptor, whereas IL-1RII serves as a decoy receptor, acting to reduce excessive IL-1. IL-1Ra is used as a therapy for autoinflammatory diseases and has been evaluated for use in autoimmune disease (Mills and Dunne, 2009). The observation that IL-1α deficiency suppressed development of arthritis in the type II collagen-induced arthritis (CIA) model (Saijo et al., 2002b) indicates that IL-1α can promote immunity to self antigens which immunologically resemble the self antigens that are often the target of endogenous anti-tumour immunity (Pardoll, 1999). Indeed, in vitro activation of
malignant T lymphoma cells to transiently express IL-1α in the cytosol and on the surface membrane (induced by treating the cells with accessory cells/mitogens) was shown to be sufficient to induce tumour regression and led to the development of a protective immune memory (Voronov et al., 1999). Treatment of a rat non-small cell lung cancer with an adenovirus expressing genes of IL-1α and IL-3, induced growth retardation (Esandi et al., 1998). These anti-tumour effects also result from transient expression of IL-1α, as adenovirus is immunogenic and is thus eradicated in mice after 1-2 weeks (Esandi et al., 1998). However, despite encouraging preclinical data, relatively few clinical trials have been performed with IL-1 in cancer patients. In a phase II clinical trial in patients with metastatic melanoma, IL-1α injected intravenously together with indomethacin induced marginal responses in patients with cutaneous metastases, while no responses were observed in patients with visceral metastases (Janik et al., 1996). In other series of clinical trials in cancer patients, IL-1 was shown to have little anti-tumour activity against melanoma, renal cell carcinoma and other malignancies (reviewed in (Veltri and Smith, 1996)). In light of the problems associated with systemic toxicity of IL-1, attempts have been made to administer IL-1 locally. Patients with low-volume metastatic colorectal carcinoma were treated in a phase I trial with a vaccine consisting of four allogeneic colon carcinoma cell lines (DLD-1, HCT116, WiDr, and T84) mixed with an adjuvant (DETOX) and IL-1α (Woodlock et al., 1999). IL-1α was administered subcutaneously (0.3-0.5 μg/m² per d for 8 d) after each vaccination in an outpatient setting. The vaccine plus subcutaneous IL-1α was administered safely with some evidence of in vivo effects. Further investigation of IL-1α-based immunotherapy of cancer is warranted. IL-1α or IL-1β may have an additional role in anti-tumour immunity via induction of T cell responses.
in particular T\textsubscript{H}17 cells. IL-1\textalpha and IL-1\textbeta promote the induction and expansion of T\textsubscript{H}17 cells and to a lesser extent T\textsubscript{H}1 cells (Sutton et al., 2006a).

Overall, the use of TLR agonists and/or cytokines as vaccine adjuvants to enhance the immune response to a tumour is a very promising and active field of investigation.
1.11 Project Hypothesis

This project addressed the hypothesis that NLR and TLR agonists were effective immunotherapeutics against murine tumours and that the efficacy of the TLR4 agonist, Monophosphoryl lipid A (MPL) as an adjuvant for a TRP-2 polyepitope-based vaccine could be enhanced by co-administration with recombinant cytokine IL-1α.

1.12 Project Aims

I. Evaluate the potential anti-tumour activity of NOD1 agonist, TriDAP against the poorly immunogenic murine B16.F10 melanoma.

II. Determine the effectiveness of a combination of a TLR4 agonist, MPL and the cytokine, IL-1α as adjuvants for a whole cell tumour vaccine or peptide tumour vaccine.

III. Evaluate the effectiveness of murine TRP-2 derived peptides (TRP-2_{180-188}, TRP-2_{58-78}, and TRP-2_{148-165}) as tumour-associated antigens in the murine B16.F10 melanoma model.

IV. Determine the effectiveness of MPL and IL-1α as adjuvants for co-administration with murine TRP-2-derived peptides (TRP-2_{180-188}, TRP-2_{58-78}, and TRP-2_{148-165}) in the B16.F10 melanoma model.
Chapter 2
Materials & Methods
2.1 Materials

2.1.1 Reagents used for tissue culture

2.1.1.1 Ammonium chloride lysis solution

0.87% (w/v) NH₄Cl dissolved in endotoxin free distilled water (dH₂O) and filter sterilized (stored at 4°C). Cell suspensions isolated from bone marrow or spleen are resuspended in 2 mls heated (37°C) ammonium chloride lysis solution for 2 min to lyse red blood cells.

2.1.1.2 Cell culture medium

Complete rosswell park memorial institute (RPMI)-1640 Medium

500 ml RPMI-1640 medium (Biosera)
10% Heat inactivated (56°C for 30 min) Foetal Calf Serum (FCS; Biosera)
100 mM L-Glutamine (Gibco)
100 μg/ml Penicillin/Streptomycin (Gibco)

Complete RPMI-1640 (cRPMI) medium was used to culture murine bone marrow derived dendritic cells (BMDC), and human monocyte-derived DC (MoDC) in vitro.

Complete dulbecco’s modified eagles medium (cDMEM)

500 ml DMEM medium (Sigma)
10% Heat inactivated FCS (Biosera)
100 mM L-Glutamine (Gibco)
100 μg/ml Penicillin/Streptomycin (Gibco)
cDMEM was used to culture the following cell types in vitro:

a) Immortalized mouse bone marrow-derived macrophage (iBMDM) cell line
   (kind gift from A. Bowie, Trinity College Dublin)

b) B16.F10 tumour cell line (American Type Culture Collection; ATCC)

c) B16.F10 cell line transfected with cDNA encoding chicken ovalbumin (OVA)
   (kind gift from Dr. Edith Lord, University of Rochester). To maintain OVA expression, the OVA-expressing B16.F10 cells (B16-OVA) were cultured in Geneticin (G418; 400 μg/ml).

**Serum-free X-Vivo 15 medium**

500 ml X-Vivo 15 (Lonza)

100 mM L-Glutamine (Gibco)

100 μg/ml Penicillin/Streptomycin (Gibco)

10^{-3}M 2-βMercaptoethanol (Sigma-Aldrich) (added for murine T cell stimulation assays only)

Serum free X-Vivo 15 medium was used in all in vitro BMDC/BMMΦ stimulation assays and in all in vitro/ex vivo T cell assays.

2.1.1.3 Freezing medium

45% Heat inactivated FCS (Biosera)

45% DMEM medium

10% Dimethyl sulfoxide (DMSO; Sigma-Aldrich)
2.1.2 PRR Agonists

<table>
<thead>
<tr>
<th>Ligand</th>
<th>Receptor</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPL</td>
<td>TLR4</td>
<td>0.001-50 μg/ml (<em>in vitro</em>) 0.01 - 100 ng/ml (<em>in vivo</em>)</td>
<td>Enzo Life Sciences</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10 μg or 25 μg /mouse (<em>in vivo</em>)</td>
<td></td>
</tr>
<tr>
<td>LPS</td>
<td>TLR4</td>
<td>0.01 - 100 ng/ml</td>
<td>Alexis Biochemicals (now integrated into Enzo Life Sciences)</td>
</tr>
<tr>
<td>CpG</td>
<td>TLR9</td>
<td>1-10 μg/ml</td>
<td>Sigma Genosys</td>
</tr>
<tr>
<td>Pam3-CSK</td>
<td>TLR2/TLR1 heterodimer</td>
<td>1-10 μg/ml</td>
<td>InvivoGen</td>
</tr>
<tr>
<td>Poly:IC</td>
<td>TLR3</td>
<td>25-100 μg/ml</td>
<td>InvivoGen</td>
</tr>
<tr>
<td>TriDAP</td>
<td>NOD1</td>
<td>1-100 μg/ml (<em>in vitro</em>) 100 μg/mouse (<em>in vivo</em>)</td>
<td>InvivoGen</td>
</tr>
</tbody>
</table>

2.1.3 Materials used in Immunization studies

<table>
<thead>
<tr>
<th>Antigen/Adjuvant</th>
<th>Concentration</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>KLH</td>
<td>2-20 μg/ml (<em>in vitro</em>) 20 μg/mouse (<em>in vivo</em>)</td>
<td>Calbiochem</td>
</tr>
<tr>
<td>MPL</td>
<td>10 μg or 25 μg /mouse</td>
<td>Enzo Life Sciences</td>
</tr>
<tr>
<td>Recombinant murine IL-1α (carrier free)</td>
<td>100 ng/mouse (<em>in vivo</em>) 100 ng/ml (<em>in vitro</em>)</td>
<td>R&amp;D Systems</td>
</tr>
<tr>
<td>OVA</td>
<td>100 μg/mouse (<em>in vivo</em>)</td>
<td>Hyglos</td>
</tr>
<tr>
<td>LPS</td>
<td>10 μg/mouse (<em>in vivo</em>)</td>
<td>Alexis Biochemicals (now integrated into Enzo Life Sciences)</td>
</tr>
<tr>
<td>OVA&lt;sub&gt;257-264&lt;/sub&gt;</td>
<td>20 μg/ml (<em>in vitro</em>)</td>
<td>Gift from Hidde Ploegh, Massachusetts Institute of Technology</td>
</tr>
<tr>
<td>OVA&lt;sub&gt;323-336&lt;/sub&gt;</td>
<td>20 μg/ml (<em>in vitro</em>)</td>
<td>New England Peptide</td>
</tr>
</tbody>
</table>
2.1.4 Tyrosine-related protein-2 (TRP-2) peptides

All peptides were synthesized by Cambridge Research Biochemicals and were dissolved in either DMSO or phosphate buffered saline (PBS).

<table>
<thead>
<tr>
<th>Name</th>
<th>Peptide Sequence</th>
<th>Source Antigen</th>
<th>T cell epitope</th>
<th>Solubilization</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>TRP-2 180-188</td>
<td>SVYDFVWL</td>
<td>Murine TRP-2</td>
<td>CD8</td>
<td>100% DMSO</td>
<td>91%</td>
</tr>
<tr>
<td>TRP-2 58-78</td>
<td>Ac-RGQCAEVQTDR</td>
<td>Murine TRP-2</td>
<td>CD4</td>
<td>100% PBS</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>PWSPYILR-NH₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TRP-2 148-165</td>
<td>Ac-AKSIHPDYVIT</td>
<td>Murine TRP-2</td>
<td>CD4</td>
<td>100% PBS</td>
<td>95%</td>
</tr>
<tr>
<td></td>
<td>QHWLG-NH₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- Cambridge Research Biochemicals (CRB) supplied the peptides as freeze-dried materials. Freeze-dried peptides were stored at -20°C. Peptides in solution were stored at -80°C.

- The peptides were purified by HPLC with a small amount of trifluoroacetic acid (TFA) as moderator and thus the peptides following HPLC purification were TFA salts. However, TFA can be toxic to cells at high concentrations. Thus, all three peptides were supplied as chloride salts (i.e. TFA counter-ion exchange with chloride request made to Cambridge Research Biochemicals).

- For peptides TRP-2 58-78 and TRP-2 148-165: the N-terminus of the peptide was acetylated (Ac) and the C-terminus was amidated (NH₂), as this would maintain the same charges of the synthetic peptides as in the native protein sequence (recommendation by Dr. Marc Devocelle, Senior Lecturer in Chemistry, Royal College of Surgeons in Ireland).
Figure 2.1 Mass Spectrophotometry purity data by HPLC for TRP-258-78 peptide (CRB Ref)

Samples were analyzed by Matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry under positive ionization using cyano-4-hydroxy-cinnamic acid as matrix. The peptide crude was purified by reversed-phase high-performance liquid chromatography (HPLC) using C18-Ace columns. Fractions were analyzed and pooled above desired purity.
Figure 2.2 Mass Spectrophotometry purity data by HPLC for TRP-2148-165 peptide (CRB Ref 29012)

Samples were analyzed by MALDI-TOF under positive ionization using cyano-4-hydroxy-cinnamic acid as matrix. The peptide crude was purified by reversed-phase HPLC using C18-Ace columns. Fractions were analyzed and pooled above desired purity.
Samples were analyzed by MALDI-TOF under positive ionization using cyano-4-hydroxy-cinnamic acid as matrix. The peptide crude was purified by reversed-phase HPLC using C18-Ace columns. Fractions were analyzed and pooled above desired purity. Liquid chromatography mass spectrometry (LCMS) analysis carried out (as shown in Figure 2.4) to provide final peak (circled in red).
Matrix-assisted laser desorption ionization (MALDI) showed multiple peaks due to lack of charge on peptide (LCMS analysis confirmed that main peak (i.e. 1197.34) was desired product). MALDI showed sodium adduct which is frequent when peptides lack basic groups.
2.1.5 Enzyme-linked immunosorbant assay (ELISA) reagents

PBS (20 X)

320g Sodium Chloride (NaCl, 1.4M)

46g Sodium hydrogen phosphate (Na$_2$HPO$_4$, 0.08M)

8g Potassium dihydrogen phosphate (KH$_2$PO$_4$, 0.01M)

8g Potassium chloride (KCl, 0.03M)

Dissolved in 2 litres of dH$_2$O and adjusted to pH 7.4

Wash buffer

500 ml 20X PBS pH 7.0

5 ml Tween 20 (Made up to 10 litres with dH$_2$O)

Phosphate citrate buffer

20.23g Citric Acid (C$_6$H$_8$O$_7$)

73.8g di-Sodium hydrogen orthophosphate dodecahydrate (Na$_3$HPO$_4$.12H$_2$O)

Made up to 2 litres with dH$_2$O, pH 5.0 (stored at 4°C)
**Substrate solution**

1. o-Phenylenediamine dihydrochloride (OPD) 10 mg tablet (Sigma)

25 ml Phosphate Citrate Buffer

7 μl Hydrogen Peroxide (H₂O₂; Sigma)

**Stop solution (1M H₂SO₄)**

26.74 ml 18M H₂SO₄

473.26 ml dH₂O

**Carbonate buffer**

4.2g NaHCO₃

1.78g Na₂CO₃

Dissolved in 500 ml dH₂O, pH 9.5 (stored at 4°C)

**2.1.6 Flow cytometry**

**FACS buffer**

2% FCS

0.1% Sodium azide

Made up in 500 ml 1X PBS. Stored at 4°C.
Blocking

Anti-CD16/CD32 (Fcγ Block; BD Pharmingen)

Table 2.1 Fluorochrome-labeled Antibodies

<table>
<thead>
<tr>
<th>Surface Marker</th>
<th>Clone</th>
<th>Fluorochrome</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3ε</td>
<td>500A2</td>
<td>V500</td>
<td>BD Horizon</td>
</tr>
<tr>
<td>CD4</td>
<td>RM4-5</td>
<td>V450</td>
<td>BD Horizon</td>
</tr>
<tr>
<td>CD8α</td>
<td>53-6.7</td>
<td>APC-eFluor 780</td>
<td>eBioscience</td>
</tr>
<tr>
<td>γδT-cell Receptor</td>
<td>GL3</td>
<td>PE</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>NK1.1</td>
<td>PK136</td>
<td>PerCP Cy5.5</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD25</td>
<td>PC61.5</td>
<td>PE-Cy7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>FoXP3</td>
<td>FJK-16s</td>
<td>APC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>IFNy</td>
<td>XMG1.2</td>
<td>PE-Cy7</td>
<td>eBioscience</td>
</tr>
<tr>
<td>IL-10</td>
<td>JES5-16E3</td>
<td>APC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>IL-17A</td>
<td>eBio17B7</td>
<td>FITC</td>
<td>eBioscience</td>
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<td>M5E2</td>
<td>FITC</td>
<td>BD Pharmingen</td>
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<tr>
<td>CD209/DC-SIGN</td>
<td>DCN46</td>
<td>PerCP Cy5.5</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD11c</td>
<td>B-ly6</td>
<td>PE</td>
<td>BD Pharmingen</td>
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<tr>
<td></td>
<td>N418</td>
<td>PE-Cy7</td>
<td>Biolegend</td>
</tr>
<tr>
<td>CD40</td>
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<td>APC</td>
<td>eBioscience</td>
</tr>
<tr>
<td>CD80</td>
<td>16-10A1</td>
<td>PE</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>CD86</td>
<td>GL1</td>
<td>FITC</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td>MHC Class II (I-A/I-E)</td>
<td>M5/114.15.2</td>
<td>PE</td>
<td>BD Pharmingen</td>
</tr>
<tr>
<td></td>
<td>M5/114.15.2</td>
<td>Pacific Blue</td>
<td>Biolegend</td>
</tr>
</tbody>
</table>
2.1.7 Magnetic Cell Sorting (MACS)

**MACS buffer**

- 2% FCS
- 5 ml 25mM Ethylenediaminetetraacetic acid (EDTA)

Made up in 500 ml 1X PBS. Stored at 4°C.

**EDTA (25mM)**

0.93g EDTA (Sigma) dissolved in 50 ml dH₂O.

2.1.8 REAL TIME RT-PCR PRIMERS

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Gene</th>
<th>Ref.Sequence</th>
<th>Product Code</th>
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<tr>
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<td>18S</td>
<td>X03205.1</td>
<td>4319413E</td>
</tr>
<tr>
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<td>IL27</td>
<td>NM_145636.1</td>
<td>Mm00461164_m1</td>
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<tr>
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<td>Nos2</td>
<td>NM_010927.3</td>
<td>Mm01309898_m1</td>
</tr>
<tr>
<td>IL-1RI</td>
<td>IL1rl</td>
<td>NM_001123382.1</td>
<td>Mm00434237_m1</td>
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</tbody>
</table>
2.2 Methods

2.2.1 Animals

Specific pathogen-free C57BL/6 (H-2^b) or C3H/HeJ (H-2^k) mice were purchased from Harlan UK Ltd. (Bicester, Oxon, UK) and bred in-house. Animals were maintained according to the regulations and guidelines of the Irish Department of Health and children. Experiments were performed under license from the Irish Department of Health and with the approval of Trinity College Dublin BioResources Ethics committee. All mice used were female 6-12 weeks old at the initiation of each experiment.

Table 2.3 MHC alleles expressed by mouse strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Appearance</th>
<th>Haplotype</th>
<th>K</th>
<th>D</th>
<th>L</th>
<th>IA</th>
<th>IE</th>
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<tbody>
<tr>
<td>C57BL/6</td>
<td>black</td>
<td>Kb</td>
<td>Db</td>
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<td>^</td>
<td>1Ab</td>
<td>^</td>
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<tr>
<td>C3H/HeJ</td>
<td>agouti</td>
<td>Kk</td>
<td>Dk</td>
<td></td>
<td>^</td>
<td>1Ak</td>
<td>IEk</td>
</tr>
</tbody>
</table>

C57BL/6 strain is null (') at L and it has the I-Eb<^b> allele but it is null for the I-Ea<^> allele (The Jackson Laboratory, 2012, ProImmune, 2012)

2.2.2 Tumour challenge model

The B16.F10 tumour cell line was maintained in cDMEM medium. Growing cells were harvested by trypsinization. The viability of cells was determined by trypan blue dye exclusion. To establish local tumours, 2 x 10^5 B16.F10 melanoma cells were subcutaneously (s.c.) inoculated into the left hind flank of C57BL/6 mice. Mice were routinely monitored for tumour growth. Tumour size was measured in two dimensions
by calipers and determined by the following formula: \((\text{width}^2) \times \text{(length)} \times n/6\), where width is the lesser value. Mice were sacrificed when tumour length measured >15 mm or when tumours displayed severe ulceration. In certain experiments, C57BL/6 mice were challenged with \(2 \times 10^5\) B16-OVA cells.

2.2.3 Tumour protection and therapy

In prophylactic experiments, C57BL/6 mice were immunized into the left hind flank with heat-shocked and irradiated (hs/irr) B16 cells \((1 \times 10^6/\text{mouse})\) with or without MPL (10 µg), with or without IL-1α (100 ng) twice at a 4-week interval. Seven d after the boost, mice were challenged with \(2 \times 10^5\) B16.F10 cells and tumour growth was monitored. hs/irr B16 cells were prepared by incubation of B16.F10 tumour cells at 43°C for 1 h followed by irradiation at 200 Gy. The irradiation dose chosen was based on published studies from this and other laboratories (Prasad et al., 2005, Jarnicki et al., 2008, Sarrabayrouse et al., 2010, Marshall et al., 2012). Alternatively, C57BL/6 mice were immunized with TRP-2 peptides \((\text{TRP-2}^{180-188}, \text{TRP-2}^{58-78}, \text{TRP-2}^{148-165}; 100 \mu\text{g/peptide})\) with or without MPL (10 µg), with or without IL-1α (100 ng) twice at a 4-week interval (peri-tumour immunization). Mice were challenged with \(2 \times 10^5\) B16.F10 cells seven d post boost immunization. Mice that completely rejected the growth of B16.F10 cells were re-challenged with \(2 \times 10^5\) B16.F10 cells s.c. into the opposite flank.

In therapeutic experiments, TriDAP (100 µg) was administered s.c. to C57BL/6 mice with the B16.F10 tumour cells on d 0 and again on d 3 and 6 (TriDAP Early schedule; peri-tumour injection). Alternatively, TriDAP (100 µg) was administered s.c. to tumour bearing mice on d 6 and subsequently every 3 d thereafter (TriDAP Late schedule; peri-
tumour injection). As a non-treatment control, mice were peri-tumourally injected with PBS on d 3 and subsequently every 3 d thereafter. In certain therapeutic experiments, C57BL/6 mice challenged with $2 \times 10^5$ B16-OVA cells were treated with s.c. peri-tumour injections of OVA (100 µg) with or without MPL (10 µg), with or without IL-1α (100 ng) on d 3, 10, and 17 post-tumour induction. Alternatively, C57BL/6 mice were challenged with $2 \times 10^5$ B16.F10 cells and treated peri-tumourally with TRP-2 peptides (TRP-2180-188, TRP-258-78, TRP-2148-165; 100 µg/peptide) with or without MPL (10 µg), with or without IL-1α (100 ng) on d 3, 10, and 17 post-tumour induction (full details of immunization schedule are given in Table 2.4).

<table>
<thead>
<tr>
<th>Group (6 mice/group)</th>
<th>Immunization d 3*</th>
<th>Immunizations d 10, 17*</th>
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<tr>
<td>1</td>
<td>PBS</td>
<td>PBS</td>
</tr>
<tr>
<td>2</td>
<td>TRP-2 peptides</td>
<td>TRP-2 peptides</td>
</tr>
<tr>
<td>3</td>
<td>TRP-2 peptides + MPL</td>
<td>TRP-2 peptides + MPL</td>
</tr>
<tr>
<td>4</td>
<td>TRP-2 peptides + MPL</td>
<td>TRP-2 peptides + MPL</td>
</tr>
<tr>
<td>5</td>
<td>TRP-2 peptides + MPL + IL-1α</td>
<td>TRP-2 peptides + MPL + IL-1α</td>
</tr>
<tr>
<td>6</td>
<td>TRP-2 peptides + MPL + IL-1α</td>
<td>TRP-2 peptides + MPL + IL-1α</td>
</tr>
</tbody>
</table>

* days post-B16.F10 tumour inoculation
TRP-2 peptides: TRP-2180-188, TRP-258-78, TRP-2148-165; 100 µg/peptide
MPL (10 µg)
IL-1α (100 ng)
2.2.4 Direct immunization with TLR agonists (MPL/LPS) and IL-1α used as adjuvants co-administered with antigen (KLH)

C57BL/6 mice (n=3) were immunized by s.c. injection into the footpad (25 µl/footpad) with KLH (20 µg/mouse) and MPL (25 µg/mouse). Mice were sacrificed 7 d later, and popliteal lymph node cells were restimulated with either KLH alone or KLH and IL-1α (100 ng/ml) for analysis of KLH-specific T cell cytokine secretion. Alternatively, mice (n=3/group) were immunized twice (d 0 and 28) in the footpad with 20 µg KLH alone or with 10 µg MPL. Popliteal LN cells were harvested on d 35 and the cells restimulated with KLH for analysis of KLH-specific T cell cytokine expression and proliferation. In another experiment, mice (n=3/group) were immunized in the footpad with 20 µg KLH with/without MPL (10 µg) with/without recombinant IL-1α (100 ng). On d 28 mice were immunized with either KLH alone or KLH and IL-1α (i.e. MPL was not injected in the booster immunization). On d 35, popliteal LN cells were restimulated with KLH for analysis of KLH-specific T cell cytokine secretion and proliferation.

2.2.5 Evaluation of the immunogenicity of TRP-2 peptides in vivo

C57BL/6 mice (n=3/group) were co-immunized s.c. twice (d 0 and 28) in the footpad with 10 µg/TRP-2 peptide (TRP-2_180-188, TRP-2_258-278 or TRP-2_148-165) and MPL (50 µg). Popliteal LN cells were harvested on d 35 and the cells were restimulated with 1-20 µg/ml TRP-2 peptide. PMA/anti-CD3 was used as a positive control and cells pulsed with an irrelevant peptide (OVA_{257-264} or OVA_{323-336}; 20 µg/ml) served as a negative control.
2.2.6 Cell Culture

Cells were cultured in an incubator at 37°C with an atmosphere maintained at 95% humidity and 5% CO₂.

2.2.6.1 Cell counting/cell viability assessment

Trypan blue method

Cell counts were performed by diluting cells (usually 1/10 dilution) in trypan blue (Sigma). A 10 μl volume of the cell suspension was then loaded onto a disposable haemocytometer (Hycor Biomedical, UK). The number of viable cells (white) and dead cells (blue) were counted using a light microscope. The number of cells per ml was calculated by the following formula: 

\[ \text{No. of cells/ml} = \text{cell number} \times 10^4 \times \text{dilution factor} \]

Ethidium bromide-acridine orange (EBAO) method

Cell counts were performed by diluting cells (1/2 or 1/10 dilution) in EBAO. A 10 μl volume of the cell suspension was then loaded onto a disposable haemocytometer (Hycor Biomedical, UK). The number of viable cells (white) and dead cells (orange) were counted using a fluorescent microscope. The number of cells per ml was calculated by the following formula: 

\[ \text{No. of cells/ml} = \text{cell number} \times 10^4 \times \text{dilution factor} \]
2.2.6.2 Culture of monocyte-derived DCs (MoDCs) from human peripheral blood monocytes

Blood packs from healthy donors were obtained with consent from the Irish Blood Transfusion Board. Blood was diluted 1 in 2 with PBS and centrifuged (787 x g at room temperature (RT) for 10 min). The white buffy coat layer containing leucocytes was removed and diluted in 100 ml PBS. 12.5 ml of cell suspension was layered over 20ml histopaque (Sigma) and cell gradients were obtained by centrifugation (787 x g at RT for 20 min). The peripheral blood mononuclear cell (PBMC) layer was carefully removed, washed in PBS and pelleted by centrifugation. PBMC were washed again and pelleted by centrifugation (238 x g at RT for 7 min) to remove platelets. Monocytes (CD14^+ cells) were isolated from PBMC using the monocyte isolation kit (catalogue # 130-091-153) and an LS column from Miltenyi Biotec according to the manufacturer's protocol. This procedure yielded predominantly CD14^+ cells with purity >95% as assessed by flow cytometric analysis. CD14^+ monocytes (1 x 10^6 cells/ml) were cultured in 6 well plates in cRPMI medium supplemented with 50 ng/ml recombinant human GM-CSF (R&D) and 70 ng/ml recombinant human IL-4 (R&D) for 7 d. One half of the culture medium was changed on d 3 and d 5, replaced with cRPMI supplemented with 50 ng/ml GM-CSF and 70 ng/ml IL-4. On d 7, non-adherent DC were removed from each well by gentle pipetting, washed in cRPMI medium and pelleted by centrifugation (283 x g at RT for 7 min). Viability of the MoDCs was assessed by EBAO method and the cells were cultured in cRPMI at 1 x 10^6 cells/ml. MoDCs were rested for several hours before stimulation.
2.2.6.3 Generation of murine bone marrow-derived DCs (BMDC) and bone marrow-derived macrophages (BMMφ)

2.2.6.3A Harvesting murine cells from bone marrow

Bone marrow-derived cells were generated from C57BL/6 mice, using a method similar to that described by Lutz and colleagues (Lutz et al., 1999). Mice were euthanized and their femurs and tibiae removed. The bone marrow was flushed out with a 27 G needle attached to a 20 ml syringe barrel containing cRPMI (or cDMEM) and cell aggregates dissociated using a 19 G needle attached to a 20 ml syringe barrel containing cRPMI (or cDMEM). The cell suspension was pelleted by centrifugation (283 x g for 5 min) and cells were resuspended in 2 ml ammonium chloride lysis solution (37°C) for 2 min to lyse red blood cells. The cells were then washed in cRPMI/cDMEM medium (centrifugation; 283 x g for 5 min) and the pellet was resuspended in 10 ml cRPMI (or cDMEM) medium. Cell viability was assessed by trypan blue staining. Cells were cultured in cRPMI medium supplemented with GM-CSF or cDMEM medium supplemented with M-CSF to generate BMDCs (B) or BMMφ (C), respectively.

2.2.6.3B Culture of BMDCs

Immature BMDC were cultured at 0.5 x 10⁶/ml in cRPMI-1640 medium supplemented with conditioned media from a GM-CSF-expressing cell line (J558-GM-CSF) to give a final concentration of 20 ng/ml of GM-CSF. After 3 d incubation, 20 ml of fresh cRPMI medium containing 20 ng/ml of GM-CSF was added to each culture flask. On d 6, cell culture supernatant was carefully decanted. 20 ml of PBS (37°C) was added to each flask followed by gentle agitation and transfer of PBS suspension to 50 ml tubes containing cRPMI. 20 ml of 37°C EDTA (0.02% Sigma) was added to each culture flask before returning the flask to the 37°C incubator for 10 min. Meanwhile, cells
removed in the PBS step were pelleted by centrifugation (283 x g for 5 min) and resuspended in cRPMI-1640 medium. Culture flasks were removed from the incubator and the EDTA solution pipetted over the layer of remaining cells, before being added to cRPMI medium and centrifuged. The cell pellet was resuspended in cRPMI medium and pooled with the pellet obtained in the PBS step. Cells were then counted and re-cultured at 0.5 x 10^6/ml in cRPMI medium supplemented with 20 ng/ml of GM-CSF. After a further 2 d of incubation, 20 ml of fresh cRPMI medium containing 20 ng/ml of GM-CSF was added to each culture flask. After a further 2 d in culture (d 10), the loosely adherent cells were harvested, by gentle pipetting of the culture medium followed by transfer to 50 ml tubes. The cells were pelleted by centrifugation and viability of the BMDC was assessed by the trypan blue method. Cells were cultured in X-Vivo medium containing 10 ng/ml of GM-CSF at the required concentrations (e.g. 1 x 10^6cells/ml) in tissue culture plates. BMDC were rested for several hours before stimulation.

2.2.6.3C Culture of BMMΦ

Immature BMMΦ were cultured at 0.5 x 10^6 cells/ml in cDMEM medium supplemented with 15% supernatant from an M-CSF-expressing cell line (L929). L929 supernatant was a kind gift from L. O'Neill, Trinity College Dublin. After 2 d incubation, 25 ml cDMEM medium containing 15% M-CSF was added to each culture flask. After a further 2 d, 25 ml cDMEM medium containing 15% M-CSF was again added to each culture flask. After a further 3 d in culture (d 7), supernatant was carefully decanted from each culture flask. 20 ml PBS was added to each flask followed by scraping of the cells using a cell scraper. The cell suspension in PBS was then transferred to 50 ml tubes containing cDMEM medium. The cells were then pelleted by centrifugation (283 x g for 5 min) and cell viability was assessed by the trypan blue method. Cells were
cultured in cDMEM medium supplemented with 10% M-CSF at the required concentrations (e.g. 1 x 10^6 cells/ml) in tissue culture plates. BMMΦ were rested for several hours before stimulation.

2.2.6.4 Cell cryopreservation and recovery

Freezing medium

45% Heat inactivated FCS (Biosera)

45% DMEM medium (Sigma)

10% DMSO (Sigma)

Cell cryopreservation

Cells were grown to 70% confluence in T175 flasks. Cells were harvested, counted and resuspended at 5 x 10^6 cells/ml in freezing medium. 1 ml of cell suspension was then immediately transferred to cryovials. Vials were placed in a Cryo freezing container and then placed at -80°C. After 24 h, vials were transferred to a liquid nitrogen storage vessel.

Cell recovery from liquid nitrogen

To recover cells from liquid nitrogen, cells were warmed in a water bath at 37°C. Once thawed, cells were washed and contents were transferred to a T75 tissue culture flask and 5 ml culture medium was added slowly. Flasks were then incubated at 37°C, 5% CO₂.
2.2.6.5 Culturing of the B16.F10 cell line / iBMDM cell line

Cells were recovered from liquid nitrogen. Cells were grown at 37°C, 5% CO₂ in T75 flask to 70% confluence, usually for 2-3 d. Cells were harvested by addition of 5 ml PBS followed by gentle agitation and decanting off the PBS solution. 5 ml of 37°C trypsin (Sigma Trypsin-EDTA solution (1X) with 0.5g porcine trypsin and 0.2g EDTA) was then added to the culture flask before returning the flask to the 37°C incubator. After 5 min, the flask was removed from the incubator and the trypsin solution pipetted over the layer of remaining cells, before being added to fresh, heated cDMEM medium in a 50 ml tube and centrifuged (283 x g for 5 min). The cell pellet was resuspended in 25 ml cDMEM medium and slowly transferred to a T125 tissue culture flask. T125 tissue culture flask was then incubated at 37°C, 5% CO₂. Once cells were grown to 70% confluence in T125 flask, cells were harvested as described above. The cell pellet was resuspended in 4 ml cDMEM medium. 1 ml cell suspension was then added slowly to each of four T175 tissue culture flasks containing 50 ml cDMEM medium (i.e. 1/4 split). T175 tissue culture flasks were then incubated at 37°C, 5% CO₂.

2.2.7 Cell stimulation assays

2.2.7.1 In vitro stimulation of human MoDCs and murine BMDC/BMMΦ

After resting period, MoDCs/BMDC/BMMΦ cultured at 0.2 x 10⁷ cells/well in 96-well tissue culture plates were stimulated in vitro in X-Vivo medium. Precise conditions for stimulation of MoDCs/BMDC/BMMΦ in vitro are outlined in each figure legend. Culture supernatants were collected after 24-48 h and cytokine concentrations were quantified by ELISA. For analysis of mRNA expression, murine BMDCs/BMMΦ (1 x
$10^6$ cells/well cultured in 24-well plates) were treated as outlined in each figure legend. Total RNA was isolated from BMDCs/BMMΦ by lysing the cells in TRIzol (Invitrogen) reagent and storing the lysates in RNase-Free tubes at -20°C. In experiments investigating murine BMDC maturation, cells ($1 \times 10^6$ cells/well) were cultured in 24-well plates for 24 h before the cells were gently removed, washed and used for flow cytometric analysis.

For co-culture experiments with purified murine naïve CD4$^+$ T cells, murine BMDC ($0.1 \times 10^6$ DC per well; cultured in 48-well plate) were stimulated with LPS (100 ng/ml) or CpG (5 μg/ml) in the presence of TriDAP (100 μg/ml). DC supernatant was collected after 24 h. Cells were washed and co-cultured with purified CD4$^+$ T cells in X-Vivo medium ($0.1 \times 10^6$ DC: $1 \times 10^6$ CD4$^+$ T cell per well) with or without anti-CD3 (1 μg/ml; BD Pharmingen) stimulation. Co-culture supernatants removed after 72 h and DC supernatant removed after 24 h were assayed for cytokine production by ELISA.

2.2.7.2 In vitro stimulation of immortilized macrophage cell line

Macrophage cell line were cultured in tissue culture plates at the required concentration. After overnight resting period, the culture medium was replaced with fresh cDMEM medium just prior to stimulation. In the investigation of the effects of TriDAP and TLR agonist (LPS and Poly:IC) synergy on iBMDM cell line cytokine production, macrophages ($0.8 \times 10^5$ cells/well) were cultured in 96-well tissue culture plates. For analysis of mRNA expression, macrophages ($0.4 \times 10^5$ cells/well cultured in 24-well plates) were treated as outlined in figure legend. Total RNA was isolated from macrophages by lysing the cells in TRIzol and storing the lysates in RNase-Free tubes at -20°C.
2.2.7.3 Stimulation of spleen and lymph node cells \textit{ex vivo}

Single cell suspensions were prepared by passing spleen/lymph node cells through a 40 μm cell strainer (BD Falcon). The cell suspension was pelleted by centrifugation (283 x g for 5 min) and cells were resuspended in 2 ml ammonium chloride lysis solution (37°C) for 2 min to lyse red blood cells. The cells were then washed and the pellet was resuspended in medium and counted. For antigen (KLH or TRP-2 peptides) recall responses, splenocytes/lymph node cells were seeded at 0.4 x 10⁶ cells/well in 96-well tissue culture plates.

In some instances, CD3⁺, CD4⁺, or CD8⁺ T cells were purified (from single cell suspensions prepared by homogenizing spleen and lymph node cells) using MACS bead (magnetic cell sorting isolation kits, Miltenyi Biotec) and an LS column, according to the manufacturer’s instructions, tested for cell purity by FACS staining, and cultured with TriDAP or co-cultured with murine BMDC.

2.2.8 Analysis of immune responses

2.2.8.1 Proliferative responses of KLH-specific T cells

T cell proliferation was assessed by monitoring [³H] thymidine incorporation into newly synthesised DNA. Briefly, co-cultured DC-T cells or lymph node cells were pulsed with 0.5 μCi of [³H] thymidine/well (Amersham Pharmacia Biotech) diluted in X-Vivo medium. After 18 h incubation at 37°C and 5% CO₂, cells were harvested onto glass fibre filter mats (Mitchell et al.) with an automatic cell harvester. Once completely dry, the filter mats were sealed in plastic sample bags with 5 ml of non-
aqueous scintillation fluid (BetaScint, Wallac). \[^{3}H\] thymidine incorporation was detected by liquid scintillation counting using a micro-β-counter (Mitchell et al.). Results are expressed as mean counts per minute (CPM) of \[^{3}H\] thymidine incorporation for triplicate cultures.

2.2.8.2 Nitrite measurement (Griess reaction)

Nitric oxide generation was monitored indirectly by levels of nitrite (NO\(_2\); a stable and nonvolatile breakdown product of nitric oxide) released into the culture medium as determined using the Griess reagent (Alexis Biochemicals) with sodium nitrite as standard. Briefly, 48 h post cell stimulation, supernatants were incubated with Griess reagent in a 1:1 dilution (50μl supernatant: 50μl Greiss reagent) at RT. The OD of triplicate wells was measured via Versamax Tunable microplate reader (Molecular Devices) at 540 nm after 10 min. Nitrite concentrations were calculated by comparison with OD\(_{540}\) values of standard solutions of sodium nitrite prepared in culture medium (nitrite standard reference curve 0–100μM).

2.2.8.3 Measurement of cytokine production by ELISA

Commerically available ELISA kits/paired antibodies were used to quantify cytokine concentrations (Tables 2.4, 2.5). High-binding 96-well microtiter plates (Greiner Bio-one) were coated, sealed and incubated overnight (O/N) at 4°C with 50 μl/well of capture (I°) antibody in coating buffer at the recommended concentration (refer to tables 2.5 and 2.6). Wells were then aspirated and washed 5 times with >250 μl/well wash buffer. The plates were blotted on absorbent paper to remove any residual buffer.
Non-specific binding sites were blocked by addition of 200 µl/well of blocking solution for 2 h at room temperature (RT). Aspiration/washing (x5) was repeated. Using the assay diluent, the top working concentration of the standard (recombinant protein) was two-fold serially diluted to make the standard curve. The seven point two-fold serial dilution from the top working standard recombinant protein for the cytokine to be measured was added to the appropriate wells (50 µl/well of standard was added). 50 µl/well of test sample(s) was then added. 50 µl of assay diluent alone was added to serve as a blank. The plate was sealed and incubated at RT for 2 h or O/N at 4°C. Aspiration/washing (x5) was repeated. 50 µl/well of detection (2°) antibody diluted in assay diluent was then added. The plate was sealed and incubated at RT: for 2 h (R&D), 1 h (BD Pharmingen, eBioscience). Aspiration/washing (x5) was repeated. 50 µl/well of HRP-conjugated streptavidin diluted in assay diluent was then added (R&D 1:200 dilution; BD Pharmingen 1:1000 dilution; eBioscience 1:250 dilution). The plate was sealed and incubated at RT in the dark: for 20 min. Aspiration/washing (x7) was repeated. 50 µl/well of substrate solution was then added to each well and incubated at RT until the standard curve was visible. R&D, BD Pharmingen substrate: one 10 mg OPD tablet + 25 ml Phosphate Citrate Buffer + 7 µl H₂O₂, eBioscience substrate: Tetramethylbenzidine (TMB). The enzyme reaction was quenched by the addition of 25 µl/well 1M H₂SO₄. Absorbance was read at 492 nm (R&D, BD Pharmingen) or 450 nm (eBioscience) on a Versamax Tunable microplate reader (Molecular Devices). Cytokine concentrations in the test sample were determined by reference to the standard curve, prepared using recombinant cytokines of known concentration, and following subtraction of the blank absorbance reading from each unknown sample.
<table>
<thead>
<tr>
<th>Supplier</th>
<th>1° Antibody Dilution</th>
<th>Coating Buffer</th>
<th>Blocking Solution</th>
<th>Assay Diluent</th>
<th>2° Antibody Dilution</th>
<th>*Top Working Standard</th>
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<td>Assay Diluent ( supplied)</td>
<td>1:500</td>
<td>4 ng/ml</td>
</tr>
</tbody>
</table>

* Recombinant cytokines used as ELISA standard

<table>
<thead>
<tr>
<th>Supplier</th>
<th>1° Antibody Dilution</th>
<th>Coating Buffer</th>
<th>Blocking Solution</th>
<th>Assay Diluent</th>
<th>2° Antibody Dilution</th>
<th>*Top Working Standard</th>
</tr>
</thead>
<tbody>
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<td><strong>R&amp;D IL-27</strong></td>
<td>1:180</td>
<td>PBS</td>
<td>1% BSA in PBS</td>
<td>1% BSA in PBS</td>
<td>1:180</td>
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<tr>
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<td>Carbonate buffer</td>
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<td>10% FCS in PB</td>
<td>1:1000</td>
<td>1 ng/ml</td>
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<tr>
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<td>1:250</td>
<td>IL-23 Coating Buffer</td>
<td>Assay Diluent (supplied)</td>
<td>Assay Diluent (supplied)</td>
<td>1:250</td>
<td>2 ng/ml</td>
</tr>
</tbody>
</table>

* Recombinant cytokines used as ELISA standard
2.2.9 Flow cytometric analysis

2.2.9.1 Flow cytometric analysis of DC expression of maturation markers

BMDC cell surface expression of maturation markers was analysed by flow cytometry using a Canto II cytometer (Koenders et al.). DCs were harvested from wells and transferred to FACS tubes. Cells were washed in FACS buffer by centrifugation (283 x g for 5 min). Cell pellets were resuspended in 100 µl FACS buffer and non-specific binding was blocked by incubation with anti-CD32/CD16 (Fcγ Block; 1 µg/ml; BD Pharmingen) for 15 min at RT before extracellular staining for surface markers with anti-CD11c, anti-CD40, anti-MHC class II, anti-CD80 and anti-CD86 (20 min incubation at 4°C in the dark). Cells were then washed in FACS buffer by centrifugation and resuspended in FACS buffer and immunofluorescence analysis performed. The flow cytometer was calibrated using the compensation function in conjunction with BD Comp Bead (BD Biosciences). Results were analysed using FlowJo software with gating set on FMOs.

2.2.9.2 Flow cytometric analysis to determine purity of human MoDC and murine T cells

The purity of isolated monocytes was determined by flow cytometry. Monocytes were stained with FITC conjugated anti-CD14 antibody (BD Pharmingen) for 20 min at RT in the dark. Cells were then washed in FACS buffer by centrifugation (283 x g for 5 min) and resuspended in FACS buffer and immunofluorescence analysis performed. CD14 positive and negative cell population gates were set using stained PBMC. Monocyte purity was routinely between 85-95%. The purity of human DC was determined by staining with anti-CD14 and anti-DC-SIGN for 20 min at RT in the dark.
Cells were then washed and resuspended in FACS buffer and immunofluorescence analysis performed. Human DC purity was >95%.

CD3⁺, CD4⁺, or CD8⁺ T cells purified using MACS bead (Miltenyi Biotec), according to the manufacturer’s instructions were tested for cell purity by FACS staining. T cells were incubated with anti-CD32/CD16 (Fcγ Block; 1 µg/ml; BD Pharmingen) for 15 min at RT before extracellular staining for surface markers with anti-CD3, anti-CD4 and anti-CD8 (20 min incubation, RT in the dark). T cells were then washed in FACS buffer by centrifugation and resuspended in FACS buffer and immunofluorescence analysis performed. T cell purity was routinely >90%.

2.2.9.3 Indirect flow cytometric analysis of TRP-2 expression on B16.F10 cells in vitro and ex vivo

B16.F10 cell surface and intracellular expression of TRP-2 antigen was analysed by indirect flow cytometry (refer to Table 2.6). Indirect labelling requires two incubation steps, firstly with anti-TRP2 primary antibody (Abcam; ab74073) and then with a compatible secondary antibody conjugated to a fluorescent dye: Alexa Fluor 647 goat anti-rabbit IgG antibody (Invitrogen; A21244). B16.F10 cells (1 x 10⁶ cells/well) were cultured in 24-well plates in vitro. After 24 h, cells were harvested from wells and transferred to FACS tubes. Alternatively, B16.F10 melanoma single-cell suspensions were prepared 16 d post tumour inoculation and 1 x 10⁶ cells transferred to FACS tubes. Analysis was performed using a CantoII cytometer (Koenders et al.). Controls included cells stained as in Table 2.6, but omitting the primary antibody.
Table 2.7 Indirect flow cytometry procedure

<table>
<thead>
<tr>
<th>(a) Blocking step</th>
<th>(b) 1st Antibody</th>
<th>(c) 2nd Antibody</th>
<th>(d) Fix</th>
<th>(e) Permeabilize</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blocking reagent 1% BSA in PBS containing 1% goat serum (1 h, RT)</td>
<td>anti-TRP2 dilution 1:200 in Blocking reagent (1 h, RT)</td>
<td>Alexa Fluor 647 goat anti-rabbit dilution 1:500 in Blocking reagent (1 h, RT)</td>
<td>4% paraformaldehyde (1 h, RT)</td>
<td>Permeabilization solution (Dako kit; 15 min, RT)</td>
</tr>
</tbody>
</table>

Surface TRP-2 expression protocol: a – b – c

Intracellular TRP-2 expression protocol: a – d – e – b – e

2.2.10 Real Time Quantitative RT-PCR

Total ribonucleic acid (RNA) was extracted from cells (in chapter 3) using the TRIzol/chloroform method. Briefly, cells were collected in 1 ml TRIzol reagent and transferred to RNase-free tubes. 200 µl chloroform was added to each tube, vortex mixed, incubated at RT for 3 min and then centrifuged (13,400 x g for 15 min at 4°C). The upper aqueous layer was transferred to fresh RNase-free tubes, and 500 µl isopropanol added to each tube, mixed vigorously by inverting the tubes for several min and then stood at RT for 10 min, before centrifugation. Supernatants were decanted and the pellet washed in 1 ml 75% ethanol in nuclease-free H2O (samples were vortexed vigorously followed by centrifugation – 5,400 x g for 5 min at 4°C). Finally, remaining ethanol was removed with a pipette and the tubes inverted and allowed to air-dry for 10 min. RNA was resuspended in 20 µl nuclease-free H2O. RNA concentration (in chapter 3) was determined spectrophotometrically using an Eppendorf BioPhotometer 6131 (Eppendorf) by diluting 2 µl RNA in 98 µl nuclease-free H2O and reading the absorbance at 260 nm and determining the 260/280 nm ratio. Total RNA was extracted from cells (in chapter 4) using the Qiagen RNeasy® Mini Kit according to the manufacturers instructions. To quantify RNA (in chapter 4), 2 µl of undiluted RNA was placed directly onto the lower measurement pedestal of the NanoDrop ND-1000.
spectrophotometer. The upper optical pedestal was lowered and the measurement was
taken. After each reading, the sample was wiped from both the upper and lower
pedestals using a clean Kimwipe. Each RNA sample was quantified in this manner.
1µg of each RNA sample was reversed transcribed into cDNA using the Applied
Biosystems High Capacity cDNA Reverse Transcription kit, according to the
manufacturer’s protocol. Transcripts were quantified by real time quantitative PCR on
an ABI 7500 Fast Real Time PCR System with Applied Biosystems predesigned
Taqman Gene Expression Assays and reagents according to the manufacturer’s
instructions. For each sample, mRNA concentration was normalised to the amount of
18S ribosomal RNA (rRNA) and is expressed as fold difference compared to cells
incubated in medium only.

2.2.11 Predicting the binding of TRP-2 peptides to MHC class II IA<sup>b</sup> using a
knowledge based threading method

The peptide sequences for the human TRP-2 molecule and the murine TRP-2 molecule
were obtained from the Swissprot database, accession numbers P40126-1 and P29812,
respectively. The amino-acid sequences QCAEVQTDTWRSGP (TRP-2<sub>60-74</sub>) and
KKSIHPDYVITTQHW (TRP-2<sub>149-163</sub>), both obtained from P29812, were threaded onto
the co-ordinates of the human CLIP<sub>87-101</sub> peptide in the template “1MUJ” using
MODPROPEP web-server (Kumar and Mohanty, 2007), a knowledge based threading
approach for modeling MHC protein-peptide complexes. As a model system,
spontaneous C57BL/6-derived B16 melanoma, a well established and widely used
tumour model, is used in this study. The T cell receptors of C57BL/6 mice (MHC
haplotype K<sup>b</sup>, D<sup>b</sup>, IA<sup>b</sup>) are restricted to H2-IA<sup>b</sup> for class II MHC because this strain has
the I-Eb allele but it is null for the I-Ea allele (The Jackson Laboratory, 2012). In
MODPROPEP, the length of the peptide that constitutes the structural template determines the length of the threaded peptides; thus in the software, one template is available to thread 15-mers on H2-IA\textsuperscript{b} (1MUJ), and one template is available to thread 29-mers on H2-IA\textsuperscript{b} (1LNU). The CLIP peptides from human and mouse share the same nine-residue core-binding region (MRMATPLLMM) but differ in their flanking regions (Zhu et al., 2003). Furthermore, studies have shown that CLIP binds the peptide-binding groove in a similar way to that of other antigenic peptides (reviewed in Zhu et al., 2003). Thus, among the structures available in MODPROPEP for H2-IA\textsuperscript{b}, the “1MUJ” template structure was selected for modelling the two epitopes (Figure 5.6). In this threading approach, binding affinity of a peptide is predicted by the total energy of interaction with contact residues. The contacts of the query peptide in the template co-crystal structure are determined according to two different criteria; i) β-carbon atoms are closer than 7.0 Å and ii) any two atoms are closer than 4.0 Å. The C-beta <7.0 Å distance criterion was used to determine the contacts of the peptide in the template structure. The binding energy values for amino acid-to-amino acid interactions were taken from the table of statistical pairwise contact potentials derived by Miyazawa and Jernigan (Miyazawa and Jernigan, 1996). Miyazawa and Jernigan developed a scoring matrix with emphasis on hydrophobic interactions. This pair-wise contact potential table was selected based on the evidence that the IA\textsuperscript{b} peptide-binding groove displays a more hydrophobic environment at pockets P1, P3, P4, P6, P7 and P9 (Zhu et al., 2003). The 3D-Mol viewer from the Vector NTI Advance 10.3.0 package (Invitrogen) was used to display the 3D protein structure models of peptides TRP-2\textsubscript{60-74} and TRP-2\textsubscript{149-} in complex with H2-IA\textsuperscript{b}. 

100
1. MHC-peptide crystal structure

2. List for each peptide position all MHC contacting residues

<table>
<thead>
<tr>
<th>Peptide Positions</th>
<th>MHC interacting residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>A E</td>
</tr>
<tr>
<td>P2</td>
<td>L L V N P I</td>
</tr>
<tr>
<td>P3</td>
<td>A</td>
</tr>
<tr>
<td>P4</td>
<td></td>
</tr>
<tr>
<td>P5</td>
<td>A</td>
</tr>
<tr>
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</tr>
<tr>
<td>P7</td>
<td>R</td>
</tr>
<tr>
<td>P8</td>
<td>R L H</td>
</tr>
<tr>
<td>P9</td>
<td>A T V F L</td>
</tr>
</tbody>
</table>

3. For each of the 20 amino acids at each peptide position compute the interaction score using a pairwise contact potential table

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>C</th>
<th>D</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.22</td>
<td>-0.26</td>
<td>0.30</td>
<td>0.43</td>
</tr>
<tr>
<td>C</td>
<td>0.26</td>
<td>-1.34</td>
<td>0.38</td>
<td>0.46</td>
</tr>
<tr>
<td>D</td>
<td>0.30</td>
<td>0.38</td>
<td>0.27</td>
<td>0.49</td>
</tr>
<tr>
<td>E</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

E.g. For P1 = A \( \cdot -0.22 + 0.43 = 0.21 \)
For P1 = C \( \cdot 0.26 + 0.46 = 0.72 \)

Structure-based scoring matrix

<table>
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<tr>
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<th>3</th>
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<th>6</th>
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</table>

Taken from (Altuvia and Margalit, 2004)

Figure 2.5 The framework of the threading algorithm applied to MHC–peptide binding prediction

2.2.12 Statistics

Statistical analyses were performed with GraphPad Prism Version 4.00 (GraphPad Software Inc., San Diego, USA). Mean values were compared by one-way analysis of variance (Mahnke et al.) and the Tukey-Kramer multiple comparisons test was used to identify significant differences between individual groups. Differences in tumour growth kinetics between groups was analysed by Kruskal-Wallis one-way ANOVA on ranks followed by Dunn’s post test for multiple pairwise comparisons (non-normally distributed data). Kaplan-Meier survival curves were analysed using a logrank test. All RT-PCR data were normalized to 18 s rRNA. P value less than 0.05 was considered to indicate statistically significant differences between treatment groups.
Chapter 3
The Immunomodulatory & Anti-tumour effects of the NOD1 Agonist TriDAP
3.1 Introduction

The NLR family member, NOD1 (CARD4), is an intracellular PRR that recognises bacterial peptidoglycan-related dipeptide γ-D-glutamyl-\(\text{meso}\)-diaminopimelic acid (iEDAP), which is produced by most Gram-negative and certain Gram-positive bacteria such as \textit{Bacillus subtilis} and \textit{Listeria monocytogenes} (Chamaillard et al., 2003, Girardin et al., 2003a). Upon recognition of their respective agonists, NOD1 and NOD2 (the best-characterised NLRs) physically associate with the adaptor protein, RIP2 (also known as RICK) through homophilic CARD-CARD interactions (Park et al., 2007, Hasegawa et al., 2008). An intermediate region located between its CARD and its kinase domain mediates RIP2 interaction with the regulatory subunit IKK\(\kappa\) (or NF-\(\kappa\)B essential modulator; NEMO), linking NOD1 and NOD2 to the phosphorylation and degradation of \(\text{I} \kappa \text{B}\alpha\), which allows the cytoplasmic release and nuclear translocation of NF-\(\kappa\)B (Inohara et al., 2000b). NF-\(\kappa\)B subsequently activates transcription of inflammatory cytokines/chemokines. Stimulation of NOD1 and NOD2 also results in the activation of mitogen-activated protein kinases (MAPKs) p38, ERK and JNK (Chen et al., 2009). However, the molecular events leading to activation of these pathways are not fully understood.

The main function of NOD1 was thought to be as a cytosolic bacterial sensor via recognition of a specific muropeptide from bacterial peptidoglycan and NOD1 deficiency in mice leads to increased susceptibility to bacterial infections (Viala et al., 2004, Chen et al., 2009). However, work by Ulevitch and colleagues have recently extended the role of NOD1 beyond its well-known function in responses to infection, providing evidence supporting a role for NOD1 in controlling estrogen responsiveness.
of MCF-7 breast cancer epithelial cell line, a xenograft model for estrogen-sensitive breast cancer tumours (da Silva Correia et al., 2006). Overexpression of NOD1 in MCF-7 cells resulted in inhibition of estrogen-dependent tumour growth in severe combined immunodeficient (SCID) mice \textit{in vivo} and reduction of estrogen-induced proliferation \textit{in vitro}, suggesting that the presence of NOD1 acts to halt estrogen-dependent tumour growth. Additional support for the contention that NOD1 may possess tumour suppressor properties has been demonstrated by Chen et al showing that NOD1 deficiency resulted in increased development of both colitis associated and Apc tumour suppressor-related colon tumours (Chen et al., 2008). However, spontaneous tumour development has not been observed in NOD1 deficient mice, suggesting that the protective role of NOD1 signalling against development of colonic tumour manifests only in the context of chronic injury and inflammation (Chen et al., 2008).

NOD1 and NOD2 have similar structure and signalling pathways. A synthetic NOD2 agonist, muramyl dipeptide (MDP)-Lys (L18) has been reported to elicit anti-tumour immunity in humans and in animal models (Yoo et al., 1994, Nitta et al., 2000, Yanagawa et al., 2000). Additionally, (MDP)-Lys co-administered with IFNβ significantly suppressed the growth of B16.F10 melanoma in mice (Fujimura et al., 2011). A recent study has shown that NOD1 stimulation in conjunction with TLR stimulation primes T\textsubscript{H}1 and T\textsubscript{H}17 immune responses \textit{in vivo} (Fritz et al., 2007) indicating that NOD1 signalling is also important in the initiation of T\textsubscript{H} differentiation and adaptive immune responses. Similarly, a role for NOD2 in the co-ordination of adaptive immune responses is implicated by the observation that (MDP)-Lys synergistically induced IL-12 production in combination with TLR agonists in human DCs \textit{in vitro}, which promoted T cells to produce IFNγ production (i.e. T\textsubscript{H}1
differentiation) (Tada et al., 2005). Collectively, this data lead to the formulation of the hypothesis in the current study that NOD1 stimulation via a commercially available NOD1 activator L-alanyl-γ-D-glutamyl-meso-diaminopimelic acid (TriDAP), which comprises the iE-DAP dipeptide and an L-Ala residue, may possess potential anti-tumour activity. B16.F10 (H-2b) melanoma is among the most aggressive, poorly immunogenic murine tumours (Fidler, 1975, Overwijk and Restifo, 2001). The reason for this low immunogenicity is still unknown, however the low expression of MHC class I molecules on B16.F10 cells may contribute to this (Overwijk and Restifo, 2001). Thus, among transplantable mouse tumours, this tumour model system provides a stringent test of the efficacy of an immunotherapeutic approach. Thus, the poorly immunogenic B16.F10 melanoma was used to test the hypothesis that the NOD1 agonist TriDAP has therapeutic potential against tumours. In support of this hypothesis, Ohteki and colleagues reported significant tumour suppression in mice prophylactically treated with OVA and the NOD1 agonist, FK565 (an iE-DAP containing compound) in an OVA-expressing EG-7 tumour model (Asano et al., 2010).

Initial studies were performed in *in vitro* cellular models to evaluate the immunomodulatory properties of TriDAP. Antigen-presenting cells such as DCs and MΦs are initiators and modulators of immune responses. APCs present internalized exogenous antigens to CD4 expressing T cells via MHC class II and endogenously synthesised antigens to CD8 expressing T cells via MHC class I molecules. In addition, DCs have the unique ability to cross-present exogenous antigen to CD8+ T cells via MHC class I, a phenomenon known as cross-priming, generating CD8+ T cell immunity to cancer. Signals mediated by NOD1 ligands (Asano et al., 2010) and TLR agonists (Datta et al., 2003, Schulz et al., 2005a) have been shown to significantly enhance the
induction of DC-mediated cross-priming. Recent studies also showed that stimulation of NOD1 (Fritz et al., 2007) and TLRs (Dabbagh and Lewis, 2003) contributes to the priming of adaptive immune responses (i.e. T helper differentiation) which is essential for mounting anti-tumour responses \textit{in vivo}. Taken together, these findings encouraged the investigation of the effects of NOD1 stimulation on antigen-presenting cells, such as DCs and MΦs. In addition, given that the tumour microenvironment is enriched in danger signals released from necrotic tumour/effector cells, the potential synergistic effects of NOD1 agonist TriDAP with TLR agonists on innate immune cells in relation to modulation of TLR signalling and cytokine release, as has previously been reported for NOD2 stimulation (Yang et al., 2001, Netea et al., 2005, Wiken et al., 2009) was also investigated. It is possible that TriDAP may not exert tumour suppressor properties in isolation, but instead act in synergy with other PAMPS, such as TLR ligands, which are commonly found in the tumour environment. To test these two possibilities, the \textit{in vitro} effect of TriDAP both alone and in synergy with TLR agonists in inducing cytokines was investigated in various experimental settings including murine BMDCs, murine BMMΦs, and human monocyte-derived DCs.

**Aim of this Chapter**

To investigate the potential anti-tumour activity of NOD1 agonist, TriDAP against the poorly immunogenic murine B16.F10 melanoma.
3.2 Results

3.2.1 Phenotype, cell yield and the proportion of CD11c\(^\text{+}\) cells from sample BMDC culture

Murine bone marrow cells were cultured in cRPMI medium supplemented with GM-CSF for 10 days. On day 10 of culture (n = 2), the cells expressed high levels of CD11c indicating that these cells were phenotypically DCs (Figure 3.1A). The total cell yield from each bone marrow culture (n = 5) was found to be consistently similar, with the exception of one culture (Figure 3.1B). To further characterize the CD11c\(^\text{+}\) BMDC generated under the culture conditions, cells were analyzed by flow cytometry for the expression of co-stimulatory molecules CD40, CD80, CD86 and MHC class II. As shown in Figure 3.1C, CD11c\(^\text{+}\) BMDC expressed levels of each of these markers.

3.2.2 NOD1 agonist TriDAP synergises with TLR agonists to promote cytokine secretion by murine DC

Different doses of TriDAP (1 – 100 \(\mu\)g/ml) were assessed for the ability to promote or modulate cytokine production from murine BMDCs co-stimulated with the TLR4 ligand, LPS and the TLR9 ligand, CpG. Stimulation of BMDC with TriDAP alone did not induce cytokine production from DC above background levels, even at concentrations as high as 100 \(\mu\)g/ml (Figure 3.2; \(p>0.05\) versus medium alone, ANOVA). In contrast, there was a significant synergistic effect of various doses of the NOD1 agonist, TriDAP on IL-12p70 responses in DCs to combined stimulation with TLR4 ligand, LPS (Figure 3.2). Post hoc analysis demonstrated that IL-12p70 secretion in response to TriDAP and LPS stimulation was significantly increased from LPS stimulation alone: at 1 \(\mu\)g/ml TriDAP (\(p<0.05\)), 10 \(\mu\)g/ml TriDAP (\(p<0.001\)), and 100
μg/ml TriDAP (p<0.001). A similar effect was seen with CpG, with TriDAP enhancing CpG-induced IL-12p70 production. There was a significant synergistic effect of higher doses (10 - 100 μg/ml) of TriDAP on IL-23 responses in DCs to combined stimulation with either TLR4 ligand, LPS or TLR9 ligand, CpG (10 μg/ml; p<0.001 and 100 μg/ml; p<0.001; Figure 3.2). TriDAP also enhanced LPS- and CpG-induced IL-1β and IL-10 production by murine DCs in a dose-dependent manner (Figure 3.2). The most significant synergistic enhancement was seen with IL-23 production (observed for LPS plus TriDAP stimulation only), which would suggest it would promote a Th17 response. This initial dose-response study with microgram/ml concentrations of the NOD1 agonist, TriDAP, demonstrated that TriDAP stimulation alone did not induce a significant amount of cytokine secretion in the absence of TLR signalling. Secondly, TriDAP was effective in augmenting LPS-induced responses at concentrations as low as 1 μg/ml, whilst the 100 μg/ml dose of TriDAP was the minimal dose to significantly enhance CpG responses, thus reflecting differential synergistic responses with different TLR agonists. Therefore the 100 μg/ml dose of TriDAP was chosen for subsequent studies. Finally, TriDAP markedly increased both pro-inflammatory as well as anti-inflammatory cytokine responses by TLR agonists, LPS and CpG, which was dose dependent and maximal at 100 μg/ml TriDAP.

The effects of TriDAP (100 μg/ml dose) alone and in combination with various doses of LPS (1-100ng/ml) or CpG (1-5μg/ml) was next investigated. Consistent with the data in Figure 3.2, TriDAP alone did not stimulate cytokine production in DCs. LPS alone (at all doses tested) induced dose dependent production of IL-12p70, IL-1β, IL-23, IL-10, IL-12p40 and TNFα, but co-stimulation with TriDAP and LPS over a range of concentrations enhanced the production of IL-12p70, IL-1β, IL-23, IL-10, IL-12p40
and TNFα by DCs. As shown in Figure 3.3, this synergistic effect was statistically significant when compared with LPS alone. TriDAP also exhibited similar synergistic activities with CpG, however, with the exception of IL-10 production, marked synergism was observed only with CpG at a concentration of 5 µg/ml. Having analysed the results shown in both Figures 3.2 and 3.3, the following doses were selected for subsequent studies in order to obtain maximal synergism: 100 µg/ml TriDAP, 100 ng/ml LPS and 5 µg/ml CpG.

3.2.3 TriDAP in synergy with LPS optimally induces IL-27p28 mRNA expression in murine DCs after 4 hours

IL-27 is a heterodimeric cytokine composed of p28 and EBV-induced gene 3 (EBI3) proteins. IL-27 induces the differentiation of Th1 cells but it also inhibits effector Th17 cells (Yoshida et al., 2009). Initially, an IL-27 ELISA was not commercially available. Therefore, the effect of TriDAP alone and in combination with TLR agonists on the induction of IL-27 could only be assessed at the transcriptional level. It has been reported that the p35 subunit of IL-12 can alternatively complex with EBI3 (Devergne et al., 1997), to form the heterodimeric cytokine, IL-35 (Niedbala et al., 2007). Therefore, because IL-35 shares EBI3 with IL-27, the p28 subunit rather than EBI3 is specific for IL-27. To this end, the mRNA expression of IL-27 p28 subunit was determined by real-time PCR as an indicator of IL-27 induction. Figure 3.4 shows the kinetics of mouse IL-27 p28 expression by BM DCs stimulated with TriDAP alone or in combination with LPS. A transient expression was observed for IL-27p28 with maximal message levels found after only 4 h. Whilst p28 message was still above non-stimulated background levels after 8 h, mRNA levels declined to almost background levels after 12 h, indicating that peak IL-27p28 expression occurred within 4 h of LPS.
and LPS plus TriDAP stimulation. These results confirm 4 hours, which was chosen in subsequent studies (Figures 3.6, 3.8), as the optimum time point for transcriptional expression of the IL-27p28 gene following stimulation with TLR agonists in synergy with TriDAP. To conclude, TriDAP enhanced LPS-induced IL-27p28 mRNA expression in murine DCs optimally after 4 hours, and as before, TriDAP stimulation alone did not induce the mRNA expression of IL-27p28.

3.2.4 Effects of pre-treatment with TriDAP on TLR agonist induced cytokine production and IL-27p28 mRNA expression

To investigate the effect of priming with TriDAP prior to TLR stimulation, murine BMDCs were incubated with 100 μg/ml TriDAP for 2 h or at the same time as stimulation with either 100 ng/ml LPS or 5 μg/ml CpG (Figure 3.5). Simultaneous stimulation with TriDAP and LPS lead to an increase of IL-12p70 and IL-23 release (p<0.05 and p<0.001, respectively) compared with pre-incubation with TriDAP. However, the difference was not statistically significant for IL-1β and IL-10 secretion. Furthermore, pre-incubation of TriDAP did not increase cytokine release compared with simultaneous stimulation with CpG.

The effects of pre-treatment with TriDAP on LPS induced IL-27p28 mRNA expression was also examined (Figure 3.6). Murine BMDCs were incubated with 100 μg/ml TriDAP for 2 h or co-stimulated with 100 ng/ml LPS. As shown in Figure 3.6, pre- or co-stimulation with TriDAP enhanced TLR-induced IL-27p28 mRNA expression. Pre-incubation with TriDAP did not increase IL-27p28 mRNA expression compared with simultaneous stimulation with LPS (p>0.05).
Because the difference between TriDAP pre-incubation and TriDAP simultaneous stimulation was not statistically significant (with the exception of IL-12p70 and IL-23 release; TriDAP and LPS co-stimulation), stimuli were added together in subsequent experiments. In addition, these results confirmed that the synergistic effects of TriDAP on the cytokine production stimulated by the TLR4 ligand, LPS and TLR9 ligand, CpG (as demonstrated in Figures 3.2 and 3.3) does not require pre-incubation with the NOD1 agonist prior to TLR ligand stimulation.

3.2.5 Synergistic effects of NOD1 agonist TriDAP with TLR agonists on the induction of cytokine production by iBMDMs

A recent report demonstrated that in mouse macrophages, iE-DAP (the minimal structure recognised by NOD1) combined with LPS generated a higher IL-6 response than LPS alone, suggesting an additive effect (Chamaillard et al., 2003). Thus, whilst it is well established that DCs are the most potent activators of naïve T cells (Janeway et al., 2005), macrophages can also function as APCs and in the context of tumours have been shown to be important in killing tumour cells. The possibility that NOD1 stimulation of murine macrophages alone or in combination with TLR agonists may induce cytokine secretion was thus evaluated. To test this, the effects of TriDAP alone and in combination with TLR agonists, LPS and Poly:IC, on the induction of cytokine production by immortalized mouse bone marrow-derived macrophages (iBMDMs) was examined (Figure 3.7). The dose of Poly:IC was based on previous research (Sweeney and Mills; personal communication; unpublished data). As previously demonstrated, TriDAP alone did not stimulate cytokine production. However, TriDAP significantly enhanced LPS- and poly:IC-induced IL-1β, TNFα, and IL-6 production in iBMDMs in
a dose-dependent manner. In contrast to BMDCs (as shown in Figures 3.2 and 3.3), TriDAP did not enhance TLR agonist-induced IL-10 production in iBMDMs.

The effect of TriDAP alone and in combination with TLR agonists on IL-27p28 mRNA expression in iBMDMs was also examined. As shown in Fig. 3.8, TriDAP enhanced LPS- and Poly:IC-induced IL-27p28 mRNA expression, at all doses of the TLR agonist tested. This is consistent with the cytokine protein data shown in Figure 3.7. These findings suggest that NOD1 stimulation in combination with TLR agonists can lead to increased cytokine production in macrophages.

3.2.6 Synergistic effects of NOD1 agonist TriDAP with TLR4 agonist LPS on the induction of cytokine secretion by human MoDCs

Human peripheral blood monocytes were cultured with IL-4 and GM-CSF for 7 days. On day 7 of culture, the cells expressed high levels of CD11c and DC-SIGN but little/no CD14, indicating that these cells were phenotypically DCs (Figure 3.9). The effects of TriDAP (at doses ranging from 1 – 100 µg/ml) alone or combined with various different doses of the TLR4 ligand, LPS on cytokine production by human MoDCs was evaluated on day 7 of culture (Figure 3.10). Cytokines produced in response to TriDAP alone, at all doses tested, were minimal (typically below assay detection limits) and indistinguishable from un-stimulated cells. Synergistic increases in IL-23, IL-10 and IL-27 production were seen with 10 µg/ml and 100 µg/ml TriDAP in combination with all doses of LPS tested. As shown in Figure 3.10 this synergistic effect was statistically significant when compared with LPS stimulation alone. IL-12p70 was not analysed due to culture supernatant volume limitations as a consequence
of limiting cell numbers. Interestingly, loss of synergistic activity was observed at the lowest dose of TriDAP (1 μg/ml), a phenomenon also seen with murine BMDCs (Figure 3.2), providing evidence that high concentrations of TriDAP are required when compared with TLR agonists to exert modulatory activity. In contrast to previous observations, human MoDC response to LPS was saturated at a dose of 0.1 ng/ml. Interestingly, TriDAP was capable of further enhancing maximal LPS responses.

Having demonstrated that 0.1 ng/ml LPS is the optimal dose for synergistic activity, the effects of TriDAP with lower doses of LPS (0.01 ng/ml) was examined to determine if synergistic activity was still evident even at sub-optimal concentrations of the TLR agonist. Co-stimulation of MoDCs with TriDAP induced a 3-fold increase in IL-10 secretion (mean 3961.7 pg/ml vs 1295 pg/ml LPS alone) and a 2-fold increase in IL-27 secretion (mean 1408 pg/ml vs 757.2 pg/ml LPS alone) over that induced by LPS alone (Figure 3.11). The effect was even more pronounced at 10 pg/ml LPS; co-stimulation of MoDCs with TriDAP induced a 3-fold and statistically significant increase in IL-10 secretion (mean 1215.9 pg/ml vs 408.6 pg/ml LPS alone) and a 4.7-fold and statistically significant increase in IL-27 secretion (mean 785 pg/ml vs 166.1 pg/ml LPS alone), indicating that TriDAP enhanced LPS-induced IL-10 and IL-27 production in human MoDCs even at sub-optimal doses of LPS. The data shown in Figure 3.10 and Figure 3.11 is from two individual donors. However, it is fully appreciated that the testing of more donors is warranted.
3.2.7 The effects of TriDAP on BMDC maturation

In addition to cytokine production, the effect of TriDAP on TLR-induced maturation of murine BMDC was determined by examining cell-surface expression of MHC class II and CD86. Immature DC expressed considerable levels of MHC Class II, but low levels of CD86 (Figure 3.12). Stimulation of DC with TriDAP only slightly enhanced cell surface expression of CD86 and MHC Class II, consistent with the observation that TriDAP stimulation alone did not induce a significant amount of cytokine secretion. In contrast, the expression of CD86 and MHC Class II on BMDCs was considerably up-regulated by stimulation with LPS and CpG. Co-stimulation with TriDAP did not alter TLR-induced up-regulation of CD86 and MHC Class II expression. It is possible that the TLR agonists alone induced substantial maturation of BMDCs without the need for additional signalling mediated by NOD1 stimulation.

3.2.8 Direct effects of TriDAP on T cells

A review of the literature has revealed that, the ability of NOD agonists to directly activate T cells in an APC-free system has so far not been examined. Thus, the effect of TriDAP alone and in synergy with TLR4 agonist LPS, on cytokine production from purified populations of CD3+CD4+ and CD3+CD8+ T cells stimulated with anti-CD3/CD28 in vitro, was investigated. Stimulation with TriDAP alone had no effect on IFNγ, IL-10, IL-4 and TNFα secretion from anti-CD3/CD28 activated T cells (Figure 3.13). Simultaneous stimulation of activated CD4+ T cells with TriDAP and LPS significantly decreased IL-4 production when compared with TLR agonist stimulation alone. This pattern was also observed with activated CD8+ T cells. The significance of suppressed IL-4 production from activated T cells stimulated with a combination of NOD1 and TLR4 agonist is not clear and warrants further investigation. TriDAP did
not modify IFNγ and TNFα production by either CD4+ or CD8+ T cells, suggesting that NOD1 does not directly enhance the production of Th1 cytokines from T cells. Similarly, TriDAP did not modify IL-10 production by anti-CD3/CD28 activated CD4+ or CD8+ T cells.

3.2.9 DC treated with TriDAP and TLR agonist suppress the secretion of IL-4 from naïve CD4+ T cells

The observation that stimulation of DCs with the NOD1 agonist TriDAP enhanced TLR-induced IL-12, a Th1 inducing cytokine and also IL-1β and IL-23, which are well established Th17 promoting cytokines (Mills, 2008b), raised the question of whether DC-derived cytokines promote the development of naïve T cells to Th1 cells (with resultant IFN-γ production) or to Th17 cells, resulting in IL-17 production. Therefore, the cytokine production from anti-CD3 stimulated CD4+ T cells (isolated from naïve mice) co-cultured with DCs stimulated with TriDAP alone, TLR agonists (LPS and CpG) alone and combinations thereof, was examined. There was no significant difference in the levels of CD4+ T cell secreted cytokines after co-culture with DCs stimulated with TriDAP alone when compared with DCs cultured in medium alone (p>0.05), as shown in Figure 3.14 (A). Despite the fact that TriDAP enhances TLR induced IL-12p70 production, DCs co-stimulated with TriDAP and TLR agonists did not enhance IFN-γ secretion from T cells as compared with DCs stimulated with TLR agonists alone (p>0.05). Similarly, co-stimulation of DCs with TriDAP and TLR agonists did not enhance IL-17 secretion from T cells when compared with DCs stimulated with TLR agonists alone (p>0.05), despite the cooperative NOD1 and TLR activation enhancing the Th17-promoting factors, IL-1β and IL-23. Surprisingly, DCs co-stimulated with TriDAP and TLR agonists suppressed IL-4 and IL-10 from CD4+ T
cells when compared with DCs stimulated with TLR agonists alone. This difference was significant for IL-4 production (p<0.01 when compared with LPS stimulation alone; <0.05 as compared with CpG stimulation alone). However, the decrease in IL-10 production did not reduce to statistical significance. The supernatants from the DC culture were assayed to confirm production of Th polarising cytokines prior to co-culture with CD4^+ T cells (Figure 3.14 (B)). TriDAP enhanced LPS-induced IL-12p70, IL-1β, IL-23, IL-10 and IL-27 secretion and CpG-induced IL-12p70, IL-23 and IL-10 secretion by DCs. Therefore, the capacity of TriDAP-TLR-co-stimulated DCs to enhance the Th1 and Th17 responses was not due to the inability to produce key cytokines needed for Th1 and Th17 cell development, because the DCs crucially secreted IL-12p70, IL-1β and IL-23. It is unclear why higher levels of DC-derived-IL-12p70 and DC-derived-IL-1β and IL-23 induced by TriDAP plus TLR agonist stimulation, did not lead to enhanced IFNγ and IL-17 production, respectively. However, a Th1 and Th17 response was evidently sustained. It is well established that Th1 cells reciprocally regulate Th2 responses, with IL-12-induced IFN-γ down-regulating the expression of transcription factor GATA-3 (Murphy et al., 2000a). In addition, IL-27 has been shown to inhibit Th2 cell development as well as Th2 cytokine production by down-regulation of GATA-3 (Hibbert et al., 2003, Lucas et al., 2003). Therefore, it is quite possible that the capacity of TriDAP-TLR-co-stimulated DCs to suppress Th2 responses could be due to the ability to induce a higher level of IL-12p70 and IL-27 (Figure 3.2) than DCs stimulated with TLR agonist alone. Further studies that neutralize IL-12p70 and IL-27 should clarify to what extent these cytokines play a role. Taken together, these preliminary findings suggest that TriDAP exerts a suppressive effect on IL-4 production induced with TLR-activated DC but does not modify Th1 and Th17 responses.
3.2.10 Effect of NOD1 agonist TriDAP on tumour growth in the B16.F10 melanoma model

To determine whether the NOD1 agonist TriDAP could modify the growth of B16.F10 melanoma cells in wild type C57BL/6 mice, TriDAP was administered either prophylactically at the injection of B16.F10 tumour cells ("early" dosing schedule) or therapeutically to mice with established tumours ("late" dosing schedule). B16.F10 cells (0.2 x 10^6) were inoculated into C57BL/6 mice on day 0. Mice were peri-tumourally injected with 100 μg of TriDAP on either days 0, 3, 6 (TriDAP early treatment) or on day 6 and subsequently every 3 days thereafter (TriDAP late treatment). Control mice were peri-tumourally injected with PBS every 3 days. Tumour growth was rapid in the PBS treated mice (Figures 3.15 A and B). Although mice in the TriDAP-late-treated group did develop tumours, the growth was significantly delayed compared with PBS treated controls (p<0.05; Figures 3.15 A and B). Interestingly, when administered on the same day as tumour inoculation (TriDAP early-treated group), treatment with TriDAP markedly accelerated tumour growth when compared with PBS treated controls. In addition, all tumour bearing mice died or had to be sacrificed within 27-30 days after early treatment with TriDAP (on days 0, 3, 6) or PBS (Figure 3.15 (C)). In contrast, administration of TriDAP therapeutically significantly (p=0.0043 versus PBS treated controls Kaplan-Meier logrank test) enhanced survival with 30% of the mice surviving after the end of the observation period (Day 31).
3.3 Discussion

The immunoregulatory role of professional APCs (predominantly DCs but also MΦs) in infection/tissue injury is initiated following sensing specific PAMPs, such as TLR agonists and NOD agonists, by PRRs, which initiate and modulate DC function to drive adaptive immune responses (Kapsenberg, 2003). This study investigated the stimulatory potential of the NOD1 agonist, TriDAP alone and in combination with various TLR agonists on DCs and MΦs. It had been suggested that NOD1 may not exert tumour suppressor properties in isolation, but instead act in synergy with other PAMPs, such as TLR ligands, which are commonly found in the tumour microenvironment. The findings reported herein clearly demonstrated that TriDAP exhibited remarkable synergistic effects with TLR3, TLR4, and TLR9 agonists to induce the production of multiple cytokines in both human and murine dendritic cells and also in murine macrophages. The synergistic effects were observed for both pro-inflammatory cytokines, with the induction of IL-12p70, IL-1β, IL-23, and TNFα, but also the anti-inflammatory cytokine, IL-10. In addition, TriDAP enhanced TLR agonist-induced IL-27 production from DCs and MΦs. IL-27 has both pro- and anti-inflammatory properties. The finding that TriDAP stimulation alone did not induce any cytokine release in murine DCs, murine macrophages, and human monocyte-derived DCs even at microgram/ml concentrations, strongly implies that the NOD1 agonist requires or provides a second signal for immune cell stimulation. Chamaillard et al. reported that stimulation of murine macrophages with iE-DAP (the minimal structure recognised by NOD1) alone induced very low concentrations of TNFα and IL-6 secretion (Chamaillard et al., 2003). However, the data in the present study as well as that reported by others (Traub et al., 2004, Fritz et al., 2005, Tada et al., 2005, van Heel et al., 2005) consistently demonstrated that NOD1 agonist alone failed to induce
significant concentration of cytokine secretion from APCs. Conversely, how it induces remarkable synergistic effects with TLR agonists is still unclear. Takahashi et al. reported that LPS, lipoteichoic acid (LTA) or peptidoglycan up-regulated the expression of both NOD1 and NOD2 mRNAs in murine macrophage RAW264.7 cells at 2 h after endotoxin treatment (Takahashi et al., 2006). In addition, the activation of NF-κB by LPS leads to NOD1 expression (Inohara et al., 2001). Furthermore, Fritz et al. reported that HEK293T cells cotransfected with NOD1 and TLR2 (together with a NK-κB-luciferase reporter construct) and then synergistically stimulated by their respective agonists had enhanced NK-κB activation compared with cells stimulated with NOD1 or TLR2 alone (Fritz et al., 2007). Taken together, these studies highlight a relationship between TLR agonist activation and NOD1 expression, implying a crosstalk between NOD1 and TLR signalling pathways. They also suggest that cooperative NOD1 and TLR activation enhances signalling through NK-κB, which leads to synergistic production of both pro- and anti-inflammatory cytokines.

In contrast to the enhancing effect of TriDAP on TLR-agonist induced cytokine secretion from APCs, cell-surface expressions of CD86 and MHC class II molecules was not enhanced following stimulation of DC with TLR agonists in the presence of TriDAP, suggesting that NOD1 recognition does not alter the maturation state of DCs. However, it is possible that the TLR agonists alone induced maximal DC maturation without the need for additional signalling mediated by NOD1 stimulation. In support of this finding, Tada et al. reported that stimulation of DC with the NOD1 agonist, FK565, or the NOD2 agonist, MDP, did not enhance LPS-induced up-regulation of CD83, CD80 or CD86 (Tada et al., 2005). In contrast, Fritz et al. reported that NOD1 as well as NOD2 agonists did synergize with LPS to induce maturation of human monocyte-
derived DC (Fritz et al., 2005). The discrepancy between these results is unclear and warrants further investigation.

Having demonstrated that a combination of TriDAP and TLR agonists synergistically triggered both T_H1-polarising and a T_H17-polarising cytokines in DCs, with production of IL-12, IL-27, IL-1β and IL-23, the effect of TriDAP ligation on the T_H1-, T_H2- and T_H17-inducing capacity of DCs was then investigated. The results revealed that, although DC treated with TriDAP alone had no effect on T cell activation, TriDAP suppressed the capacity of LPS or CpG-activated DCs to promote IL-4 (but not IFN-γ and IL-17) production by naïve CD4^+ T cells. It is well established that IL-12 promotes T_H1 development by means of a signalling pathway that involves T-bet and STAT4, while IL-4 induces GATA-3 and STAT6 activation and drives naïve CD4^+ T cells down a T_H2 differentiation pathway (Murphy et al., 2000a). T-box transcription factor T-bet plays a central role in T_H1 differentiation by maintaining expression of the IL-12 receptor (IL-12R) β2 chain after activation of CD4^+ T cells (Mullen et al., 2001, Afkarian et al., 2002). In addition, it has recently been discovered that the IL-12 family member, IL-27, promotes T_H1 polarisation by inducing expression of T-bet in a STAT1-dependent manner, resulting in IL-12Rβ2 expression on the surface of newly activated T cells and IFN-γ production (Pflanz et al., 2002, Hibbert et al., 2003, Lucas et al., 2003). Thus, IL-27 renders the cells sensitive to IL-12. Furthermore, IL-27 signals via its heterodimeric receptor (IL-27R), which consists of the receptor subunits gp130 and WSX-1 (Pflanz et al., 2004). Yoshida et al. reported that WSX-1^-/- mice are susceptible to Leishmania major infection, with impaired IFN-γ production early in infection (Yoshida et al., 2001). Taken together, it is possible that the IL-12 and IL-27 secreted by TriDAP and TLR agonist primed DCs leads to a sustained T_H1 response.
with resultant IFN-γ production. Furthermore, IFN-γ and IL-27 have been shown to inhibit Th2 cell development as well as Th2 cytokine production by down-regulation of GATA-3 (Szabo et al., 1997, Murphy et al., 2000a, Hibbert et al., 2003, Lucas et al., 2003). This may explain the suppressive capacity of TriDAP and TLR agonist primed DCs to promote IL-4 production from CD4+ T cells.

The aim of the current study was to test the potency of NOD1 agonist TriDAP against the poorly immunogenic B16.F10 melanoma. The data demonstrated that administration of TriDAP, activated immune responses against B16.F10 melanoma cells in vivo, which resulted in delayed tumour growth. Log-rank testing indicated that the delay in B16.F10 tumour growth in mice treated with TriDAP was statistically significant (p = 0.01 versus controls) with 30% of mice surviving after the end of the observation period. However, this anti-tumour effect was only observed when TriDAP was administered therapeutically (on Day 6 and every three days thereafter), as not only was treatment with TriDAP less efficacious when administered at the time of tumour inoculation (Day 0, 3, 6), tumour progression was accelerated when compared with PBS-treated control mice. Whilst this enhanced tumour growth did not reach statistical significance, this finding suggests that TriDAP could have the opposite effect (direct and/or indirect) on the inflammatory process depending on the timing of administration. This possibility is supported by recent studies in K. Mills laboratory showing that administration of TriDAP to mice at the onset of EAE significantly reduced disease severity, but exacerbated the clinical symptoms of EAE when administered after the onset of disease (Kelly and Mills; personal communication; unpublished data).
The findings of this study suggest that the anti-tumour effects of TriDAP may be mediated via its effects on immune modulation. Since TriDAP significantly delayed B16.F10 tumour growth when administered therapeutically but accelerated tumour growth when administered at the same time as B16.F10 tumour inoculation, it is possible that TriDAP exerts its anti-tumour properties via induction of T<sub>H</sub>1-promoting cytokines, IL-12 and IL-27, leading to IFN-γ production. Although IL-12 is the most potent inducer of T<sub>H</sub>1 differentiation and IFN-γ production, it has recently been discovered that IL-27, an IL-12 family member, also functions to drive naïve cells into the T<sub>H</sub>1 subset at the initial stage of differentiation. Yoshida et al. reported that WSX-1<sup>−/−</sup> (IL-27 receptor KO) mice are susceptible to L. major, an intracellular pathogen whose clearance depends largely on a T<sub>H</sub>1 response (Yoshida et al., 2001). However, impaired IFN-γ production was observed only at the early phase of L. major infection in WSX-1-deficient mice, with IFN-γ concentrations restored to those in wild type mice at later stages of infection, indicating that IL-27/WSX-1 signalling is only required at the initial stage of T<sub>H</sub>1 differentiation. Thus, IL-27 is essential for the initial production of IFN-γ and induction of T<sub>H</sub>1 responses, but it is not required for their maintenance (Yoshida et al., 2001), suggesting that TriDAP induced IL-27 is required at the early stages of the anti-tumour response. Furthermore, the anti-tumour activity of IL-12 in combination with IL-27 has recently been shown to be superior to IL-12 administration alone in an adenocarcinoma 4T1 murine model (Zhu et al., 2010), implicating the importance of these cytokines in anti-tumour immunity.

TriDAP promoted IL-1β and IL-23, cytokines known to promote the production of IL-17 from T<sub>H</sub>17 cells. However, evidence is now emerging that IL-27 also has an immunosuppressive/regulatory function unrelated to its T<sub>H</sub>1-promoting activity.
Retinoid-related orphan receptor (ROR)γt and RORA are lineage-specific transcription factors that direct T_h17 development (Yang et al., 2008a). Evidence is accumulating that IL-27 exerts differential effects on naïve versus committed T cells, in that IL-27 acts directly on CD4^ T cells to suppress T_h17 differentiation (Batten et al., 2006, Diveu et al., 2009), but once differentiated, T_h17 cells acquire resistance to suppression by IL-27 (El-behi et al., 2009). Diveu et al. reported that IL-27 can suppress T_h17-specific transcription factor RORγt, in a STAT1-dependent manner (Diveu et al., 2009). This suggests that IL-27 regulates the frequency of T_h17 cells primarily at the early differentiation stage. In support of this theory, El-behi et al. reported that RORγt and RORA expression in committed T_h17 was not affected by IL-27, and that the resistance to IL-27 was not due to the absence of a functional IL-27 receptor or reduced expression of IL-23R (El-behi et al., 2009). These findings are further supported with reports of Yoshimura et al. demonstrating limited ability of IL-27 to down-regulate IL-17A production in memory T cells (Yoshimura et al., 2006) and also by Diveu et al. describing the ineffectiveness of IL-27 in suppressing IL-17A production by in vitro-differentiated T_h17 cells stimulated with IL-23 (Diveu et al., 2009). Additionally, IL-27p28^-^ and IL-27Ra^-^ mice are more susceptible to EAE than wild type mice (Batten et al., 2006, Diveu et al., 2009). Diveu et al. demonstrated that at the onset of EAE, a higher gene expression of T_h17-related molecules (IL-23, IL-23ra, transcription factor rorc, Il-17a and Il-17ra) was observed in the CNS of IL-27p28^-^ mice compared with WT mice, however, later in established disease, the increased gene expression in IL-27p28^-^ mice was no longer observed, suggesting that IL-27 regulatory effects are exerted in the earlier phases of EAE (Diveu et al., 2009). Moreover, Batten et al. reported that IL-27Ra^-^ mice are more susceptible to EAE due to their ability to generate a robust T_h17 response (Batten et al., 2006). Furthermore, administration of
IL-27 to mice at the onset of EAE reduced disease severity, whilst IL-27 treatment in established EAE had no effect on disease course (Fitzgerald et al., 2007). The authors suggested that IL-27 may have suppressed EAE by inhibiting development of encephalitogenic $T_h17$ cells, rather than suppressing committed $T_h17$ cells. Furthermore, IL-27 has also been shown to inhibit development of Foxp3$^+$-inducible regulatory T cells (Neufert et al., 2007, Huber et al., 2008), which are known to suppress anti-tumour immune responses.

$T_h17$ cells, depending on the stage of their development, display a difference in their susceptibility to IL-27. Furthermore, IL-27 not only blocks IL-17A and IL-17F production in naïve T cells, it also inhibits $T_h17$ differentiation by suppressing, in a STAT1-dependent manner, the expression of the $T_h17$-specific transcription factor RORγt, suggesting that IL-27 regulates the frequency of $T_h17$ cells primarily at the early differentiation stage (Diveu et al., 2009). Thus, it is possible that therapeutic administration of TriDAP lead to the production of a $T_h17$ mediated response (via induction of $T_h17$-promoting factors IL-1β and IL-23) due to resistance of committed $T_h17$ cells to the effects of TriDAP-induced IL-27. Early TriDAP treatment may have accelerated tumour growth because whilst a robust $T_h17$ response is generated, TriDAP-induced IL-27 suppresses the development of $T_h17$ responses due to the inhibitory effects of IL-27.

However, IL-27 was only one of the many cytokines induced by the synergistic effects of TriDAP and TLR agonists on DCs, and thus it is possible that other cytokines and/or immune mediators may also be involved. The depletion of certain lymphocyte subsets
using *in vivo* administration of specific antibodies, and the use of mice deficient in certain immune-modulating molecules (for example IL-27 receptor (WSX-1<sup>−/−</sup>), IL-12R<sup>−/−</sup>, IL-17<sup>−/−</sup> knock-out mice) are two approaches that could be used to identify the immune cells/molecules responsible for mediating TriDAP-induced anti-tumour and pro-tumour responses to B16.F10 melanoma *in vivo*. Furthermore, the *in vitro* findings reported herein await *in vivo* verification of the synergy between NOD1 and TLR agonists on the priming of T<sub>H1</sub> and T<sub>H17</sub> responses. However, in support of the *in vitro* findings, Fritz and colleagues demonstrated that immunization of mice with ovalbumin emulsified in IFA with FK156 (NOD1 agonist) and LPS (TLR4 agonist) or FK156 and Pam<sub>3</sub>CSK<sub>4</sub> (TLR2 agonist) significantly increased antigen-specific IFN-γ and IL-17 production by CD4<sup>+</sup> T cells following ex-vivo restimulation (Fritz et al., 2007). This demonstrates that NOD1 agonists synergize with TLR agonists to prime antigen-specific T<sub>H1</sub> as well as T<sub>H17</sub> immune responses *in vivo*.

In addition to generating a T<sub>H1</sub>-type immune response, IL-12 derived from TriDAP activated DCs could also influence innate immunity, such as responses of NK and NKT cells. DCs play a predominant role in the activation of NK cells, and early NK cell recruitment to tumour lesions play a pivotal role in the innate immunity against tumour cells (Pan et al., 2004). Moreover, in animal models, NK cells were shown to be necessary for eliminating primary tumour implants (Wu et al., 1995), as well as metastasis (Tamara et al., 1997). Smyth *et al.* reported that NK cells were the primary mediators of IL-12-induced anti-tumour activity against murine RM-1 prostate tumours, and that T cells were not involved (Smyth *et al.*, 2000a). In contrast, Grant *et al.* observed that although NK cells were the primary effectors mediating early inhibition of tumour growth, NK cells alone were not sufficient to prevent mortality by RM-1
tumours following vaccination with ALVAC recombinant canarypox viruses encoding IL-2, IL-12 and TNFα (Grant et al., 2006). Moreover, in this model, the presence of both CD4⁺ and CD8⁺ T cells in the absence of NK cells was not sufficient to inhibit growth of primary tumour or following rechallenge (Grant et al., 2006). This demonstrates that co-operation is required between innate and adaptive responses to mediate optimal tumour immunity. The RM-1 murine prostate cancer cell line utilized in these studies has a similar immunogenicity profile to that of the B16.F10 melanoma cell line used in the current study; both cell lines are highly aggressive and poorly immunogenic due to low MHC class I expression. As NK cells preferentially target tumour cells with low MHC class I expression, this may suggest a prominent role for NK cells in B16.F10 tumour rejection. Thus, it is possible that the anti-tumour immune responses in mice therapeutically treated with TriDAP, involved both NK cell-mediated innate response in addition to a tumour-specific T cell response. This highlights the need for critical evaluation of effector cells other than T cells in future analysis of TriDAP treatment of murine melanoma, as co-operation between innate and adaptive immunity could be an important factor in the ability of TriDAP induced cytokines to mediate tumour immunity.

 Whilst prolonged survival of tumour-bearing mice was observed, the TriDAP late regimen therapy failed to eradicate (or lead to regression of) the aggressive B16.F10 tumours. These findings reveal the necessity to improve on the immunotherapeutic design, as TriDAP as a monotherapy could only at best achieve a slowing of the growth of established B16.F10, but was ineffective in inducing a cure of any treated mice. This is in line with a study reported recently, whereby the NOD2 agonist, MDP-Lys (L18) as a monotherapy demonstrated equivalent growth kinetics to that of untreated B16.F10
melanoma-bearing mice (Fujimura et al., 2011). However, an improved immunotherapeutic approach consisting of MDP-Lys in combination with IFN-β significantly augmented the anti-tumour effect of IFN-β administered as a monotherapy and significantly suppressed the growth of B16.F10 melanoma when compared with untreated control mice (Fujimura et al., 2011).

In this chapter, since experiments were conducted only once (but with an appropriate number of mice for statistical evaluation of the data), the data has to be interpreted with caution. Since TriDAP modulated TLR-induced DC cytokine production but when used alone did not induce quantifiable cytokine production and since TriDAP as a monotherapy did not induce regression of murine tumours, this diminished its potential for further investigation. However, the findings of the present study may assist in the development of an improved anti-tumour vaccine or immunotherapy. In terms of anti-tumour immunity, two critical findings emerged from this study with the observation that costimulation of DCs by NOD1 agonist TriDAP with TLR agonist led to a significant increase in the production of both Th1- and Th17-promoting cytokines; IL-12p70 and IL-27; and IL-1β and IL-23, respectively, which are known to play a role in tumour eradication. This suggests that functional interaction between Th1 cells and Th17 cells could potentially generate clinically relevant anti-tumour immunity. This hypothesis is explored further in the next chapter of this thesis.
Figure 3.1 Phenotype, yield and the proportion of CD11c+ cells from sample BMDC culture. Bone marrow cells derived from two C57BL/6 mice were cultured in cRPMI medium supplemented with GM-CSF and non-adherent cells harvested on day 10. (A) Percentage of CD11c+ cells analyzed by flow cytometry (n=2 BMDC cultures). (B) Total cell count on day 0 of culture and total cell count following harvest of all non-adherent cells on day 10 of culture from five independent BMDC cultures. Results are expressed as mean (±SD). (C) Surface expression of CD40, CD80, CD86 and MHC class II by bone marrow-derived CD11c+ BMDC. Non-adherent cells were harvested on day 10 of culture and labelled with antibodies specific for CD11c, CD40, CD80, CD86 and MHC class II. Dot plots show all culture-derived cells after gating out dead cells. The numbers in each profile indicate the percentage of CD11c cells staining for each marker.
Figure 3.2 The NOD1 agonist TriDAP synergises with TLR agonists to promote cytokine secretion by murine DC. DC were stimulated for 24 h with increasing concentrations of TriDAP (1-100 μg/ml) alone or in combination with either LPS (100 ng/ml) or CpG (5 μg/ml). (A) The concentrations of IL-12p70, IL-1β, IL-23 and IL-10 in culture supernatants were quantified by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. * P < 0.05; ** P < 0.01; *** P < 0.001 versus TLR agonist alone (ANOVA).
Figure 3.3 The NOD1 agonist TriDAP synergises with a range of doses of TLR agonist to promote cytokine secretion from murine DC. DC were stimulated for 24 h with TriDAP (100 ng/ml) alone or in combination with increasing concentrations of either LPS (1-100 ng/ml) or CpG (1-5 μg/ml). The concentrations of IL-12p70, IL-1β, IL-23, IL-10, IL-12p40 and TNFα in culture supernatants were analysed by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. * P < 0.05; ** P < 0.01; *** P < 0.001 versus TLR agonist alone (ANOVA).
Figure 3.4 TriDAP in synergy with LPS optimally induces IL-27p28 mRNA expression in murine DCs after 4 h. DC were stimulated for 4-12 h with TriDAP (10 μg/ml) and LPS (10ng/ml) either alone or in combination. IL-27p28 mRNA expression was evaluated at each time point by real-time PCR. Values are expressed as relative quantities (RQ) normalized to the endogenous control gene, 18 s rRNA and relative to BMDC stimulated with medium only. * P < 0.05 versus LPS alone (ANOVA). Results are from one experiment.
Figure 3.5 The effects of pre-treatment with TriDAP on TLR agonist induced cytokine production. DC were either pre-treated with TriDAP (100 μg/ml) 2 h prior to stimulation with TLR agonists, LPS (100 ng/ml) and CpG (5 μg/ml) or co-treated with TriDAP and TLR agonists or treated with TLR agonist alone. Culture supernatants were collected after 24 h and cytokine concentrations were quantified by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. * P < 0.05; ** P < 0.01; *** P < 0.001 (ANOVA).
Figure 3.6 The effects of pre-treatment with TriDAP on LPS induced IL-27p28 mRNA expression. DC were either pre-treated with TriDAP (100 μg/ml) 2 h prior to stimulation with LPS (100 ng/ml) or co-treated with TriDAP and LPS or treated with LPS alone. IL-27p28 mRNA expression was evaluated after 4 h by real-time PCR. Values are expressed as relative quantities (RQ) normalized to the endogenous control gene, 18 s rRNA and relative to BMDC stimulated with medium only. *** P < 0.001 versus LPS alone (ANOVA). Results are from one experiment.
Figure 3.7 Synergistic effects of NOD1 agonist TriDAP with TLR agonists on the induction of cytokine production by iBMDMs. Cells were stimulated for 24 h with TriDAP (100 μg/ml) either alone or with LPS (10/100 ng/ml), Poly:IC (25/100 μg/ml). Cytokine concentrations in culture supernatants were analysed by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. * P < 0.05; ** P < 0.01; *** P < 0.001 versus TLR agonist alone (ANOVA).
Figure 3.8 Synergistic effects of NOD1 agonist TriDAP with TLR agonists on IL-27p28 mRNA expression in iBMDCs. Cells were stimulated with TriDAP (100 μg/ml) either alone or with LPS (10/100 ng/ml), Poly:IC (25/100 μg/ml). IL-27p28 mRNA expression was evaluated after 4 h by real-time PCR. Values are expressed as relative quantities (RQ) normalized to the endogenous control gene, 18 s rRNA and relative to iBMDC stimulated with medium only. * P < 0.05 versus TLR agonist alone (ANOVA). Results are from one experiment.
Figure 3.9 Flow cytometric analysis identified human MoDCs as DC-SIGN+ CD14- cells. Human peripheral blood monocytes were cultured with IL-4 and GM-CSF for 7 days. On day 7 of culture, the purity of human MoDC was determined by staining with anti-CD14 and anti-DC-SIGN.
Figure 3.10 Synergistic effects of NOD1 agonist TriDAP with TLR4 agonist LPS on the induction of cytokine secretion by human MoDCs. (A) MoDC were stimulated for 24 h with increasing concentrations of TriDAP (1-100 µg/ml) either alone or with LPS (0.1 – 100 ng/ml). The concentrations of IL-23, IL-10, and IL-27 in culture supernatants were analysed by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment from a single donor. * P < 0.05; ** P < 0.01; *** P < 0.001 (ANOVA). (B) The purity of human MoDC was determined by staining with anti-CD14 and anti-DC-SIGN. Flow cytometric analysis identified MoDC’s as DC-SIGN † CD14 † cells.
Figure 3.11 Synergistic effects of TriDAP with low dose LPS on the induction of IL-27 and IL-10 from human monocyte-derived dendritic cells. MoDC were stimulated for 24 h with TriDAP (10 μg/ml) either alone or with LPS (10/100 pg/ml). The concentrations of IL-27 and IL-10 in culture supernatants were analysed by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment from a single donor (separate donor to that in Figure 3.8). ** P < 0.01; *** P < 0.001 (ANOVA).
Figure 3.12 The effects of TriDAP on maturation of BMDC. Immature BMDC were incubated with TriDAP (100 μg/ml) either alone or with LPS (100 ng/ml), CpG (5 μg/ml) or medium only. Cells were harvested after 24 h and labelled with antibodies specific for CD11c, CD86 and MHC class II. Cells were gated on CD11c. Results are from one experiment.
Figure 3.13 Direct effects of TriDAP on T cells. CD4⁺ or CD8⁺ T cell populations were purified from naïve mice and stimulated with anti-CD3 and anti-CD28 in the presence or absence of TriDAP (100 µg/ml) and LPS (100 ng/ml) either alone or in combination as shown. Culture supernatants were collected after 72 h and assayed for IFNγ, IL-10, IL-4, and TNFα production by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. *** P < 0.001 versus TLR agonist alone (ANOVA).
Figure 3.14 BMDC treated with TriDAP and TLR agonist suppress the secretion of IL-4 from treatment naïve CD4\(^+\) T cells. Murine DC were stimulated for 24 h with LPS (100 ng/ml) or CpG (5 μg/ml) in the presence of TriDAP (100 μg/ml). DC supernatant was collected after 24 h. Cells were washed and co-cultured with purified CD4\(^+\) T cell (1:10 ratio) with or without anti-CD3 (1 μg/ml) stimulation. T cell-DC co-culture supernatants removed after 72 h was assayed for IFNγ, IL-17, IL-4 and IL-10 production by ELISA (shown in A). The concentrations of IL-12p70, IL-1β, IL-23, IL-10, IL-27 and TNFα in DC supernatants removed after 24 h were quantified by ELISA (shown in B). Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. * P < 0.05; ** P < 0.01 versus TLR agonist alone (ANOVA).
Figure 3.14

B

![Graphs showing cytokine production](image)

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Plotted cytokines:
- IL-12p70 (pg/ml)
- IL-30 (pg/ml)
- IL-27 (pg/ml)
- TNF-α (pg/ml)
Figure 3.15 Effect of NOD1 agonist TriDAP on the growth of B16.F10 melanoma *in vivo*

Tumours were established in all groups (10 mice/group) on day 0 by subcutaneous injection of $2 \times 10^5$ B16.F10 cells into the left hind flank. Mice were intratumourally injected with 100 μg of TriDAP on days 0, 3, 6 (TriDAP Early) or on day 6 and subsequently every 3 days thereafter (TriDAP Late). The tumour volumes were determined every other day. Results are from one experiment, with ten mice/group. (A) Growth kinetics for each individual mouse, (B) Mean growth kinetics and (C) Percentage of mice surviving. Kaplan-Meier survival curves were analyzed by the logrank survival test. * $P < 0.05$ versus PBS treated group (Kruskal-Wallis test).
Figure 3.15

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Chapter 4
MPL and IL-1α as adjuvants for tumour vaccines
4.1 Introduction

It is well established that T cells, including αβ TCR^+CD4^+ αβ TCR^+CD8^+, and γδ TCR^+ T cells, play a crucial role in tumour immunosurveillance to eradicate tumour cells (Kaplan et al., 1998, Smyth et al., 2000c, Girardi et al., 2001, Shankaran et al., 2001, Gao et al., 2003, Dunn et al., 2006, Vesely et al., 2011). Numerous reports have examined the predictive value of tumour-infiltrating T cells, both effector and suppressor subsets, in patients with cancer. Studies have shown that tumour-infiltrating T_{reg} cells are associated with poor prognosis and decreased survival rates (Ichihara et al., 2003, Sasada et al., 2003, Fu et al., 2007, Curiel et al., 2004), whereas tumour-infiltrating effector T cells correlate with an improved prognosis in many human cancers (reviewed in (Jochems and Schlom, 2011)). These studies provide valuable information for the design of novel but most importantly effective cancer immunotherapeutics. Additionally, these studies provide the scientific and clinical rationale for developing novel immuno-enhancing strategies that promote T effector cell populations (such as T_{H1} and T_{H17} cells), but importantly do not induce the development, recruitment or expansion of T_{reg} cells, a strategy that would ultimately bias the immune response in favour of anti-tumour immunity rather than a T_{reg} cell mediated-tumour immune suppression.

Following antigen-specific activation via TCR interaction with MHC class II-antigenic peptide complex on DC, CD4^+ helper T cells differentiate into lineages with distinct cytokine expression and effector functions. In contrast, a subset of CD8^+ T cells are destined to develop into cytotoxic effector cells that secrete IFNγ and express granzyme B and perforin. IL-12, a disulfide-linked heterodimeric cytokine composed of a 35 kDa
chain (p35) and a 40 kDa chain (p40) (Kobayashi et al., 1989), is the dominant polarizing cytokine in directing the differentiation of naïve T cells into Th1 cells, which characteristically produce IFNγ (Trinchieri, 1994, Heufler et al., 1996, Trinchieri, 2003). Th1 differentiation is initiated with the activation of STAT-1 by IFNγ. STAT-1 signalling induces the expression of T-bet, which is critical for Th1 cell development by promoting the expression of IFNγ (through chromatin remodelling of the IFNγ locus) and IL-12Rβ2, whilst simultaneously suppressing GATA-3 and thus Th2 cell differentiation (Szabo et al., 2000). This step enables the activation of STAT-4 by IL-12, further enhancing the production of IFNγ. STAT-4 activation by IL-12 is an important determinant of Th1 development in CD4+ T cells (Jacobson et al., 1995); STAT-4 deficient mice have defective Th1 development (Kaplan et al., 1996) and IL-12 receptor-deficient mice have defective IFNγ production (Wu et al., 1997). IFN-γ exerts its biologic effects by interacting with an IFN-γ receptor (IFNGR) 1 and IFNGR2 (Pestka et al., 1997), which results in downstream activation of the Janus tyrosine kinase (JAK)-STAT signalling pathway which ultimately leads to the phosphorylation and subsequent dimerization of two STAT1 molecules followed by their nuclear translocation (Schindler and Plumlee, 2008).

IFNγ has potent anti-tumour activity against various experimental tumours (Giovarelli et al., 1986, Maekawa et al., 1988, Gansbacher et al., 1990), and it induces MHC class I protein expression on tumour cells, rendering these cells more immunogenic and susceptible to tumour-specific CTLs (Dighe et al., 1994, Kaplan et al., 1998). Studies have shown that induction of Th1 cells mediate effective anti-tumour immunity following immunization with antigen and certain adjuvants (that are Th1 inducers) (Ikeda et al., 2004). Tumour-specific Th1 cells can overcome strong
immunosuppression in tumour-bearing hosts and induce tumour regression in mice (Nishimura et al., 1999, Ikeda et al., 2004, Chamoto et al., 2006, Zhang et al., 2007, Wakita et al., 2009).

Activation of DC through TLR4 recognition has been shown to instruct DC to stimulate $T_{H1}$ cell differentiation via IL-12p70 production (Agrawal et al., 2003a). The lipid A portion of the TLR4 ligand LPS is known to possess strong immunostimulatory properties, inducing the maturation and activation of DC, enhancing $T_{H1}$ type responses and establishing long term protective immunity (Wysocka et al., 1995, De Smedt et al., 1996, Heufler et al., 1996). However, clinically, the use of purified LPS as an adjuvant for inducing $T_{H1}$ type responses is precluded by its toxicity. In an attempt to uncouple the immunomodulatory effects of the lipid A portion of LPS from its toxicity, Ribi and colleagues demonstrated that toxicity could be ameliorated by selective hydrolysis of the 1-phosphate group (Qureshi et al., 1985). This LPS derivative from Salmonella minnesota R595 was further detoxified by Myers et al. with the removal of an ester-linked fatty acid group (i.e. selective hydrolysis of the 3-hydroxytetradecanoyl group) (Myers et al., 1990) generating 3-O-desacyl-4'-monophosphoryl lipid A (MPL). The removal of a phosphate and a fatty acid group from lipid A produced a molecule that retained the adjuvant properties of lipid A but significantly reduced its toxicity (Baldridge and Crane, 1999, Alving, 1993). Murine studies have shown that MPL is a potent adjuvant for protein antigens primarily promoting the development of $T_{H1}$-type responses, characterised by increased IFN$\gamma$ production and induction of IgG2a antibodies (Fattom et al., 1995, Neuzil et al., 1997, Sasaki et al., 1997, Thompson et al., 1998, Moore et al., 1999). Mills and colleagues reported that injection of MPL into the peritoneal cavity of mice induced the production of IL-12 by peritoneal exudates cells.
(80% of which were macrophages) *ex vivo* (Moore et al., 1999). Collectively, these studies indicate that MPL has potent T\(_{h1}\) cell inducing capacity. Furthermore, MPL was found to be safe in various clinical vaccine trials in humans (Thoelen et al., 1998, Casella and Mitchell, 2008, Cluff, 2009). Thus, one aim of this study was to explore the potential to induce a T\(_{h1}\)-dominant tumour-antigen-specific immune response via the use of MPL as an adjuvant for tumour vaccination.

The cytokine IL-17 was cloned and described in 1995 (Yao et al., 1995a, Yao et al., 1995b). However, it was not until 2005 that IL-17 producing T\(_{h17}\) cells were classified as a new CD\(_4^+\) helper T cell subset distinct from T\(_{h1}\) and T\(_{h2}\) cells (Harrington et al., 2005). In contrast to the well established role of T\(_{h1}\) cells in promoting protective anti-tumour immune responses (Nastala et al., 1994, Zitvogel et al., 1996, Aruga et al., 1997, Tsung et al., 1997, Nishimura et al., 1999, Ikeda et al., 2004, Chamoto et al., 2006, Zhang et al., 2007, Wakita et al., 2009), less is known about T\(_{h17}\) cells in tumour immunity and immunotherapy. Physiologically, T\(_{h17}\) cells play a crucial role in host defence against certain infectious pathogens via the expression of chemokines and neutrophil recruitment (Ye et al., 2001, Ouyang et al., 2008, Iwakura et al., 2008, Tesmer et al., 2008, Khader et al., 2009). Furthermore, T\(_{h17}\) cells have been implicated in the pathogenesis of many inflammatory and autoimmune disorders (reviewed in (Iwakura et al., 2008, Tesmer et al., 2008)). However, several lines of evidence suggests that this effector T cell subset is also involved in protective anti-tumour immune responses and may be a target cell population for cancer immunotherapy.
Direct evidence for a protective role of IL-17 in anti-tumour responses was demonstrated in the B16.F10 melanoma model and the MC38 colon cancer model (Kryczek et al., 2009b, Martin-Orozco et al., 2009). The authors showed accelerated tumour growth and metastases in IL-17-deficient mice compared with wild type control mice (Kryczek et al., 2009b, Martin-Orozco et al., 2009). Moreover, transfection of murine Meth-A fibrosarcoma cells with the human IL-17 gene (Hirahara et al., 2001) or transfection of mastocytoma P815 and plasmocytoma J558L hematopoietic tumour cells with murine IL-17 gene (Benchetrit et al., 2002) were shown to suppress tumour progression in immuno-competent mice. A host-dependent mechanism involving T cells was suggested by the authors to be involved in the anti-tumour activity of IL-17, as nude mice transplanted with IL-17-transfected tumour cells did not show any difference in growth kinetics when compared with vector only transfected cells (Hirahara et al., 2001, Benchetrit et al., 2002). Adoptive transfer of TCR transgenic CD4+ T cells specific for tyrosine-related protein 1 (TRP-1; antigen expressed in normal melanocytes and in melanoma cells) polarized to a Th17 cell phenotype in vitro, was shown to induce B16.F10 melanoma eradication in immuno-competent mice (Muranski et al., 2008).

In patients with prostate cancer, a significant inverse correlation was found between the frequency of IL-17+ T cells infiltrating the tumour and the Gleason score (i.e. tumour grade), suggesting that Th17 cells mediate an anti-tumour effect in the development of prostate cancer (Sfanos et al., 2008). In addition, increased tumour ascites IL-17 and/or Th17 cells positively predict patient survival in ovarian cancer (Kryczek et al., 2009a). In gastric adenocarcinoma patients, high levels of intratumoural IL-17 expression correlate with increased survival over those with lower IL-17 expression levels (Chen et
Moreover, the accumulation of \( \text{T}_{17} \) cells in malignant pleural effusion in lung cancer patients predicted improved survival (Ye et al., 2010). Taken together, these studies provide strong evidence that \( \text{T}_{17} \) cells are involved in tumour immunity and suggest that approaches to differentiate and expand this helper T cell population may have therapeutic potential.

Notwithstanding, \( \text{T}_{17} \) cells have also been shown to have pro-tumorigenic functions. Since \( \text{T}_{17} \) cells are considered potent inducers of autoimmunity through the promotion of tissue inflammation, the resulting inflammatory mediators may promote malignant cell transformation, tumour growth and metastasis (Mantovani et al., 2008). The frequency of intratumoral IL-17-positive cells was shown to inversely correlate with patient prognosis and positively correlate with microvessel density in hepatocellular carcinoma and NSCLC patients (Zhang et al., 2009, Chen et al., 2010). Furthermore, compared with healthy volunteers, patients with gastric cancer were shown to have a higher proportion of \( \text{T}_{17} \) cells in peripheral blood (Zhang et al., 2008). Notably, the increased prevalence of \( \text{T}_{17} \) cells was associated with clinical stage (Zhang et al., 2008). Studies in mice showed that the growth of two transplanted tumours (B16.F10 melanoma and MB49 bladder carcinoma) is reduced in IL-17\(^{-}\) mice (Wang et al., 2009). Moreover, He et al. demonstrated decreased growth of mouse tumor cells (EL4 lymphoma, Tramp-C2 prostate cancer and B16.F10 melanoma) in IL-17-receptor-deficient mice (He et al., 2010). In line with these studies, Numasaki et al. transfected murine colon adenocarcinoma (MC38) and fibrosarcoma (MCA205) with murine IL-17 gene and found that the transfectants exhibited accelerated growth in wild-type C57BL/6 mice, when compared with non-transfected tumours (Numasaki et al., 2003). In a similar study, Tartour et al. reported enhanced growth of human cervical tumour
cells transfected with human IL-17 in nude mice when compared to the non-transfected tumours (Tartour et al., 1999). Moreover, Numasaki et al. demonstrated that IL-17 increases the net angiogenic activity and promotes the *in vivo* growth of human NSCLC transplanted in SCID mice via promoting CXCR2-dependent angiogenesis (Numasaki et al., 2005). Furthermore, ablation of IL-17A was reported to significantly reduce tumour development in mice bearing a heterozygote mutation in the adenomatous polyposis coli (APC) gene (Min<sup>Ap<sub>c716+/-</sub></sup> mice; a spontaneous model of intestinal tumorigenesis) (Chae et al., 2010). Another study revealed that the Th17 response directly contributes to enterotoxigenic *Bacteroides fragilis* (ETBF)-induced colon carcinogenesis (Wu et al., 2009). Notably, antibody-mediated blockade of IL-17A alone or combined blockade with the receptor for IL-23, a key cytokine amplifying Th17 responses, significantly inhibited ETBF-induced colon tumour formation in this study (Wu et al., 2009).

IL-23, an IL-12 cytokine family member, promotes the expansion and survival of Th17 cells. It has been reported that IL-23p19-deficient mice are resistant to chemically-induced skin carcinogenesis, and the resistance was associated with decreased matrix metalloproteinase (MMP) 9 expression and increased CD8<sup>+</sup> T cell infiltration into the tumour tissues (Langowski et al., 2006). In addition, transplanted tumours (B16.F10 melanoma and LL/2 lung carcinoma) are growth-restricted in hosts depleted of IL-23 or in IL-23-receptor-deficient mice (Langowski et al., 2006). TNFα is another cytokine which has been recently shown to promote tumour development. Charles et al. reported that TNFα-dependent IL-17 release lead to increased myeloid cell recruitment and increased tumour burden in a mouse model of ovarian cancer (Charles et al., 2009). Consistent with this, in patients with advanced cancer, treatment with the TNFα-
specific antibody infliximab substantially reduced plasma IL-17 levels (Charles et al., 2009). Thus, undoubtedly the role of Th17 cells in cancer is highly complex, and it remains controversial whether these cells promote tumour growth or mediate protective anti-tumour immune responses.

When naïve CD4⁺ T cells are activated via TCR engagement, the local cytokine milieu plays an important role in determining which effector T cell lineage they differentiate into, by inducing lineage-specific transcription factors. Thus, like the transcription factors T-bet (under the influence of IL-12) and GATA-3 (under the influence of IL-4) controlling the Th1 and Th2 lineages, respectively, Th17 cell development is controlled by transcription factors RORγt (the human ortholog is RORC), RORα (which have synergistic and partially redundant functions), STAT3 and interferon regulatory factor 4 (IRF4) (Ivanov et al., 2006, Brüstle et al., 2007, Harris et al., 2007, Yang et al., 2007, Yang et al., 2008b). Studies in humans and mice have identified a critical role for IL-1 in promoting the differentiation of Th17 cells (Acosta-Rodriguez et al., 2007, Wilson et al., 2007, Ben-Sasson et al., 2009, Chung et al., 2009, Ghoreschi et al., 2010, Gulen et al., 2010) but also in the expansion and maintenance of polarized effector Th17 cells (Sutton et al., 2006b, Chung et al., 2009). Importantly, it has been shown that IL-1 signalling induces the expression of RORγt and IRF4, transcription factors identified as critical for Th17 differentiation (Ivanov et al., 2006, Brüstle et al., 2007, Chung et al., 2009). In line with these studies, IL-1RI (the signalling receptor bound by both IL-1α and IL-1β) expression has been reported on human and murine T cells (Dower et al., 1986, Shirakawa et al., 1987) and approximately 20% of human CD4⁺ T cells in peripheral blood express IL-1RI of which approximately 39% coexpress naïve markers (CD45RA⁺CCR7⁺), 34% coexpress central memory markers (CD45RA⁻CCR7⁻), and
27% coexpress effector memory markers (CD45RA^CCR7^) (Lee et al., 2010). Moreover, both naïve and memory CD4^ T cells up-regulate IL-1RI expression following activation with anti-CD3 and anti-CD28, which mimics TCR engagement and costimulation (Rao et al., 2007, Chung et al., 2009). Furthermore, it has recently been reported that IL-1RI is selectively expressed on T_\text{h}17^ cells (Chung et al., 2009, Guo et al., 2009). The finding that memory CD4^ T cells express the IL-1RI suggests that IL-1 also functions in differentiated T cells and supports the described role of IL-1 in the expansion and maintenance of polarized T_\text{h}17^ cells (Sutton et al., 2006b, Chung et al., 2009).

Experimental autoimmune encephalomyelitis (EAE) is considered to be a T cell-mediated autoimmune disease model mimicking several aspects of the pathogenesis of human multiple sclerosis (MS). Initially, IFN\_\gamma-producing T_\text{h}1^ cells were thought to be crucial for the induction of EAE, however, IFN\_\gamma^ mice, IL-12^ mice and IL-12R\beta2^ mice all exhibit exacerbated EAE development (Ferber et al., 1996, Krakowski and Owens, 1996, Chu et al., 2000, Cua et al., 2003, Zhang et al., 2003). More recently, the contribution of T_\text{h}17^ cells/IL-17 to the pathogenesis of EAE has been demonstrated in mice treated with anti-IL-17-neutralizing antibody and also by the demonstration that adoptive transfer of autoantigen-specific T_\text{h}17^ cells, but not T_\text{h}1^ cells, induced EAE (Langrish et al., 2005b). Importantly, IL-1αβ double deficient mice (Matsuki et al., 2006) as well as IL-1RI^ mice (Schiffenbauer et al., 2000, Sutton et al., 2006b) exhibited significant resistance to the development of EAE, whereas IL-1Ra^ mice developed more severe EAE than wild type mice (Matsuki et al., 2006). Moreover, treatment with IL-1α exacerbated the course of EAE while administration of soluble IL-1RI or IL-1Ra significantly suppressed EAE in lewis rats (Jacobs et al., 1991, Martin
and Near, 1995). Furthermore, whilst profound attenuation of EAE was observed in IL-1αβ double deficient mice, IL-1α−/− or IL-1β−/− mice developed EAE in a manner similar to wild type mice (Matsuki et al., 2006), indicating that the presence of either IL-1α or IL-1β alone is sufficient to initiate development of disease. Collectively, these studies indicate an important role of IL-1 in EAE. Sutton et al. further showed that the induction of autoantigen-specific Th17 cells was abrogated in IL-1Rα−/− mice, suggesting that IL-1 plays an important role in the induction of autoantigen-specific Th17 cells, contributing to the development of EAE (Sutton et al., 2006b).

Other autoimmune disorders have also provided evidence that IL-1 signalling drives pathogenic IL-17-producing Th17 cells. Higher levels of Th17 cells and IL-17 expression have been reported in biopsy samples from patients with a deficiency of the interleukin-1 receptor antagonist (syndrome termed DIRA), an endogenous inhibitor of IL-1 which maintains homeostatic regulation of IL-1 activity (Aksentijevich et al., 2009). In mice, increased percentage of Th17 cells and high levels of IL-17 were observed in IL-1Rα−/− mice which spontaneously develop arthritis due to unopposed excess IL-1 signalling (Koenders et al., 2008). Furthermore, is has been shown that IL-17 production by IL-1Rα−/− T cells was significantly enhanced compared with wild type T cells (Nakae et al., 2003). These studies suggest a crucial role for IL-1 in the development of autoantigen-specific Th17 cells and thus the pathogenesis of arthritis in IL-1Rα−/− mice. Furthermore, Th17 cells are abundant in the skin of mice with a missence mutation in the NLRP3 (NACHT, LRR, and PYD domain-containing protein 3) inflammasome, which results in excessive production of IL-1β (Meng et al., 2009). Taken together, the identification of IL-1 as a key regulator of Th17 differentiation provides support for the hypothesis in the current study which is to explore the potential
to induce differentiation and expansion of a $T_H17$-tumour-antigen-specific immune response as well as a $T_H1$-tumour-antigen-specific response via administration of IL-1$\alpha$ in combination with MPL as adjuvants for tumour vaccination.

IFN$\gamma$-producing $T_H1$ cells and IL-17-producing $T_H17$ cells appear to function cooperatively in the optimal host protection against *Klebsiella pneumoniae* and against *Mycobacterium tuberculosis* (reviewed in (Chen and O'Shea, 2008)), suggesting that in some circumstances $T_H1$ and $T_H17$ cells collaborate in host defense. However, to date, the complimentary contributions of $T_H1$ and $T_H17$ cells to anti-tumour immunity remains unclear. In light of this, the current study aimed to investigate the adjuvant properties of MPL and recombinant IL-1$\alpha$ for their combined ability to mediate a mixed $T_H1/T_H17$ response to a model antigen.

In this study a model antigen and tumour vaccine/antigen preparations were used to determine the efficacy of the adjuvant combination of MPL and IL-1$\alpha$ in generating $T_H1$ and $T_H17$ responses and in protecting against B16.F10 tumours in mice. Initially, experiments were conducted using keyhole limpet hemocyanin (KLH), as the model antigen enabled an investigation of the generation of $T_H1$ and $T_H17$ cells using an antigen-specific model. Tumour studies were performed using heat-shocked and irradiated (Hs/Irr) tumour cells and the surrogate antigen, ovalbumin (OVA) as a source of tumour-rejection antigen(s) in prophylactic and therapeutic vaccination settings, respectively.
Aims of this Chapter

I) To investigate the adjuvant properties of TLR4 agonist, MPL and recombinant IL-1α for their combined ability to mediate a mixed T\textsubscript{H}1/T\textsubscript{H}17 response to a model antigen.

II) To determine the effectiveness of a combination of a TLR4 agonist, MPL and the cytokine, IL-1α as adjuvants for a whole cell tumour vaccine or peptide tumour vaccine.
4.2 Results

4.2.1 MPL induces secretion of the Th1-promoting cytokine IL-12p70 by murine DC

To confirm that LPS derivative, MPL, promotes the production of a Th1-inducing cytokine profile in DC, DC were stimulated with medium alone or with increasing concentrations of MPL (0.001–50 µg/ml) for 48 h. In comparison with medium-treated cells, MPL induced significant levels of the pro-inflammatory cytokines IL-12p70, IL-1β, IL-23 and TNFα at doses ranging from 0.1-50 µg/ml (Figure 4.1A). MPL also induced production of the immunosuppressive cytokine IL-10. However, IL-10 secretion was induced at low concentrations of MPL and less effectively at higher concentrations (p<0.05 10 µg/ml vs 1 µg/ml; p<0.001 25 µg/ml vs 1 µg/ml; p<0.001 50 µg/ml vs 1 µg/ml; p<0.01 25 µg/ml vs 10 µg/ml; p<0.001 50 µg/ml vs 25 µg/ml), with maximum IL-10 induced at 1 µg/ml of MPL (Figure 4.1A). This effect does not appear to have been due to cell toxicity as MPL induced an array of cytokines at all doses tested (Figure 4.1A). The different dose response curve for IL-10 production was also reflected in an increasing IL-12p70:IL-10 ratio with increasing concentrations of MPL (50 µg/ml ratio 1.349215 > 25 µg/ml ratio 0.9532129 > 10 µg/ml ratio 0.6883973 > 1 µg/ml ratio 0.569823; Figure 4.1B). MPL at concentrations less than 0.1 µg/ml did not induce any significant cytokine production. Based on these data the 10 µg/ml suboptimum dose of MPL was used in subsequent experiments. Of relevance to tumour therapeutics, MPL showed a dose-dependent decrease in IL-10 production in DC, and the IL-12p70:IL-10 ratio increased in a dose-dependent manner. This indicates that higher doses of MPL (≥10 µg/ml) promote production of Th1-inducing cytokines (IL-12p70 and TNFα) from DC rather than immunosuppressive cytokines characterised by
a dominance for IL-10 secretion. Furthermore, MPL also induced IL-1β and IL-23 production in DC, indicative of Th17-polarizing capacity.

4.2.2 MPL-induced DC cytokine production is dependent on functional TLR4
C3H/HeJ mice are LPS unresponsive due to a point mutation (proline→histidine) in TLR4 (Poltorak et al., 1998, Qureshi et al., 1999). Thus, DCs derived from C3H/HeJ mice are tolerant (i.e. do not respond) to TLR4 ligand activation. The results in Figure 4.2B show that the cytokine-inducing effect of MPL was abolished in DCs derived from TLR4-defective C3H/HeJ mice. The same pattern of responses was also observed with LPS stimulation. In contrast, CpG and Pam3-CSK, TLR9 and TLR2 agonists, respectively, induced cytokine production by DCs from C3H/HeJ mice. In contrast, DCs derived from C57BL/6 mice responded to all TLR agonists examined. These results demonstrate that TLR4 is essential for the recognition of MPL as well as LPS.

4.2.3 MPL induces maturation of murine BMDC
Optimal activation of naïve T cells requires TCR occupancy by MHC-peptide complex on DC and the interaction between costimulatory molecules, such as CD80, CD86, and CD40, on DC and their respective receptors on T cells (Schwartz, 1992, Lenschow et al., 1996). T cell activation in the absence of costimulation leads to antigen-specific hyporesponsiveness, T cell anergy or apoptosis (Linsley and Ledbetter, 1993, Chambers and Allison, 1997). LPS induces DC maturation in vivo (De Smedt et al., 1996). As MPL is a detoxified derivative of LPS, it was investigated whether MPL also induced DC maturation. DC were cultured for 24 h in the presence of MPL (10-50 μg/ml), LPS (100 ng/ml), or medium only. Expression of MHC class II, the costimulatory molecules
CD80 and CD86, and the TNF-receptor family member CD40 was analysed by flow cytometry. DCs cultured with medium alone expressed detectable levels of all four surface markers analysed, with particularly high levels of MHC class II expression (Figure 4.3). Culture of DC in the presence of MPL resulted in a significant increase in expression of all of the membrane markers, consistent with MPL-induced maturation of DCs. CD40, CD80 and CD86 up-regulation was particularly evident, because of the relatively low constitutive expression of these markers, whereas MHC class II up-regulation was more variable. A very similar pattern of activation was observed following culture with LPS. These observations indicate that the LPS derivative, MPL induced maturation of DC in vitro.

4.2.4 Stimulation of DC with MPL induces the production of chemokine CXCL10

CXCL10 is a CXC chemokine that specifically activates a receptor, CXCR3. CXCL10 functions to chemoattract CXCR3-positive cells, including activated T lymphocytes (CD4+ T_{H1} and CD8+ T cells), NK cells and APCs towards inflammatory and neoplastic regions (Reviewed in (Groom and Luster, 2011)). CXCR3 is not expressed on naïve T cells, but is rapidly upregulated following DC-induced T-cell activation (Groom and Luster, 2011). Knowing the potent role of CXCL10 in the chemoattraction of activated lymphocytes and NK cells, the expression of CXCL10 was analyzed in MPL-treated DC (Figure 4.4). DC cultured in medium alone did not produce any CXCL10. In contrast, MPL induced significant CXCL10 production in DC (p<0.001). Positive control of CXCL10 production was given by stimulation with LPS (100 ng/ml). Thus, MPL signalling triggers CXCL10 production by DC in vitro.
4.2.5 MPL promotes the induction of antigen-specific T cells that secrete IFNγ

To investigate the activity of MPL as an adjuvant *in vivo*, mice were immunized with model antigen KLH in the presence of MPL and T cell response evaluated *ex vivo*. C57BL/6 mice were immunized subcutaneously in the footpad twice (day 0 and 28) with 20 µg KLH either alone or with 10 µg MPL. The MPL dose of 10 µg/mouse was chosen based on the findings of previous studies in the laboratory (Moore et al., 1999). Popliteal LN cells were isolated seven days post booster immunisation and cultured with increasing concentrations of the antigen, KLH. LN cells from control mice immunized with PBS did not induce proliferation or secrete any cytokines in response to KLH stimulation *ex vivo* (Figures 4.5A and B). Immunization of mice with KLH alone induced T cells that secreted high levels of IL-10 and IL-4, but no IFNγ or IL-17, cytokine profiles characteristic of T_{h2} and T_{r1}-type cells. Coadministration of KLH with MPL as the adjuvant generated T cells that secreted high levels of IFNγ, but lower concentrations of IL-10 and IL-4 than that observed following immunization with KLH only (Figures 4.5A and C). The ratio of KLH-specific IFNγ:IL-10 was 4.5 from mice immunized with KLH in the presence of MPL compared with a ratio of 0.3 from mice immunized with KLH alone (Figure 4.5C). These findings are consistent with the induction of T_{h1} cells with MPL as an adjuvant for a coadministered antigen *in vivo*. However, coadministration with MPL failed to prime KLH-specific T_{h17} cells; IL-17 was below the limit-of-detection in antigen restimulation LN cells from mice immunized with KLH in the presence of MPL (Figures 4.5A). Antigen-specific proliferation was monitored by [³H]-thymidine incorporation. Coadministration of MPL with KLH significantly enhanced the antigen-specific proliferation of T cells (p<0.001 at all antigen doses tested) when compared with immunization with KLH only (Figure 4.5B). These findings demonstrate that MPL promotes the induction of T_{h1}
cells specific for the coadministered antigen. Furthermore, coadministration with MPL enhanced proliferation and significantly decreased IL-10 and IL-4 production. Similar results were observed in a repeat experiment; however, the decrease in IL-10 production did not reduce to statistical significance (Figures 4.5 D, E, F). Taken together, these findings clearly demonstrate that adjuvant MPL shifts T cell responses induced with an antigen from a Th2 and Th1-type response to a Th1 dominant response, implicating MPL as a powerful Th1-driving adjuvant in vivo.

4.2.6 IL-1α induces IL-17 and IFNγ secretion by activated CD4⁺ T cells and antigen-specific T cells in vitro

IL-1RI–deficient mice are resistant to the development of EAE, indicating that IL-1RI is necessary for the induction of immune responses that mediate EAE in mice (Sutton et al., 2006b). Importantly, defective IL-1RI signalling was associated with a severe defect in the autoantigen-specific IL-17-producing and to a lesser extent autoantigen-specific IFNγ-producing T cell populations (Sutton et al., 2006b), suggesting a major role for IL-1 in promoting T₇ Th cell responses and a modest role for IL-1 in promoting THI cell responses in vivo. This is in line with a report which showed that IL-1RI mRNA was expressed at higher levels in T₇ Th cells when compared with THI cells (Chung et al., 2009). In this study the effect of IL-1α on T₇ Th and THI cell differentiation in purified murine CD4⁺ T cells was investigated in vitro. CD4⁺ T cells were activated in the presence of anti-CD3/CD28 antibodies with or without IL-1α. CD4⁺ T cells activated in the presence of anti-CD3/CD28 antibodies with IL-12 was used as control for THI cell differentiation. IL-1α significantly enhanced anti-CD3/CD28 induced IL-17 and IFNγ secretion from CD4⁺ T cells (Figure 4.6), cytokine
profiles consistent with the differentiation of T\textsubscript{h}17 and T\textsubscript{h}1 cells, respectively. Importantly, TCR and costimulation signalling in the presence of IL-1\textalpha induced low or undetectable levels of IL-10 production from CD4\textsuperscript{+} T cells, suggesting that IL-1\textalpha does not mediate differentiation of IL-10-producing T\textsubscript{h}1-like cells. Interestingly, IL-12 enhanced anti-CD3/CD28 induced IL-10 production by CD4\textsuperscript{+} T cells.

To investigate the effect of IL-1\textalpha on \textit{in vivo} primed antigen-specific memory T cells \textit{in vitro}, C57BL/6 mice were immunized s.c. in the footpad with KLH (20 \textmu g) coadministered with MPL (25 \textmu g) as an adjuvant. 7 d later, popliteal LN cells were restimulated with either antigen (KLH) alone or antigen and IL-1\textalpha. IL-1\textalpha significantly enhanced IL-17 and IFN\textgamma secretion by \textit{in vivo} primed KLH-specific T cells over that induced by antigen stimulation alone (Figure 4.7). Importantly, IL-1\textalpha had no effect on the production of IL-10 from antigen-specific T cells (Figure 4.7). It was initially thought that TGF\beta was essential for T\textsubscript{h}17 cell development and that studies which were unable to demonstrate an essential role for TGF\beta in T\textsubscript{h}17 differentiation were due to the fact that the levels of TGF\beta in serum-containing cultures had been underestimated (reviewed in (Annunziato and Romagnani, 2011)). However, a study by Ghoreschi et al. has firmly demolished any role for TGF\beta in T\textsubscript{h}17 differentiation (Ghoreschi et al., 2010). All studies reported in this thesis were performed in serum-free medium thus negating any effects of TGF\beta in cultures, and in agreement with Ghoreschi and colleagues, firmly establishes the critical role for IL-1 in T\textsubscript{h}17 cell development a process which occurs in absence of TGF\beta signalling. Collectively, this data demonstrates that IL-1\textalpha acts directly on T cells to enhance cytokine production, as T cells stimulated in the absence of APC with anti-CD3 and anti-CD28 produced
increased amounts of IL-17 and IFNγ upon treatment with IL-1α. IL-1α also enhanced IL-17 and IFNγ production from in vivo primed antigen-specific T cells. Taken together, these findings confirm recent reports that IL-1 promotes the differentiation of Th17 cells and to a lesser extent Th1 cell differentiation (Sutton et al., 2006b).

4.2.7 Direct effects of IL-1α on murine BMDC

To confirm that IL-1α is acting on T cells and not on DC, DC were treated with IL-1α and the production of IL-12p70 and IL-10 was assessed after 24 h. DC treated with MPL was used as control. IL-1α did not induce the production of IL-12p70 or IL-10 from DC (Figure 4.8). Furthermore, IL-1α had no effect on MPL-induced IL-12p70 or IL-10 secretion from DC. These findings suggest that IL-1α exerts its effects on T cells and not on DC.

4.2.8 IL-1α and MPL as adjuvants promote the induction of antigen-specific T cells that secrete IFNγ and IL-17

To test the capacity of IL-1α and MPL to prime antigen specific Th1 and Th17 responses in vivo, C57BL/6 mice were immunized s.c. in the footpad with KLH (20 μg) with or without MPL (10 μg) and with or without recombinant IL-1α (100 ng), or with PBS only. On day 28 mice were boosted with either PBS alone, KLH alone or KLH and IL-1α (i.e. no MPL in booster immunization). Since the role of IL-1α may be masked by the potent effects of MPL, it was only given in the primary immunization. Seven days after the second immunization, mice were sacrificed and popliteal LN cells were restimulated with increasing concentrations of antigen (KLH at 2-50 μg/ml) in vitro. Cytokine concentrations were determined in supernatants removed after 72 h and
antigen-induced proliferation was assessed by the addition of $[^3]H$-thymidine for the last 18 h of culture. LN cells from control mice immunized with PBS did not induce proliferation or secrete any cytokines in response to KLH stimulation ex vivo (Figure 4.9A and C). Immunization of mice with KLH alone induced T cells that secreted high levels of IL-4 and IL-10, but no IFNγ or IL-17 (Figure 4.9A), cytokine profiles characteristic of Th2 or Tr1-type cells. In contrast, coadministration of KLH in the presence of IL-1α and MPL generated T cells that secreted significantly high levels of IFNγ and IL-17 (p<0.001 and p < 0.001 compared with KLH only immunized mice, respectively), cytokine profiles consistent with the induction of Th1 and Th17 cells, but also generated a significant decrease in IL-10 and IL-4 production (p<0.01 and p<0.001 compared with KLH only immunized mice, respectively). This cytokine profile suggests a shift in T cell responses induced with an antigen alone from a Th2 and Tr1-type dominant response to a Th1 and Th17 response induced by IL-1α and MPL as adjuvants for coadministered antigen. Consistent with the results shown in Figure 4.5A, coadministration of antigen with only MPL as adjuvant generated T cells that secreted significantly high levels of IFNγ (p<0.001 compared with KLH only immunized mice). However, a significant increase in IFNγ was detected in supernatants from KLH-stimulated LN cells from mice immunized with KLH in the presence of IL-1α and MPL (p<0.001 compared with KLH and MPL immunized mice). This is supported by the more significant lower concentration of IL-10 and IL-4 detected in supernatants from KLH-stimulated LN cells from mice immunized with KLH in the presence of IL-1α and MPL, when compared with mice immunized with KLH and MPL (Figure 4.9A). This is also reflected by an increase in the ratio of IFNγ:IL-10 from mice immunized with KLH in the presence of IL-1α and MPL over that observed for mice immunized with KLH and MPL (Figure 4.9B). Furthermore,
consistent with the results shown in Figure 4.5A, coadministration of antigen with MPL generated T cells that secreted undetectable levels of IL-17. In contrast, coadministration of antigen in the presence of IL-1α and MPL generated T cells that secreted significantly high levels of IL-17 (p<0.001 compared with KLH and MPL immunized mice). Significantly enhanced antigen-specific proliferation was observed in LN cells from mice immunized with KLH in the presence of IL-1α and MPL and KLH in the presence of MPL when compared with LN cells from mice immunized with KLH only (Figure 4.9C). Taken together, these findings consolidate the in vitro data shown in Figures 4.6 and 4.7, demonstrating that IL-1α promotes the induction of T<sub>H17</sub> cells specific for the coadministered antigen, but also reveals that IL-1α enhances the MPL-induced induction of T<sub>H1</sub> cells specific for the coadministered antigen. IL-1α and MPL as adjuvants coadministered with an antigen bias toward a shift in T cell responses away from a T<sub>H2</sub> and T<sub>R1</sub>-type response primarily induced by the cognate antigen towards a T<sub>H1</sub> and T<sub>H17</sub> type response. Therefore, immunization with antigen coadministered with IL-1α and MPL as adjuvants may provide optimal conditions for the generation of T<sub>H1</sub> and T<sub>H17</sub> cells in vivo.

4.2.9 IL-1α and MPL as adjuvants promote the induction of T cells that secrete IFNγ and IL-17

To examine the capacity of IL-1α and MPL to promote the induction of T cells secreting IFNγ and IL-17 in vivo, C57BL/6 mice (n=3/group) were immunized s.c. in the footpad with KLH (20 μg) with or without MPL (10 μg) and with or without recombinant IL-1α (100 ng). On day 28 mice were boosted with either KLH alone or KLH and IL-1α (i.e. no MPL in booster immunization). Since the role of IL-1α may be masked by the potent effects of MPL, it was only given in the primary immunization.
Seven days after the second immunization, mice were sacrificed and popliteal LN cells were restimulated *ex vivo* with PMA and ionomycin for 6 h, with brefeldin A being added in the last 4 h. LN cells were then stained with antibodies specific for surface CD3, CD4, CD8 and γδ TCR and intracellular IFNγ, IL-17 and IL-10 and analyzed by FACS. The frequency of IL-17⁺ CD4⁺ T cells (Figure 4.10A) and IL-17⁺ γδ T cells (Figure 4.11) was increased in mice immunized with KLH in the presence of IL-1α and MPL. In comparison to mice immunized with KLH alone, immunization with KLH in combination with MPL or MPL and IL-1α did not enhance the frequency of IFNγ⁺ CD4⁺ T cells (Figure 4.10A), IFNγ⁺ CD8⁺ T cells (Figure 4.10B) or IFNγ⁺ γδ T cells (Figure 4.11). Evidently, however, the frequency of these populations was sustained in all immunized groups. In comparison to mice immunized with KLH alone or KLH in combination with MPL, the frequency of IL-10-secreting CD4⁺ T cells was slightly increased in mice immunized with KLH in the presence of IL-1α and MPL (Figure 4.10A). IL-10-secreting CD8⁺ T cells or γδ⁺ T cells was not induced in any immunized group (Figures 4.10B and 4.11). Taken together, these findings demonstrate that IL-1α and MPL as adjuvants coadministered with an antigen promote the induction of CD4⁺ T cells that secrete IFNγ and IL-17, cytokine profiles consistent with the induction of T₁₁ and T₁₁₁₇ cells. Thus, immunization with antigen coadministered with IL-1α and MPL as adjuvants may provide optimal conditions for the generation of T₁₁ and T₁₁₁₇ cells *in vivo*. IL-1α and MPL as adjuvants coadministered with an antigen also promoted the induction of CD8⁺ T cells that secrete IFNγ and γδ⁺ T cells that secrete IFNγ and IL-17.

4.2.10 IL-1α enhances MPL-induced nitrite (NO₂⁻) formation in macrophages

Nitric oxide (NO), a diatomic highly reactive free radical molecule, is a critical mediator of macrophage cytotoxicity (Hibbs et al., 1987) and is important in host
defence against pathogens (Green et al., 1991, Nathan and Hibbs, 1991, Nathan, 1992, Beckerman et al., 1993, Schmidt and Walter, 1994). NO is also associated with the cytotoxic activity of macrophages against tumour cells (Farias-Eisner et al., 1994, Xie et al., 1995, MacMicking et al., 1997, Juang et al., 1998, Xu et al., 1998, Garbán and Bonavida, 1999) and as a consequence NO-inducing agents/stimuli and NO-generating cells have attracted much attention in cancer immunotherapeutic approaches (Pipili-Syntos et al., 1995, Xie et al., 1995, Juang et al., 1998, Xu et al., 1998, Xu et al., 2002, Le et al., 2005, Kiziltepe et al., 2007, Bonavida et al., 2008, Bonavida and Baritaki, 2011). Therefore, this study investigated the possibility that MPL and/or IL-1α could promote NO production in macrophages in vitro. BMMΦs were treated with medium, MPL (10 μg/ml), IL-1α (0.1-1 μg/ml), or cotreated with MPL and IL-1α, and after 48 h, the concentration of nitrite (NO$_2^-$) secreted from cells was measured by the Greiss reaction with sodium nitrite as the standard (Green et al., 1982, Guevara et al., 1998). The accumulation of NO$_2^-$ provides a sensitive index of NO production in cells because NO$_2^-$ is a stable end-product of NO generation. The results showed that MPL stimulated NO production by murine BMMΦs within 48 h of culture. In contrast, NO production by BMMΦs was not observed following treatment with IL-1α, even at the higher dose tested (1 μg/ml). However, the combination of MPL with increasing concentrations of IL-1α (0.1-1 μg/ml) resulted in a synergistic effect, which significantly enhanced the concentration of NO released by BMMΦs in a dose-dependent manner, compared with MPL treatment alone (p<0.001; Figure 4.12A). This effect was completely abolished by treatment with IL-1Ra, which inhibits IL-1RI occupancy in BMMΦs, with levels of NO production indistinguishable from that induced by MPL alone following co-incubation with IL-1Ra (p>0.05; Figure 4.12B). To confirm this finding, BMMΦs derived from IL-1RI$^+$ and wild type mice were
treated with medium, MPL (10 μg/ml), IL-1α (100 ng/ml), or cotreated with MPL and IL-1α for 48 h. NO production was induced in wild type BMMΦs following treatment with MPL, and enhanced by IL-1α (p<0.001; Figure 4.13). While MPL induced NO production in IL-1Rα−/− BMMΦs, the synergistic effect of IL-1α on MPL-induced NO production was abolished in IL-1Rα−/− BMMΦs (Figure 4.13). These results demonstrate that IL-1α enhanced MPL-induced NO production in BMMΦs. Moreover, the synergistic effect of IL-1α on NO production was completely abolished by blocking IL-1RI signalling (IL-1Ra treatment) or by defective IL-1RI signalling (treatment of IL-1Rα−/− BMMΦs), suggesting that the IL-1α enhanced MPL-induced NO production is the direct effect of IL-1 signalling in BMMΦs.

4.2.11 IL-1α enhances MPL-induced iNOS mRNA in macrophages

NO is derived from the oxidation of one molecule of L-arginine at a guanidino nitrogen to produce Nω-OH-L-arginine as an intermediate, which is further oxidized to yield one molecule each of NO and L-citrulline (MacMicking et al., 1997, Nathan and Xie, 1994). These two sequential monooxygenase reactions are catalyzed by several isoforms of the nitric oxide synthase (NOS) enzyme; endothelial NOS (eNOS), neuronal NOS (nNOS) both of which are constitutively expressed and inducible NOS (iNOS; gene symbol NOS2), the expression of which is inducible. To determine whether IL-1α enhanced MPL-induced NO production in BMMΦs is due to iNOS induction, BMMΦs were examined for the ability to induce iNOS upon treatment with MPL and IL-1α. BMMΦs were treated with medium, MPL (10 μg/ml), IL-1α (500 ng/ml), or cotreated with MPL and IL-1α for 18 h. A significant level of iNOS mRNA was observed by RT-PCR in MPL-treated BMMΦs (p<0.001 compared with untreated cells; Figure 4.14). In contrast, iNOS mRNA expression was not detected in BMMΦs upon treatment with IL-
1α similarly to medium treated cells. However, the combination of MPL with IL-1α resulted in a synergistic enhancement of iNOS expression in BMMΦs compared with either treatment alone (p<0.001; Figure 4.14). This effect was completely abolished by treatment with IL-1Ra, with iNOS expression levels indistinguishable from that induced by MPL stimulation alone following co-incubation with IL-1Ra (p>0.05; Figure 4.15A). These results demonstrate that IL-1α enhances functional iNOS expression in BMMΦs upon treatment with MPL. Furthermore, the synergistic effect of IL-1α on iNOS expression was completely abolished by blocking IL-1RI signalling suggesting that the IL-1α enhanced MPL-induced iNOS mRNA expression is the direct effect of IL-1 signalling on BMMΦs.

4.2.12 MPL promotes the induction of the type 1 interleukin-1 receptor (IL-1RI) mRNA in macrophages

IL-1α binds and signals via the IL-1RI. The data shown in Figures 4.12 – 4.15 suggests that the IL-1α enhanced MPL-induced iNOS mRNA expression and NO production is the effect of IL-1 signalling on BMMΦs. However, this IL-1 signalling effect on BMMΦs is only triggered following co-stimulation of TLR4. Thus, it is possible that TLR4 activation may induce IL-1RI expression on BMMΦs. BMMΦs were treated with medium, MPL (10 μg/ml), IL-1α (500 ng/ml), or cotreated with MPL and IL-1α. After 18 h, IL-1RI mRNA expression in treated cells was evaluated by RT-PCR. IL-1RI mRNA expression was not detected in BMMΦs upon treatment with IL-1α. In contrast, a significant level of IL-1RI mRNA was observed in MPL-treated BMMΦs (p<0.001; Figure 4.16). However, the combination of MPL with IL-1α resulted in a synergistic effect, which significantly enhanced the induction of IL-1RI expression in BMMΦs over that induced by MPL stimulation alone (p<0.001; Figure 4.16). These
findings suggest that TLR4 stimulation via recognition of agonist MPL promotes the induction of IL-1RI mRNA expression in BMMΦs rendering the cells responsive to IL-1α. This then enables IL-1α signalling to further enhance the upregulation of its own receptor in BMMΦs.

4.2.13 Prophylactic immunization of mice with a tumour vaccine in combination with MPL and IL-1α promote anti-tumour immunity

To evaluate the significance of MPL and IL-1α induced differentiation and expansion of antigen-specific IFNγ and IL-17 –secreting cell populations in the induction of anti-tumour immunity in vivo, C57BL/6 mice were immunized s.c. into the left hind flank with heat-shocked and irradiated B16 cells (Hs/Irr B16; 1x10⁶/mouse) with or without MPL (10 μg/mouse) with or without IL-1α (100 ng/mouse) twice at a 28-day interval. Seven days after the second injection, 2x10⁵ B16.F10 tumour cells were implanted s.c. into each group of mice. Tumour growth was rapid in the untreated mice and those treated with Hs/Irr B16 cells alone, and there was no significant difference in the tumour volumes between these two groups (p>0.05; Figures 4.17A and B). In contrast, mice immunized with Hs/Irr B16 cells and MPL showed a significant decrease in tumour growth (p<0.001; Figures 4.17A and B) and significant increase in survival (p<0.001; Figure 4.18) when compared with untreated control mice. However, the most effective tumour suppression (p<0.001; Figures 4.17A and B) and enhancement in survival (p<0.001; Figure 4.18) compared with untreated control mice was observed in mice immunized with Hs/Irr B16 cells coadministered with IL-1α and MPL. Indeed, 44% (4/9) of mice immunized with Hs/Irr B16 cells in the presence of IL-1α and MPL completely rejected the tumour challenge and 75% (3/4) of these mice were resistant to rechallenge with B16.F10 tumour cells (p<0.001 compared with tumour only mice;
Figures 4.17 C and D), suggesting induction of potent and functionally active effector memory T cell responses in these mice. Tumours that grew in mice from the rechallenge in Figure 4.17D (i.e. n=1 mouse immunized with Hs/Irr B16 in combination with MPL and IL-1α as adjuvants and n=1 mouse immunized with Hs/Irr B16 coadministered with MPL as adjuvant) were stained with antibodies specific for surface CD3, CD4, CD8, and NK1.1 and intracellular IFNγ, IL-17 and IL-10 and analyzed by FACS. The frequency of IFNγ+ and IL-17+ CD4+ T cells was increased in the mouse immunized with Hs/Irr B16 in combination with MPL and IL-1α as adjuvants compared with untreated control mice (Figure 4.17E). This increased frequency of IL-17+ CD4+ T cells was also observed in the mouse immunized with Hs/Irr B16 in combination with MPL (Figure 4.17E). In comparison to the untreated mice, immunization with Hs/Irr B16 in combination with MPL and IL-1α enhanced the frequency of IFNγ+ CD8+ T cells (Figure 4.17F) and IFNγ+ NK cells (Figure 4.17G). Importantly, in comparison to the untreated mice, the frequency of IL-10-secreting CD4 and CD8 T cells was not increased in the mouse immunized with Hs/Irr B16 in combination with MPL and IL-1α (Figures 4.17 E and F, respectively). There was a slight decrease in IL-10+ NK cells in the mouse immunized with Hs/Irr B16 in combination with MPL and IL-1α compared with untreated mice (Figure 4.17G). Taken together, these findings suggest that the induction of tumour-antigen-specific effector T cell responses induced by the heat-shocked and irradiated B16 cell-tumour vaccine was significantly augmented by adjuvants MPL and IL-1α leading to effective anti-tumour immunity characterised by reduced tumour growth and increased survival. Furthermore, 33% (3/9) of mice in the MPL and IL-1α immunized group rejected a second rechallenge with tumour, suggesting the development of long-term anti-tumour immunity induced by MPL and IL-1α as adjuvants for coadministered tumour vaccine.
4.2.14 MPL adjuvant therapy or MPL and IL-1α co-adjuvant therapy in the presence of antigen-specific vaccination suppresses tumour growth

From a clinical prospective, it is extremely important to evaluate the protective effect of tumour vaccines using a therapeutic approach. OVA was used as a surrogate tumour antigen and mice were challenged with OVA-expressing B16.F10 cells. C57BL/6 mice (n = 7/group) were inoculated s.c. with 2x10^5 B16.F10-OVA cells. The tumour-bearing mice were treated on days 3, 10 and 17 post-tumour inoculation with s.c. injection of OVA protein (100 μg/mouse), with or without MPL (10 μg/mouse), with or without IL-1α (100 ng/mouse). Control mice received treatment with PBS. In earlier studies, K. Mills reported significant therapeutic efficacy by various different immunotherapeutic approaches utilizing this immunization schedule (Jarnicki et al., 2008, Toomey et al., 2008, Conroy et al., 2012, Marshall et al., 2012). Tumour growth was rapid in the PBS treated mice (Figures 4.19 A and B). Immunization of tumour-bearing mice with OVA antigen alone showed a minor but insignificant decrease in tumour growth when compared with control PBS-treated mice (p>0.05; Figures 4.19 A and B). Immunization with OVA in the presence of MPL enhanced the anti-tumour effect compared with OVA alone (Figures 4.19 A and B) and showed a non-significant increase in survival compared with PBS-treated or OVA-treated mice (Figure 4.19C). The anti-tumour effect of immunization with OVA and MPL was further augmented by coadministration of IL-1α, with a highly significant decrease in tumour growth (p<0.01 compared with PBS-treated mice; Figures 4.19 A and B) and an increase in survival when compared with all other groups (Figure 4.19C). However, this enhanced survival did not reach statistical significance. These findings demonstrate that therapeutic immunization with a model tumour-rejection antigen combined with MPL adjuvant therapy enhanced the anti-tumour effect of the tumour-rejection antigen in vivo against
the poorly immunogenic B16.F10 melanoma and importantly, this anti-tumour effect was significantly augmented by coadministration with IL-1α.
4.3 Discussion

Tumour-infiltrating lymphocytes have been reported to be associated with better prognosis (reviewed in (Yu and Fu, 2006, Jochems and Schlom, 2011)). However, it is now emerging that it is in fact the balance between effector and suppressor tumour-infiltrating lymphocytes, rather than their magnitude that is the critical determinant for mediating an effective anti-tumour immune response and thus for prognosis (Curiel et al., 2004, Galon et al., 2006, Yu and Fu, 2006). T_{reg} cells maintain immune homeostasis by controlling peripheral tolerance to host-derived antigens, including TSAs and TAAs, and thus function to suppress the host spontaneous anti-tumour immune response. Furthermore, several studies have shown that depletion of T_{reg} cells or suppressing their function enhances the efficacy of immunotherapy (Dannull et al., 2005, Viehl et al., 2006, Mahnke et al., 2007, Xu et al., 2009) suggesting that T_{reg} cells also suppress specific anti-tumour immunity induced by adaptive immunotherapy or cancer vaccines. Therefore, the hypothesis tested in this project was that skewing the population of tumour-infiltrating cells to favour helper CD4^+ T cells over the regulatory CD4^+ T cells within the tumour microenvironment would allow the induction of protective T_{H1}, T_{H17} and CTL responses that promote rejection of pre-established tumours.

Immunological adjuvants are substances designed to selectively enhance the four signals required for the initiation and activation of immune responses. The first signal (signal 0), is based on the stimulation of PRRs expressed on APCs, such as DCs and macrophages, by binding to PAMPs. As a result of this activation, APCs mature and present antigens in association with MHC molecules. T lymphocytes then become
activated through the interaction of the TCR with the MHC-antigenic peptide complex (signal 1) and a co-stimulatory signal(s) that is not antigen-specific but is required for T lymphocyte activation mainly through CD80 and CD86 binding to CD28 receptor or the CD40-CD40L interaction (signal 2). Immunocompetent cells may also determine the type of immune response by the release of pro- or anti-inflammatory cytokines that orientate T cell differentiation towards the Th1, Th2, Th17 or Treg functional pathways (polarizing cytokine signal 3). Adjuvants which target innate immunity include, cytokine-inducing PRR binding adjuvants, such as TLR agonists. Adjuvants that directly activate adaptive immunity include recombinant proteins such as cytokines, chemokines and co-stimulatory molecules.

In this study, MPL, the detoxified derivative of TLR4 ligand, LPS, was utilized as an adjuvant to act, via modulation of DC activity, in promoting the differentiation and expansion/maintenance of antigen-specific IFNγ-producing CD4⁺ Th1 cells, which have a well established role in promoting effective anti-tumour immunity (Nishimura et al., 1999, Ikeda et al., 2004, Chamoto et al., 2006, Zhang et al., 2007, Wakita et al., 2009). Kryczek et al. reported that Th17 cells were the only cell type expressing IL-17 in ovarian cancer ascites and that the levels of IL-17 in ovarian cancer ascites fluid was positively correlated with IL-1α and IL-1β but not with TGFβ, IL-6, IL-23 or IL-21 (Kryczek et al., 2009a), other cytokines which have been reported in the literature to be associated with Th17 cell development (reviewed in (Mills, 2008a)). These findings support the concept that IL-1 plays a selective and crucial role in Th17 cell development. Thus, recombinant IL-1α was utilized in this study as an adjuvant to promote the differentiation and expansion/maintenance of antigen-specific IL-17-producing CD4⁺ Th17 cells, which have only recently emerged, by several lines of
evidence, as a population with important anti-tumour activity (Hirahara et al., 2001, Benchetrit et al., 2002, Muranski et al., 2008, Sfanos et al., 2008, Kryczek et al., 2009a, Kryczek et al., 2009b, Martin-Orozco et al., 2009, Ye et al., 2010, Chen et al., 2011).

MPL, a derivative of LPS, exhibits only 0.1 to 1% of the toxicity of LPS (Ribi, 1984), which has led to clinically approved use of MPL as a vaccine adjuvant in humans (Thoelen et al., 1998, Casella and Mitchell, 2008, Cluff, 2009). As shown in this study, DCs exposed to MPL dose-dependently produced high levels of IL-12p70, whereas IL-10 production was decreased in a dose-dependent manner. As reflected in the IL-12p70:IL-10 ratio, higher doses of MPL (≥10 μg/ml) induced a shift in DC production towards a cytokine profile consistent with Th1-inducing capacity (i.e. increased IL-12p70 with decreased IL-10 induction). In line with this finding, Mitchell and colleagues reported that MPL activates Src homology-2 domain containing inositol 5-phosphatase 1 (SHIP1) in a MyD88-dependent manner and also reported that plasma concentrations of IL-10 were significantly higher in MPL-treated SHIP1-deficient mice compared with wild type mice (Cekic et al., 2011). SHIP1 is a lipid phosphatase that hydrolyzes the 5' phosphate of phosphatidylinositol 3,4,5-trisphosphate to generate phosphatidylinositol 3,4-bisphosphate, a process that blocks phosphoinositide 3-kinase (PI3K)-mediated membrane localization of Akt (Backers et al., 2003), and therefore counteracts the effects of the PI3K/Akt pathway which has been shown to lead to downstream activation of the IL-10 promoter (Martin et al., 2005). Martin et al. demonstrated that PI3K activation via TLR4 stimulation leads to activation of Akt, which subsequently mediates Ser9 phosphorylation of Glycogen synthase kinase (GSK)3β resulting in its inhibition (Martin et al., 2005). GSK3β inhibition augments the binding of CREB to the nuclear coactivator CBP causing the release of CREB to the
nucleus, where it binds and activates the IL-10 promotor (Martin et al., 2005). Supporting this, Martin et al. showed that inhibition of PI3K using LY294002, which abrogates the ability of LPS to inactivate GSK3β via Ser9 phosphorylation, resulted in a significant decrease in IL-10 but substantial increase in IL-12p40 compared with that of monocytes treated with LPS alone (Martin et al., 2005). Mice given the GSK3 inhibitor SB216763 2 h before LPS challenge showed a significant enhancement of IL-10 but reduction in IL-12p40 and IFNγ in the plasma 12 h post-LPS challenge compared with LPS only treated mice (Martin et al., 2005). In agreement with that reported, a study conducted in this laboratory found that inhibition of PI3K significantly suppressed secretion of IL-10 by BMDCs stimulated with LPS (Marshall et al., 2012). Collectively, these studies suggest that the ability of TLR4 agonist, MPL to differentially regulate IL-12 and IL-10 production from DC, is dependent on inhibition of the PI3K pathway via activation of SHIP1, which leads to reducing IL-10 substantially, while augmenting IL-12.

DC maturation, which involves upregulation of MHC class II and co-stimulatory molecules such as CD80, CD86, and CD40, is critically involved in naïve T cell activation and differentiation into T helper cell subsets. Importantly, T cell activation in the absence of costimulation leads to antigen-specific hyporesponsiveness, T cell anergy or apoptosis (Linsley and Ledbetter, 1993, Chambers and Allison, 1997). The findings of the present study showed that MPL enhanced the expression of MHC class II and co-stimulatory molecules CD80, CD86, and CD40 on DC in vitro, indicating maximal DC maturation is induced following MPL stimulation. In line with these in vitro findings, Moore et al. reported a significant increase in the numbers of peritoneal exudates cells expressing CD80 6 and 24 h after injection of MPL compared with
untreated control mice (Moore et al., 1999). In another study, Moser and colleagues showed that CD86 was strongly upregulated on CD11c+ DC following i.v. injection of MPL (De Becker et al., 2000). MPL has also been reported to augment anti-CD3-induced expression of CD40 ligand (CD40L) by CD4+ T cells (Ismaili et al., 2002). Taken together with the findings of the present study, this suggests that MPL enhances the CD40-CD40L interaction by upregulating CD40 expression on DC and by augmenting CD40L expression on CD4+ T cells induced by TCR engagement, thereby leading to optimal maturation of DC. Therefore, MPL enhances MHC class II and costimulatory molecule expression on DCs, which correlates with an enhanced ability of MPL stimulated-DCs to present antigen.

Immunization experiments performed in the present study demonstrated that coadministration of MPL with a model antigen KLH promoted the induction of antigen-specific T cells that secreted high levels of IFNγ, significantly greater than that induced by immunization with antigen alone. Conversely, antigen-specific IL-10 and IL-4 producing T cells were lower in LN cells from mice immunized with KLH and MPL compared with KLH only, indicating that MPL as an adjuvant shifts T cell responses induced with an antigen from a Th2 and Tr1-type response to a Th1 dominant response. Moreover, coadministration with MPL significantly augmented proliferation of antigen-specific T cells over that induced by immunization with antigen alone. Studies from this laboratory have shown that immunization with the whole cell pertussis vaccine, which is composed of high concentrations of Bordetella pertussis LPS, induced TLR4-dependent IL-1 and IL-23 production from DC, which ultimately leads to the induction of IL-17 production by murine antigen-specific memory T cells (Higgins et al., 2006). Furthermore, immunization with antigen in combination with LPS as adjuvant promotes
the generation of Th17 cells in murine small intestinal lamina propria (McAleer et al., 2010). However, in contrast with these reports, the current study demonstrated that immunization of antigen (KLH) with the LPS derivative, MPL, did not promote the induction of Th17 cells; IL-17 was undetectable in LN cells upon antigen recall ex vivo, despite the finding that MPL induced IL-1 and IL-23 production in DC in vitro, which is indicative of Th17-polarizing capacity. The explanation for this was not explored in the current study and warrants further investigation. Taken together, the findings reported herein demonstrate that MPL is a potent mediator of IL-12 production in DC, inducing optimal DC maturation in vitro and as an adjuvant MPL promotes a Th1-biased immune response in vivo.

The results of the current study showed that IL-1α significantly elevated IL-17 secretion from anti-CD3 and anti-CD28 activated CD4+ T cells in vitro, consistent with the role of IL-1α in activating Th17 cells. Furthermore, IL-1α significantly enhanced IL-17 secretion by antigen-activated memory T cells ex vivo. Importantly, IL-1α had no effect on the production of IL-10 from anti-CD3 and anti-CD28 activated CD4+ T cells or from antigen-specific memory T cells, suggesting that IL-1α does not support the generation of IL-10-producing T_{reg} cells. IL-1α also significantly enhanced IFNγ production from activated CD4+ T cells and from antigen-specific memory T cells, suggesting that IL-1α also plays a role in Th1 differentiation.

The examination of IL-1α and MPL as adjuvants for the antigen KLH showed the induction of potent antigen-specific T cells that secreted IFNγ and IL-17, cytokine profiles consistent with the generation of Th1 and Th17 T cells, respectively. The Th1
and Th17 responses were stronger with the combination of MPL and IL-1α than with MPL alone. Furthermore, the IL-1α and MPL adjuvant combination induced weaker antigen-specific −IL-10 and −IL-4 secreting T cells, compared with MPL alone. This suggests that the IL-1α and MPL adjuvant combination is more effective than MPL in tipping the balance away from Th2 and Tr1 type dominant response induced with the antigen alone towards a biased Th1 and Th17 dominant response. In support of these findings, both naïve and memory CD4+ T cells up-regulate IL-1R1 expression following activation with anti-CD3 and anti-CD28, which mimics TCR engagement and costimulation (Rao et al., 2007) and IL-1RI+ naïve CD4+ T cells and IL-1RI+ memory CD4+ T cells have been shown to produce IL-17 and IFNγ in response to IL-1 (Rao et al., 2007, Lee et al., 2010). MOG-specific −IL-17 and −IFNγ production was shown to be decreased in IL-1R−/− T cells consistent with another report (Sutton et al., 2006b) and increased in IL-1Ra−/− T cells compared with wild type T cells (Matsuki et al., 2006). Furthermore, IL-1 dramatically increased the frequency of IL-17-producing cells and modestly increased the frequency of IFNγ-producing cells, in response to OVA, among OT-II cells (enriched for CD4+ T cells) transferred to IL-1R−/− or wild type recipient mice (Ben-Sasson et al., 2009). Therefore, IL-1 augmented the differentiation of IL-17-producing and IFNγ-producing cells in OT-II cells primed in IL-1R−/− recipients or wild type recipients (Ben-Sasson et al., 2009). Taken together, these findings indicate that IL-1 plays a crucial role not only in Th17 differentiation but also to a more modest extent in Th1 differentiation.

To investigate if IL-1α was mediating its effects on T cells indirectly via DC, the production of IL-12p70 and IL-10 in DC in response to IL-1α in the presence and absence of MPL was investigated. As shown in the current study, IL-1α did not induce
the production of IL-12p70 or IL-10 from DC directly, nor did it exert any effect on MPL-induced cytokine induction in DC, suggesting that IL-1α exerts its effects on T cells directly. Further evidence supporting a direct effect of IL-1 on T cells has been shown in two elegant studies recently. Using the adoptive transfer of IL-1RI^+ OT-II cells into wild type recipient mice, Ben-Sasson et al. demonstrated that the major effect of IL-1 was on the responding CD4^+ T cells (Ben-Sasson et al., 2009). Chung et al. demonstrated that IL-1RI signalling deficiency on T cells but not on DC resulted in a great reduction in IL-17 production, suggesting that the IL-1 signal in CD4^+ T cells rather than DC is required for T_H17 cell polarization (Chung et al., 2009). Taken together, these findings suggest that IL-1 acts directly on CD4^+ T cells.

Chemokines constitute a family of chemoattractant cytokines which induce leukocyte migration into inflammatory sites or regulate lymphocyte trafficking through lymphoid tissues by activating receptors on the target cell (Yoshie et al., 2001, Murphy et al., 2000b) and some patterns of chemokine receptor expression can be associated with well established T cell phenotypes and pathways of T cell differentiation. CXCR3 chemokine receptor has been shown to be expressed preferentially on T_H1 cells (Bonecchi et al., 1998, Yamamoto et al., 2000, Kim et al., 2001) and its expression is driven by T-bet (Sundrud et al., 2003, Lord et al., 2005). Importantly, CXCR3 expression on T cells has been shown to induce their recruitment to tumour tissues in colorectal cancer and breast cancer (Musha et al., 2005, Chu et al., 2007). The results reported herein have demonstrated that MPL induces the secretion of the CXCR3 ligand CXCL10 from murine DC in vitro. This suggests that MPL, via the production of CXCL10 in DC, would likely favour the recruitment of T_H1 cells from the systemic circulation into the tumour microenvironment in vivo, however, such possibility
remains to be examined in the B16.F10 melanoma model. Furthermore, MPL may exert anti-angiogenic properties, albeit indirectly, conferred by its downstream mediator CXCL10. Studies suggest that solid tumours require the development of an adequate blood supply (through angiogenesis; formation of new capillaries from pre-existing blood vessels) to grow beyond 1-2 mm in diameter, in order to obtain sufficient nutrients, prevent hypoxia-induced necrosis and to metastasise to secondary sites (Folkman, 1990). The angiogenic process is regulated by the balance of endogenous stimulatory angiogenic factors (such as basic fibroblast growth factor; bFGF and vascular endothelial growth factor; VEGF) and inhibitory angiostatic factors (such as CXCL9, CXCL10, CXCL11, thrombospondin-1 and platelet factor-4) (Fidler, 1994, Strieter et al., 2005). There is considerable evidence that CXCL10 mediates anti-angiogenic effects \textit{in vivo}. CXCL10 is a potent inhibitor of angiogenic factor bFGF-induced neovascularization of matrigel injected subcutaneously into athymic mice (Angiolillo et al., 1995). In addition, CXCL10 dose-dependently suppressed endothelial cell differentiation into tubular capillary structures \textit{in vitro} (Angiolillo et al., 1995). Growth in nude mice of CXCL10-retrovirally transduced A375 human melanoma cells was markedly diminished compared to parental cells and the growth inhibition was associated with a marked reduction in microvessel density (Feldman et al., 2002). Furthermore, IL-12 mediated regression of renal cell carcinoma in a murine model was reported to be CXCR3 ligand-dependent, with the finding that the antitumor effect of IL-12 was lost when CXCR3 ligands, CXCL10 and CXCL9, were depleted (Tannenbaum et al., 1998). Thus, the angiostatic activity of MPL-mediated CXCL10 may also play a role in non-specific anti-tumour activity \textit{in vivo}. 

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Recently, a pattern of chemokine responsiveness/chemokine receptor expression that is characteristic of IL-17-producing T cells has been identified. Up-regulation and stable expression of CCR6 chemokine receptor has been reported to be a fundamental feature of T\(_{\text{H}}\)17 cells (Annunziato et al., 2007, Pêne et al., 2008, Singh et al., 2008, Reboldi et al., 2009) and CCR6 has been shown to be important in regulating the recruitment of T\(_{\text{H}}\)17 cells into inflammatory tissues (Hirota et al., 2007, Pêne et al., 2008, Yamazaki et al., 2008). Importantly, studies have shown that neutralization of CCR6 suppresses development of EAE and rheumatoid arthritis in mice (Hirota et al., 2007, Liston et al., 2009), autoimmune diseases in which T\(_{\text{H}}\)17 cells play a crucial role in the pathogenesis of disease (Mills, 2008a, Tesmer et al., 2008). To date, CC chemokine CCL20 is the sole ligand of CCR6 and CCR6 is also the sole receptor of CCL20 (Schutyser et al., 2003). CCL20 has been shown to direct the migration of CCR6\(^+\) cells (Liao et al., 1999). Moreover, high levels of CCL20 are found in human tumour microenvironments (Bell et al., 1999). A study in SKG mice, a strain that spontaneously develops T cell-mediated autoimmune arthritis, showed that T\(_{\text{H}}\)17 cells express CCR6 and synoviocytes express CCL20, suggesting CCL20-induced recruitment of T\(_{\text{H}}\)17 cells to the inflamed joints (Hirota et al., 2007). *In vitro* migration assay showed that CCR6\(^+\)CD4\(^+\) SKG T cells migrated in response to CCL20 in a dose-dependent manner, an effect that was blocked by anti-CCR6 monoclonal antibody (Hirota et al., 2007). Furthermore, CD4\(^+\) SKG T cells that had migrated in response to CCL20 were significantly enriched for IL-17\(^+\)CD4\(^+\) T cells, but not for IFN\(\gamma\)\(^+\)CD4\(^+\) T cells, indicating the preferential migration of T\(_{\text{H}}\)17 cells in response to CCL20 (Hirota et al., 2007). In support of this finding, Ye et al. showed that malignant pleural effusion (from patients with lung cancer) was chemotactic for T\(_{\text{H}}\)17 cells, and this activity was partially blocked by anti-CCL20 (Ye et al., 2010). Importantly, IL-1\(\beta\) has been shown
to be a potent inducer of CCL20 production by RA synoviocytes (obtained from patients with RA) and a synergistic effect was observed with IL-1β in combination with TNFα (Chabaud et al., 2001). This was complemented by another study showing that IL-1β-stimulated fibroblast-like synoviocytes (obtained from the synovial fluid of human RA) recruit CCR6+ mononuclear cells including CCR6+ T_h17 cells in a CCL20-dependent manner (Tanida et al., 2009). CCL20 production was up-regulated by IL-1α and TNFα in human melanoma cell lines (A375, A2058, and SK-MEL-2), suggesting that CCL20 release may be stimulated in malignant melanocytes by proinflammatory cytokines, IL-1α and TNFα (Hasan et al., 2006). The contribution of IL-1α alone to the induction of CCL20 production in melanoma cells was not determined in this study (Hasan et al., 2006). Collectively, these studies suggest that in addition to its crucial role in T_h17 differentiation, IL-1 may also play a role in the recruitment of CCR6+ T_h17 cells to the tumour microenvironment by inducing secretion of CCL20 from stromal cells within the tumour milieu, as has been described in other inflammatory tissues (Hirata et al., 2010) and/or directly from murine melanoma cells in agreement with that reported for human melanoma cells (Hasan et al., 2006). This notion was not explored in this study and warrants further investigation. Additionally, CCL20 induction has been demonstrated in peripheral blood mononuclear cells, DCs, and MΦs stimulated with LPS (Hromas et al., 1997, Rossi et al., 1997, Schutyser et al., 2000). Whether the removal of a phosphate and a fatty acid group from the lipid A portion of LPS, generating the MPL molecule, would impair/diminish its ability to induce CCL20 from DC/MΦ, is a matter of investigation. However, it is quite possible that MPL, like its parent molecule, might also induce the secretion of CCL20 from DC/MΦ, and consequently function in concert with IL-1α in the recruitment of CCR6+
T_{H17} cells to the tumour microenvironment. This concept warrants further investigation.

NO is a critical mediator of macrophage function, and its expression is associated with the cytotoxic activity of macrophages against transformed cells (Farias-Eisner et al., 1994, Xie et al., 1995, MacMicking et al., 1997, Juang et al., 1998, Xu et al., 1998, Garbán and Bonavida, 1999). It was determined in this study that iNOS mRNA was induced in macrophages in vitro in response to MPL. Surprisingly, however, whilst IL-1\(\alpha\) stimulus alone had no effect, IL-1\(\alpha\) enhanced MPL-induced iNOS expression in macrophages, a synergistic effect which was blocked by treatment with IL-1Ra. It was shown in this study that NO production followed iNOS mRNA expression. NO production was induced in macrophages in response to MPL and consistent with enhanced iNOS expression levels, IL-1\(\alpha\) enhanced MPL-induced NO production in macrophages. In agreement with the effects of IL-1 signalling-neutralisation on iNOS mRNA expression, this synergistic effect was completely abolished by treatment with IL-1Ra. This was further confirmed in IL-1RI-deficient macrophages, with NO production being induced in response to MPL similarly to wild type macrophages, however, the synergistic effect of IL-1\(\alpha\) on MPL-induced NO production was completely abolished in IL-1RI\(^{-/-}\) macrophages. These findings indicate that TLR4 agonist MPL and cytokine IL-1\(\alpha\) synergize for enhanced iNOS induction and NO production in macrophages. In an attempt to delineate the mechanism by which IL-1\(\alpha\) enhances MPL-induced NO production whereas IL-1\(\alpha\) stimulus alone had no inducible effect, the expression levels of IL-1RI was examined in treated macrophages. The results reported herein have shown that TLR4 stimulation via agonist MPL promotes the induction of IL-1RI mRNA expression in macrophages rendering the cells
responsive to IL-1α, thereby enabling IL-1α signalling to further enhance the up-regulation of its own receptor in macrophages. Consequently, the following sequential model of events is proposed: macrophages are rendered responsive to IL-1α via TLR4 agonist MPL-induced up-regulation of IL-1RI expression in macrophages. Once responsive to the effects of IL-1α, macrophages further increase the expression levels of IL-1RI in response to IL-1α signalling making the cells even more sensitive to the effects of IL-1α with consequential enhancement of iNOS induction leading to enhanced NO production in macrophages treated with IL-1α in combination with MPL in vitro. This sequential model proposed, however, warrants further investigation.

Within a tumour microenvironment, NO can be produced by macrophages, endothelial cells, stromal cells but also by the tumour cells themselves. Indeed, a number of studies have demonstrated an inverse correlation between NOS expression and cancer progression (reviewed in (Williams and Djamgoz, 2005)). Various mechanisms have been proposed for the anti-tumour properties of NO, including i) NO-induced apoptosis in tumour cells mediated by activation of caspases, upregulation of Fas on tumour cells rendering the cells sensitive to FasL-mediated CTLs and activation of the mitochondrial intrinsic apoptotic pathway; ii) NO-induced DNA double-strand breaks, which are the most cytotoxic DNA lesions that lead to cell apoptosis, and NO-induced DNA damage response pathways; and iii) anti-angiogenic effects of NO (Garbán and Bonavida, 1999, Xu et al., 2002, Le et al., 2005, Williams and Djamgoz, 2005, Kiziltepe et al., 2007, Snyder et al., 2009). It will be important to assess in vitro the sensitivity of B16.F10 tumour cells to lysis mediated by murine macrophages treated with MPL in the presence and absence of IL-1α. Further, it must be determined whether iNOS can be induced in macrophages within the tumour microenvironment in response to
immunization with MPL or MPL and IL-1α in combination with a tumour-antigen(s) \textit{in vivo}. An extension of these studies would then lead to examining the importance of macrophage-associated NO expression to B16.F10 tumour progression, in which tumour-bearing mice would be immunized with tumour antigen in combination with MPL and IL-1α. These mice would also receive treatment with a NOS inhibitor such as Nω-nitro-L-arginine methyl ester (L-NAME). These studies outlined will certainly receive attention in the future.

It was demonstrated in this study that the induction of anti-tumour effector responses induced by prophylactic injection of heat-shocked and irradiated B16.F10 tumour cells, was significantly augmented by adjuvants MPL and IL-1α leading to effective anti-tumour immunity characterised by reduced tumour growth and increased survival. Furthermore, 33% (3/9) of mice immunized with an antigen in combination with MPL and IL-1α rejected a second rechallenge with tumour, suggesting the development of long-term anti-tumour immunity induced by immunization with antigen in combination with MPL and IL-1α as adjuvants. In a therapeutic setting, immunization of tumour-bearing mice with OVA combined with MPL as an adjuvant showed a decrease in tumour growth and an increase in survival compared with mice immunized with OVA without adjuvant. In contrast, immunization with OVA combined with MPL and IL-1α as adjuvants significantly delayed tumour growth, when compared with mice immunized with antigen only. The greater potency of MPL and IL-1α adjuvants for tumour-antigen vaccine in the prophylactic compared with the therapeutic setting underlines the major obstacle of inducing immune responses once a tumour is already developing. This may also reflect the suppressive effects of the tumour on the induction of effector T cell responses.
While MPL has been well established as an attractive candidate as an adjuvant for cancer vaccines (Cluff, 2009), the effectiveness of IL-1α as a cancer vaccine adjuvant has to date been little explored with only one phase I study of adjuvant IL-1α immunotherapy with an allogenic colon cancer vaccine for Dukes' D colon or rectal cancer (Woodlock et al., 1999) being published to date. Furthermore, to the best of my knowledge this is the only study reported utilizing both MPL and IL-1α as adjuvants combined in an active specific immunotherapy approach. This suggests the potential for MPL and IL-1α used in combination as adjuvants for cancer vaccines has to date not been fully appreciated and thus this current project set out to explore the effectiveness of this combination of adjuvants, which with the exception of one phase I study (Woodlock et al., 1999), represents an unexplored and exciting adjuvant combination for cancer vaccines.

In conclusion, MPL and IL-1α enhanced the anti-tumour effector function of vaccine-induced cells in both prophylactic and therapeutic immunization settings, leading to suppression of tumour growth and enhanced survival. The *in vitro* and *in vivo* findings reported herein demonstrated that MPL and IL-1α as adjuvants promote the induction of KLH (model antigen)-specific Th1 and Th17 cells, and thus this adjuvant combination may also promote the induction of tumour-antigen-specific Th1 and Th17 cells which may mediate anti-tumour immune responses. Helper T cells may also provide help for the induction and maintenance of CTL-specific anti-tumour responses, and collectively these T cells suppress tumour growth. Additionally, MPL-mediated CXCL10 and the IL-1α and MPL-induced NO production in macrophages may also play a role in non-specific anti-tumour activity *in vivo* and thus contribute to suppression of tumour growth.
Figure 4.1 MPL promotes cytokine secretion by murine DC in a dose-dependent manner. BMDC were stimulated for 48 h with increasing concentrations of MPL (0.001–50 μg/ml). (A) The levels of IL-12p70, IL-10, IL-23, IL-1α, IL-1β, and TNFα in culture supernatants were quantified by ELISA. (B) The relationship between IL-12p70 and IL-10 secretion. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. * P < 0.05; ** P < 0.01; *** P < 0.001 versus medium, + P < 0.05; ++ P < 0.01; +++ P < 0.001 versus MPL, ANOVA.
Figure 4.2 MPL induced cytokine production by DC is TLR4-dependent
BMDC from C57BL/6 (A) or C3H/HeJ (B) mice were stimulated for 24 h with either MPL (µg/ml; TLR4 agonist), LPS (100 ng/ml; TLR4 agonist), Cpg (5 µg/ml; TLR9 agonist) or Pam3-CSK (10 µg/ml; TLR2 agonist). The concentrations of IL-12p70, IL-10, IL-23 and IL-1β in culture supernatants were quantified by ELISA. Results are expressed as mean values ±SD for n=3 replicates from one experiment. * P < 0.05; ** P < 0.01; *** P < 0.001 versus medium, ANOVA.
Figure 4.3 TLR agonist MPL induces DC maturation

Immature DC were either unstimulated or incubated with MPL (10/25/50 ng/ml) or LPS (100 ng/ml). Cells were harvested after 24 h and labelled with antibodies specific for CD11c, CD40, CD80, CD86 and MHC class II. Surface phenotype was evaluated by flow cytometry. Cells were gated on CD11c. (A), (B) and (C) show results from three independent experiments. (A) and (B) CD40, CD80, CD86 and MHC class II were evaluated. (C) Immature DC were either unstimulated or incubated with MPL (10 μg/ml). Cells were labelled with antibodies specific for CD11c, CD86 and MHC class II.
Figure 4.3
Figure 4.3

C

Untreated
MPL

CD86
MHC II
Figure 4.4 MPL induces CXCL10 secretion by murine BMDC
BMDC were either unstimulated or incubated for 24 h with MPL or LPS. The concentration of CXCL10 in culture supernatants was quantified by ELISA. (A) and (B) show results from two independent experiments. (C) Results are mean values from two experiments; (A) and (B). Results are expressed as mean (±SD). 
*** P < 0.001 versus medium, ANOVA.
Figure 4.5 MPL as an adjuvant promotes the induction of antigen-specific T cells that secrete IFN-γ. C57BL/6 mice were immunized s.c. twice (day 0 and 28) in the footpad with PBS, KLH (20 µg) alone, or KLH with MPL (10 µg). Popliteal lymph nodes were harvested on day 35 and the cells were restimulated with KLH (2-50 µg/ml) or medium only. (A and D) Supernatants were collected after 72 h, and IFN-γ, IL-10, IL-17 and IL-4 concentrations were determined by ELISA. (B and E) Proliferation was assessed after 72 h by monitoring [3H] thymidine incorporation. (C and F) The relationship between IFN-γ and IL-10 secretion. * P < 0.05; ** P < 0.01; *** P < 0.001 versus KLH alone. ANOVA. (A, B, C) and (D, E, F) show results from two independent experiments. Results represent the mean (±SD) of three mice/group.
Figure 4.5

D

![Graph D](image)

E

![Graph E](image)

F

![Graph F](image)
Figure 4.6 IL-1α induces IFNγ and IL-17 secretion by activated CD4+ T cells

Purified CD4+ T cells with or without anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) stimulation were treated with IL-1α or IL-12 at the indicated concentrations. Supernatants were assayed for cytokine production by ELISA after 72 h. * P < 0.05; ** P < 0.01; *** P < 0.001 versus anti-CD3/-CD28 stimulation alone, ANOVA. (A) and (B) show results from two independent experiments. (C) Results are mean values from two experiments; (A) and (B). Results are expressed as mean (±SD).

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<td>IL-12 (μg/ml)</td>
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Figure 4.6
Figure 4.7 IL-1α promotes IL-17 and IFNγ secretion by antigen-specific T cells
C57BL/6 mice were immunised s.c. in the footpad with KLH (20 µg) and MPL (25 µg). Popliteal lymph nodes were harvested after 7 d and the cells were restimulated with either KLH (2-10 µg/ml) alone or KLH and IL-1α (100 ng/ml). Supernatants were collected after 72 h, and IL-17, IFNγ and IL-10 concentrations were determined by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. ** P < 0.01; *** P < 0.001; n.s., not significant versus KLH alone, ANOVA.
Figure 4.8 Effects of IL-1α on IL-12 and IL-10 production by murine DC
BMDC were stimulated with IL-1α (0.1 μg/ml), MPL (10 μg/ml), or both for 24 h. The concentrations of IL-12p70 and IL-10 in culture supernatants were quantified by ELISA. n.s., not significant versus MPL alone, ANOVA. (A) and (B) show results from two independent experiments. (C) Results are mean values from two experiments; (A) and (B). Results are expressed as mean (±SD).
Figure 4.9 IL-1α and MPL as adjuvants promote the induction of antigen-specific T cells that secrete IFN-γ and IL-17. C57BL/6 mice were immunized s.c. in the footpad with 20 μg KLH with/without MPL (10 μg) with/without recombinant IL-1α (100 ng), or with PBS. On Day 28 mice were immunized with either PBS, KLH alone or KLH and IL-1α (i.e. no MPL in boost immunization). Popliteal lymph nodes were harvested on day 35 and the cells were restimulated with KLH (2-50 μg/ml) or medium only. (A) Supernatants were collected after 72 h, and IFN-γ, IL-10, IL-17 and IL-4 concentrations were determined by ELISA. (B) The relationship between IFN-γ and IL-10 secretion. (C) Proliferation was assessed after 72 h by monitoring [3H]thymidine incorporation. Results represent the mean values (±SD) of three mice/group from one experiment. * P < 0.05; ** P < 0.01; *** P < 0.001 versus KLH alone, + P < 0.05; ++ P < 0.01; +++ P < 0.001 versus KLH with MPL, ANOVA.
Figure 4.10 MPL and IL-1α as adjuvants promote the induction of T cells that secrete IFNγ and IL-17
C57BL/6 mice (n=3/group) were immunised in the footpad with 20 µg KLH with/without MPL (10 µg) with/without recombinant IL-1α (100 ng). On Day 28 mice were immunised with either KLH alone or KLH and IL-1α (i.e. no MPL in boost immunization). Popliteal lymph nodes (pooled from each group) were harvested on day 35 and the cells were restimulated with PMA (20 ng/ml) and ionomycin (200 ng/ml) for 6 h, with Brefeldin A (5 µg/ml) being added in the last 4 h. Intracellular IFNγ and IL-10 was determined in CD4 (A) and CD8 (B) T cells. Intracellular IL-17 was also determined in CD4 T cells (A). Results are from one experiment.
Figure 4.11 MPL and IL-1α as adjuvants promote the induction of T cells that secrete IFNγ and IL-17. C57BL/6 mice (n=3/group) were immunised in the footpad with 20 μg KLH with/without MPL (10 μg) with/without recombinant IL-1α (100 ng). On Day 28 mice were immunised with either KLH alone or KLH and IL-1α (i.e. no MPL in boost immunization). Popliteal lymph nodes (pooled from each group) were harvested on day 35 and the cells were restimulated with PMA (20 ng/ml) and ionomycin (200 ng/ml) for 6 h, with Brefeldin A (5 μg/ml) being added in the last 4 h. Intracellular IFNγ, IL-17 and IL-10 was determined in γδ T cells. Results are from one experiment.
Figure 4.12 IL-1α enhances MPL-induced NO$_2^-$ formation in macrophages

BMMΦ were stimulated for 48 h with (A) MPL (10 µg/ml) and IL-1α (0.1/1 µg/ml) either alone or in combination, (B) MPL (10 µg/ml), IL-1α (0.1 µg/ml), or both with or without IL-1RA (25 µg/ml). Nitric oxide production was determined in culture supernatants by measuring NO$_2^-$ (a stable metabolite of nitric oxide) accumulation using the Greiss reagent assay. Results are expressed as mean (±SD). *** P < 0.001; n.s., not significant versus MPL alone; +++ P < 0.001 versus MPL + IL-1α (0.1 µg/ml), ANOVA. (A) Results are the mean of four experiments, with n = 3 replicates per experiment. (B) Results are the mean of three experiments, with n = 3 replicates per experiment.
Figure 4.13 Synergistic effect of IL-1α on MPL-induced NO$_2^-$ is lost in IL-1R$^-$/ macrophages. Wild type or IL-1R$^-$ BMMΦ were stimulated for 48 h with MPL (10 µg/ml), IL-1α (0.1 µg/ml), or both. Nitric oxide production was determined in culture supernatants by measuring NO$_2^-$ (a stable metabolite of nitric oxide) accumulation using the Greiss reagent assay. (A) and (B) show results from two independent experiments. (C) Results are mean values from two experiments; (A) and (B). Results are expressed as mean (±SD). ** P < 0.01; *** P < 0.001; n.s., not significant versus MPL alone, ANOVA.
Figure 4.14 IL-1α enhances MPL-induced iNOS mRNA in macrophages
BMMΦ were treated with IL-1α (0.5 μg/ml) in the presence and absence of MPL (10 μg/ml) or treated with medium alone. (A) iNOS mRNA expression was evaluated after 18 h by real-time PCR. Values are expressed as relative quantities (RQ) normalized to the endogenous control gene, 18 s rRNA and relative to BMMΦ stimulated with medium only. Shown is the 260/280 nm ratio and 260/230 nm ratio of each RNA sample as determined by the NanoDrop ND-1000 spectrophotometer. (A) and (B) show results from two independent experiments. (C) Results are mean values from two experiments; (A) and (B). *** P < 0.001 versus untreated cells; +++ P < 0.001 versus MPL only, ANOVA.
**Figure 4.15 IL-1α enhanced MPL-induced iNOS mRNA in macrophages is inhibited by IL-1RA.** Wild type BMMΦ were treated with IL-1α (0.5 µg/ml), MPL (10 µg/ml), or both with or without IL-1RA (25 µg/ml). (A) iNOS mRNA expression was evaluated after 18 h by real-time PCR. Values are expressed as relative quantities (RQ) normalized to the endogenous control gene, 18 s rRNA and relative to BMMΦ stimulated with medium only. (B) 260/280 nm ratio and 260/230 nm ratio of each RNA sample as determined by the NanoDrop ND-1000 spectrophotometer. *** P < 0.001; n.s., not significant versus MPL alone, ANOVA. Results are from one experiment.
Figure 4.16 MPL promotes induction of the type 1 interleukin-1 receptor mRNA in macrophages
BMMΦ were treated with IL-1α (0.5 µg/ml) in the presence and absence of MPL (10 µg/ml) or treated with medium alone. IL-1R1 mRNA expression was evaluated after 18 h by real-time PCR. Values are expressed as relative quantities (RQ) normalized to the endogenous control gene, 18 s rRNA and relative to BMMΦ stimulated with medium only. Shown is the 260/280 nm ratio and 260/230 nm ratio of each RNA sample as determined by the NanoDrop ND-1000 spectrophotometer. (A) and (B) show results from two independent experiments. (C) Results are mean values from two experiments; (A) and (B). *** P < 0.001 versus IL-1α alone; +++ P < 0.001 versus MPL alone, ANOVA.
Figure 4.17 Prophylactic immunization of mice with a tumour vaccine in combination with MPL and IL-1α promote anti-tumour immunity. Mice were immunized s.c. into the left hind flank with heat-shocked and irradiated B16 cells (1 \times 10^6) alone or with MPL (10 μg), and IL-1α (100 ng) twice at a 4-week interval. Seven days after the boost, mice were challenged with 2×10^5 B16.F10 cells s.c. into the left hind flank. The tumour volumes were determined every other day. (A) Tumour volumes for each mouse. (B) Mean (±SEM) tumour growth rates for nine mice per group. (C and D) Mice (treated as in A) that completely rejected the growth of B16.F10 cells were re-challenged on day 41 with 2×10^5 B16.F10 cells s.c. into the opposite flank (n=4 mice Hs/Irr B16 + MPL + IL-1α group; n=1 mouse Hs/Irr B16 + MPL group, n=4 mice tumour only group). Re-challenge growth kinetics shown in C (tumour volumes for each mouse) and D (mean tumour growth rates). * P < 0.05; ** P < 0.01; *** P < 0.001 versus untreated tumour only group (Kruskal-Wallis test). Tumours that grew in mice from the re-challenge in (B) (i.e. n=1 mouse Hs/Irr B16 + MPL + IL-1α group; n=1 mouse Hs/Irr B16 + MPL group, n=4 mice tumour only group) were isolated and cells were stimulated with PMA (20 ng/ml) and ionomycin (200 ng/ml) for 6 h, with brefeldin A (5 μg/ml) being added in the last 4 h. Intracellular IFNγ and IL-10 was determined in CD4 T cells (E), CD8 T cells (F) and NK cells (G). Intracellular IL-17 was also determined in CD4 T cells (E). The gating strategy is also shown. Results are from one experiment.
Figure 4.17

B

Days after tumour challenge

Tumour volume (mm$^3$)

- Untreated
- Hs/Irr B16
- Hs/Irr B16 + MPL
- Hs/Irr B16 + MPL + IL-1 $\alpha$
Figure 4.17

C

Untreated Tumour Only

Days

Tumour volume (mm³)

Days

Hs/Irr B16 + MPL

Hs/Irr B16 + MPL + IL-1α

D

Tumour volume (mm³)

Days after tumour Re-challenge

213
Figure 4.17

F

\[
\begin{array}{cc}
\text{Untreated} & \text{Hs/IrrB16} + \text{MPL} & \text{Hs/IrrB16} + \text{MPL + IL-1α} \\
\text{% CD8^+IFNγ} & & \\
\end{array}
\]

G

\[
\begin{array}{cc}
\text{Untreated} & \text{Hs/IrrB16} + \text{MPL} & \text{Hs/IrrB16} + \text{MPL + IL-1α} \\
\text{% IFNγ^+NK cells} & & \\
\end{array}
\]

\[
\begin{array}{cc}
\text{Untreated} & \text{Hs/IrrB16} + \text{MPL} & \text{Hs/IrrB16} + \text{MPL + IL-1α} \\
\text{% IL-10^+ NK cells} & & \\
\end{array}
\]
Figure 4.18 Prophylactic immunization of mice with a tumour vaccine in combination with MPL and IL-1α significantly increases survival.
Mice were immunized s.c. into the left hind flank with heat-shocked and irradiated B16 cells (1 \times 10^6) alone or with MPL (10 μg), and IL-1α (100 ng) twice at a 4-week interval. Seven days after the boost, mice were challenged with 2\times10^5 B16.F10 cells s.c. into the left hind flank. Shown are the Kaplan-Meier survival plots of tumour-bearing mice (9 mice/group). Survival curves were analyzed by the logrank survival test. Results are from one experiment.
Figure 4.19 Therapeutic immunization with a tumour vaccine combined with MPL or MPL and IL-1α suppresses tumour growth

Tumours were established in all groups on day 0 by subcutaneous injection of $2 \times 10^5$ B16/OVA cells into the left hind flank. Mice were treated in the region of the tumour on days 3, 10, and 17 with the indicated combinations of OVA (100 μg), MPL (10 μg), and IL-1α (100 ng). Tumour volumes for each mouse (A), tumour volume expressed as the mean ($\pm$SEM) for seven mice per group (B) and survival (C) were monitored. Kaplan-Meier survival curves were analyzed by the logrank survival test. ** $P < 0.01$ versus PBS treated group (Kruskal-Wallis test). Results are from one experiment.
Chapter 5
Identification of TRP-2 epitopes and their use as peptide vaccines in combination with MPL and IL-1α as adjuvants
5.1 Introduction

Identification and characterisation of tumour-associated antigens that are recognised by CD8^+ CTLs and CD4^+ T_H cells in the context of MHC classes I and II, respectively, has been one of the most significant advances in the field of tumour immunology and have assisted in the design of active immunization strategies for the treatment of cancer. Since the cloning of MAGE-1 in 1991, the first gene reported to encode a human tumour antigen recognized by T cells (van der Bruggen et al., 1991), molecular characterization of novel tumour-associated antigens has rapidly evolved, with a comprehensive but continuously growing list of tumour-associated antigens in the cancer immunity peptide database, http://cancerimmunity.org/peptide (van der Bruggen et al., 2012). Tumour antigens have been discovered by a number of different strategies including i) screening tumour-derived cDNA libraries with autologous tumour-specific CTL clones (van der Bruggen et al., 1991), ii) by the use of biochemical methods which involves extraction of antigenic peptides from MHC/peptide complexes expressed on the surface of tumour cells, followed by their fractionation by chromatography, screening by tumour specific T cells and the use of mass spectrometry to sequence the antigenic peptide (Cox et al., 1994, Skipper et al., 1999), iii) reverse immunology, which involves the epitope prediction phase (tumour-specific proteins are analysed for the presence of known HLA binding motifs by computer prediction algorithms) and the epitope validation phase (candidate peptides are tested by binding and stability assays in vitro) (Bocchia et al., 1995, Asemissen et al., 2006), iv) a strategy called SEREX (serological identification of antigens by recombinant expression cloning), which depends on the in vivo generation of anti-tumour antibodies, used in the identification of the NY-ESO-1 antigen (Chen et al., 1997), involves screening patient anti-sera against a tumour-derived cDNA library expressed in
bacteria. Tumour antigens can be classified as i) Cancer-testis antigens (alternative designation germline antigens), result from re-activation of genes normally silent in adult tissues but are transcriptionally activated in different tumours. Cancer-testis antigens are expressed in testis, however, cells of the testis do not express class I and II HLA molecules and thus the testis is not a target for an autoimmune reaction. ii) Differentiation antigens are antigens shared by the tumour and the normal cells from which the tumour arose. Most of these antigens have been identified in melanomas and normal melanocytes. iii) Widely occurring, overexpressed antigens are antigens generally expressed at low levels in normal tissues but overexpressed in tumours. iv) Unique and shared tumour-specific antigens arise from point mutations or splicing alterations of normal genes (reviewed in (Novellino et al., 2005)).

TRP-2 (alternatively called DOPAchrome tautomerase; DCT), a member of the tyrosinase-related protein (TRP)-family, which also includes tyrosinase and TRP-1, is a DOPAchrome tautomerase melanogenic enzyme catalyzing a distal step in the melanin synthesis pathway in specialised organelles in melanocytes termed melanosomes and shares 40% amino acid sequence identity with tyrosinase and TRP-1 (Jackson et al., 1992, Tsukamoto et al., 1992, Bouchard et al., 1994, Yokoyama et al., 1994). Human TRP-2, located on human chromosome 13 (13q32), encodes a protein with 519 amino acids and shares 84% amino acid identity with its mouse counterpart (Bouchard et al., 1994, Yokoyama et al., 1994). Murine TRP-2, which encodes a protein with 517 amino acids, is located at the slaty locus on mouse chromosome 14 (14 58.0 cM) (Jackson et al., 1992). TRP-2 catalyzes the conversion of DOPAchrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Tsukamoto et al., 1992). In the absence of the enzyme, DOPAchrome spontaneously converts to 5,6-dihydroxyindole (DHI) with little or no
DHICA being formed (Tsukamoto et al., 1992). Since DHI is toxic to cells, while DHICA is a less toxic intermediate, it is thought that in addition to its function in melanin pigmentation, TRP-2 may also act as a detoxifying enzyme protecting the viability of melanocytes from the severe cytotoxic effects of DHI (Pawelek and Lerner, 1978, Tsukamoto et al., 1992, Urabe et al., 1994). In addition to its activity in the melanin biosynthetic pathway, TRP-2 has been identified as a non-mutated melanoma differentiation antigen recognized by tumour-reactive CTLs in both humans and mice (Wang et al., 1996, Bloom et al., 1997, Parkhurst et al., 1998, Wang et al., 1998). Rosenberg and colleagues demonstrated that human TRP-2 is recognized as a melanoma-associated antigen by a CTL clone derived from a population of tumour infiltrating lymphocytes (Wang et al., 1996). This CTL clone recognized the hTRP-2_{197-205} peptide of TRP-2 in the context of HLA-A31 (Wang et al., 1996). This report was the first description of TRP-2 as a tumour antigen in humans and it was swiftly followed by the identification of the analogous tumour antigen in murine melanoma. Bloom et al. identified CTLs reactive with the B16 melanoma recognizing a non-mutated product of the TRP-2 gene found by screening a cDNA library from B16 with tumour-reactive CTLs (Bloom et al., 1997).

Melanoma is a malignant tumour derived from epidermal melanocytes and can occur in any tissue harbouring these specialized melanin producing cells. TRP-2 is classified as a melanoma differentiation antigen because the gene is expressed specifically in the cells of the melanocytic lineage, the normal cell type from which melanoma is derived. TRP-2 mRNA expression, determined by quantitative reverse transcription-PCR, was detected in 89% (31/35) of metastatic tumour specimens and it was shown that elevated mRNA copy levels of TRP-2 significantly correlated with an improved overall survival.
of late-stage IV melanoma patients (Takeuchi et al., 2003). In terms of autoreactive T cells targeting TRP-2 antigens leading to an adverse autoimmune reaction, TRP-2 expression is confined to cells of the pigment cell lineage, namely melanocytes and melanoblasts and cells of the retinal pigment epithelium (RPE) (Steel et al., 1992, Mackenzie et al., 1997). During embryonic development, TRP-2 is expressed in migratory melanoblasts early (embryonic day 10 (E10)) after they emerge from the neural crest, whereas tyrosinase and TRP-1 are expressed later in development (i.e. 4 days later than TRP-2 expression) (Steel et al., 1992). TRP-2 is also expressed in the dorsal telencephalon of the forebrain where pigment and melanocytes have not been identified, but the function of TRP-2 in the developing telencephalon is unclear, but may serve as detoxifying enzyme within neuronal tissue (Steel et al., 1992, Zhao and Overbeek, 1999). Thus, this confined expression suggests that TRP-2 is a good candidate antigen for vaccination against melanoma.

The selection of tumour cell variants that no longer present the antigens that are the targets of the effector T cells (termed antigen loss variants) represents a mechanism of tumour escape from both spontaneous and vaccine-induced anti-tumour immune responses. Antigen loss can result from a mutation or deletion in the gene coding for the antigenic peptide. A search of GeneCards® (an integrated database of human genes that includes automatically-mined genomic, proteomic and transcriptomic information, disease relationships, single-nucleotide polymorphisms (SNPs), gene expression, and gene function (Crown Human Genome Center, 2012)) revealed that human TRP-2 has not yet been associated with a human pigmentary disease and no such mutation has been found in the human TRP-2 gene, thus making it possible that TRP-2 is an indispensable enzyme. Whereas many mutations in humans have been found in the
genes encoding tyrosinase and TRP-1, which have been associated with two different hypopigmentary diseases, OCA (oculocutaneous albinism) types I (OCA1) and III (OCA3) respectively (Toyofuku et al., 2001a, Toyofuku et al., 2001b, Sarangarajan and Boissy, 2001). Antigen loss in melanoma cells can also result from the switching off of the genes encoding the melanoma differentiation antigen. However, complete lack of TRP-2 in melanoma cells (due to switching off the TRP-2 gene) might have a severe effect on the viability of melanoma cells as in the absence of the enzyme; DOPAchrome spontaneously converts to the cytotoxic metabolite, DHI. It has thus been proposed that TRP-2 may maintain cell viability of melanocytes and also melanoma cells which derive from melanocytes by controlling the concentration of DHI, a toxic intermediate of melanin biosynthesis (Pawelek and Lerner, 1978, Tsukamoto et al., 1992, Urabe et al., 1994). This suggests that TRP-2 is a critical detoxifying enzyme indispensable for melanoma cell viability. Antigen loss in tumour cells adversely effects the efficacy of tumour vaccines and thus it is critically important in terms of avoiding the generation of antigen loss variants in tumour cells that candidate target antigen(s) selected for vaccination approaches against tumours represents an antigen whereby the encoding gene if switched off/deleted in the tumour cell, it would severely effect the viability of the tumour cell and consequently the progression of the tumour in the host. TRP-2 appears to satisfy this requirement (i.e. TRP-2 is a detoxifying enzyme indispensable for melanoma cell viability) providing further evidence that TRP-2 is a good candidate antigen for inclusion in a vaccine for melanoma.

It is widely accepted that dysfunctions of homeostatic regulation of stem cells may result in conversion into cancer stem cells (CSCs), defined as a population showing high tumour-initiating ability, and the capacity to self-renew and generate differentiated
progeny; the so called cancer stem cell theory (Reya et al., 2001). These properties suggest that CSCs are at the origin of tumours and are essential for tumour maintenance, recurrence, and distant metastasis (Reya et al., 2001, Polyak and Hahn, 2006), suggesting that targeting CSCs may be important in controlling cancer. Indeed, recent studies have shown that CSCs are resistant to standard cancer treatments, including chemotherapy and radiotherapy (Park et al., 2009). Importantly, however, CD8$^+$ CTLs can efficiently recognize CSCs (Inoda et al., 2011, Hirohashi et al., 2012). Thus, antigen-specific cancer vaccines that target CTL activation or adoptive transfer of CTLs may be promising approaches to eradicate CSCs.

The existence of melanoma stem cells has been recently suggested in both human and murine melanomas (Dou et al., 2007, Schatton et al., 2008, Dou et al., 2009) and it has been proposed that melanoma stem cells are generated from melanocyte stem cells via the accumulation of genetic changes and that they may have similar phenotypic properties to melanocyte stem cells (Sabatino et al., 2009). Nishimura et al. identified melanocyte stem cells as TRP-2-positive and c-kit (a receptor for stem cell factor) signalling independent cells in the lower permanent portion of the hair follicle (Nishimura et al., 2002). Moreover, Osawa and colleagues demonstrated that most markers involved in melanocyte development, including tyrosinase, TRP-1, sox10, lefl1, kit, mitf, were absent in the melanocyte stem cells, whereas TRP-2 and pax3 were significantly expressed (Osawa et al., 2005). These studies indicate that TRP-2 protein expression marks the melanocyte stem cell and in accordance with the cancer stem cell hypothesis, it is possible that like the melanocyte stem cells from which it originated, TRP-2 may be similarly expressed by melanoma stem cells. However, this speculation has so far not been confirmed. Nevertheless, the evidence to date would suggest that
CTLs targeting TRP-2 may participate in the recognition of melanoma stem cells via the speculated expression of TRP-2 in melanoma stem cells. Taken together, these findings suggest that TRP-2 may be a tumour-associated antigen, capable of generating anti-tumour immune responses. TRP-2 was thus chosen as the candidate antigen for the novel tumour vaccination approach in this study because of its demonstrable expression in normal melanocytes and in melanocyte stem cells (with the possibility of also being expressed in melanoma stem cells which relates to the so-called cancer stem cell theory), its high and consistent level of expression in metastatic melanoma lesions, its proposed function as a critical detoxifying enzyme indispensable for melanocyte/melanoma cell viability and the suggestion that TRP-2 is an indispensable melanogenic enzyme in humans relating to the fact that mutations in the \( TRP-2 \) gene have not been identified in humans to date.

Tumour-induced and/or vaccine-induced expansion of \( T_{\text{reg}} \) cells is an obstacle to successful cancer vaccines and immunotherapies (Shimizu et al., 1999b, Sakaguchi, 2000, Sutmuller et al., 2001, Jarnicki et al., 2006, Conroy et al., 2012). It has been speculated that the generation and maintenance of \( T_{\text{reg}} \) cells at tumour sites require the presence of target antigen (Thornton and Shevach, 2000b, Shevach, 2002, Wang et al., 2004, Nishikawa et al., 2005), either directly presented to T cells by tumour cells (Wang et al., 2005a) and/or cross-presented by DCs (Heath and Carbone, 2001, Yamazaki et al., 2003). It is widely accepted that \( T_{\text{reg}} \) cells require antigen-specific activation through TCR engagement, but mediate suppression in an antigen-nonspecific bystander fashion (Thornton and Shevach, 2000b). Baatar et al. demonstrated the existence of two distinct subsets of \( T_{\text{reg}} \) cells in human peripheral blood, memory-type (CCR4\(^{+}\)CD45RA\(^{-}\)CD45RO\(^{+}\)) and naïve-type (CCR4\(^{-}\)CD45RA\(^{+}\)) \( T_{\text{reg}} \) cells. They
proposed that CCR4<sup>+</sup>CD45RA<sup>-</sup> T<sub>reg</sub> cells (which also expressed CCR7 and CD62L, markers associated with homing to secondary lymphoid organs) migrate to lymphoid organs to acquire the TCR-dependent activation signals from antigen-expressing APCs and to differentiate into circulating memory-type CCR4<sup>+</sup> T<sub>reg</sub> cells, which are then primed to suppress and regulate immune responses (Baatar et al., 2007).

Evidence is mounting that T<sub>reg</sub> cells recognise specific epitopes within self antigens. CD4<sup>+</sup>CD25<sup>+</sup>GITR<sup>-</sup>FoxP3<sup>+</sup> T<sub>reg</sub> cells recognising peptides derived from LAGE-1 (Wang et al., 2004) and ARCT1 (Wang et al., 2005a) have been cloned from the TIL of a melanoma patient. Moreover, an epitope within α-Foetoprotein (AFP<sub>46-55</sub>) has been shown to activate the expansion of inducible T<sub>reg</sub> cells (Alisa et al., 2008). Furthermore, Vence et al. demonstrated that patients with metastatic melanoma display IL-10 secreting CD4<sup>+</sup> T<sub>reg</sub> cells specific for tumour-associated antigens, including TRP-1, NY-ESO-1, gp100 and survivin (Vence et al., 2007). Vence and colleagues were the first to demonstrate that NY-ESO-1-specific T<sub>reg</sub> cell epitopes are found along the entire length of the NY-ESO-1 protein: NY-ESO-1<sub>66-85</sub>, NY-ESO-1<sub>180-105</sub>, NY-ESO-1<sub>120-145</sub> and NY-ESO-1<sub>160-180</sub> (Vence et al., 2007). This suggests that like the NY-ESO-1 protein, TRP-2 would similarly contain T<sub>reg</sub> cell epitopes within the protein sequence in addition to CTL and helper epitopes (Figure 7 (Vence et al., 2007)) and therefore vaccinating with the whole protein would activate not only TRP-2-specific CD8<sup>+</sup> CTLs and CD4<sup>+</sup> T<sub>H</sub> cells (essential for mediating anti-tumour immune responses) but it would also activate TRP-2-specific T<sub>reg</sub> cells which maintain tolerance to the self-derived TRP-2 antigen and as a consequence breaking tolerance to TRP-2 would be extremely difficult to induce by vaccination. Therefore, peptide sequences derived from the TRP-2 melanoma differentiation antigen recognised by effector and not regulatory T cells may
be more suitable than the entire TRP-2 protein sequence in the design of a tumour vaccine.

A vast body of evidence supports the importance of the collaboration between CD4^+ helper T cells and CD8^+ CTLs so called CD4 help, which is required for the generation of primary CTL responses, but it is also critical for generating functional long-lived CD8^+ T cell memory (Williams and Bevan, 2007). Therefore, the epitope-based vaccine in the current study was designed to incorporate both CD8^+ CTL and CD4^+ T_H cell epitopes satisfying the requirement of CD4 T cell help for optimal CTL responses.

TRP-2 peptides were combined with adjuvants MPL and IL-1α identified in the previous chapter as an effective adjuvant combination for tumour vaccines. It is proposed that the addition of MPL and IL-1α as adjuvants will enhance the immunogenicity of the peptides, which are generally weakly immunogenic, to overcome tolerance to self-derived antigenic peptides. MPL and IL-1α should promote T_H1 and T_H17 cells and not T_reg cells, thereby shifting the ratio of effector to regulatory T cells in the tumour microenvironment in favour of anti-tumour effector T cells.

**Aims of this Chapter**

I) To determine whether the newly identified HLA-DRB1^*0301-restricted CD4^+ T cell epitopes derived from human TRP-2, were CD4^+ T cell epitopes in mouse restricted by the H2-I^A^b MHC class II allele.
II) To investigate the immunogenicity and antigenicity of the peptides TRP-2\textsubscript{58-78} and TRP-2\textsubscript{48-165}.

III) To evaluate the effectiveness of murine TRP-2 derived peptides (TRP-2\textsubscript{180-188}, TRP-2\textsubscript{58-78}, and TRP-2\textsubscript{48-165}) as tumour-associated antigens in the murine B16.F10 melanoma model.

IV) To determine the effectiveness of MPL and IL-1α as adjuvants for co-administration with murine TRP-2-derived peptides (TRP-2\textsubscript{180-188}, TRP-2\textsubscript{58-78}, and TRP-2\textsubscript{48-165}) in the B16.F10 melanoma model.
5.2 Results

5.2.1 B16.F10 melanoma cells express TRP-2 \textit{in vitro} and \textit{in vivo}

The murine melanoma B16 is a spontaneously arising melanoma of C57BL/6 mice propagated by Dr. Isaiah Fidler (Fidler, 1970, Fidler, 1973). In this study, B16.F10 cells grown overnight in culture were used for flow cytometric analysis, which confirmed the expression of TRP-2 in B16.F10 cells \textit{in vitro} (Figure 5.1). Established B16.F10 melanoma from five tumour-bearing mice were harvested and analysed for the expression of TRP-2 \textit{ex vivo} by flow cytometry. Consistent with B16.F10 cells growing in culture, these cells maintain expression of TRP-2 upon injection into syngeneic C57BL/6 mice (Figure 5.2), suggesting that TRP-2 may be a useful target antigen for melanoma vaccine studies.

5.2.2 TRP-2\textsubscript{180-188} peptide is a murine CD8\textsuperscript{T} T cell epitope

CD8\textsuperscript{T} T cells recognize processed peptides in the context of MHC class I antigens (HLA in human, H2 in mouse) expressed on the surface of APCs. TRP-2\textsubscript{180-188} peptide is recognized by melanoma-reactive CTLs in both human (Parkhurst et al., 1998) and mouse (Bloom et al., 1997) and murine TRP-2\textsubscript{180-188} has shown efficacy in numerous tumour vaccination studies in the B16 melanoma model (Jérôme et al., 2006, McCormick et al., 2006, Kou et al., 2007, Mansour et al., 2007, Cho and Celis, 2009). Therefore, the H2-K\textsuperscript{b}-restricted TRP-2\textsubscript{180-188} epitope was selected as the CD8\textsuperscript{T} T cell epitope. In order to confirm that TRP-2\textsubscript{180-188} is a CD8\textsuperscript{T} T cell epitope in mice, C57BL/6 mice were immunized s.c. twice in the footpad (days 0, 28) with 100 \textmu g of TRP-2\textsubscript{180-188} and 50 \textmu g MPL. Seven days after the booster immunization, popliteal LN cells from injected mice were restimulated with TRP-2\textsubscript{180-188} (1-20 \textmu g/ml), or with...
irrelevant peptide OVA_{257-264}, or with PMA/αCD3 as a positive control. Immunization with TRP-2_{180-188} peptide and MPL generated LN T cells that secreted significant concentrations of IFNγ in response to TRP-2_{180-188} for all doses tested (p<0.001 compared with medium). In contrast, very low and insignificant levels of IL-10 was detected from T cells from mice immunized with TRP-2_{180-188} and MPL (p>0.05; Figures 5.3). Incubation of LN cells with OVA_{257-264} did not lead to any detectable cytokine release indicating the response was specific to the immunizing peptide TRP-2_{180-188}. This finding demonstrates that TRP-2_{180-188} as a TRP-2-derived epitope could be recognized by tumour-specific T cells, probably CD8^+ cells. This study provides proof-of-principle that a T cell repertoire to self antigen TRP-2_{180-188} exists and that this repertoire can be targeted by vaccination.

5.2.3 Selection of T helper epitopes from the TRP-2 antigen

Tumour regression in mice has been shown to depend on both helper T cells and CTL functions (Ossendorp et al., 1998, Toes et al., 1999). Therefore, the identification of tumour epitopes recognized by CD4^+ T cells is critically important in the design of tumour vaccines. Peptides derived from human TRP-2 (TRP-2_{60-74} and TRP-2_{149-163}) were recognized by CD4^+ T cells from HLA-DRB1*0301-transgenic mice injected with either human TRP-2 protein or adenovirus encoding human TRP-2 (Paschen et al., 2005, Osen et al., 2010). HLA-DRB1*0301-transgenic mice express HLA-DRB1*0301 molecule on an IA^0 H2 background and thus TRP-2_{60-74} and TRP-2_{149-163} are HLA-DRB1*0301-restricted CD4^+ T cell epitopes (Paschen et al., 2005, Osen et al., 2010). In the current study, the murine sequences of the newly identified HLA-DRB1*0301-restricted CD4^+ T cell epitopes derived from human TRP-2 (TRP-2_{60-74} and TRP-2_{149-163}) were examined as putative CD4^+ helper epitopes in mice. TRP-2_{180-188}
188 is an epitope recognized by both human and murine CD8\(^{+}\) T cells presented by the MHC class I haplotypes HLA-A*0201 and H2-K\(^{b}\), respectively. Thus, it was speculated in this study that TRP-2\(_{60-74}\) and TRP-2\(_{149-163}\) may also be recognized by both human and murine CD4\(^{+}\) helper T cells in a similar fashion. The peptide sequences for the human TRP-2 molecule and the murine TRP-2 molecule were obtained from the Swissprot database, accession numbers P40126-1 and P29812, respectively (Figure 5.4). The TRP-2\(_{180-188}\) (SVYDFFVWL) epitope has an identical amino acid sequence in both human and murine TRP-2 protein sequences (Figures 5.4A and B). However, murine TRP-2\(_{60-74}\) (QCAEVQTDTRPWSGP) displays three non-conservative amino acid substitutions compared with human TRP-2\(_{60-74}\) (QCTEVRADTRPWSGP; Table 5.1 and Figures 5.4A and B). Similarly, murine TRP-2\(_{149-163}\) (KKSHPDYVTTQHW) displays one non-conservative and one conservative amino acid substitution compared with human TRP-2\(_{149-163}\) (KKRHPDYVTTQHW; Table 5.1 and Figures 5.4A and B). The binding of peptides to MHC class II is a critical step in activating T\(_{H}\) cell responses. The findings by Paschen and colleagues (Paschen et al., 2005, Osen et al., 2010) confirms that human TRP-2\(_{60-74}\) and human TRP-2\(_{149-163}\) must bind within the peptide-binding groove of the HLA-DRB1*0301 molecule in order to be presented to the TCR on the reactive CD4\(^{+}\) T cell. However, it must be determined in the current study firstly whether murine TRP-2\(_{60-74}\) and TRP-2\(_{149-163}\) bind to the MHC class II molecule expressed in the strain of mouse used in the current study and secondary whether the amino acid differences in the sequences of murine TRP-2\(_{60-74}\) and murine TRP-2\(_{149-163}\) will impact positively/negatively on their ability to serve as CD4\(^{+}\) T cell epitopes.
5.2.4 Predicting the binding of murine TRP-2 peptides (TRP-2_{60-74} and TRP-2_{149-163}) to MHC class II IA^b using a knowledge based threading approach

For a peptide to stimulate a T_{H} cell response, it must bind within the peptide-binding groove of the MHC class II molecule. Many different computational prediction methods can be applied to assist in predicting peptide-MHC binding that can later be experimentally validated. A knowledge based threading approach was used in this study to predict whether TRP-2 peptides TRP-2_{60-74} and TRP-2_{149-163} bind within the groove of the MHC class II IA^b molecule. The T cell receptors of C57BL/6 mice (MHC haplotype; K^b, D^b, IA^b) are restricted to H2-IA^b for class II MHC (The Jackson Laboratory, 2012). The amino acid sequences of peptides TRP-2_{60-74} and TRP-2_{149-163} were threaded onto the template structure 1MUJ (MHC class II complex H2-IA^b/human CLIP(87-101)peptide) using MODPROPEP web server (Kumar and Mohanty, 2007). The “1MUJ” template structure was selected for modelling the two epitopes (Figure 5.5).

The epitope QCAEVQTDTRPWSGP (TRP-2_{60-74}) was predicted to be a strong binder to H2-IA^b (Figure 5.6). Threading of the above epitopic sequence into the groove of the IA^b MHC molecule revealed the binding energy to be -138.44 which is very close with that of the template peptide of MHC-peptide complex (1MUJ) which was -161.66. The second epitope KKSICHDPYVITQHW (TRP-2_{149-163}) was also predicted to be a strong binder to H2-IA^b (Figure 5.7) with binding energy -140.2 (compared with template peptide that has binding energy of -161.66). The results of the knowledge based threading approach is dependent on the template structure used, as a peptide ranks high if its binding pattern is similar to the template peptide i.e. the threading algorithm verifies the probability of peptide sequence to adopt a particular fold in the MHC groove using binding energy score. The similar binding energy patterns suggest that these two epitope sequences should efficiently fit in the groove of the MHC class II IA^b
molecule. How efficiently the peptide binds in the groove of the MHC molecule is an important factor to determine its binding affinity.

Whilst the threading approach has predicted these two peptides TRP-260-74 and TRP-2149-163 as strong binders to the MHC class II 1A^b molecule and thus identified the peptides as good vaccine candidates, it must be appreciated that this approach is a mere prediction and thus it is important that these peptides are validated for their immunogenicity *in vivo*. In an attempt to increase the probability that the MHC class II 1A^b molecule binds and presents TRP-2 CD4^+ T cell epitopes to the available antigen-specific CD4^+ T cell repertoire, TRP-260-74 (15-mer) was extended by two amino acid residues towards the N-terminus and four amino acid residues towards the C-terminus (upon recommendation by Cambridge Research Biochemicals; the peptide synthesis company) to generate TRP-258-78 (21-mer; Figure 5.4B). Similarly, TRP-2149-163 (15-mer) was extended by one amino acid residue towards the N-terminus and two amino acid residues towards the C-terminus (in accordance with Cambridge Research Biochemicals recommendations) to generate TRP-2148-165 (18-mer; Figure 5.4B). Accordingly, this would also improve the peptide solubility.

### 5.2.5 Analysis of peptide immunogenicity and antigenicity *in vivo*

To evaluate whether peptides TRP-258-78 and TRP-2148-165, could elicit peptide-specific IFNγ-producing cells as an indicator of the immunogenicity of the TRP-2 peptides, C57BL/6 mice (n=3/group) were immunized s.c. twice in the footpad (days 0, 28) with 10 µg of TRP-258-78 or TRP-2148-165 and 50 µg MPL. Seven days after the booster immunization, popliteal LN cells from immunized mice were restimulated with
increasing concentrations of the immunizing peptide (1-20 μg/ml), or with irrelevant peptide OVA<sub>323-336</sub>, or with PMA/αCD3 as a positive control. IFNγ and IL-10 were quantified by ELISA after 72 h stimulation with peptides (Figures 5.8, 5.9). Peptide-specific IFNγ T cell production could be detected in mice immunized with TRP-2<sub>58-78</sub> and MPL (Figure 5.8), whereas T cells from immunized mice did not respond to OVA<sub>323-336</sub> peptide indicating the responses were specific to the immunizing peptide (Figures 5.8, 5.9). Importantly, very low to undetectable levels of TRP-2<sub>58-78</sub>-specific IL-10 production was observed in LN cells from mice immunized with TRP-2<sub>58-78</sub> and MPL, suggesting that this peptide with MPL as adjuvant did not induce T<sub>REG</sub> cells. Similarly, LN cells from mice immunized with TRP-2<sub>148-165</sub> peptide and MPL generated T cells that secreted high concentrations of IFNγ but undetectable levels of IL-10 in response to TRP-2<sub>148-165</sub> restimulation ex vivo (Figure 5.9). Interestingly, LN cells from mice immunized with TRP-2<sub>148-165</sub> and MPL secreted IFNγ at a concentration range of 6.7 – 9.5 ng/ml in response to restimulation with TRP-2<sub>148-165</sub> ex vivo, whereas LN cells from mice immunized with TRP-2<sub>58-78</sub> and MPL secreted IFNγ at a slightly lower concentration range of 3.1 – 4.1 ng/ml in response to restimulation with TRP-2<sub>58-78</sub> ex vivo, indicating that the responses against peptide TRP-2<sub>148-165</sub> are stronger. Nonetheless, these findings indicate that immunization with peptides TRP-2<sub>58-78</sub> and TRP-2<sub>148-165</sub> can generate peptide specific T cells. These results confirm that peptides TRP-2<sub>58-78</sub> and TRP-2<sub>148-165</sub> bind to MHC, predicted by the threading approach to be MHC class II H2-IA<sup>b</sup>-restricted T cell epitopes. Whilst the T cell subset was not confirmed in this study (by co-staining for CD4 and IFNγ with subsequent analysis by flow cytometry), it is speculated from the prediction results of the threading approach in section 5.2.4, that the TRP-2 peptide reactive T cells are of the subset CD4<sup>+</sup>. However, it is appreciated, that verification of this speculation is warranted.

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5.2.6 T cells from B16.F10 melanoma-bearing mice respond to the TRP-2\textsubscript{58-78} and TRP-2\textsubscript{148-165} epitopes

A successful approach in the identification of whether an epitope will be immunogenic in a cancer vaccine is to determine whether T cells from tumour-bearing mice respond to the peptides. Thus, it was investigated whether murine T cells reactive to peptides TRP-2\textsubscript{58-78} and TRP-2\textsubscript{148-165} could be detected in B16.F10 melanoma-bearing mice. Tumours were established in three mice by s.c. injection of $2 \times 10^5$ B16.F10 cells into the left hind flank. 16 days later, spleen cells were harvested and restimulated \textit{ex vivo} with either TRP-2\textsubscript{58-78} (1 - 50 µg/ml), TRP-2\textsubscript{148-165} (1 - 50 µg/ml) or irrelevant peptide OVA\textsubscript{23-336} (50 µg/ml). After 72 h, spleen cell supernatant was analyzed for IFN\textsubscript{γ} by ELISA. Spleen cells from one B16.F10 tumour-bearing mouse secreted significant concentrations of IFN\textsubscript{γ} in response to both epitopes, TRP-2\textsubscript{58-78} (Figure 5.10A; $p<0.001$ for all peptide restimulation doses tested compared with medium) and TRP-2\textsubscript{148-165} (Figure 5.10B; $p<0.001$ for all peptide restimulation doses tested compared with medium). However, IFN\textsubscript{γ} secreting TRP-2\textsubscript{58-78}-reactive T cells and TRP-2\textsubscript{148-165}-reactive T cells were not detected in the other two B16.F10 tumour-bearing mice (Figures 5.10A and B). One possibility for this observation is that the frequency of TRP-2\textsubscript{58-78}-specific T cells and TRP-2\textsubscript{148-165}-specific T cells in these mice might have been too low for detection by ELISA, and perhaps more sensitive techniques such as tetramer staining might be required for their detection. Alternatively, prime and boost immunization with the peptides might be required for their detection. Consistent with this possibility, it has been reported that spleen cells from B16.F10 tumour-bearing mice immunized with a DC vaccine pulsed with hs/irr B16 cells secreted IFN\textsubscript{γ} in response to TRP-2\textsubscript{180-188} peptide (Marshall et al., 2012). Depletion of CD25$^+$ T\textsubscript{reg} cells from peripheral blood mononuclear cells isolated from melanoma patients has been

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shown to enhance detection of antigen-specific CD4\(^+\) T cell responses (Osen et al., 2010) and thus it is possible that depletion of T\(_{reg}\) cells from the spleen cell population in the current study might allow peptide-specific IFN\(\gamma\) producing T cells to become detectable. These findings confirm the results in the previous section (5.2.5) demonstrating that TRP-2\(_{58-78}\) and TRP-2\(_{148-165}\) epitopes are bound in complex with MHC (predicted to be MHC class II H2-IA\(^b\)) for presentation to the TCR on the reactive T cell. Importantly, these results indicate that tolerance to self-derived melanoma-differentiation antigen TRP-2 is incomplete and that TRP-2\(_{58-78}\) and TRP-2\(_{148-165}\) specific T cells escape thymic central tolerance and also homeostatic tolerance mechanisms in the periphery providing proof-of-principle that T cell repertoires specific for epitopes TRP-2\(_{58-78}\) and TRP-2\(_{148-165}\) exist in the periphery, which can be targeted by vaccination. Collectively, these results provide scientific rationale for the inclusion of epitopes TRP-2\(_{58-78}\) and TRP-2\(_{148-165}\) into a tumour vaccine.

5.2.7 Prophylactic immunization of mice with a TRP-2 polyepitope-based vaccine in combination with MPL and IL-1\(\alpha\) adjuvant co-therapy promotes anti-tumour immunity and increases survival

The efficacy of a TRP-2 polyepitope-based vaccine (composed of epitopes TRP-2\(_{180-188}\), TRP-2\(_{58-78}\) and TRP-2\(_{148-165}\)) in combination with MPL and IL-1\(\alpha\) as adjuvants against B16.F10 melanoma was first evaluated in a prophylactic setting. C57BL/6 mice were immunized s.c. into the left hind flank with TRP-2\(_{180-188}\), TRP-2\(_{58-78}\), TRP-2\(_{148-165}\) (100 \(\mu\)g/peptide) alone or with MPL (10 \(\mu\)g) or MPL and IL-1\(\alpha\) (100 ng) twice at a 28 d interval. Seven days after the booster immunization (d 35), mice were challenged subcutaneously with \(2\times10^5\) B16.F10 cells in PBS in the left hind flank. Tumour growth was rapid in the untreated mice, whereas there was significantly delayed tumour growth.
after B16.F10 cell challenge in all three treatment groups (p<0.001 for each individual treatment group compared with untreated mice; Figures 5.11 A, B and C). There was a significant enhancement in survival in treated mice over that observed in untreated control mice (p<0.001 for each individual treatment group compared with untreated mice; Figure 5.11D). Immunization with the TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α as adjuvants provided the most significant protection against B16.F10 cell challenge. However, the protection was only marginally superior to the other vaccine protocols; immunization with TRP-2 polyepitope-based vaccine alone or vaccine combined with MPL as adjuvant (p>0.05). Tumour draining lymph node cells (n=6 mice/group) were stained with antibodies specific for surface CD3, CD4, CD8, γδTCR and NK1.1 and intracellular IFNγ, IL-17 and IL-10 and analyzed by FACS. The frequency of IL-17γ and IL-17γIFNγγ CD4+ T cells was increased in mice immunized with the TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α as adjuvants compared with all other groups (Figure 5.11E). However, these increased frequencies did not reach statistical significance. There was a slight increase in IL-17γγδ T cells in mice immunized with the TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α compared with untreated mice (Figure 5.11G). This increased frequency of IL-17γγδ T cells was also observed in mice immunized with the TRP-2 polyepitope-based vaccine in combination with MPL or TRP-2 vaccine alone (Figure 5.11G). However, these increased frequencies did not reach statistical significance. In comparison to the untreated mice, immunization with the TRP-2 vaccine alone or in combination with MPL or MPL and IL-1α did not enhance the frequency of IFNγγ CD4+ T cells (Figure 5.11E) or IFNγγ CD8+ T cells (Figure 5.11F). Evidently, however, the frequency of these populations was sustained in all vaccinated groups. A similar pattern of responses was observed with IFNγ-secretating γδ T cells and
NK cells (Figures 5.1 IG and H, respectively). Importantly, in comparison to the untreated mice, the frequency of IL-10-secretion CD4, CD8, γδ T cells or NK cells was not increased in mice immunized with the TRP-2 polypeptide-based vaccine in combination with MPL and IL-1α (Figures 5.11 E, F, G, and H, respectively). A similar pattern was also observed in mice immunized with the TRP-2 vaccine alone or in combination with MPL. Furthermore, mice immunized with the TRP-2 vaccine alone or in combination with MPL or MPL and IL-1α had decreased numbers of CD4⁺CD25⁺Foxp3⁺ T cells in comparison to untreated mice (Figure 5.11 I). These results suggest that protection induced with the TRP-2 vaccine alone or in combination with MPL or MPL and IL-1α was associated with the selective induction of effector T cell responses in vivo. It is difficult to quantify the full effect of the TRP-2 polypeptide-based vaccine in combination with MPL and IL-1α as adjuvants on T cell responses since T cells were assessed in all mice (both treated and untreated) when tumours were at end-stage. An earlier time point on, possibly d 20, would be more appropriate for assessing the effect of the vaccine on T cell responses.

5.2.8 MPL and IL-1α as adjuvants enhance the efficacy of TRP-2 polypeptide-based vaccine against B16.F10 melanoma in a therapeutic setting

To investigate whether the TRP-2 polypeptide-based vaccine with MPL and IL-1α as adjuvants were effective therapeutically against established tumours, C57BL/6 mice (n = 6/group) were inoculated s.c. with 2x10⁵ B16.F10 cells. Tumour-bearing mice were treated on days 3, 10 and 17 post-tumour inoculation with s.c. injection of TRP-2(180-188), TRP-2(38-78), TRP-2(148-165) (100 μg/peptide) with or without MPL (10 μg/mouse) with or without IL-1α (100 ng/mouse) (groups 2, 4 and 6; Figure 5.12). Another experimental group received TRP-2 peptides and MPL on day 3 but only received TRP-2 peptides on
days 10 and 17 (group 3 Figure 5.12). Another experimental group received TRP-2 peptides, MPL and IL-1α on day 3 but only received TRP-2 peptides and IL-1α on days 10 and 17 (group 5 Figure 5.12). Tumour growth was rapid in the PBS treated control group; all tumour-bearing mice had to be sacrificed within 18-22 days (Figures 5.12 A and B). Immunization of mice with the TRP-2 polyepitope-based vaccine alone (group 2) showed a minor but insignificant decrease in tumour growth when compared with PBS-treated control mice (p>0.05; Figures 5.12 A and B). Treatment with the TRP-2 polyepitope-based vaccine in combination with MPL as adjuvant (group 4) enhanced the anti-tumour effect compared with the TRP-2 vaccine alone (Figures 5.12 A and B) and showed an increase in survival compared with PBS-treated mice (p<0.05) or mice immunized with the TRP-2 vaccine alone (Figure 5.12C). However, the TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α as adjuvants provided the most significant protection against B16.F10 tumour growth. The protection was significantly superior to all other vaccine protocols tested (Figures 5.12 A and B). Furthermore, therapeutic administration of the TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α as adjuvants significantly enhanced survival (p<0.001 versus PBS treated mice; Figure 5.12C) with 33% (2/6) of the mice surviving after the end of the observation period (day 50). Importantly, protection was lost when adjuvant MPL was omitted from the vaccine protocol in the booster immunizations on days 10 and 17 (groups 3 and 5), with protection reducing to that observed with the TRP-2 polyepitope-based vaccine alone (Figures 5.12 A and B). This finding suggests that a prime immunization with MPL is insufficient for optimal polarization of anti-tumour T_h1 responses, booster immunizations being required (i.e. group 3 versus group 4). To conclude, therapeutic administration of the TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α as adjuvants induced protective anti-tumour
immunity *in vivo* associated with a significant delay in B16.F10 tumour growth and a significant increase in survival, with some mice exhibiting complete tumour rejection.
5.3 Discussion

The most important factors in the design of peptide-based therapeutic vaccines are the choice of target antigen and the selection of appropriate immunological adjuvant(s) to enhance the immunogenicity of the peptides. Upon activation, iDCs mature into potent activators of antigen-specific T cells (Guermonprez et al., 2002). Furthermore, activation of DCs favors cross-presentation, allowing presentation of exogenous antigens via MHC class I (Melief et al., 2002). Thus, the first strategy utilized in this study to enhance the potency of the peptide-based vaccine was the use of MPL to activate DC via ligation of TLR4. The second strategy utilized in this study to enhance the potency of the peptide-based vaccine was the induction of peptide-specific CD4\(^+\) T helper cell immune responses polarizing to specific T\(_{H1}\) subtypes via a T\(_{H1}\)-polarizing adjuvant (MPL) and a T\(_{H17}\)-polarizing adjuvant (IL-1\(\alpha\)). The transmembrane melanosomal glycoprotein, TRP-2, which catalyzes the conversion of DOPAchrome to DHICA in melanin biosynthesis, was shown in this study to be highly expressed in B16.F10 mouse melanoma cells in agreement with previously published data (Bloom et al., 1997). This antigen, also expressed in human melanoma (Wang et al., 1996, Parkhurst et al., 1998) was thus selected as the tumour-rejection antigen to target in this therapeutic vaccine.

CD8\(^+\) T cells have long been defined as the ultimate effector cells in anti-tumour immunity owing to their capability to directly kill malignant cells via a number of mechanisms, including the granule exocytosis pathway and the death receptor pathway. Indeed, adoptive T cell therapy using CD8\(^+\) CTL clones targeting melanocytic differentiation antigens has shown to induce regression of patients' metastatic
melanoma (Yee et al., 2002, Mitchell et al., 2002, Mackensen et al., 2006). A number of novel CD8+ T cell epitopes encoded by TRP-2 have been identified including epitopes presented by HLA-A*0201 (TRP-2180-188 (Parkhurst et al., 1998), TRP-2265-273 (Sun et al., 2000), TRP-2360-368 (Noppen et al., 2000), TRP-2255-263 (Harada et al., 2001). TRP-2476-484 (Noppen et al., 2000); HLA-A*31/ HLA-A*33 (TRP-2197-205 (Wang et al., 1996, Wang et al., 1998)) and HLA-Cw*8 (TRP-2187-195 (Castelli et al., 1999)). However, the frequency of HLA-A*31, HLA-A*33 and HLA-Cw*8 alleles in the Caucasian population are low compared with that of HLA-A*0201, which is the most commonly expressed class I HLA allele in Caucasians, with a frequency of 44% (van der Bruggen et al., 2012). A vaccine approach designed for melanoma patients expressing the HLA-A*0201 allele would be applicable for a significant fraction of patients and it could be modified to cover other alleles if proven successful. Moreover, Parkhurst et al. showed that peptide TRP-2180-188 binds to the HLA-A*0201 molecule with a 50% inhibition concentration (IC50) value of 36 nM, which is indicative of high affinity binding (Parkhurst et al., 1998). Furthermore, human TRP-2180-188 peptide recognized by CTLs in the context of HLA-A*0201 is unusual in that it is identical to a defined epitope from TRP-2 recognized by murine melanoma-reactive CTLs (Bloom et al., 1997). The octamer TRP-2181-188 was identified as the major reactive epitope within TRP-2 recognized by B16-reactive CTL lines in the context of H2-Kb and a CTL line raised from splenocytes by repeated in vitro stimulation with TRP-2181-188 peptide (VYDFFVWL) effectively treated three day established B16 pulmonary metastases (Bloom et al., 1997). However, later studies revealed the protective and therapeutic anti-tumour capacity of TRP-2-specific murine CTLs in the B16 melanoma model by vaccination with the nonamer (TRP-2180-188: SVYDFFVWL) generated by extending the 8-mer in the NH2 terminal direction (Mansour et al., 2007, Cho and Celis, 2009).
Importantly, TRP-2\textsubscript{180-188} conforms exactly to the H2-K\textsuperscript{b} binding motif: F/Y at pocket 5 and L/M at pocket 9 (MHC motif viewer (Rapin et al., 2008)). Furthermore, immunization with an alpha-based virus-like replicon particle encoding TRP-2 (Avogadri et al., 2010) or DCs treated with hs/irr B16 cells, TLR5 ligand flagellin and PI3 kinase inhibitor (Marshall et al., 2012) induced IFN\(\gamma\)-secreting CD8\textsuperscript{+} T cells that were responsive to the TRP-2\textsubscript{180-188} peptide. Therefore, because TRP-2\textsubscript{180-188} is recognized by melanoma-reactive CTLs in both human and mouse and also the fact that the H2-K\textsuperscript{b}-restricted TRP-2-specific CTL epitope aa180-188 (SVYDFFVWL) has been previously used in vaccination studies against B16.F10 melanoma (Jérome et al., 2006, McCormick et al., 2006, Kou et al., 2007, Mansour et al., 2007, Cho and Celis, 2009), this CD8\textsuperscript{+} T cell epitope was selected for inclusion into the tumour vaccine. Indeed, it was confirmed in this study that immunization with the H2-K\textsuperscript{b}-restricted epitope TRP-2\textsubscript{180-188} generated LN cells secreting significant concentrations of IFN\(\gamma\), providing scientific rationale for the inclusion of this epitope into the tumour vaccine. Importantly, this result is consistent with previously published reports (Bronte et al., 2000, Cho and Celis, 2009) indicating that tolerance to self-derived antigen TRP-2 is incomplete and that TRP-2\textsubscript{180-188}-specific T cells escape thymic central tolerance and also homeostatic tolerance mechanisms in the periphery providing proof-of-principle that a T cell repertoire to self antigen TRP-2\textsubscript{180-188} exists and that this can be targeted by vaccination.

While the induction of cognate CD4\textsuperscript{+} helper T cells by vaccination has not been pursued as extensively as vaccine-induced CTL responses via MHC class I-restricted epitopes, the critical role of CD4 help in the priming and maintenance of CTL responses is now well established. CD4\textsuperscript{+} T helper cells provide critical signals during
the priming of CTL responses but they also play an essential role in promoting CD8⁺ memory T cell development (Williams and Bevan, 2007). In support of this, studies have demonstrated an essential role for CD4⁺ T helper cells in the induction of CTL responses to peptide vaccines (Shirai et al., 1994, Ahlers et al., 1996, Ahlers et al., 2001). These findings are consistent with a clinical study describing the complete remission of stage IV metastatic melanoma upon adoptive transfer of ex vivo expanded autologous tumour antigen (NY-ESO-1₁₅₇₋₁₇₀ peptide)-specific CD4⁺ T cell clones (Hunder et al., 2008). However, a major obstacle for the development of optimal cancer vaccines inducing both CD8⁺ CTL and CD4⁺ helper populations is the lack of/limited identification of MHC class II-restricted epitopes from tumour antigens (like TRP-2) that can stimulate CD4⁺ T cells. Paschen and colleagues found that peptides derived from human TRP-2 (TRP-2₆₀₋₇₄ and TRP-₂₁₄₉₋₁₆₃) could be recognized by CD4⁺ T cells from HLA-DRB₁*0301-transgenic mice injected with either recombinant human TRP-2 protein or recombinant adenovirus encoding human TRP-2 (Paschen et al., 2005, Osen et al., 2010). HLA-DRB₁*0301-transgenic mice express HLA-DRB₁*0301 molecule on an IA<sup>00</sup> H₂ background (i.e. do not express endogenous murine MHC class II molecules) and thus TRP-2₆₀₋₇₄ and TRP-₂₁₄₉₋₁₆₃ are HLA-DRB₁*0301-restricted CD4⁺ T cell epitopes (Paschen et al., 2005, Osen et al., 2010). Paschen and colleagues confirmed the processing and presentation of TRP-2₆₀₋₇₄ and TRP-₂₁₄₉₋₁₆₃ by human target cells and also confirmed the presence of TRP-2₆₀₋₇₄- and TRP-₂₁₄₉₋₁₆₃- specific CD4⁺ T cells in peripheral blood lymphocytes from HLA-DRB₁*0301⁺ melanoma patients and HLA-DRB₁*0301⁺ healthy donors (Paschen et al., 2005, Osen et al., 2010). Therefore, this study set out to predict whether the newly identified HLA-DRB₁*0301-restricted CD4⁺ T cell epitopes derived from human TRP-
2, could function analogously as CD4$^+$ T cell epitopes in mouse restricted by the H2-IA$^b$ MHC class II allele.

Efforts have been made to predict epitopes of tumour antigens using computational MHC-peptide binding prediction models based on either sequence data or structure data. Sequence-based methods rely on the sequence of peptides that are known to bind to a specific MHC allele using binding assays (Rudensky et al., 1991, Hammer et al., 1993). An alternative to the motif-based prediction is weight matrix methods (Schuler et al., 2007, Hakenberg et al., 2003, Peters and Sette, 2005) and Support Vector Machine learning approaches (Donnes and Kohlbacher, 2006). In these approaches, the binding affinity of each peptide residue is scored using a matrix (matrices must be generated for each specific HLA allele) taking into consideration the contribution of residues at other positions; the sum of contributions by all residues gives predicted binding values. However, sequence-based methods have limitations: i) they make the assumption that different peptide positions contribute in an additive manner to the binding affinity and overlook the interaction between different peptide side chains; ii) their predictive power is directly dependent on the amount of experimental data of binding peptides; for each combination of MHC allele and peptide length, they require a large set of peptides that were tested experimentally. Therefore, these approaches are not feasible in situations where insufficient experimental binding sequence data are available (Zhao et al., 2007). It is proposed that a minimum of 200 peptides with characterized binding affinity is required to derive an accurate description of the binding motif for MHC class II alleles (Nielsen et al., 2010). Only 14 HLA-DR allotypes and 2 HLA-DQ allotypes in the immune epitope database (IEDB; one of the main databases hosting data describing the binding specificities for MHC molecules)
meets this criterion, leaving that vast majority of the different human HLA class II molecules (> 4000) and the mouse H2 class II alleles uncovered (Nielsen et al., 2010). This poses a series problem when attempting to predict the binding of peptide epitopes to an under-characterized or uncharacterized MHC allotype (which is generally the case for mouse H2 class II alleles), rendering such generated predictions random or close to it. Thus, because the applicability of the current publicly available sequence-based methods is in most cases limited to HLA-DR, these prediction methods could not be utilized in the current study for the prediction of epitopes TRP-260-74 and TRP-2149-163 due to the limited/lack of coverage of the mouse H2 class II allele of interest (i.e. H2-IA b).

Structure-based prediction methods such as the threading approach (Altuvia et al., 1995, Altuvia and Margalit, 2004) can address the shortcomings of sequence-based prediction methods, as these approaches, which do not require binding data, use three-dimensional (3D) structures of peptide-MHC complexes whereby predictions are based on the physical principles of intermolecular interactions. Thus, such methods can be used to determine the binding of a query peptide to under/un-characterised MHC allotypes such as non-human H2-IA b. The threading approach replaces the peptides in the existing co-crystal template structure available in the Protein Data Bank (PDB) with the new query peptide to form a new structure. In 2007, Kumar and Mohanty developed an online web server, MODPROPEP, for knowledge-based modeling of peptides in complex with MHC class I and class II proteins (Kumar and Mohanty, 2007). The physical, chemical compatibility between query peptide and MHC groove is estimated using pair-wise potential matrix and the overall binding score is obtained by adding all pair-wise values.
for residues in the MHC groove with the corresponding peptide residues at every position. The threading approach is based on i) the availability of structural templates for peptides bound to different MHC alleles obtained from the PDB, ii) the pair-wise potential matrix used (i.e. binders are selected using statistical pairwise potentials), and iii) the criterion for determining peptide and MHC positions that are in contact. The frequency of amino acid pair residues that are in contact in protein structures is assumed to represent the interaction preference between different types of residues. This interaction preference between two amino acids is expressed by its comparison with their affinity to a "reference state". Two most commonly used pair-wise potential matrices are those deduced by Miyazawa and Jernigan (Miyazawa and Jernigan, 1996) who used solvent as a reference state with the resultant matrix putting much emphasis on hydrophobic interactions and Betancourt and Thirumalai (BT) (Betancourt and Thirumalai, 1999) who used the amino acid threonine as the reference state with the resultant matrix superior at describing hydrophilic interactions. The MODPROPEP server has the option of evaluating the binding score of query peptides with the MHC molecule using residue-based statistical energy function by MJ, BT, or other user-defined residue-based schemes. In this study, using the MODPROPEP server (with selection of the Miyazawa and Jernigan pair potential matrix) murine TRP-2 derived epitopes, TRP-2\textsubscript{60-74} and TRP-2\textsubscript{149-163}, were predicted to be strong binders to the class II MHC H2-IA\textsuperscript{b} molecule (the MHC allele expressed by the C57BL/6 strain), suggesting their potential as TRP-2 derived CD4\textsuperscript{+} helper epitopes.

The immunogenicity and antigenicity of the amino acid extended peptides TRP-2\textsubscript{58-78} and TRP-2\textsubscript{148-165} was confirmed in the current study with the finding that LN cells from
peptide (TRP-258-78/TRP-2i4g.|65) immunized mice and spleen cells from B16.F10 melanoma-bearing mice recognized peptides TRP-258-78 and TRP-2i48-i65 determined by an indirect measure of IFNγ secretion by peptide-reactive T cells in recall responses ex vivo. It must be confirmed in future studies that epitopes TRP-258-78 and TRP-2i48-i65 are indeed restricted to H2-IAb. A HLA-DR peptide binding assay protocol has been described (Southwood et al., 1998, Manici et al., 1999). These binding assays are inhibition based assays with peptide binding expressed as inhibitory concentration (IC50) values relative to an indicator peptide i.e. IC50 is the concentration of competitor peptide (for example TRP-258-78 or TRP-2i48-i65) required to inhibit 50% binding of the indicator peptide. This protocol could be applied to evaluate peptide binding to H2-IAb; purified H2-IAb molecule, a source cell line expressing H2-IAb allele and an iodinated (125I)-radiolabelled peptide that binds H2-IAb would be required. Alternatively, antibodies to H2-IAb are commercially available, and thus it could be tested in future studies whether they could be used as blocking antibodies (which to date has not been demonstrated in the literature) to neutralize the binding of peptide TRP-258-78 or TRP-2i48-i65 to H2-IAb and measuring the antibody effects on T cell effector function (i.e. peptide cytokine secretion) in an effort to confirm restriction to H2-IAb. It must also be verified in future studies that TRP-258-78/TRP-2i48-i65 MHC complex is specific for CD4+ T cells (i.e. that the responding T cell is a CD4+ T cell).

This study showed that the murine TRP-2-derived CTL epitope (TRP-2180-188) and the newly identified murine TRP-2-derived TH epitopes (TRP-258-78 and TRP-2i48-i65) were immunogenic in vivo and induced anti-tumour immunity, indicating their efficacy as a melanoma vaccine. In the prophylactic setting, the TRP-2 polyepitope-based vaccine
(TRP-2180-188, TRP-258-78 and TRP-2148-165) in combination with MPL and IL-1α as adjuvants provided the most significant protection against subcutaneous B16.F10 melanoma challenge. However, the degree of protection was only marginally superior to the TRP-2 polypeptide-based vaccine alone or vaccine in combination with MPL as adjuvant. In contrast, therapeutic immunization with the TRP-2 polypeptide-based vaccine coadministered with MPL and IL-1α as adjuvants eradicated tumours in 33% of treated mice and significantly (p<0.001) enhanced survival compared with untreated mice. Furthermore, immunization with the TRP-2 polypeptide-based vaccine in combination with MPL and IL-1α as adjuvants was significantly superior to the TRP-2 polypeptide-based vaccine alone (p<0.001) or in combination with MPL (p<0.01) in promoting protective anti-tumour immunity translated as delayed tumour growth and increased survival. The discrepancy between results obtained in the prophylactic and therapeutic settings is unknown and was not explored in this study and warrants further investigation. It is possible that the differential prophylactic versus therapeutic vaccination effects on tumour growth may be due to differential penetration of tumour by T cells and differential tumour microenvironments (tumour cell composition, nontransformed cellular infiltrates, stroma etc.) promoting a differential inflammatory response (i.e. inflammation is regarded as a promoter to carcinogenesis) and this may not have been apparent in the prophylactic study in the previous chapter as it is possible that heat-shocking (which releases heat-shock proteins) and irradiation of B16.F10 cells used as tumour-rejection-antigen source may render these cells more immunogenic, and thus masking the differential effect as observed in the current study. However, it is unlikely that a prophylactic melanoma vaccine would ever be clinically developed as the potential side effect of vitiligo (an autoimmune disease that causes depigmentation of patches of skin resulting from loss of function or death of melanocytes) which is
frequently associated with melanoma regression would not be accepted in healthy individuals and particularly as TRP-2 peptide reactivity has been linked to this autoimmune depigmentation (Rosenberg and White, 1996, Cho and Celis, 2009), it is not likely to be traded for the possibility of protection in healthy individuals who may never develop melanoma. This autoimmune phenomenon would however be acceptable in patients with established melanoma, the prognosis of such disease being very poor in most cases and a disease which is resistant to standard radiation and chemotherapy regimes. Thus, the focus of this study was the development of a therapeutic vaccine designed to target established tumours, a more clinically relevant approach.

This study showed that omitting the Th1-polarizing adjuvant MPL from the booster immunizations on days 10 and 17 (i.e. MPL with TRP-2 vaccine prime immunization day 3 but only TRP-2 vaccine on days 10 and 17) resulted in a degree of protection similar to that of TRP-2 polyepitope vaccine alone, providing indirect evidence that MPL is an effective adjuvant for the development of anti-tumour immune responses. However, it also suggests the requirement for adjuvant MPL to promote the breaking of tolerance to the self-derived antigen TRP-2, which is subject to mechanisms of peripheral tolerance homeostatically essential for limiting the activity of the self-reactive repertoire that escapes central tolerance. However, immunization with the TRP-2 polyepitope-based vaccine in combination with MPL as an adjuvant lacked the tumour eradicating potency of the TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α as adjuvants, demonstrating that the combined adjuvant approach was most effective in enhancing the potency of the TRP-2 polyepitope-based vaccine and thus in breaking tolerance to the TRP-2 epitopes. Omitting the Th1-polarizing
adjuvant MPL from the booster immunizations on days 10 and 17 from the immunization injection (i.e. group 5) resulted in a degree of protection similar to that of TRP-2 polyepitope-based vaccine alone, whereas the same immunization administered with MPL at all three immunizations (d3, 10, 17) eradicated tumours in 33% of treated mice (i.e. group 6). This finding suggests that both the Th1-polarizing adjuvant MPL and the Th17-polarizing adjuvant IL-1α are both required for optimal protection. It has recently been reported that a high frequency of polyfunctional CD4\(^+\)IFN\(\gamma\)^+IL-17\(^+\), CD4\(^+\)IFN\(\gamma\)^+IL-17\(^+\)IL-2\(^+\), CD4\(^+\)IFN\(\gamma\)^+IL-2\(^+\) T cells infiltrate the tumours of mice treated with DCs pulsed\(^{in vitro}\) with hs/irr B16.F10 cells, TLR5 agonist flagellin and a class I PI3K inhibitor (Marshall et al., 2012). The protection mediated by this vaccine was shown to require both IFN\(\gamma\) and IL-17; the protective effect was completely abrogated in IL-17\(^{-}\) mice and in mice treated with an anti-IFN\(\gamma\) neutralizing antibody (Marshall et al., 2012). Furthermore, the CD4\(^+\) tumour-infiltrating lymphocytes from vaccine treated IL-17\(^{-}\) mice was composed of a higher frequency of cells secreting IFN\(\gamma\) compared with vaccine treated wild-type mice (Marshall et al., 2012). This is consistent with another study showing that the tumour-infiltrating CD4\(^+\) T cells from B16.F10 tumours in IL-17\(^{-}\) mice produced more IFN\(\gamma\) relative to wild-type control mice whereas the frequency of B16.F10 tumour-infiltrating CD4\(^+\)IL-17\(^+\) T cells was increased in IFN\(\gamma\)^+ mice (Wang et al., 2009). The study by Marshall et al. showed that the CD4\(^+\) tumour-infiltrating lymphocytes from vaccine treated IFN\(\gamma\) depleted mice was composed of a higher frequency of cells secreting IL-17 compared with vaccine treated wild-type, however neither CD4\(^+\) tumour-infiltrating lymphocyte population (i.e. IFN\(\gamma\)-secreting T cells in IL-17\(^{-}\) mice or IL-17-secreting T cells in IFN\(\gamma\)^+ mice) could mediate protection, both cytokine secreting CD4\(^+\) T cells being required for tumour
protection (Marshall et al., 2012). Thus, this recent study by Marshall et al. supports the findings in the current study.

A novel tumour vaccine approach was described in this thesis consisting of adjuvants MPL and IL-1α combined with two newly identified TRP-2 T\textsubscript{H} epitopes, TRP-2\textsubscript{58-78} and TRP-2\textsubscript{148-165} and the CD8\textsuperscript{+} CTL epitope TRP-2\textsubscript{180-188}. In the B16.F10 mouse model of melanoma, therapeutic immunization with the TRP-2-polyepitope based vaccine in combination with MPL and IL-1α as adjuvants delayed tumour growth and increased survival, with some mice exhibiting complete tumour rejection. This study demonstrated that MPL and IL-1α were effective adjuvants to enhance TRP-2-specific immunity induced by a TRP-2-polyepitope cancer vaccine. Furthermore, the combined adjuvant approach may enhance the activation of innate immune mechanisms. In conclusion, the findings reported in this study lend itself to clinical exploration but they also have practical importance to the design of future vaccine strategies against cancer in humans.
Figure 5.1 Cultured B16.F10 cells highly express TRP-2
B16.F10 cells were incubated with the primary and secondary antibodies against TRP-2 or with the secondary antibody alone as a control (indicator of non-specific staining). Shown is flow cytometric analysis of TRP-2 expression on the surface of B16.F10 cells and in intracellular compartments. Results are from one experiment.
Figure 5.2 TRP-2 expression on B16.F10 melanoma cells ex vivo

Tumours were established in five mice by s.c. injection of $2 \times 10^6$ B16.F10 cells into the left hind flank. On day 16, tumours were harvested and $1 \times 10^6$ cells were transferred to FACS tubes. Melanoma cells were then incubated with the primary and secondary antibodies against TRP-2 or with the secondary antibody alone as a control (indicator of non-specific staining). (A) Shown is the flow cytometric analysis of TRP-2 expression on the surface of melanoma cells. (B) The bar graphs represent the mean fluorescence intensity (MFI) values. Results are from one experiment.
Figure 5.3 Immunization with TRP-2_{180-188} peptide and MPL induces IFNγ-secreting TRP-2_{180-188}-specific LN cells
C57BL/6 mice were immunised s.c. twice (day 0, 28) in the footpad with TRP-2_{180-188} (10 μg) and MPL (50 μg). Popliteal lymph nodes were harvested on day 35 and the cells were restimulated with 1-20 ng/ml TRP-2_{180-188}. PMA/αCD3 was used as a positive control and an irrelevant peptide (OVA_{257-264}) served as a negative control. Supernatants were collected after 72 h, and peptide-specific IFNγ and IL-10 concentrations were determined by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. *** P < 0.001; n.s., not significant versus medium, ANOVA.
**A**

**tyrosinase-related-protein-2 [Homo sapiens]**

Swissprot database, accession number P40126-1

>gi|545619|GenBank|AAC60627.1| Length=519 AA, Mass(Da)=59,145

**Isoform 1** (P40126-1) has been chosen as the 'canonical' sequence

**Isoform 2** (P40126-2) differs from the canonical sequence as follows:

393-393: V → VISHNLVATNILEHVRKATKELFSLHV

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1  MSLHGWFL SCLGCKILPG AQOQGFPRVCM TVDSLWNEKC CPPRGAEAS VCGSQQQRGQ  60-74

61  CTVEVRADTRP WSGPYILRNQ DDRELFWFRF EHTCCTCGN FAGYNGDCKX FGTQQPNQVC 149-163

121  KKAPWLRHI HSLSPQEREQ FLGALDLARK RHVPDYVITT QHMLGLLGPL GTQPQFANC  180-188

181  YVDFFWVLLHY YSVRDTLLGP GRFRAIDFS HQGAPFVTWV RHHLLCILRD LQHRGNESF  241

241  ALPYWNPATG RNEDCVDCTQ LFGAARPDDP TLISRNSRFV SWETVCSLDD YNHLVTLNC

301  GYEGLLRRN QMGRSMKLP TLKDIRCCLS LQKFDNPPF QNSTFSFNRA LEGFSDKADG

361  LQSVQMSLHN LVSHFNLGTN ALPHSAANDP IFVVLHSDTD AIFDEWKKRF NPPADWPQ  421

421  LAPYGHNRMY NMPFPPFPVT NELFLTDSTQ LGYSAIDLP VSVEEPTGWP TTLLVVMGTL  481

481  VALVGLFVLL AAPQYRIILK GYTPIMETHL SSKRTEEA

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**B**

**tyrosinase-related-protein-2 [Mus musculus]**

Swissprot database, accession number P29812

>gi|44890807|GenBank|AAH67064.1| Length=517 AA, Mass(Da)=58,510

1  MGLVGWGLLL GLCCLGILLR ARAQPFPRVCM TLDGVLWNEKC CPPRGPEATN ICGFLEGROQ  60-74

61  CAEVQDTRP WSGPYILRNQ DDRELFWFRF EHTCCTCGN FAGYNGDCKX FGTQQPNQVC 149-163

121  KKAPWLRHI HSLSPQEREQ FLGALDLARK RHVPDYVITT QHMLGLLGPL GTQPQFANC  180-188

181  YVDFFWVLLHY YSVRDTLLGP GRFRAIDFS HQGAPFVTWV RHHLLCILRD LQHRGNESF  241

241  ALPYWNPATG RNEDCVDCTQ LFGAARPDDP TLISRNSRFV SWETVCSLDD YNHLVTLNC

301  GYEGLLRRN QMGRSMKLP TLKDIRCCLS LQKFDNPPF QNSTFSFNRA LEGFSDKADG

361  LQSVQMSLHN LVSHFNLGTN ALPHSAANDP IFVVLHSDTD AIFDEWKKRF NPPADWPQ  421

421  LAPYGHNRMY NMPFPPFPVT NELFLTDSTQ LGYSAIDLP VSVEEPTGWP TTLLVVMGTL  481

481  VALVGLFVLL AAPQYRIILK GYTPIMETHL SSKRTEEA

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**Figure 5.4 FASTA protein sequence of Human TRP-2 and Murine TRP-2**

(A) Highlighted in red on human TRP-2 sequence are TRP-2 (60-74) and TRP-2 (149-163)

(B) Highlighted in red on murine TRP-2 sequence are TRP-2 (58-78) and TRP-2 (148-165) with the additional amino acids underlined and highlighted blue Highlighted in green in both the human and murine TRP-2 sequences is TRP-2 (180-188). The epitope amino acid sequence is identical in both species.
Table 5.1 Amino acid differences between Human and Murine TRP-2 protein sequences

<table>
<thead>
<tr>
<th>Residue No.</th>
<th>HUMAN</th>
<th>MOUSE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amino Acid</td>
<td>Type</td>
</tr>
<tr>
<td>62</td>
<td>Threonine</td>
<td>Hydrophilic</td>
</tr>
<tr>
<td>65</td>
<td>Arginine</td>
<td>Electrically charged (positive)</td>
</tr>
<tr>
<td>66</td>
<td>Alanine</td>
<td>Hydrophobic</td>
</tr>
<tr>
<td>151</td>
<td>Arginine</td>
<td>Electrically charged (positive)</td>
</tr>
<tr>
<td>152</td>
<td>Valine</td>
<td>Hydrophobic</td>
</tr>
</tbody>
</table>

1 Amino acids are classified by the chemical nature of their side chains. Polar (or hydrophilic) amino acids have side chains that interact with water. Non-polar (or hydrophobic) amino acids have side chains that do not interact with water. Another group of amino acids are those with ionisable side chains: electrically charged (negative); electrically charged (positive).
Figure 5.5 Structure of murine class II MHC I-Ab in complex with a human CLIP peptide \((87, 101)\), the “1MUJ” template structure available in MODPROPEP selected for modelling the two TRP-2 derived epitopes for binding to the H2-IA\(^b\) molecule.

Structure extracted from the Protein Data Bank, DOI: 10.2210/pdb1muj/pdb
Figure 5.6 The epitope sequence QCAVQT DTRPW SGP (TRP-2_{50-74}) was predicted to be a relatively strong binder with H2-IA^b. Structural peptide threading approach was applied by using MODPROPEP online web server and the potential interaction energies of the epitope sequence with the MHC molecule IA^b was evaluated using Miyazawa and Jernigan pair potential matrix. Atoms highlighted in blue represents the TRP-2 epitope sequence bound within the groove of MHC class II IA^b.
Figure 5.7 The epitope sequence KKSIHPDYVITTTQHW (TRP-2_{149-163}) was predicted to be a relatively strong binder with H2-IA^b. Structural peptide threading approach was applied by using MODPROPEP online web server and the potential interaction energies of the epitope sequence with the MHC molecule IA^b was evaluated using Miyazawa and Jernigan pair potential matrix. Atoms highlighted in blue represents the TRP-2 epitope sequence bound within the groove of MHC class II IA^b.
Figure 5.8 Immunization with TRP-2_{58-78} peptide and MPL promotes the generation of IFN\(\gamma\)-secreting TRP-2_{58-78}-specific T cells

C57BL/6 mice were immunised s.c. twice (day 0, 28) in the footpad with TRP-2_{58-78} (10 \(\mu\)g) and MPL (50 \(\mu\)g). Popliteal lymph nodes were harvested on day 35 and the cells were restimulated with 1-20 \(\mu\)g/ml TRP-2_{58-78}. PMA/anti-CD3 was used as a positive control and an irrelevant peptide (OVA_{323-336}) served as a negative control. Supernatants were collected after 72 h, and peptide-specific IFN\(\gamma\) and IL-10 concentrations were determined by ELISA. Results are expressed as mean values \(\pm\)SD for \(n=3\) sample replicates from one experiment.

* \(P < 0.05\); *** \(P < 0.001\) versus medium, ANOVA.
Figure 5.9 Immunization with TRP-2148-165 peptide and MPL promotes the generation of IFNγ-secreting TRP-2148-165-specific T cells. C57BL/6 mice were immunised s.c. twice (day 0, 28) in the footpad with TRP-2148-165 (10 µg) and MPL (50 µg). Popliteal lymph nodes were harvested on day 35 and the cells were restimulated with 1-20 µg/ml TRP-2148-165. PMA/anti-CD3 was used as a positive control and an irrelevant peptide (OVA323-336) served as a negative control. Supernatants were collected after 72 h, and peptide-specific – IFNγ and –IL-10 concentrations were determined by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. *** P < 0.001 versus medium, ANOVA.
Figure 5.10 T cells from mice with B16.F10 melanoma respond to the TRP-2\(_{58-78}\) and TRP-2\(_{148-165}\) epitopes

Tumours were established in three mice by s.c. injection of 2 x 10\(^{5}\) B16.F10 cells into the left hind flank. On day 16, spleen cells were isolated and restimulated with either (A) TRP-2\(_{58-78}\) 1, 10 and 50 μg/ml or (B) TRP-2\(_{148-165}\) 1, 10 and 50 μg/ml. Spleen cells pulsed with an irrelevant peptide (OVA\(_{23-336}\); 50 ng/ml, designated O on graph) or medium (designated M on graph) served as negative control. Supernatants were collected after 72 h. and peptide-specific IFN\(_{γ}\) concentrations were determined by ELISA. Results are expressed as mean values ±SD for n=3 sample replicates from one experiment. ***P < 0.001 versus medium, ANOVA.
Figure 5.11 Prophylactic immunization of mice with a TRP-2 polyepitope-based vaccine in combination with MPL and IL-1α as adjuvants promotes anti-tumour immunity and increases survival

Mice were immunized s.c. into the left hind flank with TRP-2_180-188, TRP-2_58-78, and TRP-2_148-165 (100 μg/peptide) alone or with MPL (10 μg) or MPL and IL-1α (100 ng) twice at a 28 d interval. Seven days after the booster immunization, mice were challenged with 2x10^5 B16.F10 cells s.c. into the left hind flank. The tumour volumes were determined every other day. (A) Mean (±SEM) tumour growth rates for 18 mice per group, combined from two experiments. Tumour volumes for each mouse shown in B (first experiment) and C (second experiment). *** P < 0.001 versus untreated tumour only group (Kruskal-Wallis test). (D) Survival plots of tumour-bearing mice treated as in (A). Kaplan-Meier survival curves were analyzed by the logrank survival test. *** P < 0.001 versus untreated tumour only group. Tumour draining lymph nodes were isolated from 6 mice/group treated as in (A) when the tumours grew to end-point and cells were stimulated with PMA (20 ng/ml) and ionomycin (200 ng/ml) for 6 h, with brefeldin A (5 μg/ml) being added in the last 4 h. Intracellular IFNγ and IL-10 was determined in CD4 T cells (E), CD8 T cells (F), γδ T cells (G) and NK cells (H). Intracellular IL-17 was also determined in CD4 T cells (E) and γδ T cells (G). (I) Tumour draining lymph node cells (n=6 mice/group) were stained directly ex vivo with antibodies specific for surface CD3 and CD4 and intracellular Foxp3. The gating strategy is also shown.
Figure 5.11

B

Untreated Tumour Only

TRP-2 Peptides

TRP-2 Peptides + MPL

TRP-2 Peptides + MPL + IL-1\(\alpha\)
Figure 5.11
Figure 5.11

D

![Graph showing percent survival over days for different treatments: Tumour Only, TRP-2 Peptides, TRP-2 + MPL, TRP-2 + MPL + IL-1α. The graph indicates significantly higher survival rates for the combined treatments compared to Tumour Only.](image-url)
Figure 5.11 Gating Strategy
Figure 5.11

E

F

270
Figure 5.11

G

H

I
Figure 5.12 MPL and IL-1α adjuvant co-therapy enhances the efficacy of TRP-2 polypeptide-based vaccine against B16.F10 melanoma in a therapeutic setting

Tumours were established in all groups on day 0 by subcutaneous injection of $2 \times 10^5$ B16.F10 cells into the left hind flank. Mice were immunized on days 3, 10, and 17 in the region of the tumour as indicated. (A) Tumour volumes for each mouse, tumour volume (B) expressed as the mean ($\pm$SEM) for six mice per group and survival (C) were monitored. Kaplan-Meier survival curves were analyzed by the logrank survival test. The symbols in (A) indicate significant differences between TRP-2 + MPL + IL-1α (d3, 10, 17) treatment group versus other treatment groups; *** $P < 0.001$ versus PBS treated group; +++ $P < 0.001$ versus TRP-2 treated group; ^^^ $P < 0.01$ versus TRP-2 + MPL (d3, 10, 17) treated group; ΔΔΔ $P < 0.01$ versus TRP-2 + MPL (d3) treated group; ΩΩΩ $P < 0.01$ versus TRP-2 + MPL + IL-1α (d3) treated group (Kruskal-Wallis test). The asterisks in (B) indicates significant differences (* $P < 0.05$; *** $P < 0.001$) between PBS treated group versus any other treatment group. Results are from one experiment.
Figure 5.12

B

- PBS
- TRP-2 Peptides (d3, 10, 17)
- TRP-2 + MPL (d3), TRP-2 (d10, 17)
- TRP-2 + MPL (d3, 10, 17)
- TRP-2 + MPL + IL-1α (d3), TRP2 + IL-1α (d10, 17)
- TRP-2 + MPL + IL-1α (d3, 10, 17)

Days after tumour inoculation

Tumour volume (mm³)

C

- PBS
- TRP-2 (d3, 10, 17)
- TRP-2 + MPL (d3), TRP-2 (d10, 17)
- TRP-2 + MPL (d3, 10, 17)
- TRP-2 + MPL + IL-1α (d3), TRP2 + IL-1α (d10, 17)
- TRP-2 + MPL + IL-1α (d3, 10, 17)

Percent survival

Days
Chapter 6
General Discussion
Vaccines against infectious diseases represent one of the most successful interventions in medical science. Vaccination strategies are either prophylactic, aimed at preventing disease or therapeutic, aimed at resolving established disease. Most vaccines for infectious diseases are prophylactic vaccines and are generally designed to induce long-lasting neutralising antibodies, which bind to the pathogens and prevent them binding to host cells or enhance their phagocytosis by monocytes resulting in pathogen clearance and prevention of disease. However, the development of vaccines for the prevention or treatment of malignant diseases is much more difficult, as cancer is a multi-stage disease driven by genetic instability, generating a multitude of unpredictable mutations and resultant heterogeneous cell phenotypes. Therefore, it is preferable to commence vaccination once the cancer has developed. Tumour specific adaptive immunity elicited with vaccines has traditionally focused primarily on eliciting a CD8\(^{+}\) CTL response, often involving vaccination with a synthetic peptide comprising a class I binding CTL epitope, due to the fact that CTLs have the capacity to directly eradicate tumour cells via granzyme/perforin and FasL ligation apoptosis inducing mechanisms. However, it has become clear that activation of CD8\(^{+}\) effector T cells alone is insufficient to mediate tumour clearance as CD4\(^{+}\) T cells are critical in generating primary CTL responses, licensing DCs and promoting protective CD8\(^{+}\) memory T cell development (Schoenberger et al., 1998, Williams and Bevan, 2007), a phenomenon termed CD4 help. Furthermore, IFN\(\gamma\)-secreting CD4\(^{+}\) T\(_{H1}\) cells also have a direct effect on tumour via IFN\(\gamma\) production. IFN\(\gamma\) has shown potent anti-tumour activity against various experimental tumours (Giovarelli et al., 1986, Maekawa et al., 1988, Gansbacher et al., 1990), and it induces MHC class I protein expression on tumour cells, rendering these cells more immunogenic and susceptible to tumour-specific CTLs (Dighe et al., 1994, Kaplan et al., 1998). Moreover, T\(_{H1}\) cells can impair tumour angiogenesis directly by
inhibiting endothelial cell proliferation via IFNγ or indirectly through the induction of anti-angiogenic chemokines CXCL9 and CXCL10 (Brandacher et al., 2006). IFNγ may also play a direct role in tumoricidal activity by generating inducible nitric oxide synthase (Brandacher et al., 2006). Thus, in recent years, increasing importance has centred on the stimulation of a CD4+ T helper cell response concurrent with the activation of CTL in cancer immunotherapy.

The first step in the design of peptide-based therapeutic cancer vaccines is the choice of target antigen(s). TRP-2 is a transmembrane melanosomal glycoprotein shown to have DOPAchrome tautomerase enzymatic activity catalyzing the conversion of DOPAchrome to DHICA in melanin biosynthesis and its expression is restricted to melanocytes, melanoblasts, melanocyte stem cells, retinal pigmented epithelium, and telencephalon (Steel et al., 1992, Bouchard et al., 1994, Mackenzie et al., 1997, Zhao and Overbeek, 1999, Nishimura et al., 2002, Osawa et al., 2005). TRP-2 was identified as a melanoma antigen in humans by cloning CTL responses from the tumour infiltrating lymphocytes of melanoma patients (Wang et al., 1996, Wang et al., 1998, Parkhurst et al., 1998). The results of the present study demonstrate that TRP-2 is also expressed in B16.F10 murine melanoma, which is in agreement with previously published data (Bloom et al., 1997, Schreurs et al., 2000), and therefore represents a tumour rejection antigen for the B16.F10 melanoma.

TRP-2 was chosen as the target antigen for the tumour vaccination approach in this study because of its demonstrable expression in B16.F10 melanoma and in melanocyte stem cells (with the possibility of also being expressed in melanoma stem cells which
relates to the so-called cancer stem cell theory), its limited tissue distribution, and its proposed function as a critical detoxifying enzyme indispensable for melanocyte/melanoma cell viability. The class I H2-K\(^b\)-binding peptide SVYDFFVWL which derives from an evolutionary conserved region of both human and murine TRP-2 corresponding to amino acids 180-188 was selected from the literature as a good candidate CTL epitope for incorporation into the cancer vaccine. This epitope was identified as a melanoma rejection antigen in C57BL/6 mice using a K\(^h\)-restricted CD8\(^T\) T cell line specific for B16 melanoma cells (Bloom et al., 1997). Indeed, as demonstrated in this study, immunization with peptide TRP-2\(_{180-188}\) was able to stimulate IFN\(\gamma\)-producing CD8\(^T\) T cells specific for the H2-K\(^b\)-binding peptide.

There have been a number of attempts to identify peptide epitopes of tumour antigens that bind HLA class II molecules in humans, but such studies are limiting in mice. In the present study, a knowledge-based threading approach was used to predict whether the equivalent sequences in murine TRP-2 were analogous to the two identified HLA-DRB1*0301-restricted CD4\(^T\) T cell epitopes derived from human TRP-2 (Paschen et al., 2005, Osen et al., 2010). Thus, the threading approach was used to predict whether these analogous epitopes could bind H2-IA\(^b\) (the class II molecule expressed by the C57BL/6 strain used in the current study) and subsequently elicit the T helper cell response. Indeed, the threading approach predicted these two murine epitopes as strong binders to the H2-IA\(^b\) molecule. Confirmation that these epitopes were indeed recognized by T cells was done by testing the immunogenicity of the two peptides in C57BL/6 mice. B16.F10 tumour-bearing mice were used as a source of tumour-specific T cells to test the antigenicity of the predicted peptide epitopes. Spleen cells from B16.F10 tumour-bearing mice secreted IFN\(\gamma\) in response to TRP-2\(_{58-78}\) and TRP-
2_{148-165} peptide restimulation *ex vivo* indicating the presence of T cells specific for epitopes in both of the peptides. This suggests that tumour-bearing mice have circulating T cells specific for the epitopes or the peptides and that tolerance to these two epitopes is incomplete. Furthermore, immunization with peptides TRP-2\textsubscript{58-78} and TRP-2\textsubscript{148-165} with MPL as adjuvant induced T cells that secreted IFNγ *ex vivo* in response to the homologous H2-IA\textsuperscript{b}-binding peptides. The peptides were predicted (via the threading approach) as epitopes for CD4\textsuperscript{+} T cells, however, it can not be ruled out that such peptides could also contain embedded CD8\textsuperscript{+} T cell epitopes, as the exact responding T cell (CD4 and/or CD8) was not determined by flow cytometry in the current study and thus such a possibility warrants future exploration. Nevertheless, collectively these findings suggested that these two T cell epitopes were suitable for testing in the cancer vaccine, and thus TRP-2\textsubscript{58-78} and TRP-2\textsubscript{148-165} peptides were used as the antigen for testing in a tumour vaccine *in vivo*.

The activity of T helper effectors depends not only on the specificity of their TCR, but also on the cytokine conditions imposed upon them during their differentiation. Therefore appropriate *in vivo* polarization of CD4\textsuperscript{+} T helper subsets is critical for the induction of tumour-specific effector T\textsubscript{H} cells rather than T\textsubscript{Reg} cells which suppress the function of effector CTL and T\textsubscript{H} cells in the tumour microenvironment and prevent tumour eradication. In this study the peptides with identified class II and class I epitopes were used as tumour-rejection antigen in combination with the adjuvants MPL and IL-1α, shown to enhance T\textsubscript{H1} and T\textsubscript{H17} responses.
The vaccine used in this study consisted of a class I (Kb)-restricted peptide representing the CTL epitope TRP-2180-188 and two peptides shown to contain Th cell epitopes derived from TRP-2 (TRP-2258-278 and TRP-2148-165), administered in combination with TLR4 agonist MPL and recombinant cytokine IL-1α as adjuvants. Immunization of mice prophylactically significantly delayed growth of B16.F10 melanoma and enhanced survival. However, complete regression of tumour was not observed in any mice. In contrast, therapeutic administration of the vaccine significantly delayed tumour growth and resulted in significant protection against the tumours, with 33% of mice exhibiting complete tumour rejection. Importantly, omitting MPL in the booster immunization was far less effective than immunization with the TRP-2 peptides administered with MPL and IL-1α in both primary and booster doses. This indicates that both MPL and IL-1α are both required as adjuvants to overcome tolerance to the self-derived TRP-2 peptides and thus for optimal protection. This highlights an important collaboration between MPL, which promotes IFNγ-producing Th1 cells, and IL-1α, which promotes IL-17-secreting Th17 cells, in mediating optimal anti-tumour immune responses. Furthermore, immature DCs activated with TLR agonists (such as MPL as shown in this study) mature into potent activators of antigen-specific T cells (Guermonprez et al., 2002). Thus, omitting MPL from the booster immunizations eliminated the DC maturing capability of MPL from the vaccine, which also contributed to the observed loss of protection in those mice.

In recent years, evidence has accumulated that CD4+ T cells significantly contribute to protection against tumours in vivo (Hung et al., 1998, Ossendorp et al., 1998, Pardoll and Topalian, 1998, Marzo et al., 2000). CD4+ T cells mediate protective anti-tumour immunity via a multitude of mechanisms. CD40 ligand (CD40L) is primarily restricted
to CD4⁺ T₇ cells (van Kooten and Banchereau, 2000). The interaction of CD40L on activated tumour-specific CD4⁺ T₇ cells with CD40 expressed on DCs sustains the anti-tumour activity of CTL by conditioning DCs to effectively prime CD8⁺ CTLs, but it is also critical for generating functional long-lived CD8⁺ T cell memory (Bennett et al., 1998, Ridge et al., 1998, Schoenberger et al., 1998, Ahlers et al., 2001, Bourgeois and Tanchot, 2003, Janssen et al., 2003, Shedlock and Shen, 2003, Sun and Bevan, 2003). Additionally, the engagement of CD40 on DC by CD40L expressed on T cell prolongs MHC/antigen complex presentation, up-regulates the expression of costimulatory molecules on DC, and enhances the production of IL-12 (Caux et al., 1994, Cella et al., 1996, Manickasingham and Reis e Sousa, 2000, van Kooten and Banchereau, 2000, Frleta et al., 2003). The TLR4 agonist MPL has been reported to augment anti-CD3-induced expression of CD40L on CD4⁺ T cells (Ismaili et al., 2002). Melief and colleagues demonstrated that treatment of tumour-bearing mice with agonistic anti-CD40 monoclonal antibody lead to improved CTL activation and tumour eradication and that MPL had similar effects on anti-tumour immune responses to that observed with anti-CD40 monoclonal antibody treatment (van Mierlo et al., 2004).

It has also been reported that IL-1 is required for the induction of CD40L on naïve T cells (Nakae et al., 2001). The expression levels of CD40L on CD4⁺ DO11.10 T cells after stimulation with OVA₃₂₃-₃₃₉ peptide in the presence of IL-1α/β⁻⁻ APC were low compared with wild type APCs, whereas CD40L expression was enhanced when IL-1Ra⁻⁻ APC were used (Nakae et al., 2001). Wild type, IL-1α/β⁻⁻, and IL-1Ra⁻⁻ APCs were all shown to express similar levels of CD40, CD80 and CD86. Nakae et al. also showed that the reduced CD40L expression on CD4⁺ DO11.10 T cells co-cultured with IL-1α/β⁻⁻ APC pulsed with OVA₃₂₃-₃₃₉ could be rescued by the addition of recombinant
IL-1 (Nakae et al., 2001). These findings are supported by a subsequent study showing that the expression of CD40L on T cells was greatly reduced in IL-1α/β−/− mice (Saijo et al., 2002a). Importantly, CD40 antibodies have been shown to induce strong CTL priming, and to show regression of established tumours (Diehl et al., 1999, French et al., 1999, Sotomayor et al., 1999, van Mierlo et al., 2002, Murphy et al., 2003, van Mierlo et al., 2004, Fransen et al., 2011) and genetic manipulation of DCs or tumour cells with CD40L has been shown to enhance their capacity to induce sustained anti-tumour immune response (Kikuchi and Crystal, 1999, Briones et al., 2002, Yurkovetsky et al., 2006). Collectively, these studies highlight the importance of CD4+ help for CD8+ T cell responses.

T helper cells also recruit NK cells and macrophages to the tumour site, key effector cells of anti-tumour immunity (Kennedy and Celis, 2008). Anti-tumour responses mediated by NK cells also require CD4+ T cell help (Perez-Diez et al., 2007, Kennedy and Celis, 2008). TH cells can also directly activate the tumoricidal activity of macrophages; TH1 cell-derived IFNγ has been shown to activate NO production by intra-tumoural macrophages (Hung et al., 1998), a critical mediator of the cytotoxic activity of macrophages against tumour cells. TH cells can also directly induce apoptosis in tumour cells by FasL ligation (Schattner et al., 1996), by mechanisms involving TRAIL (Thomas and Hersey, 1998), or via a granzyme-perforin-dependent pathway (Echchakir et al., 2000). Consistent with these studies, TH1 and TH17 cells have been reported to express granzyme A (Annunziato et al., 2007), indicating their potential to mediate apoptosis via the granzyme-perforin-dependent pathway. TH and TH17 cells can also enhance/promote B cell production of IgM, IgG, and IgA, providing evidence that they mediate B cell help for the production of these antibodies.
Thus, Th cells may play a critical role in the development of an endogenous antibody response targeting tumour cells. Although TRP-2 is primarily expressed in melanosomes, it is not confined solely to this intracellular location; the polypeptide matures in the endoplasmic reticulum and a small proportion of TRP-2 can be found in the plasma membrane (Negroiu et al., 2003), therefore providing a target for antibodies. Indeed, therapeutic vaccination with alphavirus-based virus-like replicon particles (VRP) encoding TRP-2 elicited protective humoral immunity against TRP-2 in the B16 melanoma model (Avogadri et al., 2010). Mice immunized with VRP-TRP-2 developed TRP-2 specific serum IgG reactive to a region spanning amino acids 51-101 of murine TRP-2 (Avogadri et al., 2010). The TRP-2 derived peptide incorporated into the vaccine in the current study (i.e. TRP-258-78) lies within the region spanning amino acids 51-101 of murine TRP-2, suggesting its potential to also induce TRP-2 specific IgG. Thus, it is possible that Th cells elicited by the vaccine in this study may provide B cell help for the production of protective TRP-2 specific IgG antibodies. This suggests that the novel vaccine in this study could potentially elicit TRP-2-specific humoral immunity as well as cellular immunity. This possibility was not explored in the current study and warrants future investigation. Indeed, cell-mediated immunity has been shown to act in conjunction with humoral responses to elicit an anti-tumour response (Wolpoe et al., 2003).

Sipuleucel-T (Provenge®), an autologous active cellular immunotherapy, has recently been approved by the FDA as the first therapeutic vaccine for the treatment of established tumours (i.e. hormone-resistant metastatic prostate cancer), suggesting that the promise of active immunotherapeutic approaches for cancer may finally be fulfilling its potential. An integrated analysis of 225 patients in two randomized, double-blind,
placebo-controlled phase III clinical trials, D9901 and D9902A, (147 patients in the vaccines arm and 78 patients in the placebo arm), demonstrated a statistically significant 4.3 month improvement in overall survival (23.2 months versus 18.9 months, respectively), with a 33% reduction in the risk of death (Higano et al., 2009). Another double-blind, placebo-controlled, phase III trial (341 patients in the vaccines arm and 171 patients in the placebo arm), demonstrated a 4.1 month improvement in median survival (25.8 months in the sipuleucel-T group versus 21.7 months in the placebo group) (Kantoff et al., 2010). In the sipuleucel-T group, there was a 22% reduction in the risk of death as compared with the placebo group (Kantoff et al., 2010).

A major drawback to this immunization approach is the requirement for large numbers of clinical grade autologous PBMC for patient-specific therapy, a process that can be expensive. Several studies have demonstrated that the use of TLR agonist as adjuvant in combination with DC expressing tumour antigens can improve the efficiency of DC-based vaccines (Pulendran, 2004, Jarnicki et al., 2008, Marshall et al., 2012). Thus, targeting DC in vivo with a TLR agonist (such as MPL shown in the current study) may prove to be a more feasible approach. CTLA-4 (CD152) is a molecule that is constitutively expressed by naturally occurring T_{reg} cells proposed to play a functional role in their suppressive activity (Kolar et al., 2009b). The anti-CTLA-4 blocking antibody ipilimumab (a humanized monoclonal IgG1κ antibody) was recently approved by the FDA and European Commission for the treatment of metastatic melanoma (Graziani et al., 2012). However, the life-threatening immune-related adverse effects of ipilimumab therapy in association with the outrageous cost of this treatment, indicates the need for the development of alternative approaches.
The approach described in this study exploits the ability of a pathogen-derived molecule, MPL, to target and activate the DC \textit{in vivo} for optimal priming of tumour-antigen-specific T cells. Additionally, this approach, by utilizing MPL (a Th1-inducer) and recombinant IL-1\(\alpha\) (a Th17-inducer) as adjuvants, also targets CD4\(^+\) T cells \textit{in vivo}. Overcoming the immunosuppression mediated by T_{reg} cells is a particular challenge as these cells govern peripheral immune tolerance, preventing immune responses against self-derived antigens. The approach in this study, by utilization of MPL and IL-1\(\alpha\) as adjuvants, provides a new strategy to skew the population of tumour-infiltrating cells to favour helper CD4\(^+\) T cells over the regulatory CD4\(^+\) T cells within the tumour microenvironment, providing an attractive approach to overcome tumour immunosuppression and poor immunogenicity of tumour antigens. Importantly, MPL and IL-1\(\alpha\) are safe in human individuals (Woodlock et al., 1999, Cluff, 2009) and both products are relatively inexpensive, indicating that the approach in the current study could prove a more viable approach. Furthermore, this is the first study to report the utilizing of both MPL and IL-1\(\alpha\) as adjuvants combined in an active specific immunotherapy approach, suggesting that the potential for MPL and IL-1\(\alpha\) used in combination as adjuvants for cancer vaccines has until now, not been fully appreciated. The results shown in this study indicate that this combination of adjuvants represents an exciting adjuvant combination for cancer vaccines.

In summary, the significant new findings of this study are that MPL is a potent mediator of IL-12 production in DC, inducing optimal DC maturation and as an adjuvant MPL promotes a Th1-biased immune response \textit{in vivo}. However, the administration of IL-1\(\alpha\) in combination with MPL as adjuvants for a model antigen was more effective than MPL as an adjuvant alone in tipping the balance away from Th2 and Th1 type dominant
response induced with the antigen alone towards a biased Th1 and Th17 dominant response, characterised by an increased induction of antigen-specific T cells that secrete IFNγ and IL-17 and a decreased induction of antigen-specific -IL-10 and -IL-4 secreting T cells. Using a knowledge-based threading approach, two novel murine TRP-2-derived Th epitopes, TRP-258-78 and TRP-2148-165, were identified and the immunogenicity and antigenicity of the novel epitopes was confirmed in this study with the finding that LN cells from peptide (TRP-258-78/TRP-2148-165) immunized mice and spleen cells from B16.F10 melanoma-bearing mice recognized peptides TRP-258-78 and TRP-2148-165 determined by an indirect measure of IFNγ secretion by peptide-reactive T cells in recall responses ex vivo. This study showed that the murine TRP-2-derived CTL epitope (TRP-2180-188) in combination with the newly identified murine TRP-2-derived Th epitopes (TRP-258-78 and TRP-2148-165) were immunogenic in vivo and induced anti-tumour immunity, indicating their efficacy as a melanoma vaccine. Importantly, this study showed that MPL and IL-1α were effective adjuvants to enhance TRP-2-specific immunity induced by the TRP-2-polyepitope cancer vaccine.

In conclusion, a melanoma vaccine consisting of peptide epitopes derived from the melanocytic self-antigen TRP-2 administered with the adjuvants MPL (detoxified LPS) and recombinant IL-1α was able to break peripheral T cell tolerance and induce protective immunity against B16.F10 melanoma in C57BL/6 mice. Therapeutic immunization provided significant protection against tumour progression and significantly improved survival, with some mice exhibiting complete tumour rejection. The success of vaccine formulation strategies in murine models makes the development of a therapeutic anti-cancer vaccine a realistic goal. Thus, the findings reported in this
study lend itself to clinical exploration but they also have practical importance to the design of future vaccine strategies against cancer in humans.

**Future Directions**

This study demonstrated that treatment of mice with the NOD1 agonist TriDAP enhanced the growth of B16.F10 melanoma when administered early in the establishment of tumours but had an anti-tumour effect when used against established B16.F10 tumours is an extremely interesting finding which requires further investigation. Since TriDAP enhanced IL-23 production by TLR-activated DCs, it is possible that the effect of TriDAP could be at least in part mediated by activation of \( \text{T}_{\text{H}}17 \) cells, which are known to have pro- and anti-tumour effects especially early and late in the growth of tumours respectively.

This study identified two novel murine TRP-2-derived \( \text{T}_{\text{H}} \) epitopes, TRP-2\(_{58-78}\) and TRP-2\(_{148-165}\), based on a combination of a knowledge based threading approach for predicting peptide-MHC class II H2-IA\(^b\) binding, and immunization of mice with peptide and screening melanoma-bearing mice spleen cells for peptide recognition. These peptides together with a previously defined CD8\(^+\) T cell epitope were shown to be effective immunogens and when used with a novel adjuvant combination based on a TLR4 agonist and the cytokine IL-1\(\alpha\), induced effective anti-tumour immunity when given therapeutically to mice with B16.F10 tumours. Complete eradication of tumours in a proportion of the mice suggests the induction of potent anti-tumour immune responses but also memory T cell induction. However, the fact that tumours were detectable in some mice also suggests immune regulatory processes that should be
identified and targeted for combination immunotherapy in the future. Follow-up studies with this vaccine preparation could include dose escalation studies and comparison of the timing and spacing of vaccine doses. The adjuvant combination used in this study could also be evaluated in vaccine preparations for other cancer types. Finally, since corresponding TRP-2 T cell epitopes have already been identified in humans, these peptides are now very useful tools for the evaluation of peptide based vaccines in mice with the potential for generating data that can be translated to humans.
Chapter 7
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